At the Crossroads of Inflammation and Lipid Metabolism: Modulation of Macrophage Functionality by Long-chain Metabolites of Vitamin E and Related Compounds

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13’-COOH	13’-carboxychromanol
13‘-OH	13’-hydroxychromanol
5-LOX	5-lipoxygenase, arachidonate 5-lipoxygenase
ANGPTL4	angiopoietin-like 4
ANS	anilinonaphthalene-8-sulfonic acid
AOM	azoxymethane
ApoE	apolipoprotein E
ATGL	adipocyte triglyceride lipase
AVED	ataxia with vitamin E deficiency, familial isolated vitamin E deficiency
C18	18 carbon atoms chain
C6 cells	C6 glioma cell line
CCL2	chemokine (C-C motif) ligand 2, monocyte chemoattractant protein 1
CD36	cluster of differentiation 36, fatty acid translocase
CEHC	carboxyethyl hydroxychroman
CB (CNR1/2)	cannabinoid receptor
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CRD	chronic respiratory disease
CRISPR	clustered regularly interspaced short palindromic repeats
CVD	cardiovascular diseases
CYP	cytochrome P450
CYP3A4	CYP family 3 subfamily A member 4
CYP4F2	CYP family 4 subfamily F member 2
DSS	dextran sodium sulphate
Dusp1 / MKP-1	dual specificity protein phosphatase 1, MAPK-phosphatase 1
EAR	estimated average requirements
ERK	extracellular signal-regulated kinases
FABP	fatty-acid-binding-proteins
GA	δ-garcinoic acid
Gal4	gal4 transcription factor
GC-MS	gas chromatography-mass spectrometry

GPCR	G protein-coupled receptors
GSK3787	4-chloro-N-(2-{[5-trifluoromethyl)-2-pyridyl]sulfonyl}ethyl)benzamide
GW0742	4-[2-(3-fluoro-4-trifluoromethyl-phenyl)-4-methyl-thiazol-5-ylmethylsulfanyl]- 2-methyl-phenoxy}-acetic acid
GW9662	2-chloro-5-nitro-N-phenylbenzamide
HbA1c	glycated haemoglobin / haemoglobin A1c
HCT-116	human colon cancer cells
HDL	high-density lipoprotein
HEK293	human embryonic kidney cells
HepG2	human liver cancer cell line
HPLC	high performance liquid chromatography
HPLC/EC	high performance liquid chromatography with electrochemical detection
HT-29	human colon adenocarcinoma cells
ICM	intermediate-chain metabolites
IL1α	interleukin 1α
IL1β	interleukin 1β
IL6	interleukin 6
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
LCM	long-chain metabolites
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
mPGES-1	microsomal prostaglandin E synthase 1
MS/MS	mass spectrometry/mass spectrometry
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCD	non-communicable diseases
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS2	inducible nitric oxide synthase
NPC1L1	niemann-pick C1-like protein 1
Nrf2	nuclear factor erythroid 2-related factor 2

OECD	Organization for Economic Co-operation and Development
oxLDL	oxidized low-density lipoprotein
p38 MAPK	p38 mitogen-activated protein kinases
PARP-1	Poly [ADP-ribose] polymerase 1
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E2
P-gp/MDR1	xenobiotics transporter P-glycoprotein 1, multidrug resistance protein 1
PI3K	phosphoinositide 3-kinases
PKB/Akt	protein kinase B
PKC	protein kinase C
PLIN2	perilipin 2, adipose differentiation-related protein (ADRP)
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
PPAR	peroxisome proliferator-activated receptors
PTGER / EP	prostaglandin E_2 receptors
PTGS2	prostaglandin-endoperoxide synthase 2 (also known as COX-2)
PUFA	poly-unsaturated fatty acids
PXR	pregnane X receptor
QTOF	quadrupole time-of-flight
RAW264.7	murine macrophage cell line
RDA	recommended daily allowance
RXR	retinoid X receptor
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SCM	short-chain metabolites
siRNA	small interfering RNA
SR-BI	scavenger receptor B1, HDL receptor
T3	tocotrienol
t8-iso-PGF2α	8-iso-prostaglandin F2α
TAP	tocopherol-associated proteins
TBARS	thiobarbituric acid reactive substances
TE	tocopherol equivalents
THP-1	THP-1 human monocytic cell line
TLR	toll-like receptor

Tnf	tumor necrosis factor, tumor necrosis factor ligand superfamily member 2, tumor necrosis factor α
TOH	tocopherol
UPLC	ultra-performance liquid chromatography
USA / US	United States of America
VLDL	very low-density lipoprotein
WHO	World Health Organization
α-TTP	α-tocopherol transport protein

1 The great framework: non-communicable diseases as consequence of meta-inflammation

The current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic keeps the world on their toes and all eyes are on the corresponding communicable coronavirus disease 2019 (COVID-19). This exceptional situation distracts from the minor importance of communicable diseases in 'normal times', achieved by the progress of mankind in understanding pathological mechanisms as well as of biological, medical and hygiene knowledge. The historically high standard of living led to the situation (before SARS-CoV-2) that communicable diseases were of minor importance in about 90 % of the countries worldwide (Bennett et al. 2018a). In contrast, about 70 % of global deaths were ascribed to non-communicable diseases (NCD) in 2016 (Bennett et al. 2018a). Among these, cancer, cardiovascular diseases (CVD), diabetes and chronic respiratory diseases (CRD) accounted for about 80 % of deaths (Bennett et al. 2018a). It is of note that all of these diseases have been linked to vitamin E status (refer to the chapter 'Vitamin E and non-communicable diseases'). Albeit citizens of low and middle-income are currently at main risk to die from NCD, recent progress - and prognoses on the future impact - of NCD in the United States of America (USA) show that this issue is growing again in highly developed countries (Bennett et al. 2018a). However, in Europe, reduction in premature deaths from NCD is continuing (WHO/Europe 2020b). Unfortunately, this does not necessarily translate into healthy lives. On estimation, EU citizens spend about one-fifth to a quarter of their lives in ill health (Eurostat 2020), mainly due to chronic disease. This problem will obviously aggravate since the prevalence for obesity, a main risk factor for the development of chronic disease, is rising in Europe (WHO/Europe 2020a).

We are thus facing the problem that the growing prosperity and biomedical knowledge enable us to confidently manage NCD while the same growing prosperity (or already high prosperity in highly developed countries) promotes an unhealthy way of life, abetting the development and progression of NCD (Jakovljevic and Milovanovic 2015). This lifestyle is characterized on the one hand by a lack of physical activity and on the other hand by widespread noxious eating habits summarized as 'Western Diet' (Kopp 2019). The Western Diet is generally characterized as high in calories and sugars, *trans* and saturated fat, salt and food additives. Permanent low physical activity combined with high caloric intake from foods of low nutrient-density is highly health-threatening and so the increase in life expectancy is for the first time ever predicted to decline. Albeit this represents a prognosis for the USA (Olshansky et al. 2005), other western high-income countries show comparable developments: The mentioned lifestyle is obviously related to the growing proportion of overweight and obese adults and, more important and more alarming, of overweight and obese children (Ludwig 2016). A similar trend like in the USA is seen in Germany, where already 15.4 % of children were classified as overweight and 6 % as obese in 2017 (Schienkiewitz et al. 2018). Among adults, 67 % of men and 53 % of women were classified as overweight and 23.3 % of men and 23.9 % of women in Germany as obese, with increasing prevalence especially in young adults (Mensink et al. 2013).

At present, researchers are even stating that 'we face a pandemic of lifestyle-associated diseases' (Christ and Latz 2019), since more than one-third of the world's adult population is classified as overweight or obese (WHO 2020). Albeit the negative effect of the current SARS-CoV-2 pandemic can hardly be estimated (OECD 2020), the middle class in emerging countries like China and India is predicted to grow in the long term (OECD 2010, 2019), resulting in more people following a 'western-like' lifestyle and thus likely becoming overweight

and obese. Researchers already summarize that ‘a tipping point has been reached beyond which technological advances may no longer compensate’ (Ludwig 2016) for the negative consequences of overweight and obesity in the USA. In consequence, the ‘pandemic of lifestyle-associated diseases’ (analogously of NCDs) can most probably only be stopped by prevention, information and education, and governmental regulation.

However, to date governments of highly developed countries generally avoid to force food industry to produce and distribute more healthy products albeit effective tools like taxes on sugar-sweetened beverages are at hand (it is of note that sugary drink taxes are charged in some countries like France, Norway and the United Kingdom). However, the isolated application of such an intervention is unlikely to effectively reduce the prevalence of obesity and associated NCD (Fernandez and Raine 2019). Hence, tools should be combined in an overall strategy to generate a higher impact on people’s behavior. Additional approaches comprise, among others, the nutritional education of children and young adults.

The current SARS-CoV2 pandemic demonstrates again the bitter truth of the dictum ‘there is no glory in prevention’. Here, in extreme cases, effective preventive measures are even retrospectively declared as gratuitous by sceptics. Basically, prevention suffers from the inherent problem that success is not directly visible, whereas the causality of successful treatment of an existing acute problem is obvious to human beings. This also describes a basic problem in nutritional sciences. Findings in this field are generally translated into recommendations for healthier diets. This rather represents a preventive approach than a treatment option, especially if diseases have progressed so far that they cause severe health problems. Taking into account that humans have difficulties to understand and address problems in the distant future if subjectively perceived as ‘abstract’ (Trope and Liberman 2010) (here, future health problems as consequences of a permanent noxious diet) and governments’ hesitations to force healthy eating habits, treatment of NCD rather than prevention will play an important role in the near and distant future. Cancer, CVD, CRD and diabetes represent the most important NCD (Bennett et al. 2018a). These diseases are all characterized by (chronic) inflammatory processes as well as alterations in the (nutrient) metabolism. The connection between these processes is so well-established that the term ‘meta-inflammation’ (also termed ‘metaflammation’ or ‘metinflammation’) was coined and diseases in this context are also designated as immunometabolic diseases (Hotamisligil 2017). This dissertation (henceforth referred to as ‘thesis’) and all included manuscripts relate to this highly relevant scientific field.

Meta-inflammation is defined as ‘low-grade, chronic inflammation orchestrated by metabolic cells in response to excess nutrients and energy’ (Gregor and Hotamisligil 2011) and thus closely linked to nutritional status and diet. Vitamin E represents a valuable constituent of health-preserving and health-promoting diets as outlined in the following sections. Based on this knowledge, vitamin E has been proposed to have beneficial effects in people suffering from metabolic syndrome (the prime example for the connection of obesity, inflammation and consequent diseases) and all major NCD as outlined in the chapter ‘Vitamin E and non-communicable diseases’. However, studies on this issue often lead to contradictory or unexpected results. The long-chain metabolites (LCM) of vitamin E, the central compounds investigated in this thesis, might serve as explanatory approach for these contradictions (refer to the chapters ‘Vitamin E and non-communicable diseases’ and ‘Future perspectives’). As outlined above, prevention of disease by following a healthy diet and consequently reaching adequate levels of vitamin E and thus of the hypothetical bioactive LCM is desirable, but not sufficient in the context of NCD. This thesis focusses therefore on both physiological functions and pharmacological functions of the LCM and garcinoic acid (GA, referring to δ-GA throughout

the entire thesis) as a related substance (refer to the chapter ‘Physiologic and pathophysiologic implications of the works in the context of NCD and meta-inflammation’). Given their promising biological functions, GA and the LCM may serve as lead compounds for the development of pharmacological treatment strategies for NCD and principally for all diseases with inflammation or disordered lipid metabolism in their pathophysiology. Finally, most of our research was carried out in macrophage models, since macrophages are key players in virtually all inflammatory diseases and thus all major NCD (Li et al. 2018; Ponzoni et al. 2018; Russo and Lumeng 2018; Schultze et al. 2015).

2 Vitamin E

Almost 100 years ago, in 1922, an enigmatic dietary substance being vital for the fertility of rats was found and initially termed ‘X’ (please refer to the section ‘Aim of the work’) for a brief review of the history of vitamin E research). During the following years of intense research, ‘X’ was identified as α-tocopherol (α-TOH) and several lipid-soluble molecules with similar structural (and functional) properties were found and characterized later and summarized under the hypernym ‘vitamin E’ (refer to the chapter ‘Structures and related structures’). However, parts of the scientific community argue that only α-TOH can be regarded as the ‘one (real) vitamin E’ since it represents the only structure which was shown to serve as treatment for ataxia with vitamin E deficiency (AVED), a disease due to a genetic defect in the α-tocopherol transport protein (α-TTP) and consequently extremely low levels of α-TOH in humans suffering from this disease (Azzi 2018, 2019). Still, this view is a matter of debate in the scientific community and has not yet become established. Thus, the term ‘vitamin E’ is used as hypernym for the different lipophilic structures outlined in the following chapter throughout this thesis. Vitamin E is associated to fats in dietary sources due to the lipophilic character of the molecules summarized as ‘vitamin E’. Thus, especially plant oils represent good sources for vitamin E. Here, wheat germ oil, sunflower oil, soybean oil and corn oil contain high levels of vitamin E (Shahidi and Camargo 2016). Vitamin E shows a relatively high stability in food. However, stability depends on storage conditions as well as the matrix and the processing of the food (Chapman et al. 2009). Further, vitamin E is susceptible to heat (Réblová 2006) and light as well as atmospheric oxygen and is quickly oxidized (Pignitter et al. 2014). Despite the good availability of food containing vitamin E and its relatively high stability, a large part of the world population does not reach the recommended daily intake (Péter et al. 2019). Based on the recommended level for vitamin E intake (here represented by the recommended daily allowance for US adults of 15 mg/d), 82 % are below this level, globally, with 91 % in North and South America, 79 % in the Asia-Pacific region and 80 % in Europe (Péter et al. 2019). However, since the recommended level is based on values that are experimentally necessary to avoid peroxide-induced hemolysis in the blood taken from vitamin E-deficient individuals (Péter et al. 2015), the relevance of the recommended level and the strategy of determination is questionable (Azzi 2018). In addition, although recommended supply is not reached by most people, the consequent undersupply remains mostly without symptoms. Consequently, a revision of the reference values for vitamin E intake is repeatedly brought into question (Novotny et al. 2012; Raederstorff et al. 2019). Notwithstanding this, the following sections will provide an overview of the current view on structural features, recommendations, intake and insufficiency as well as on the metabolic fate and other facets of vitamin E.

2.1 Structures and related structures

In 1936, Herbert Evans, Oliver Emerson and Gladys Emerson reported on the isolation of an alcohol having the properties of vitamin E from wheat germ oil (Evans et al. 1936). This alcohol was analyzed to have the chemical formula $C_{29}H_{50}O_2$ and the name ‘α-tocopherol’ (from the

greek terms ‘tokos’ meaning ‘childbirth’ and ‘*phero*’ meaning ‘to bear’) was proposed based on the reported biological function. Later, the chemical formula was suggested to represent a ‘chroman derivative with a side chain composed of isoprene residues’ which ‘should be thought’ as a condensation product from trimethylhydroquinone and phytol (Karrer et al. 1938a). Albeit the exact structure was proposed but not known at that time, the proposed chroman derivative is today known as ‘α-tocopherol’ and determines the fundamental structure for all compounds classified as ‘vitamin E’ (Figure 1). Characteristics of all vitamin E structures are the chroman ring system and the isoprenoid-like side chain. The substitution of the chroman ring*, i.e.* the number and position of methyl groups determines the classification as ‘α, β, γ, or δ-form’ and the saturation of the side chain determines the classification as ‘tocopherols’ or ‘tocotrienols’. The hallmark of the tocotrienols (T3) is the unsaturated side chain. The exact chemical structures of the eight known forms of vitamin E are presented in Figure 1**.** Natural forms of vitamin E occur either in the *RRR* configuration, in the case of TOH, or in the *R* configuration in the case of T3. Besides, there are further naturally occurring vitamin E-related structures, forming a less consistent group with members like terrestrian tocomonoenols or marine-derived TOH (refer to Manuscript V (Manuscript V: Kluge et al. 2016)).

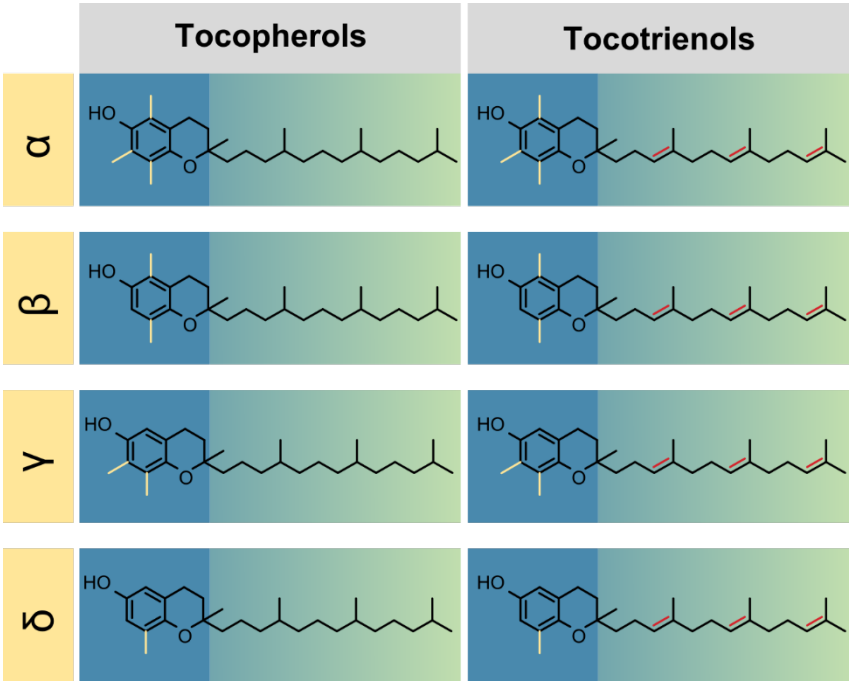

Figure 1: Structures of the eight known forms of tocopherols and tocotrienols generally summarized as ‘vitamin E’. Blue square: Chroman ring system; Green: phytyl-like side chain; Yellow: methyl groups determining α-, β-, γ- or δ-forms; Red: double-bonds determining tocotrienol forms

2.2 Recommendations, intake, status and insufficiency of vitamin E

Human metabolism preferentially retains α-TOH (refer to the chapter ‘Distribution and storage’) and quickly eliminates all other TOH and T3. For this reason, α-TOH is the most abundant form in human plasma and considered as the most biologically active form (Burton and Traber 1990; Traber et al. 2010b). Consequently, recommendations for the intake of vitamin E are established based on the α-form. For this reason, recommendations are given as mg per day, either in the form of α-TOH or international units (IU), as for example in the USA, or as tocopherol equivalents (TE), as for example in Germany. Both consider that the different forms of vitamin E show different efficacy in the setting (*vide infra*) used to estimate the recommendations. Hence, one IU of vitamin E is equivalent to 1 mg of the ‘synthetic’ dl-α-tocopheryl acetate and equivalent to 0.67 mg of the ‘natural’ d-α-tocopherol. The principle of

‘tocopherol equivalents’ also considers the forms of vitamin E. Here, calculation of the amount of a form representing one TE is based on the biological activity in the rat resorption-gestation test (Kamal-Eldin and Appelqvist 1996). One TE represents 1 mg of α-TOH but, for example, γ-TOH shows only 10 % of the activity of α-TOH, hence 10 mg of γ-TOH are equivalent to 1 mg α-TOH and also represent one TE (Table 1). In Germany, a daily intake of 12 to 15 mg TE per day is recommended. However, in the USA, 12 mg/d of Vitamin E represents the Estimated Average Requirements (EAR) and 15 mg/d is the Recommended Daily Allowance (RDA). However, the meaningfulness of these values as recommendations are still a matter of debate. Reasons for this include *inter alia* that recommendations do not sufficiently take into account factors like sex and age, since they are almost exclusively based on a study on men (Meydani et al. 2018) and that large parts of the population show an underconsumption of vitamin E with respect to the recommendations (*vide supra*) which does not result in detrimental health outcomes. The latter fact was recently again supported by an analysis which states that the mean vitamin E intakes in Europe were between 7.8 and 16.0 mg α-TOH per day and were 6.3 mg/d for US women and 7.8 mg/d for US men, respectively (Péter et al. 2019).

At present the plasma concentration of α-TOH representing an ‘adequate’ status is set at 12 µmol/l. However, this value is based on experimental data from a project conducted between 1953 and 1967. In experiments on the blood of vitamin E depleted and later repleted men, the level of α-TOH needed to prevent hydrogen-peroxide induced hemolysis was estimated. By setting the cut-off to ≤ 12 % hemolysis, a plasma concentration of 12 µmol/l was determined as the lowest effective concentration. Based on this value, the EAR and RDA were calculated (*vide supra*) (Meydani et al. 2018). However, while hydrogen peroxide-induced red blood cell hemolysis may have been the best approach at that time, this assay is now outdated and the field of vitamin E research has evolved. A large body of evidence on non-antioxidant functions, distribution throughout the body and in individual tissues and cell-types as well as health-promoting effects are now available that justify a reconsideration of the recommended plasma concentrations and consequent intake recommendations. Notwithstanding this, insufficiency of vitamin E is characterized by adverse effects at plasma levels of α-TOH below 8 µmol/l comprising the development of ataxia, peripheral neuropathy and skeletal myopathy and below 12 µmol/l comprising miscarriage and increased erythrocyte fragility. Consequently, the cut-off value to indicate a vitamin E deficiency is set at 12 µmol/l (Péter et al. 2019). Further, low plasma levels are considered to be involved in the development of Alzheimer’s disease and mild cognitive impairments (Mangialasche et al. 2012). On the other hand, plasma levels of more than 30 µmol/l are now suggested to represent a desirable level in order to provide health benefits, *inter alia* to decrease the risk for non-communicable diseases (Wright et al. 2006; Péter et al. 2019). Moreover, levels of more than 45 µmol/l are associated with benefits for cognitive and immune function and cardiovascular and liver health (Péter et al. 2019). Interestingly, high intake of vitamin E provokes an increase of the metabolization to the LCM and consequently an increase in their plasma levels (Ciffolilli et al. 2015; Giusepponi et al. 2017). Hypothetically this could contribute to the observed beneficial effects of α-TOH with respect to non-communicable diseases. Further, uptake of vitamin E is generally considered as being safe up to a level of 800 mg/d (Azzi et al. 2005) or even 1000 mg/d (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds 2000). While there is evidence from animal models and some human studies that a high vitamin E intake interferes with vitamin K functions and consequently can reduce blood clotting and promote bleeding (Podszun and Frank 2014; Zondlo Fiume 2002), a recent meta-analysis did not reveal evidence for an increase in all-cause mortality by vitamin E supplementation up to 5000 IU/d (Köpcke 2019).

Table 1: Biological activity of selected vitamin E forms and transfer to tocopherol equivalents.

Form	Activity in rat-resorption-gestation test	Amount representing 1 TE [mg]
α-TOH	100 %	1
β-TOH	50 %	2
γ-TOH	10 %	10
δ-TOH	3 %	33.3
α-T3	30 %	3.3

TE: tocopherol equivalents. Adapted from Kamal-Eldin and Appelqvist (1996).

2.3 Ataxia with vitamin E deficiency

The most severe consequence of a prolonged vitamin E insufficiency is the development of a phenotype resembling Friedreich's ataxia, a disease predominantly characterized by the degeneration of nerve tissue. Ataxia with vitamin E deficiency (AVED) is consequently also known as Friedreich-like ataxia and further as familial isolated vitamin E deficiency. As the latter implicates, AVED is a genetic disease inherited in an autosomal recessive manner. The genetic defect is located at chromosome 8 and results in the synthesis of defective α-TTP (*i.e.* a loss-of-function mutation). Clinically, AVED is characterized by progressive ataxia, clumsiness of the hands, loss of proprioception and areflexia. Other symptoms vary in their severity among the different pathogenic variants (Schuelke 2005). However, diagnostic criteria comprise a Friedreich's ataxia-like phenotype combined with markedly reduced plasma α-TOH (< 4.0 µmol/l (Cavalier et al. 1998; Mariotti et al. 2004)) and no evidence for lipid malabsorption or defective lipoprotein metabolism. Obviously, the loss of functional α-TTP causes neurological manifestations. However, knowledge on the specific role of α-TOH in the central nervous system (CNS) is sparse (Ulatowski et al. 2014). Knockout or loss of α-TTP results in axonopathy, neuromuscular injuries, reduced myelination, attenuated axonal transport and increased markers of oxidative stress in the CNS (Southam et al. 1991; Cuddihy et al. 2008). Further, cerebellar atrophy and loss of cerebellar Purkinje neurons was reported in humans (Sokol 1988; Yokota et al. 2000). Manifestation of the ataxic phenotype is ascribed to injured cerebellar Purkinje cells, since these are critical for motor output (Knogler et al. 2019). Interestingly, Ulatowski and co-workers recently reported that vitamin E is essential for Purkinje neuron integrity. However, they conclude that the underlying molecular mechanism is unclear and there is a need for further work regarding the role of vitamin E in the CNS (Ulatowski et al. 2014). Thus the beneficial effect of vitamin E cannot be ascribed to its prominent anti-oxidative function and leaves room for speculations that vitamin E contributes to proper function of nerve cells by other mechanisms, hypothetically including the LCM (refer to the chapter 'Future perspectives'). Strikingly, only α-TOH has been tested and been shown to prevent AVED. For this reason, Angelo Azzi repeatedly postulated that only α-TOH can be called "vitamin E" (Azzi 2018, 2019). However, since the β-, γ- and δ-forms exert the same anti-oxidative properties like α-TOH, this function cannot be the gist of the matter with respect to AVED. Interestingly, Azzi uses the metabolic activation of vitamin D as an example to support his case. This is essentially what we postulate for the LCM of α-TOH (Manuscript III: Schubert et al. 2018). Taking into account that the defective α-TTP in AVED patients cannot properly retain and distribute supplemented α-TOH in the body and that supplemented α-TOH hardly increases α-TOH levels in the brain of α-TTP-deficient subjects (Ulatowski et al. 2014), LCM as explanatory approach become more likely (refer to 'Future perspectives').

2.4 The metabolic fate of vitamin E

2.4.1 Bioavailability

The main sources of vitamin E are vegetable oils, nuts and seeds. However, due to the different dominance of foods in the diets across the world, different vitamers (*i.e.* different structural forms with a similar vitamin function) play key roles. For example, α-TOH represents the major form of vitamin E in the European diet, while γ-TOH represents the major form in the US. Since vitamin E is a hypernym for lipid-soluble structures, its digestion and uptake largely follows that of other dietary lipids, as outlined in the following chapters. However, according to Borel et al. (Borel et al. 2013), the bioavailability of vitamin E is generally determined by the following factors:

- **Species**, *i.e.* the individual vitamer
- **Linkage**, *i.e.* the molecular connection to other molecules
- **Amount**, *i.e.* the quantity of vitamin E consumed
- **Matrix**, *i.e.* the composition of the food
- **Effectors** of absorption and bioconversion, e.g. lipids and fiber
- **Nutrient status** of the individual
- **Genetic factors**
- **Host-related factors**, e.g. age and sex
- **Interactions**, *i.e.* combined effects based on mathematical models

The most important factors in healthy individuals determining the bioavailability of vitamin E is likely the food matrix in which vitamin E is embedded and the amount of fat provided with the meal. The former decides on the bioaccessibility, *i.e.* the release from the food matrix, and the latter facilitates the extraction of the lipid-soluble vitamin E from the matrix, stimulates biliary secretion and supports micelle formation (Reboul 2017).

2.4.2 Absorption

Micelle formation is a crucial step in the absorption of vitamin E, since it allows the presentation of lipid-soluble molecules to the enterocytes in the hydrophilic environment of the intestine (Gallo-Torres 1970; Sokol et al. 1983). The release of vitamin E from the food matrix starts already in the stomach by the action of gastric lipases (Borel et al. 2013). In the intestine, pancreatic enzymes are suggested to be involved in the absorption of vitamin E (Iqbal and Hussain 2009) by hydrolyzing vitamin E esters (which are the main form in artificial supplements but also occur in natural sources) to free vitamin E (Lauridsen et al. 2001; Flory et al. 2019). However, different mechanisms have been suggested to be involved in the hydrolysis of vitamin E esters (Reboul 2017). Notwithstanding this, vitamin E is incorporated into mixed micelles along with other lipid digestion products with the help of bile acids in the duodenum (Reboul 2017; Flory et al. 2019). The mixed micelles are assumed to dissociate at the brush border membrane (Reboul 2017) and the released vitamin E can then be absorbed, either by passive diffusion (as initially suggested) or by the action of the relatively unspecific transport proteins scavenger receptor class B type 1 (SR-BI) (Reboul et al. 2006), Niemann-pick C1-like protein 1 (NPC1L1) (Narushima et al. 2008) and cluster of differentiation 36 (CD36) (Goncalves et al. 2014). Interestingly, a metabolic conversion of vitamin E is suggested to occur already in the small intestine (Bardowell et al. 2012), allowing to hypothesize on a local action of LCM in the respective tissue. The trafficking of vitamin E across the enterocyte is poorly described. Based on the hydrophobicity, vitamin E is suggested to localize into membranes, cytosolic lipid droplets or to be bound to specific transport proteins like tocopherol-associated proteins (TAP) (Zimmer et al. 2000; Zingg et al. 2008). Most of the vitamin E is then incorporated into chylomicrons and released to the lymph (Reboul 2017). Chylomicrons are suggested to play a significant role in the supply of skeletal muscles and adipose tissue with vitamin E, however, the main distribution route of vitamin E is the transport via chylomicron

remnants to the liver (Herrera and Barbas 2001; Mardones and Rigotti 2004). In addition, a minor distribution pathway via intestinal high-density lipoprotein (HDL) has been suggested for vitamin E (Grebenstein et al. 2014; Niesor 2015).

2.4.3 Distribution and storage

The liver represents the central organ for the sorting, distribution, and metabolism of vitamin E. All eight vitamin E forms are absorbed to a similar extent and are transported to the liver (Flory et al. 2019), where the transporting chylomicron remnants are taken up. While all vitamers are taken up by the liver, α-TOH is the predominant form found in blood (Ford et al. 2006; Zerbinati et al. 2015) and in extrahepatic tissues (Traber 2013). Consequently, a discrimination of the different forms of vitamin E occurs in the liver. This discrimination is largely ascribed to the action of α-TTP. α-TTP is highly expressed in the liver but is also found in other tissues like the central nervous system (Sato et al. 1993; Ulatowski and Manor 2015). Based on the high affinity of α-TTP for *RRR*-α-TOH (SRR-α-TOH shows merely a relative affinity of 11 % and γ-TOH of 9 % of that of *RRR*-α-TOH, respectively), α-TTP is thought to preferentially retain α-TOH in cells and protect it from degradation (Hosomi et al. 1997; Meier et al. 2003). However, another aspect may be the preferential metabolization of non-α-TOH-forms independent of α-TTP (*vide infra*), leading to the discrimination of the forms (Grebenstein et al. 2014; Flory et al. 2019). In consequence, α-TTP contributes to the maintenance of sufficient α-TOH concentrations in blood and extrahepatic tissues (Traber et al. 2004). This function is realized by another aspect of α-TTP action, *i.e.* the transport of α-TOH to cellular membranes (Qian et al. 2005) and hence the likely role in the incorporation of α-TOH into VLDL particles (Traber et al. 2004). Subsequently, VLDL is secreted into the blood and α-TOH is distributed throughout the body. Supplementation studies in animals lead to the conclusion that α-TOH levels increase in virtually all tissues (e.g., red blood cells, heart, muscle, lung, brain, liver and adipose tissue). However, liver and adipose tissue accumulate α-TOH very rapidly which is not surprising due to the lipophilicity of vitamin E. Interestingly, liver levels decrease very rapidly after stopping supplementation, while levels in adipose tissue decrease very slowly. This lead to the conclusion that the liver is the major short-term storage organ while adipose tissue is the major long-term storage organ for α-TOH (Machlin and Gabriel 1982). However, it is argued that no tissue acts to release α-TOH ‘on demand’ (Traber et al. 2010a). The concentrations of α-TOH are rather related to the lipid content of the tissue. Notwithstanding this, adipose tissue levels have been used as markers for the long-term vitamin E status in humans (Traber et al. 2010a).

2.4.4 Metabolism

The liver is the central organ for vitamin E metabolization, albeit extrahepatic metabolization has also been reported, as indicated above. The knowledge on hepatic metabolism of vitamin E and the consequent formation of the LCM was repeatedly and extensively reviewed in the manuscripts included in this thesis (Manuscript V: Kluge et al. 2016; Manuscript III: Schubert et al. 2018; Manuscript VI: Schmölz et al. 2018). Thus, the hepatic metabolism of vitamin E is briefly outlined in the following and the reader is referred to the respective manuscripts (especially Manuscript VI) for detailed information.

In principle, all vitamin E forms follow the same metabolic route, since the end product of the catabolism, carboxyethyl hydroxychroman (CEHC), has been found for the different vitamers in urine (Chiku et al. 1984; Schultz et al. 1995; Swanson et al. 1999). First steps of the metabolism take place at the endoplasmic reticulum. Here, cytochrome P450 (CYP) family 4 subfamily F member 2 (CYP4F2), which is also called tocopherol-ω-hydroxylase functionalizes the TOH by a hydroxylation at the terminal (ω-) position of the side chain (Sontag and Parker 2002). This step may involve further enzymes like CYP family 3 subfamily A member 4 (CYP3A4) (Parker et al. 2000) and is regarded as the rate-limiting step of vitamin E metabolism (Flory et al. 2019). Further, parts of the scientific community argue, that CYP4F2 rather than

α-TTP is the central enzyme leading to the discrimination of the vitamers. This hypothesis is based on good evidence on the different affinity of CYP4F2 to the vitamers and consequently different catabolic rates (Flory et al. 2019). However, the formed 13'-hydroxychromanol (13'-OH) is transferred by a yet unidentified mechanism to the peroxisome. Here, the structure undergoes an ω-oxidation leading to the 13'-carboxychromanol (13'-COOH). This step is assumed to be mediated by alcohol and aldehyde dehydrogenases. Due to the functionalization with the carboxyl moiety, the TOH molecule can subsequently be catabolized like a branched-chain fatty acid by β-oxidation. Five cycles of β-oxidation, each shortening the structure by two carbon units, finally leads to the end product T-3'-COOH (also termed carboxyethyl hydroxychroman (CEHC)). While the first cycle is believed to take place in the peroxisome, the following cycles are carried out in mitochondria. Again, the transport mechanism remains elusive. Tocotrienols follow the same metabolic route in the same cellular compartments. However, due to their unsaturated side chain, three saturation steps are necessary during the β-oxidation from 13'-carboxytrienol (13'-T3-COOH) to 3'-COOH. The metabolic route was in large parts verified by the analytical detection of all proposed metabolites (reviewed in (Manuscript VI: Schmölz et al. 2018)) (Figure 2). Remarkably enough, the metabolism of vitamin E not necessarily leads to the formation of CEHC and the subsequent excretion of the metabolite. The LCM of vitamin E have repeatedly been found in human blood (Wallert et al. 2014; Pein et al. 2018; Giusepponi et al. 2019) and it was thus hypothesized that they may represent the metabolically activated biologically active form of vitamin E (Manuscript III: Schubert et al. 2018). This hypothesis is currently systematically examined by various research groups.

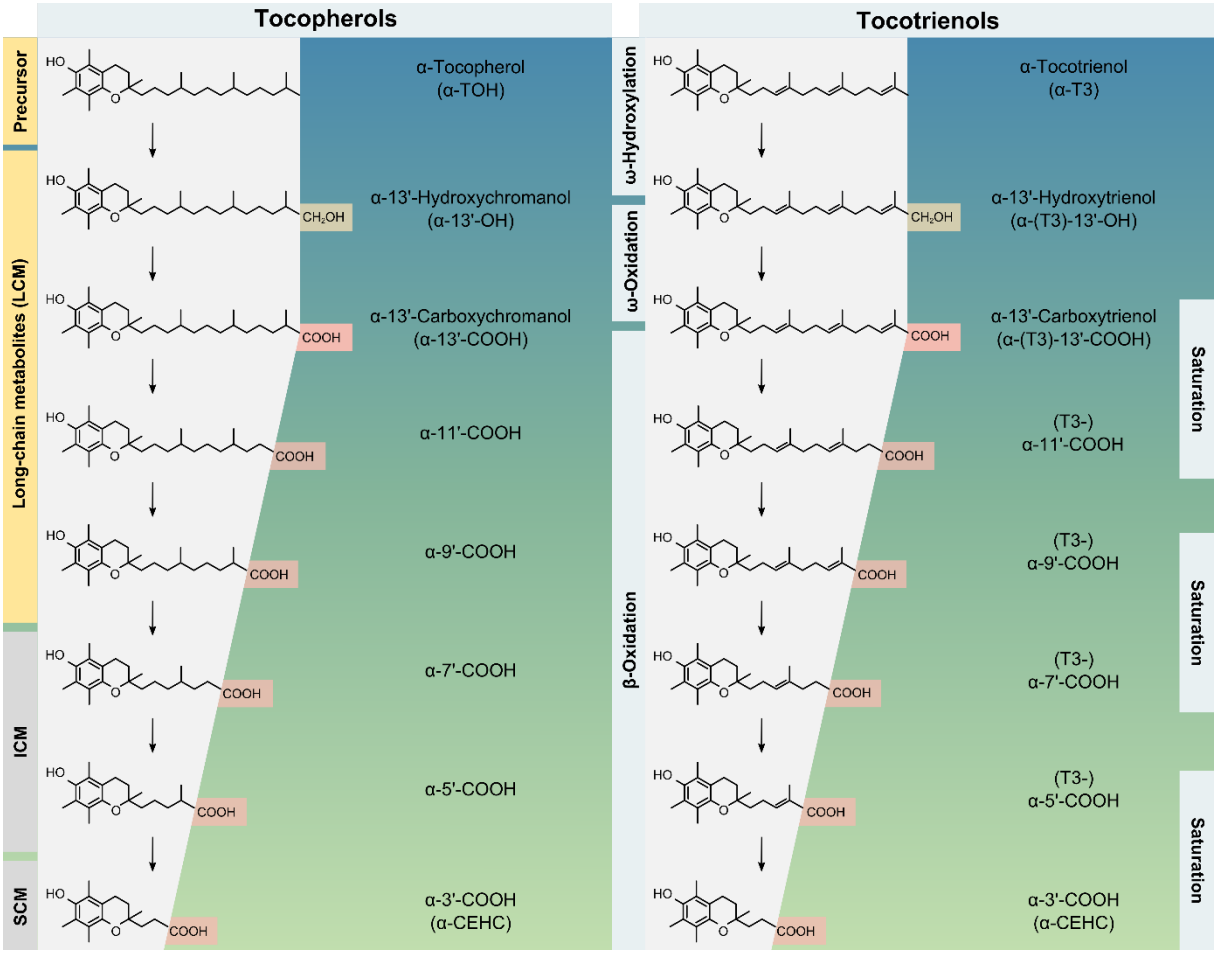

Figure 2: Representative illustration of the hepatic metabolism of vitamin E on the example of α-tocopherol and α-tocotrienol.
α-T3: α-tocotrienol; α-TOH: α-tocopherol; CH_2OH/OH: hydroxyl group; COOH: carboxyl group, ICM: intermediate-chain metabolites, LCM: long-chain metabolites; SCM: short-chain metabolites

2.4.5 Excretion

In principle, the catabolism of vitamin E is intended to render the lipid-soluble vitamin more hydrophilic and enable an excretion via aqueous liquids and thus finally the urine. By shortening of the side chain (*vide supra*) the hydrophilic metabolites 5'-COOH and CEHC are formed. These are found in blood and excreted via urine, partly as glucoside conjugates (Stahl et al. 1999; Zhao et al. 2010). However, all vitamin E metabolites including the precursors were found in feces of mice (Bardowell et al. 2012; Jiang et al. 2013) and humans (Zhao et al. 2010). Interestingly, the intestinal flora seems to be unable to metabolize vitamin E to the indicated metabolites (Zhao et al. 2010), indicating that the metabolites are indeed actively excreted via the fecal route. This leads to the conclusion that the lipid-soluble forms of vitamin E (thus presumably including the LCM) undergo the enterohepatic circulation (Mustacich et al. 1998) and the excreted amount in feces is lost from this process. To sum up, the water-soluble short-chain metabolites (SCM) are excreted via urine while the other metabolites are circulating in the body (refer to the chapter 'Long-chain metabolites of vitamin E') and are finally secreted via the bile and excreted via feces.

2.5 Vitamin E and non-communicable diseases

As mentioned earlier, virtually all major NCD are characterized by sustained inflammatory processes (refer to the chapter 'The great framework: non-communicable diseases as consequence of meta-inflammation'). Frequently, these inflammatory processes are triggered and prolonged by long-lasting obesity. In this case, the inflammatory trigger is metabolic and caused by the excess consumption of nutrients. Specialized metabolic cells regard this as an insult and respond by initiating an inflammatory program, building the interface between metabolic input and inflammatory output (Gregor and Hotamisligil 2011). This is believed to initiate a vicious cycle starting with metabolic signals leading to the initiation of an inflammatory response which in turn damages metabolic homeostasis and thus further promotes inflammation. It is therefore not surprising, that obesity and the resulting metabolic syndrome are risk factors for the major NCD (refer to the chapter 'The great framework: non-communicable diseases as consequence of meta-inflammation'), *i.e.* CVD and diabetes (Wild and Byrne 2006; O'Neill and O'Driscoll 2015), cancer (Avgerinos et al. 2019) and asthma (CRD) (Li et al. 2020).

Interestingly, vitamin E has been related to all these disease patterns. Initially, this was based on the knowledge of the prominent anti-oxidative function of vitamin E. However, as the field of vitamin E evolved, several non-antioxidant properties were elucidated. It became clear, that vitamin E can modulate the action of key enzymes in signal transduction pathways like peroxisome proliferator-activated receptors (PPAR), nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), mitogen-activated protein kinase (MAPK), nuclear factor erythroid 2-related factor 2 (Nrf2), lipoxygenases, protein kinase C (PKC), protein kinase B (PKB/Akt), phosphoinositide 3-kinases (PI3K) and others, consequently modulating cellular responses like inflammatory responses, survival, secretion of mediators and migration (Zingg 2015).

In the case of CVD, promising results including the reduction of the formation, area and progression of atherosclerotic lesions (Libinaki et al. 2017; Praticò et al. 1998; Meydani et al. 2014; Cyrus et al. 2003) as well as reduced mortality from atherosclerotic processes (Meydani et al. 2014) in apolipoprotein E (ApoE) and LDL receptor (Ldlr) knockout mouse models using normal and high-fat diets were obtained. Further, evidence was provided for a beneficial role of vitamin E with respect to the prophylaxis of cardiomyopathy and heart failure in the diabetes model of streptozotocin-treated rats (Hamblin et al. 2007), with respect to myocardial infarction by reducing infarct size and mortality in a model of infarction induction by ligation in rats (Sethi et al. 2000) and with respect to preserving cardiac function in an ischemia reperfusion model in C57BL/6 mice (Wallert et al. 2019). However, results from clinical trials in humans lead to

controversial findings. While beneficial effects of vitamin E with respect to the risk of mortality from heart failure (Eshak et al. 2018), the risk of myocardial infarction (Stephens et al. 1996; Boaz et al. 2000) and the occurrence of coronary heart disease (Rimm et al. 1993; Stampfer et al. 1993; Knekt et al. 1994; Kushi et al. 1996; Todd et al. 1999) were reported in some studies, other studies found no beneficial effects regarding the overall risk of heart failure (Chae et al. 2012), incidence of ischemic CVD (Hercberg et al. 2004) and cardiovascular outcomes in high-risk patients (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico 1999; Yusuf et al. 2000; Lonn et al. 2005). Further, conflicting results were reported from the Woman's Health Study. Here, no benefit of vitamin E supplementation regarding major cardiovascular events was found but a decreased cardiovascular mortality was reported (Lee et al. 2005). Taken together, while animal models provide evidence for beneficial effects of vitamin E in several steps of the pathogenesis and for the outcome of CVD, health-promoting effects of vitamin E are still questionable in this respect.

Based on the emerging recognition of a chronic low-grade inflammation being associated with the development of type 2 diabetes mellitus, oxidative stress comes into focus of diabetes treatment. The link between hyperglycaemia, hyperglycaemic-induced stress, inflammation and the development and progression of diabetes is based on good evidence (Oguntibeju 2019). Consequently, vitamin E, known as strong antioxidant, was suggested to modulate the pathophysiology of diabetes and possible beneficial effects were examined in human trials. Indeed, beneficial effects were repeatedly reported for vitamin E as supplement for individuals suffering from type 2 diabetes mellitus. Beneficial effects reported comprise the reduction of biomarkers of oxidative stress (Wu et al. 2007; Vafa et al. 2015), the improvement of fasting blood sugar, hemoglobin A1c (HbA1c), insulin levels and the insulin response (Vafa et al. 2015; Rafraf et al. 2016; El-Aal et al. 2018), an improved lipid profile (El-Aal et al. 2018) and vascular function (Alshiek et al. 2017). Further, vitamin E was repeatedly reported to have beneficial effects in patients with diabetic nephropathy by lowering serum creatinine (Tan et al. 2018), improving glutathione levels and the lipid profile (Aghadavod et al. 2018; Khatami et al. 2016) and having favorable effects with respect to inflammation and oxidative stress (Khatami et al. 2016). However, inflammatory markers seem to be differentially regulated with respect to the applied vitamin E isomer (Wu et al. 2007; Haghighat et al. 2014). Interestingly, gestational diabetes has also been proposed to be associated with meta-inflammation (Pantham et al. 2015). Here, vitamin E was also reported to have beneficial effects with respect to lipid profile, glycemic control and oxidative stress markers in gestational diabetes when applied in combination with omega-3 polyunsaturated fatty acids or magnesium (Taghizadeh et al. 2016; Jamilian et al. 2017; Maktabi et al. 2018). While there is a large body of evidence for benefits of vitamin E in diabetes treatment, the role of vitamin E in the development of diabetes is less clear. Some studies reported that low plasma levels of vitamin E are associated with a higher risk of type 2 diabetes mellitus (Salonen et al. 1995) and *vice versa* (Arnlöv et al. 2009) or that a higher vitamin E intake is associated with a reduced risk of type 2 diabetes mellitus (Montonen et al. 2004), respectively. However, in other studies no association between vitamin E intake and risk of diabetes in a heterogeneous Korean population (Quansah et al. 2017) and in male smokers was found (Kataja-Tuomola et al. 2011). Further, supplementation with vitamin E did not reduce the risk for diabetes in male smokers (Kataja-Tuomola et al. 2008) in women with high risk of CVD (Song et al. 2009) and older adults (Song et al. 2011). To sum up, while vitamin E intake and status as predictor for diabetes development appears questionable, vitamin E supplementation (independent of a distinct form) as therapeutic strategy in diabetes seems valuable.

The possible role of vitamin E for cancer prevention has been studied extensively in humans and the current knowledge was recently reviewed by Yang et al. (Yang et al. 2020) updating their review from 2010 (Ju et al. 2010). For detailed information, the reader is referred to these comprehensive works. In the following, a brief overview of the cancer types and effects of

vitamin E comprised in this recent review is given. Based on the number of human studies, the role of vitamin E is best examined for prostate cancer, colorectal cancer, lung cancer and pancreatic cancer. While predominantly beneficial results, such as inverse associations between intake and serum levels of vitamin E and prostate cancer were reported, supplementation studies did not reveal any association with respect to this cancer type. Further beneficial effects were reported for vitamin E intake and the risk of pancreatic cancer. However, no clear association of vitamin E and the risk of lung cancer, bladder cancer and colorectal cancer can be found based on the results of the indicated human trials. The same can be stated for total cancer risk, since respective studies either lead to the finding that supplementation with vitamin E has no effect on cancer incidence, that dietary vitamin E is a predictor of cancer mortality or that there is an inverse correlation between intake and mortality in women. However, meta-analyses on the association of vitamin E intake or blood levels, respectively, and different cancer types found beneficial effects. Inverse associations of vitamin E intake or blood levels and the risk of lung cancer, pancreatic cancer, bladder cancer, head and neck cancer, esophageal cancer, cervical cancer, breast cancer and renal cancer were found in meta-analyses on cohort and case control studies. Hence, it appears surprising that a meta-analysis on 69 prospective studies revealed no relevant association between dietary vitamin E intake and total cancer incidence. However, the same analysis lead to the result that an increase of 5 µg/ml serum α-TOH is linked to a 9 % decrease in total cancer rate (Aune et al. 2018). In conclusion it is argued that the consumption of a diet rich in vitamin E, mostly from plant-based foods is beneficial with respect to cancer prevention. Supplementation would be beneficial in people with an insufficiency of vitamin E since this condition promotes carcinogenesis and cancer risk. Studies reporting no beneficial effect of vitamin E supplementation are likely biased by a study population which is already sufficient in vitamin E (also representing an important factor for consideration when interpreting studies on vitamin E and other NCD). In conclusion, an adequate dietary intake of vitamin E lowers the cancer risk, but the beneficial role of vitamin E supplementation likely depends on the nutritional status and maybe on the form of vitamin E.

Chronic respiratory diseases are a major group of NCD. Beside chronic obstructive pulmonary disease (COPD), a disease pattern which may also be related to vitamin E (Janciauskiene 2020), asthma is the other main disease pattern within the group of CRD and likely more important, based on the number of cases according to the WHO (251 million suffering from COPD and 339 million suffering from asthma in 2016, respectively). The association of maternal vitamin E intake and infant asthma is well-known. Several studies on this issue reported a benefit of a higher maternal vitamin E intake during pregnancy with respect to different types of wheezing in the offspring (Martindale et al. 2005; Litonjua et al. 2006; Devereux et al. 2006; Stone et al. 2019). Further, vitamin E serum levels in children themselves also seem to be associated with asthma incidence (Kalayci et al. 2000). The picture in adults is less clear. Early studies based on estimation of the dietary intake of vitamin E found modest beneficial effects of a higher intake with respect to the incidence of asthma and measures of lung function (Troisi et al. 1995; Dow et al. 1996). However, the authors of a review on dietary factors and their influence on asthma and COPD in 1999 concluded that the ‘beneficial protective effect of vitamin E intake on symptoms of asthma or of COPD is still small’ (Smit et al. 1999). Later reports on the relevance of vitamin E in asthma treatment led to further conflicting results. Supplementation of vitamin E in combination with corticosteroids offered no beneficial effect (Pearson et al. 2004). In contrast, supplementation of mild atopic asthmatics with vitamin E lead to a reduced allergic inflammation and reduced airway hyper-responsiveness (Hoskins et al. 2012). However, in 2009 it was concluded that dietary vitamin E intake is not generally associated with asthma (Allen et al. 2009). Interestingly, it was later argued that the discrepancies in the outcome of vitamin E trials maybe due to the eaten or applied vitamin E form. Good evidence was provided for α-TOH being beneficial while γ-TOH

likely exerts harmful functions (reviewed in (Cook-Mills and Avila 2014)). This may even serve as explanation for different outcomes of respective studies in different countries, based on the predominant eating habits (Tabak et al. 1999). However, while good evidence is provided, this hypothesis needs confirmation in the future. To sum up, sufficient vitamin E intake during pregnancy likely positively affects lung health of the offspring, while the role of vitamin E in the prevention and treatment of adult asthma is less clear.

Taken together, beneficial effects of vitamin E have been reported with respect to NCD. However, the issue appears highly complex. While good evidence is available for vitamin E in the prevention of different types of cancer and of asthma in infants, the role in prevention of CVD and diabetes is questionable. Notwithstanding this, vitamin E, irrespective of the form appears beneficial as treatment option in diabetes. This may be based on the antioxidant function, which is shared by all TOH and T3 (Müller et al. 2010) and less on other properties like anti-inflammatory functions, which are different among the different vitamin E structures ((Manuscript XI: Wallert et al. 2020) and chapter 'Biological activity'). In support of this, the type of vitamin E is suggested to be relevant for the outcome of vitamin E prevention and treatment with respect to cancer and asthma (*vide supra*) and may also explain the conflicting results obtained from studies on vitamin E and the risk for CVD. Our work on the naturally formed α-TOH long-chain metabolites may thus explain conflicting results obtained from studies on NCD prevention and treatment in the future. Further, the second research object comprised in this work, δ-tocotrienolic acid (garcinoic acid, GA), is an interesting lead structure for the development of treatments as the reported differential effects of different vitamin E structures with respect to NCD show.

3 Long-chain metabolites of vitamin E

Tocopherols and T3 are similarly metabolized (refer to the chapter 'Metabolism'). The functionalization of the structures in the first metabolic steps leads to the formation of TOH or T3 with a hydroxyl or a carboxyl moiety at the terminal carbon of the side chain. Based on the length of the side chain and the position of the functional group, the resulting metabolites are termed 13'-carboxychromanols (13'-COOH) or 13'-hydroxychromanols (13'-OH). Technically, the shorter 11'- and 9'-metabolites are also classified as LCM. However, there is virtually no interest in these metabolites to date and scientific focus is on the 13'-metabolites. Metabolites with shorter side chains are either classified as intermediate-chain (ICM) or short-chain metabolites. However, the term 'long-chain metabolite' (LCM) is used in this thesis exclusively to describe 13'-COOH or 13'-OH. While Sontag and Parker initially described the occurrence of these metabolites around millennium, first functions of the TOH metabolites were described around 2010 (refer to the chapter 'Aim of the work'). Consequently, this thesis contributed in part to the current knowledge (especially the biologic activity) on the LCM, as well as the related GA, outlined in the following sections.

3.1 Analytical approaches

Reliable and sensitive analytical approaches to detect the LCM in various matrices like serum and plasma, tissues and feces as well as cells and cell culture supernatants form the basis for serious research on this class of substances. Accordingly, various techniques have been developed and reported to realize the sensitive analysis of LCM in different sample types (Table 2). The first LCM reported to be determined analytically were the LCM of *RRR*-γ-TOH in 2002 (Sontag and Parker 2002). Their formation by HepG2 liver carcinoma cells (HepG2) from the precursor and the occurrence in microsomes obtained from rat liver was confirmed by the use of a gas chromatography-based mass spectrometric approach (Sontag and Parker 2002). However, the field of LCM research and concomitantly the analytical techniques have considerably evolved during the past almost 20 years. For the sake of clarity, this section will

focus on the techniques to analyze the α-TOH-derived LCM and a brief note on the interesting related compound GA can be found at the end of this section. A more comprehensive view on the analytical approaches for metabolites of vitamin E is provided in Manuscript IV (Manuscript IV: Kluge et al. 2019).

In 2010, Zhao et al. reported on the finding of the α-TOH-derived LCM with carboxyl moiety in the feces of mice and human. To achieve this, they extracted the LCM from the samples by using ethanol and hexane. The detection and identification was subsequently carried out by using a high-performance liquid chromatography with electrochemical detection (HPLC/EC) system and a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) system, respectively (Zhao et al. 2010). These results lead to the conclusion that the TOH-derived LCM undergo the enterohepatic circulation. Later in the same year, Birringer et al. reported the first biological activities of α-TOH-derived LCM and concluded that they may affect colon health. The study focussed on the synthesis of LCM by liver cells. Accordingly, cell culture supernatants of HepG2 cells were analyzed. The analytical strategy comprised the extraction of the supernatant and subsequent analysis by reverse-phase HPLC and quantification using ultraviolet detection (Birringer et al. 2010). While biological activities of the LCM came to light, the relevance for human physiology remained elusive. A big step in this regard was taken by the detection of the LCM in human blood. A respective approach was first reported in 2014 by Wallert et al. (Wallert et al. 2014). Here, serum of a healthy male volunteer with no supplementation of α-TOH was successfully used as sample for α-13'-COOH determination. First, the serum was treated with glucuronidase and sulfatase with the intention to increase the yield of LCM. Subsequently, LCM were extracted using hexane and dichloromethane and analyzed using a quadrupole-time-of-flight liquid chromatography/mass spectrometry with electrospray ionization in positive mode (QTOF LC/MS (ESI(+))) system and a 18 carbon atoms chain (C18) column (Wallert et al. 2014). With this report systemic availability of the LCM was proven. One year later, the detection of α-13'-COOH and α-13'-OH in serum of supplemented volunteers was reported (Ciffolilli et al. 2015). Here, LCM were extracted using hexane and were analyzed using a C18 column in an HPLC-ECD system. Assay specificity was confirmed by gas chromatography-mass spectrometry (GC-MS) (Ciffolilli et al. 2015). However, the determined concentrations were in the nanomolar range, emphasizing the need for detection methods with high sensitivity. Accordingly, analytical approaches have constantly been refined. Torquato et al. (Torquato et al. 2016b) and Giusepponi et al. (Giusepponi et al. 2017) reported on the refinement of strategies to detect α-13'-COOH and α-13'-OH from human serum and plasma by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) after lipid-lipid extraction. Especially the latter report is of interest as the description of a method for simultaneous detection of the LCM and the precursors is provided in detail (Giusepponi et al. 2017). This facilitates the analytical procedures and may provide new insights into the relation of the LCM and the precursors. However, the sensitive analytics suggests that the LCM may have been formerly detected as an entirety comprised of different isomers. Since it is unknown whether LCM isomers exert different functions (or which respective isomer is biologically active) the complexity of LCM research is increased with this work. Notwithstanding this, the opportunity to detect individual isomers even in concentrations below 1 nM in human blood (Giusepponi et al. 2017) is of high relevance for LCM research. In line with this, Pein et al. reported on a method to detect α-13'-COOH with high sensitivity. Here, LCM were extracted using methanol and chloroform and subsequently separated on an ultra-performance liquid chromatography (UPLC) and analyzed by MS/MS. This method was applied to samples from human plasma, murine plasma, peritoneal exudates and cells and the lower limit of quantification for α-13'-COOH was specified as 1 nM (Pein et al. 2018). Taken together, several analytical approaches with high sensitivity have been developed to detect the LCM in different matrices like human and murine blood as well as murine exudates and cells. This development opens up new ways for LCM

research (as discussed in the chapter ‘Future perspectives’) and progress is still made as a recent update of the procedure by Giusepponi et al. (Giusepponi et al. 2017) enabling the simultaneous detection of TOH, their metabolites, selected poly-unsaturated fatty acids (PUFA) and potentially selected inflammatory markers shows (Giusepponi et al. 2019).

Table 2: Overview of analytical approaches successfully used to detect α-tocopherol long-chain metabolites and garcinoic acid in different matrices.

Compound	Matrix	Extraction	Analytical Approach	Reference
α-13’-COOH α-13’-OH	Cell culture supernatant	Liquid-liquid	HPLC-UV	(Birringer et al. 2010)
α-13’-COOH	Human serum		QTOF LC/MS (ESI(+))	(Wallert et al. 2014)
α-13’-OH	Human serum		HPLC-ECD / GC-MS	(Ciffolilli et al. 2015)
α-13’-COOH α-13’-OH	Human serum		LC-MS/MS	(Torquato et al. 2016b)
α-13’-COOH α-13’-OH	Human serum Human plasma		LC-MS/MS	(Giusepponi et al. 2017)
α-13’-COOH	Human plasma		UPLC-MS/MS	(Pein et al. 2018)
α-13’-COOH α-13’-OH	Human serum Human plasma		LC-MS/MS	(Giusepponi et al. 2019)
Garcinoic acid	Murine plasma		GC-MS	(Marinelli et al. 2020)

Detailed information can be found in the chapter ‚Analytical approaches‘. ESI: Electron spray ionization, GC: gas chromatography, HPLC: High performance liquid chromatography, LC: liquid chromatography, MS: mass spectrometry, QTOF: quadrupole-time-of-flight, UV: ultraviolet detector

Besides, techniques to analyze the levels of the so far less noticed δ-tocotrienol derivative GA have recently been reported for different sample matrices. GA levels were successfully determined in mouse brain and plasma samples (Marinelli et al. 2020). To achieve this, samples were treated with glucuronidase and sulfatase and subsequently purified. After methylation and silylation, samples were analyzed by GC-MS (Marinelli et al. 2020). Furthermore, GA was repeatedly analytically determined after isolation of the compound (Manuscript X: Wallert et al. 2019) or when used as internal standard for the determination of LCM (Pein et al. 2018). Hence, analytical approaches to support GA research are already at hand.

3.2 Isolation and (semi-)synthesis

Besides specific and sensitive analytics to identify and determine characteristics like purity or concentration of the LCM, a second crucial factor for LCM research is the availability of sufficient amounts of the substances to carry out experimental *in vivo* and *in vitro* studies. Accordingly, different strategies to obtain LCM have been developed (extensively reviewed in manuscript V (Manuscript V: Kluge et al. 2016)). The first approach is the isolation of the LCM from cells capable of metabolizing the respective vitamin E precursors (Jiang et al. 2008; Jiang et al. 2011). While this approach appears relatively simple and straightforward, it holds some drawbacks. The metabolization by the cells naturally leads to a wide variety of molecules, including LCM, ICM and SCM with varying chain length. These can further occur conjugated or unconjugated, finally leading to a mixture of metabolites that makes subsequent purification and concentration steps necessary to obtain individual metabolites in sufficient amount and purity. For this reason, an alternative strategy was developed to generate individual metabolites with high purity and good yield. This strategy is based on GA (δ-tocotrienolic acid) as starting point for the semi-synthesis of the LCM. Garcinoic acid can be obtained by isolation from different natural plant sources (Manuscript V: Kluge et al. 2016) whereby seeds of *Garcinia kola* as source in combination with our isolation method described in Manuscript X (Manuscript X: Wallert et al. 2019) lead to the highest reported yield (2.5 g GA from 100 g seeds) of highly pure (>99 %) GA. In principle, also a complete chemical synthesis route for

GA is available (Maloney and Hecht 2005) and could be used as starting point for LCM synthesis and studies on GA itself. However, the semi-synthesis of α-LCM and δ-LCM from isolated GA (Mazzini et al. 2009; Birringer et al. 2010) represents a feasible approach to obtain LCM in good yield and purity. The LCM α-13'-COOH is obtained in two steps from GA. First, hydrogenation of GA in a platinum-catalyzed reaction saturates the side chain and leads to δ-13'-COOH. Second, the methylation of the chroman ring by tin (II) chloride ($SnCl_2$)-catalyzed permethylation of δ-13'-COOH yields α-13'-COOH. The respective hydroxyl metabolites α-13'OH and δ-13'-OH can be obtained by a reduction using lithium aluminium hydride ($LiAlH_4$) (Manuscript V: Kluge et al. 2016). Based on a study on the anti-inflammatory potential, LCM seem to be equally efficient irrespective of the source, *i.e.* the isolation from cell culture supernatants or the chemical (semi-)synthesis (Jang et al. 2016). Thus, using semi-synthetically produced LCM in basic research on LCM is currently 'state of the art'. However, the development in the analytical strategies (as outlined in the preceding chapter) may shed new light on the role of individual isomers of the LCM and could make new approaches for the synthesis of LCM necessary.

3.3 Biological activity

First biological effects of the LCM of TOH have been reported around 2010 (Jiang et al. 2008; Birringer et al. 2010; Jiang et al. 2011). Within the last ten years, considerable progress was made with respect to (potential) biological effects of the LCM. The current state of the scientific knowledge on the biological activity of the LCM can be roughly categorized as follows: effects on lipid metabolism, anti-inflammatory effects, anti-carcinogenic effects and other effects comprising potential interactions with pharmaceuticals and regulatory feedback mechanisms. We recently extensively reviewed the anti-inflammatory and anti-carcinogenic properties of the LCM (Manuscript XI: Wallert et al. 2020). Further, the progress in terms of the effects of LCM on lipid metabolism was largely made by our group in collaboration with other groups (Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021). Accordingly, this chapter is intended to provide a brief overview of the known effects of LCM. For detailed information the reader is referred to the respective manuscripts included in this thesis (Manuscript IV: Kluge et al. 2019; Manuscript XI: Wallert et al. 2020; Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021) and to the respective reviewing Chapters 8 ('Physiologic and pathophysiologic implications of the works in the context of NCD and meta-inflammation') to 11 ('Contribution to the development of the field') of this thesis.

3.3.1 Effects on lipid metabolism

In 2014, the first effects of LCM on the lipid metabolism of cells were reported (Wallert et al. 2014). Here, α-13'-COOH and α-13'-OH were shown to induce CD36 gene expression and protein levels in THP-1 macrophages and peripheral blood mononuclear cells (PBMC), an effect strongly contrasting the action of the precursor α-TOH. Since CD36 is regarded as a major receptor responsible for oxidized low-density lipoprotein (oxLDL) uptake by macrophages, the role of the LCM in the modulation of this process was examined. Surprisingly, pre-incubation of THP-1 with the LCM prior oxLDL loading attenuated the uptake by about one-fifth. In line with this, neutral lipid accumulation was reduced in LCM-treated cells. An explanatory approach for this phenomenon was provided by the reduced phagocytosis due to LCM treatment (Wallert et al. 2014). Based on these results it was hypothesized that the LCM might influence the progress of foam cell formation, a detrimental hallmark of atherosclerosis. Further, foam cell formation is linked to lipotoxicity, a process induced by saturated fatty acids and modified lipoproteins ultimately leading to an induction of inflammatory processes and cell death (Ertunc and Hotamisligil 2016). Based on the finding that α-13'-COOH reliably induced perilipin 2 (PLIN2) gene and protein expression in macrophages, we hypothesized that the LCM may protect from lipotoxicity (Manuscript VII: Schmölz et al. 2018). Indeed, PLIN2 induction by α-13'-COOH could be shown to contribute

to the protection from stearic acid-induced lipotoxicity. This effect is probably mediated by enhancing the lipid storage capacity of the macrophages (*i.e.* the number and size of lipid droplets). Another facet of foam cell formation was addressed in our recently accepted work on the effect of α-13'-COOH on LPL and related proteins (Manuscript IX: Kluge et al. 2021). The LCM α-13'-COOH potently and reliably induces angiopoietin-like 4 (ANGPTL4) gene expression in THP-1 macrophages. The role of ANGPTL4 as endogenous negative regulator of LPL lead us to examine whether α-13'-COOH can suppress the lipid accumulation induced by the uptake of lipoproteins. In this study, very-low density lipoprotein (VLDL) was used as lipoprotein and it could indeed be shown that α-13'-COOH protects against excessive lipid accumulation induced by VLDL oversupply (Manuscript IX: Kluge et al. 2021). Taken together, there is good evidence for beneficial effects of LCM on macrophage lipid metabolism at several levels. This has predominantly implications for effects of LCM on atherosclerosis pathophysiology (Lee and Choi 2020) but also for meta-inflammation (Ertunc and Hotamisligil 2016) and due to the centrality of macrophages for various NCD (Schultze et al. 2015; Russo and Lumeng 2018; Ponzoni et al. 2018; Li et al. 2018).

3.3.2 Anti-inflammatory effects

As outlined in the chapter 'The great framework: non-communicable diseases as consequence of meta-inflammation', inflammation is underlying virtually all NCD. Thus, interest in compounds beneficially affecting this process is high. Accordingly, vitamin E and structurally related compounds were repeatedly examined regarding their anti-inflammatory potential (Manuscript XI: Wallert et al. 2020). A brief overview of known anti-inflammatory effects of the LCM, with focus on the α- and δ-forms as these are central to this thesis, is given in the following. The LCM are best investigated with respect to their effects on inducible nitric oxide synthase (NOS2) and prostaglandin-endoperoxide synthase 2 (PTGS2/COX-2). Accordingly, the TOH-derived LCM α-13'-OH, δ-13'-OH, α-13'-COOH and δ-13'-COOH have all been shown to suppress the expression of NOS2 gene and protein and subsequently the production of nitric oxide (synonym: NO) by RAW264.7 mouse macrophages in response to lipopolysaccharide (LPS) stimulation (Ciffolilli et al. 2015; Wallert et al. 2015; Schmölz et al. 2017b). It is of note that the carboxyl LCM are more effective than their hydroxyl counterparts in terms of NOS2 and NO suppression (indicated by their higher suppressive effect in half of the concentration). With respect to PTGS2, suppression of gene and protein expression and of the enzymatic activity have been shown. In detail, α-13'-OH and α-13'-COOH were reported to suppress the gene expression (Ciffolilli et al. 2015; Wallert et al. 2015), and α-13'-COOH further to suppress the protein expression (Wallert et al. 2015) in LPS-stimulated RAW264.7. In line with this, the related δ-13'-COOH was shown to inhibit PTGS2 activity in IL1β-stimulated A549 lung adenocarcinoma cells (Jiang et al. 2008) and the activity of the isolated enzyme (Jiang et al. 2008; Jang et al. 2016). The production of the enzymatic product prostaglandin E_2 (PGE_2) by RAW264.7 in response to LPS is inhibited by α-13'-COOH (Ciffolilli et al. 2015) and by α-13'-COOH (Wallert et al. 2015). However, the latter could not be reproduced in human monocytes stimulated with LPS (Pein et al. 2018), indicating that the relevance for human physiology of PTGS inhibition by LCM in murine cells needs further investigation. The same work revealed that α-13'-COOH inhibits 5-lipoxygenase (5-LOX) and suppresses 5-LOX product formation in human polymorphonuclear leukocytes (PMNL) and monocytes and in activated human blood (Pein et al. 2018). Strikingly, these findings could be transferred to *in vivo* models and showed beneficial effects in murine peritonitis and airway hyperreactivity (Pein et al. 2018). Another, less well investigated aspect of anti-inflammatory actions of LCM is the effect on cytokine expression and release (refer also to chapter 'Long-chain metabolites versus LPS Elucidation of mechanistic aspects and a reliable target'). Hence, it was merely reported that α-13'-OH and α-13'-COOH suppresses the gene expression of *interleukin 1 beta (Il1b), tumor necrosis factor (Tnf)* and *interleukin 6 (Il6)* in LPS-stimulated RAW264.7 (Ciffolilli et al. 2015; Wallert et al. 2015). For the first time, data on the effect of a LCM (α-13'-COOH)

on the cytokine release are provided in Manuscript II of this thesis (Manuscript II: Schubert et al. 2020). The results of this work suggest that interleukins 1α and 1β and the chemokine (C-C motif) ligand 2 (CCL2) represent relevant protein targets of α-13'-COOH. The reader is referred to the respective manuscript as well to the review of the manuscript within this thesis (refer to the chapter 'Manuscripts and methods focusing on inflammatory processes').

The anti-inflammatory effects of GA have also been examined regarding the above-mentioned experimental targets. The work with the most comprehensive results in this respect is Manuscript X of this thesis with data on the inhibition of Nos2 gene and protein expression, NO production, PGE_2 release and gene expression of *Il1b, Il6* and *Tnf* in response to LPS by RAW264.7 by GA (Manuscript X: Wallert et al. 2019). Further, GA was shown to inhibit the activity of isolated PTGS2 (Jang et al. 2016) and the release of PGE_2 from LPS-stimulated human monocytes (Pein et al. 2018). However, activity of isolated PTGS2 was not affected by GA in this setup (Pein et al. 2018). Moreover, GA was reported to inhibit microsomal prostaglandin E synthase 1 (mPGES-1) activity (Alsabil et al. 2016) and 5-LOX activity and product formation (Pein et al. 2018; Dinh et al. 2020). Taken together, data on the anti-inflammatory action of GA is promising but further research is needed to resolve existing contradictions.

3.3.3 Anti-carcinogenic effects

Anti-carcinogenic properties of the LCM are largely ascribed to their effects on the viability of cancer cell lines and on their effects on apoptosis and necrosis-related proteins like poly [ADP-ribose] polymerase 1 (PARP-1) and caspases (for detailed information, the reader is referred to Manuscript XI (Manuscript XI: Wallert et al. 2020)). While the balance of cell division and cell death is crucial for organism homeostasis, cancer cells usually show a reduced rate of apoptosis which is related to the occurrence of malignancies, metastases and resistance to anticancer drugs (Wong 2011). Thus, induction of apoptosis and reduction of viability of cancer cells are favorable outcomes. Apparently, the here (based on their relevance for this thesis) considered α- and δ-hydroxyl metabolites hardly reduce the viability of cancer cell lines while the α- and δ-carboxyl metabolites effectively reduce the viability of several cell lines. Accordingly, α-13'-OH and δ-13'-OH were reported to have no effect on the viability of THP-1 macrophages up to 100 µM (Wallert et al. 2014) and on HepG2 cell viability up to 50 µM (Birringer et al. 2010). Merely Mazzini et al. reported a reduced viability of C6 glioma cells by 10 µM of α-13'-OH (Mazzini et al. 2009). In contrast, α-13'-COOH substantially reduced the viability of THP1 macrophages at a concentration of 7.4 µM (Wallert et al. 2014) and of HepG2 cells at a concentration of 13.5 µM (Birringer et al. 2010). The counterpart δ-13'-COOH was reported to markedly reduce the viability of HCT-116 human colon cancer cells (HCT-116) and HT-29 human colon adenocarcinoma (HT-29) cells (Jang et al. 2016), HepG2 cells (Birringer et al. 2010) and C6 cells (Mazzini et al. 2009) at or below 10 µM and of THP-1 macrophages at 11.1 µM (Schmölz et al. 2017b). A comprehensive work regarding the induction of cleavage of PARP-1 and caspases was published by Birringer et al. in 2010 (Birringer et al. 2010). At a concentration of 20 µM α-13'-OH did not induce the cleavage of PARP-1, caspase-9, caspase-7 and caspase-3 while α-13'-COOH induced the cleavage of these factors in HepG2 cells. Both δ-13'-OH and δ-13'-COOH induced the cleavage of these factors with the exception of no cleavage of caspase-3 with δ-13'-OH treatment (Birringer et al. 2010). Induction of PARP-1 and caspase-9 cleavage have later been also reported to be induced by δ-13'-COOH and GA in HCT-116 cells at a concentration of 20 µM (Jang et al. 2016). Furthermore, viability of HCT-116 cells and HT-29 cells is reduced at a concentration of 16 µM and 17 µM, respectively (Jang et al. 2016). Already 10 µM of GA lead to a substantial reduction of the viability of glioma C6 cells (Mazzini et al. 2009). To sum up, the hydroxyl LCM appear as being less toxic to cancer cells than their carboxyl counterparts and the δ-metabolites seem to be generally more toxic than the α-LCM. However, the relevance of these data for cancer treatment and

prevention must be demonstrated in future studies. Both preventive actions and targeted application of the LCM are conceivable. One promising *in vivo* study was reported to date in this respect. Here, integration of GA in the diet of BALB/c mice reduced azoxymethane (AOM) and dextran sodium sulphate (DSS)-induced colon tumor growth (Jang et al. 2016).

3.3.4 Effects on xenobiotic metabolism and a potential positive feedback loop

This section briefly comprises some less investigated biological activities and characteristics of the LCM and GA. Recently, GA was reported to be a selective agonist of pregnane X receptor (PXR) (Bartolini et al. 2020). PXR is regarded as the master xenobiotic receptor regulating the expression of drug-metabolizing enzymes and drug transporters in order to detoxify and eliminate xenobiotics. However, despite this well-established role of PXR, this receptor is further implicated in the inflammatory response, cell proliferation and cell migration (Oladimeji and Chen 2018). Interestingly, α-13'-COOH has previously been shown to induce the activity of PXR and the expression of the xenobiotics transporter P-glycoprotein (P-gp or MDR1) (Podszun et al. 2017). While Podszun et al. concluded that induction of PXR activity should be avoided since it limits the bioavailability of concurrently taken drugs, Bartolini et al. argue that PXR activation by GA is a favorable action with respect to diseases like Alzheimer's disease and chronic inflammatory diseases such as atherosclerosis, inflammatory bowel disease, non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (Podszun et al. 2017; Bartolini et al. 2020). Another interesting aspect of PXR activation is the induction of CYP3A4 (Bartolini et al. 2020). In this context the report on the induction of CYP4F2 by α-13'-OH in HepG2 cells is also of interest (Torquato et al. 2016a). Both CYP are suggested to catalyze the conversion of the vitamin E precursors to their respective LCM (refer to the chapter 'Metabolism'). Consequently, the LCM might induce a positive feedback loop via PXR leading to an enhanced formation of LCM. However, the physiological relevance and pharmacological relevance of these effects needs confirmation in the future.

3.3.5 Structure-specific effects and potency of individual LCM

A detailed investigation of the structure-related effects of the LCM and their precursors as well as their substructures strongly suggests that the entire LCM molecule is necessary to exert specific and potent functions in macrophages (Schmölz et al. 2017b). Here, α- and δ-LCM (hydroxyl and carboxyl metabolites) and α-CEHC and pristanic acid, resembling the chromanol ring and the side chain of the LCM, respectively, were compared to α-TOH and δ-TOH. Readouts were CD36 expression in THP-1 macrophages and NOS2 expression and NO production in LPS-stimulated RAW264.7 macrophages. While all LCM induced CD36 gene and protein expression, the substructures α-CEHC and pristanic acid showed no effect in this regard. However, LPS-induced NOS2 expression and NO-release were suppressed by δ-TOH, α- and δ-13'-OH, α- and δ-13'-COOH and pristanic acid, while α-TOH and α-CEHC showed no effect. Notwithstanding this, α-13'-COOH showed the most potent effects and was used in the lowest concentration (5 μM) of all examined structures (TOH: 20 μM, alcohol metabolites: 10 μM, pristanic acid and α-CEHC: 5 μM and 10 μM). With respect to CD36 protein induction, α-13'-COOH and δ-13'-COOH were comparably effective, but α-13'-COOH appeared substantially more effective in suppressing NOS2 protein expression in response to LPS. However, the overall conclusion of this work is that the effects of the LCM depend on the presence of the chromanol ring, regardless of the substitution pattern, and on the modification of the side chain (Schmölz et al. 2017b). Nevertheless, some effects like the inhibition of NOS2 expression and NO release may resemble that of fatty acids (refer also to chapter 'Integrating metabolism and inflammation') due to the carboxyl moiety of the LCM. Apparently, the chromanol ring in the structure can augment the effects.

4 Aim of the work

In 1922, Katharine Scott Bishop and Herbert McLean Evans reported on the identification of 'X', an essential factor for the reproduction of rats, in *Science* (Evans and Bishop 1922). Today, this report is recognized as the birth of the scientific field of vitamin E research since the enigmatic substance 'X' was later termed 'vitamin E' as suggested by Barnett Sure (Sure 1924). The following almost 100 years of vitamin E research are characterized by different milestones. In 1936, 'vitamin E' was identified as an alcohol and the name 'α-tocopherol' was proposed for this structure (Evans et al. 1936). In the following years, efforts were made to identify the structure of α-TOH, leading to a 'unique event in the organic-biochemical history' at that time: A complex molecule was synthesized before its exact structure was known (Sebrell and Harris 1954; Karrer et al. 1938a; Karrer et al. 1938b). After having identified the exact structure of α-TOH, as well as the existence of the structurally related β- and γ-TOH, scientific work focussed on the elucidation of the biological activities of these molecules. First attempts to show vitamin E's significance in human health were carried out by dietary deprivation over a period of about six years. Albeit a true deficiency in the lipid-soluble vitamin E is hard to establish, it was noted that low TOH plasma concentrations were associated with an elevated erythrocyte hemolysis (Horwitt et al. 1963). However, clinical and physiological parameters remained normal in these studies, questioning the classification of tocopherol as 'vitamin'. A milestone in clarifying the question of the vitamin status of tocopherol was reached by the identification of the α-TOH-specific α-TTP (Catignani and Bieri 1977). Mutations in the gene encoding α-TTP cause very low plasma concentrations of α-TOH in humans and lead to a severe neurodegenerative disease termed 'ataxia with isolated vitamin E deficiency' (Ben Hamida et al. 1993). The fact that α-TOH is the only compound known to serve as cure for this disease confirms its classification as vitamin in humans (Azzi 2018). Through the years of research, vitamin E has also been proposed to moreover play roles in disease patterns like infections, anemia, CVD, cognitive impairments, cancer and other age-related diseases (Traber 2014; Galli et al. 2017). These functions can hardly be explained solely by the prominent antioxidant properties of tocopherols and thus focus shifted to the non-antioxidant properties of vitamin E since the 1980s (Mahoney and Azzi 1988; Zingg and Azzi 2004). In this context, the identification of bioactive metabolites formed during the metabolism of tocopherols opened up a new promising research field (Parker et al. 2000; Birringer et al. 2001; Sontag and Parker 2002). However, first biological effects of the LCM were reported as late as around 2010 (Jiang et al. 2008; Birringer et al. 2010; Jiang et al. 2011). Interestingly, the work of Qing Jiang focussed on the γ- and δ-forms of vitamin E, based on the argument that these forms are predominantly metabolized (Jiang 2014). Conversely, α-TOH is preferentially retained by the α-tocopherol transport protein in humans (Schmölz et al. 2016) and formation of LCM from this form of vitamin E may thus be of higher physiological relevance.

Strikingly, merely the α-LCM have been detected in human plasma to date, indicating a physiological relevance of these forms (Wallert et al. 2014; Ciffolilli et al. 2015; Pein et al. 2018; Giusepponi et al. 2019). However, at the beginning of the work on this thesis, the knowledge on the bioactivity of the LCM of vitamin E was rather limited, especially when focussing on the most promising α-LCM α-13'-COOH. It was merely reported that:

- α-13'-COOH induces CD36 expression but reduces oxLDL uptake and phagocytosis in macrophages (Wallert et al. 2014)
- α-13'-COOH affects the inflammatory response of macrophages to LPS with respect to the release of prostaglandin and NO as well as cytokine gene expression (Wallert et al. 2015)

- α-13'-COOH exerts pro-apoptotic effects in liver cells involving ROS formation, mitochondrial stress, caspases and PARP-1 (Birringer et al. 2010)

Although knowledge on the functions of α-13'-COOH was very limited at that time, these reports opened up three promising and specific research areas for vitamin E-derived LCM:

- Lipid metabolism of macrophages
- Inflammatory response of macrophages
- Cellular stress response in liver cells

Albeit the available knowledge on biological functions was sparse, the identification of α-13'-COOH in human plasma in 2014 (Wallert et al. 2014) was very promising with respect to the hypothesis that the LCM represent the biologically active metabolites mediating functions of vitamin E. Given the inconsistencies in the results of vitamin E studies (refer to the chapter 'Vitamin E and non-communicable diseases'), first biological activities of vitamin E-derived LCM provided an explanatory approach for these findings. This reasoned the interest in this class of molecules and justified the continuation of the efforts to clarify their biological functions.

The overall aim of this work was to broaden and evolve the knowledge from the three scientific studies and thus the research areas outlined above. The efforts made to achieve this aim can be subdivided into the following three aspects:

- Dissemination and delineation of the potential of vitamin E-derived LCM
- Metabolism and regulatory functions of the vitamin E-derived LCM
- Specific effects of vitamin E-derived LCM

The contributions of the manuscripts included in this thesis to evolve the knowledge from the three studies outlined above as starting point in the specific research fields and the contribution to further expand the basis for future research on this new and promising area of vitamin E research are presented in the chapter 'Contributions of the manuscripts to achieve the aims of the work'.

5 Manuscript overview

This thesis comprises eleven manuscripts including six original research articles and five review articles or book chapters, respectively. Hereof, six publications are contributions with the author of this thesis as first author and five contributions as co-author. Furthermore, the author of this thesis co-authored two additional works (Manuscripts AI and AII; detailed information can be found in the Appendix) that are not directly related to the central topic of research on vitamin E-derived LCM but are of relevance in the broader context of research on lipid metabolism and inflammation, as will be discussed in the following chapters. All manuscripts are related to vitamin E and related structures in the context of lipid metabolism and/or inflammation. An overview on the studies and their relation can be found in the chapter ‘Relation of the works included in this thesis’. General characteristics and the essence of the individual works are provided in the following.

5.1 Manuscript I

The peroxisome proliferator – activated receptor (PPAR)-γ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662) triggers perilipin 2 expression via PPARδ and induces lipogenesis and triglyceride accumulation in human THP-1 macrophages

Martin Schubert*, Stefanie Becher*,

Maria Wallert, Marten B. Maeß, Masoumeh Abhari,

Knut Rennert, Alexander S. Mosig, Silke Große, Regine Heller, Michael Grün,

and Stefan Lorkowski

*** authors contributed equally**

Published in:

Molecular Pharmacology

(2020):97;212-225

DOI: 10.1124/mol.119.117887.

Date of acceptance: December 6th 2019

Contribution to the manuscript:

- Research design
- Conduction of experiments
- Data analysis
- Statistical analyses
- Creation of plots and graphics
- Writing of the manuscript

Personal contribution to the manuscript: 40 %

Essence of Manuscript I and contribution to this thesis:

PPAR are key regulators of lipid and glucose metabolism and inflammation, consequently representing valuable targets for the treatment and prevention of various diseases. Thus, reliable tools to investigate PPAR functions are of high relevance to the scientific community. This article provides data on the profound off-target effect of the well-known and widely used PPARγ antagonist GW9662 involving PPARδ and consequently affecting the lipid metabolism of macrophages. GW9662 is *inter alia* shown to significantly induce lipogenesis, triglyceride accumulation and lipid droplet formation accompanied by the induced expression of respective marker genes in human macrophages. These marker genes are described as PPARγ target genes and GW9662 thus acts in a PPARγ agonist fashion. In consequence, Manuscript I provides profound data underlining the limited usability of GW9662 especially in macrophages. This has implications for PPAR research in general and thus for the work on inflammatory and metabolic processes as well as for the research on LCM and related compounds. Macrophages represent a central cellular model in LCM research and PPAR act like the LCM at the crossroads of inflammation and lipid metabolism. Prospectively, PPAR will play a role in LCM research and the knowledge on the dominant role of PPARδ, on the effects of GW9662 and on a usable alternative derived from this work will help in future studies on LCM and PPAR. The entire manuscript can be found on page 35 of this thesis.

5.2 Manuscript II

The vitamin E long-chain metabolite α-T-13'-COOH is a reliable suppressor of CCL2 / MCP-1 and modulates regulatory mechanisms of MAPK and NFκB signaling and the inflammatory response of macrophages

Martin Schubert,

Stefan Kluge, Marc Birringer, Stefan Lorkowski

In preparation for publication in:

Molecular Nutrition and Food Research

Contribution to the manuscript:

- Research design
- Conduction of experiments
- Data analysis
- Statistical analyses
- Creation of plots and graphics
- Writing of the manuscript

Personal contribution to the manuscript: 80 %

Essence of Manuscript II and contribution to this thesis:

The α-tocopherol-derived LCM α-13'-COOH was previously shown to suppress the inflammatory response of murine macrophages to LPS. However, the mechanism underlying the observed suppression of cytokine gene expression remained elusive and data on cytokine secretion was missing. This work provides first data on relevant mechanistic aspects related to the previous findings and on the effect of α-13'-COOH on cytokine secretion in response to LPS. Comparison of a short (1 h) and a long (24 h) pre-treatment phase with α-13'-COOH revealed that an adaptive response within the 24 h is needed for the anti-inflammatory effects except for the suppression of CCL2 gene expression and protein secretion. The adaptive response of murine macrophages to α-13'-COOH is characterized by the fast activation of ERK and p38 MAPK and the subsequent induction of the gene expression of negative feedback regulators of MAPK kinase and NFκB including the 'master regulators' *Dusp1* and *Tnfaip3.* The reliable and potent suppression of CCL2 gene expression and protein secretion in both long-term and short-term pre-treatment with α-13'-COOH before LPS stimulation highlights this cytokine as a reliable target of α-13'-COOH. Taken together, good evidence for an adaptive response involving a feedback loop to suppress MAPK and NFκB activity leading to a diminished response to LPS is provided. Further, CCL2, having a broad clinical relevance, was identified as a reliable and highly interesting target of α-13'-COOH in inflammatory processes. Consequently, this work provides a solid base for future investigations on and the understanding of the effects of α-13'-COOH in inflammatory and immunological disorders and thus NCD. The entire manuscript can be found on page 49 of this thesis.

5.3 Manuscript III

Long-chain metabolites of vitamin E: metabolic activation as a general concept for lipid-soluble vitamins?

Martin Schubert*, Stefan Kluge*,

Lisa Schmölz, Maria Wallert, Francesco Galli, Marc Birringer, Stefan Lorkowski

*authors contributed equally

Published in:

Antioxidants

(2018):7(1);10

DOI: 10.3390/antiox7010010

Date of acceptance: January 11th 2018

Contribution to the manuscript:

- Devisal of the concept and hypotheses
- Literature research and data extraction
- Conception of the manuscript
- Writing of the manuscript

Personal contribution to the manuscript: 45%

Essence of Manuscript III and contribution to this thesis:

The metabolic conversion of the vitamins A and D to their biologically active metabolites is well-known, well-described and accepted. As certain biological functions of the vitamin E-derived LCM are known and their occurrence in human serum indicates a physiological relevance, the hypothesis of the metabolic activation as a general principle for lipid-soluble vitamins was formulated. This hypothesis was substantiated by an extensive review on the metabolism of vitamin E and the effects of the LCM on inflammatory processes, in the context of cancerogenesis and chemoprevention and on cellular lipid homeostasis and the comparison of these effects with the known properties of vitamin A, D, K and their respective metabolites. Further, the known interactions of metabolites of lipid-soluble vitamins with specific and less specific receptors was reviewed and provides a good basis for the development of strategies to identify a (putative) receptor for LCM in the future. Taken together, the available knowledge on the vitamin E LCM was summarized and an interesting direction for future research in the field of LCM as well as lipid-soluble vitamins in general was suggested. The entire manuscript can be found on page 99 of this thesis.

5.4 Manuscript IV

Bioactivity of vitamin E long-chain metabolites

Stefan Kluge*, Martin Schubert*,

Lisa Schmölz, Maria Wallert, Marc Birringer, Stefan Lorkowski

***authors contributed equally**

Published in:

Vitamin E in Human Health (Springer Nature)

(2019):96;61-79

DOI: 10.1007/978-3-030-05315-4_6

Date of acceptance: March 11th 2019

Contribution to the manuscript:

- Devisal of the concept
- Literature research and data extraction
- Conception of the manuscript
- Writing of the manuscript

Personal contribution to the manuscript: 45%

Essence of Manuscript IV and contribution to this thesis:

The available knowledge on the vitamin E-derived LCM was summarized in this work as part of the book 'Vitamin E in Human Health' which is intended to provide an up-to-date science-based view on the essential nutrient vitamin E. Focus of this work was on the formation and distribution of the LCM by hepatic catabolism, the development and state of the art of analytical approaches for LCM and the known regulatory actions of LCM on biological processes. This comprehensive review on the available analytical strategies and known biological functions of the LCM provides valuable starting points for the development of new hypotheses and useful experimental strategies in the field of LCM research. The entire manuscript can be found on page 119 of this thesis.

5.5 Manuscript V

Garcinoic acid: a promising bioactive natural product for better understanding the physiological functions of tocopherol metabolites

Stefan Kluge*, Martin Schubert*,

Lisa Schmölz, Marc Birringer, Maria Wallert, Stefan Lorkowski

***authors contributed equally**

Published in:

Studies in Natural Products Chemistry

(2016):51;435-481

DOI: 10.1016/B978-0-444-63932-5.00009-7

Date of acceptance: February 24th 2016

Contribution to the manuscript:

- Devisal of the concept
- Literature research and data extraction
- Conception of the manuscript
- Writing of the manuscript

Personal contribution to the manuscript: 45%

Essence of Manuscript V and contribution to this thesis:

The African plant *Garcinia kola* is of high relevance in African traditional ethnomedicine. Garcinoic acid represents a key anti-inflammatory component of the seeds of this plant. With this work, the knowledge on natural sources of GA, approaches for extraction of GA from plant material and the biological effects was summarized. As GA structurally represents δ-tocotrienolic acid, this structure plays a key role in the research on LCM. This role is emphasized in this work by reviewing the isolation and synthesis of GA and the subsequent strategies to semi-synthesize the LCM α-13'-COOH, δ-13'-COOH, α-13'-OH and δ-13'-OH from GA. Semi-synthesizing the LCM from GA is currently the most feasible approach to generate sufficient amounts of LCM for *in vitro* and *in vivo* experiments and thus a crucial step in LCM research. Further, the biological activity of vitamin E, GA and the LCM was reviewed in this work and the potential of GA as anti-inflammatory compound was illustrated. This served as basis for later experimental studies on the anti-inflammatory potential of GA. The entire manuscript can be found on page 138 of this thesis.

5.6 Manuscript VI

The hepatic fate of vitamin E

Lisa Schmölz*, Martin Schubert*, Stefan Kluge*,

Marc Birringer, Maria Wallert, Stefan Lorkowski

***authors contributed equally**

Published in:

Vitamin E in Health and Disease

(2018):1;1-30

DOI: 10.5772/intechopen.79445

Date of acceptance: November 5th 2018

Contribution to the manuscript:

- Devisal of the concept
- Literature research and data extraction
- Conception of the manuscript
- Writing of the manuscript

Personal contribution to the manuscript: 30%

Essence of Manuscript VI and contribution to this thesis:

The liver represents the central organ in the metabolism of vitamin E. Accordingly, understanding the mechanisms of the metabolism in this organ is crucial in vitamin E and LCM research. This work thus summarized the knowledge on the physiological hepatic handling, comprising the uptake, trafficking, storage, metabolism and release of vitamin E. Further, factors influencing the hepatic handling like vitamin E itself, modulators of CYP activity, aging, gender, genetics and pathophysiological factors are reviewed. Taken together, this work provides a comprehensive view on the hepatic fate of vitamin E, which is of high relevance for all scientist in the field of vitamin E research and especially in the field of LCM research as the liver represents the main organ of LCM formation. The entire manuscript can be found on page 186 of this thesis.

5.7 Manuscript VII

Long-chain metabolites of vitamin E: interference with lipotoxicity via lipid droplet associated protein PLIN2

Lisa Schmölz,

Martin Schubert, Jasmin Kirschner, Stefan Kluge, Francesco Galli,

Marc Birringer, Maria Wallert, Stefan Lorkowski

Published in:

BBA Molecular and Cell Biology of Lipids

(2018):1863(8);919–927

DOI: 10.1016/j.bbalip.2018.05.002

Date of acceptance: May 3rd 2018

Contribution to the manuscript:

- Contribution to the writing of the manuscript (critical evaluation)
- Supervision of experimental work and data analyses of Jasmin Kirschner

Personal contribution to the manuscript: 10%

Essence of Manuscript VII and contribution to this thesis:

Foam cell formation from macrophages by taking up high amounts of lipids is a key process in the pathology of atherosclerosis, the disease underlying heart infarction and stroke. Once the lipid loading capacity of cells is exceeded, lipotoxicity, a process ultimately leading to cell death is induced. With this work, the potential of the LCM α-13'-COOH to prevent stearic acid-induced lipotoxicity in human macrophages was highlighted. The underlying mechanism can at least partly be ascribed to the enhanced lipid storage capacity indicated by the induction of PLIN2 by α-13'-COOH. Interestingly, the precursor α-TOH caused the opposing effect. This observation strengthens the hypothesis of the metabolic activation of vitamin E to the biologically beneficial LCM. With this work a new facet on beneficial effects of LCM in the context of lipid metabolism was provided. The entire manuscript can be found on page 215 of this thesis.

5.8 Manuscript VIII

Simple and rapid real-time monitoring of LPL activity *in vitro*

Stefan Kluge,

Lisa Boermel, Martin Schubert, Stefan Lorkowski

Published in:

MethodsX

(2020):7;100865

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Date of acceptance: March 10th 2020

Contribution to the manuscript:

- Contribution to the writing of the manuscript (critical evaluation)
- Guidance on statistical analyses of the data

Personal contribution to the manuscript: 10%

Essence of Manuscript VIII and contribution to this thesis:

Lipoprotein lipase (LPL) represents a key enzyme in the regulation of (cellular) lipid homeostasis and is well-known for its function to release free fatty acids from triglyceride-rich lipoproteins like VLDL and chylomicrons. Consequently, LPL contributes to the control of plasma triglyceride levels which are considered as risk factor for cardiovascular diseases. Thus, simple and reliable methods to examine LPL activity are of high relevance in various research fields. With this work, a method for the real-time monitoring of LPL activity in cell cultures is provided. The advantages of this approach over other currently existing methods can be summarized as follows: Most commercial kits are not applicable to cells as they are optimized for plasma samples. Commercial kits for *in vitro* application require lysis of the cells and thus do not allow – as opposed to the method presented in this work – subsequent analyses of the cells. The approach presented in this work further allows the simultaneous investigation of up to three different compounds of interest and the real-time recording of LPL activity up to 24 h. In conclusion, a useful method for the examination of LPL activity is provided with this work that will benefit in the research on lipoproteins in general and on the effects of LCM on the lipid metabolism. The entire manuscript can be found on page 224 of this thesis.

5.9 Manuscript IX

The vitamin E long-chain metabolite α-13'-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system

Stefan Kluge,

Martin Schubert, Lisa Börmel, Stefan Lorkowski

Published in:

BBA Molecular and Cell Biology of Lipids

(2021):1866 [online publication ahead of print]

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Date of acceptance: January 2nd 2021

Contribution to the manuscript:

- Contribution to the writing of the manuscript (critical evaluation)
- Guidance on statistical analyses of the data

Personal contribution to the manuscript: 10%

Essence of Manuscript IX and contribution to this thesis:

Focus of this work is on the process of macrophage foam cell formation, a hallmark in the pathogenesis of atherosclerosis. The striking finding of a highly potent induction of gene expression of ANGPTL4 – an endogenously produced inhibitor of LPL activity - by α-13'-COOH lead to the examination of the effects of the LCM α-13'-COOH and the precursor α-TOH on the LPL system in macrophages. While α-TOH has no effect on the gene expression of ANGPTL4, a reduction of LPL gene expression is found. Interestingly, α-13'-COOH shows no effect on LPL gene expression. However, both substances lead to a reduced amount of LPL protein in macrophages, apparently by different molecular mechanisms, and consequently reduce the activity of LPL. Notwithstanding this, treatment with α-13'-COOH but not with α-TOH protected the cells from excessive lipid accumulation in the presence of excessive supply of VLDL. In conclusion, this work provides evidence for a formerly unknown regulatory property of the vitamin E-derived LCM α-13'-COOH, distinct from its precursor. This strengthens on the one hand the hypothesis of the metabolic activation of vitamin E with the LCM as biologically active substances and on the other hand the suggested role of vitamin E and its LCM in NCD, especially in CVD. The entire manuscript can be found on page 233 of this thesis.

5.10 Manuscript X

The vitamin E derivative garcinoic acid from *Garcinia kola* nut seeds attenuates the inflammatory response

Maria Wallert*, Julia Bauer*,

Stefan Kluge, Lisa Schmölz, Yung-Chih Chen, Melanie Ziegler, Amy K. Searle,

Alexander Maxones, Martin Schubert, Maria Thürmer, Helmut Pein, Andreas Koeberle,

Oliver Werz, Marc Birringer, Karlheinz Peter, Lorkowski S.

*** authors contributed equally**

Published in:

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Date of acceptance: March 10th 2019

Contribution to the manuscript:

- Contribution to the writing of the manuscript (critical evaluation)

Personal contribution to the manuscript: 5%

Essence of Manuscript X and contribution to this thesis:

This work provides experimental evidence for the anti-inflammatory properties of the vitamin E-related GA (δ-T3-13'-COOH) and introduces an optimized method for the extraction of this compound from seeds of *Garcinia kola* and the subsequent purification. *In vitro* studies using LPS-stimulated murine macrophages revealed strong anti-inflammatory properties of GA. Pre-treatment with GA suppresses the induction of several pro-inflammatory genes like cytokines and Ptgs2 and Nos2. Further, the protein expression of the latter two is suppressed and formation of respective products like nitric oxide and prostaglandins is diminished. Based on these promising findings, the anti-atherosclerotic potential of GA was examined in $Apoe^{-/-}$ mice receiving a high-fat diet *ad libitum*. However, weekly administration of GA did not lead to convincing beneficial findings. Favorable findings in this model were restricted to a significant reduction of nitrotyrosinylation in plaques and evidence for immune-regulatory properties of GA by altering lymphocyte sub-populations in blood and spleen. Taken together, the promising *in vitro* findings could not be successfully transferred to an *in vivo* model of a disease with inflammatory background except for the suppression of nitric oxide formation. However, future studies are needed to clarify if the high-fat diet prevents beneficial effects of the lipid-soluble GA. This would provide valuable information on the role of vitamin E, its metabolites and related structures in NCD which are often caused by detrimental dietary habits. The entire manuscript can be found on page 290 of this thesis.

5.11 Manuscript XI

Diversity of chromanol and chromenol structures and functions: an emerging class of anti-inflammatory and anti-carcinogenic agents

Maria Wallert*, Stefan Kluge*,

Martin Schubert, Andreas Koeberle, Oliver Werz, Marc Birringer, Stefan Lorkowski

***authors contributed equally**

Published in:

Frontiers in Pharmacology

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Contribution to the manuscript:

- Contribution to the writing of the manuscript (design and structure, critical evaluation)

Personal contribution to the manuscript: 5%

Essence of Manuscript XI and contribution to this thesis:

This work focusses on highlighting the potential of chromanol and chromenol structures for the prevention and treatment of diseases with an inflammatory component including cancer. On that account, available data on the effects of selected chromanols and chromenols on signaling pathways involved in inflammation, apoptosis, cell proliferation and carcinogenesis was summarized. Prominent and promising molecular targets like 5-LOX and NFκB as well as potential modes of action of chromanols and chromenols are discussed and putative low and high-affinity targets are suggested in this context. This work summarizes the available knowledge on vitamin E, its (long-chain) metabolites and related structures and outlines the potential of this class of compounds as lead structures for future anti-inflammatory and anti-carcinogenic therapeutic approaches. The entire manuscript can be found on page 300 of this thesis.

6 Manuscripts

The manuscripts included in this thesis are reproduced in full on the following pages. Please find the page numbers of the respective manuscripts at the end of the summarizing short descriptions of the respective manuscripts in the chapter ‘Manuscript overview’. In view of the size of this thesis, supplemental information is only given for the manuscripts that have not yet been published. Please find the supplemental information of already published manuscripts in the respective online resources indicated in the manuscripts.

Supplemental material to this article can be found at:
http://molpharm.aspetjournals.org/content/suppl/2019/12/23/mol.119.117887.DC1

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MOLECULAR PHARMACOLOGY

The Peroxisome Proliferator–Activated Receptor (PPAR)-γ Antagonist 2-Chloro-5-Nitro-N-Phenylbenzamide (GW9662) Triggers Perilipin 2 Expression via PPARδ and Induces Lipogenesis and Triglyceride Accumulation in Human THP-1 Macrophages[S]

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor family, playing pivotal roles in regulating glucose and lipid metabolism as well as inflammation. While characterizing potential PPARγ ligand activity of natural compounds in macrophages, we investigated their influence on the expression of adipophilin [perilipin 2 (PLIN2)], a well-known PPARγ target. To confirm that a compound regulates PLIN2 expression via PPARγ, we performed experiments using the widely used PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662). Surprisingly, instead of blocking upregulation of PLIN2 expression in THP-1 macrophages, expression was concentration-dependently induced by GW9662 at concentrations and under conditions commonly used. We found that this unexpected upregulation occurs in many human and murine macrophage cell models and also primary cells. Profiling expression of PPAR target genes showed upregulation of several genes involved in lipid uptake, transport, and storage as well as fatty acid synthesis by GW9662. In line with this and with upregulation of PLIN2 protein, GW9662 elevated lipogenesis and increased triglyceride levels. Finally, we identified PPARδ as a mediator of the substantial unexpected effects of GW9662. Our findings show that: 1) the PPARγ antagonist GW9662 unexpectedly activates PPARδ-mediated signaling in macrophages, 2) GW9662 significantly affects lipid metabolism in macrophages, 3) careful validation of experimental conditions and results is required for experiments involving GW9662, and 4) published studies in a context comparable to this work may have reported erroneous results if PPARγ independence was demonstrated using GW9662 only. In light of our findings, certain existing studies might require reinterpretation regarding the role of PPARγ.

SIGNIFICANCE STATEMENT

Peroxisome proliferator–activated receptors (PPARs) are targets for the treatment of various diseases, as they are key regulators of inflammation as well as lipid and glucose metabolism. Hence, reliable tools to characterize the molecular effects of PPARs are indispensable. We describe profound and unexpected off-target effects of the PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662) involving PPARδ and in turn affecting macrophage lipid metabolism. Our results question certain existing studies using GW9662 and make better experimental design of future studies necessary.

Introduction

A common strategy for the elucidation of signaling pathways mediating the effects of a compound of interest is the use of specific chemical agonists or antagonists for signaling proteins. GW9662, known as a selective peroxisome proliferator (PPAR)γ antagonist, is widely accepted to demonstrate participation of PPARγ in signaling cascades (Leesnitzer et al., 2002; Lea et al., 2004; Nielsen et al., 2006).

PPARγ, together with PPARα and PPARδ (also termed PPARβ), constitute the PPAR subfamily of nuclear receptors. PPARs are activated by a variety of molecules and act mainly as sensors for fatty acids and fatty acid–derived metabolites (Lefebvre et al., 2006; Tontonoz and Spiegelman, 2008; Neels and Grimaldi, 2014). As transcription factors, PPARs regulate various genes implicated in lipid and glucose metabolism, inflammation, proliferation, and differentiation (Varga et al., 2011; Vrablík and Češka, 2015).

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The PPAR subtypes differ in their expression levels in tissues, their affinity for their respective ligands, and their biologic functions. PPARα and PPARδ are related to lipid metabolism because of their key role in the regulation of fatty acid oxidation and lipoprotein metabolism (Luquet et al., 2005; Lefebvre et al., 2006). Furthermore, anti-inflammatory effects and regulatory roles in glucose metabolism have been reported for both isoforms (Lefebvre et al., 2006; Neels and Grimaldi, 2014; Magadum and Engel, 2018). While PPARδ is ubiquitously expressed (most abundantly in tissues with high fatty acid metabolism) (Neels and Grimaldi, 2014), PPARα is mainly found in liver and muscles (Lefebvre et al., 2006). PPARγ is predominantly expressed in adipose tissue and plays a central role in adipogenesis (Wang, 2010). In addition, PPARγ exerts considerable effects on lipid and glucose metabolism and insulin sensitivity (Tontonoz and Spiegelman, 2008). Based on the knowledge of their physiologic roles, PPARs represent interesting targets for the treatment of various diseases. In brief, fibrates, agonists of PPARα, have been used as hypolipidemic drugs to treat hypertriglyceridemia (Vrablík and Češka, 2015); PPARδ is regarded as a promising target for the treatment of metabolic syndrome because of its positive effects on serum cholesterol and lipid profiles (Neels and Grimaldi, 2014); and the insulin-sensitizing effects of thiazolidinediones, a class of PPARγ ligands, are used for the treatment of type 2 diabetes mellitus (Tontonoz and Spiegelman, 2008; Hong et al., 2018).

Crucial for the use of PPARs as pharmacological targets is the understanding of their modes of action. Thus, several models have been described for PPAR-dependent gene regulation (Varga et al., 2011) as well as the functional regulation of PPARs (Brunmeir and Xu, 2018). The most common model for PPAR-dependent gene regulation is the direct transcriptional regulation by heterodimerization of a PPAR isoform with retinoid X receptors. In the absence of ligands, the heterodimer is bound to the so-called PPAR response element in the promoter of target genes. In this state, the heterodimer binds a corepressor complex and represses the transcription of target genes. Upon ligand binding, the corepressor complex is replaced by coactivators, resulting in the transcription of PPAR target genes. Chemical antagonists bind to the respective PPAR isoform, leading to the maintenance of the corepressor complex and to the prevention of agonist binding (Harmon et al., 2011). Thus, chemical antagonists are considered as useful tools in PPAR research, especially for the verification of possible PPAR ligands.

A commonly used chemical antagonist in PPAR research is GW9662. GW9662 has been shown to covalently and irreversibly bind to the PPARγ ligand binding pocket (Leesnitzer et al., 2002; Brust et al., 2018). The binding of activating ligands is thereby prevented, and the signal transduction via PPARγ is interrupted. Surprisingly, GW9662 did not act as expected in our hands when used in commonly reported concentrations and incubation periods in the THP-1 macrophage model system. Here, GW9662 did not diminish the expression of the PPARγ target genes perilipin 2 (PLIN2) and cluster of differentiation 36 (CD36) induced by established PPARγ agonists, such as rosiglitazone or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ2). Unexpectedly, these target genes were upregulated in THP-1 macrophages by GW9662 itself. This effect was not restricted to THP-1 cells but was also observed in several other human and murine macrophage cell lines. Our study clearly shows that the suitability of GW9662 as a tool for the investigation of PPARγ-dependent signaling pathways is questionable. This work aims, therefore, to characterize: 1) the effect of GW9662 on gene and protein expression as well as overall cellular function of macrophages and 2) the underlying mechanism of the unexpected effects of GW9662 in macrophages.

The importance of PPARs as therapeutic targets in several diseases requires scrupulous investigation of PPAR signaling using reliable experimental tools. Our study is exploratory research that will therefore help to correctly interpret the variety of results obtained by the use of GW9662 in the future, especially in macrophage model systems.

Materials and Methods

Ethics Statement. Mononuclear cells were isolated from buffy coats obtained from peripheral blood of anonymized healthy male and/or female volunteers. Blood donors were informed about the aim of the study and gave written informed consent. The procedure was approved by the ethics committee of the University Hospital Jena (2446-12/08) and conducted according to the ethical principles defined by the declaration of Helsinki. Animal procedures, including isolation of peritoneal macrophages, were approved by the Animal Care and Use Committee of Thuringia (permit number 02-003/09) and were in line with the National Institutes of Health guidelines for the care and use of laboratory animals.

Reagents. Chemicals were from Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), and Merck Chemicals (Darmstadt, Germany) if not otherwise indicated. Rosiglitazone, 15dPGJ2, 4-chloro-*N*-[2-[[5-(trifluoromethyl)-2-pyridinyl]sulfonyl]ethyl]benzamide (GSK3787), and 2-chloro-5-nitro-N-4-pyridinyl-benzamide (T0070907) were purchased from Cayman Chemical Company (Biomol, Hamburg, Germany). GW9662 was purchased either from Sigma-Aldrich or Enzo Life Sciences (Lörrach, Germany). Key findings were confirmed using different lots of GW9662 obtained either from Sigma-Aldrich or Enzo Life Sciences.

Cell Culture. Murine RAW264.7 and J774A.1 cells as well as human THP-1 and U937 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained as recommended by the supplier and cultured at 37°C in a humidified 5% CO_2 air atmosphere. THP-1 and U937 monocytes were differentiated into macrophages using 100 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) in the presence of 50 μM β-mercaptoethanol (Sigma-Aldrich) in RPMI 1640 medium (PAA, Cölbe, Germany) supplemented with 10% FBS Gold (PAA) and antibiotics (PAA) for 96 hours as previously described (Robenek et al., 2005). Murine cells were

ABBREVIATIONS: 15dPGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; CD36, cluster of differentiation 36; COX, cyclooxygenase; FABP, fatty acid–binding protein; GPR, Global Pattern Recognition; GSK3787, 4-chloro-*N*-[2-[[5-(trifluoromethyl)-2-pyridinyl]sulfonyl]ethyl] benzamide; GW9662, 2-chloro-5-nitro-N-phenylbenzamide; J774, murine macrophages cell line from BALB/C mice; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PLIN2, perilipin 2; PMA, phorbol-12-myristate-13-acetate; PPAR, peroxisome proliferator–activated receptor; Ppib, peptidyl-prolyl cis-trans isomerase B; RAW264.7, murine leukemic monocyte macrophage cell line; RPL37A, ribosomal protein L37a; RT-qPCR, quantitative real-time reverse-transcription PCR; siRNA, small interfering ribonucleic acid; T0070907, 2-chloro-5-nitro-N-4-pyridinyl-benzamide; THP-1, human monocytic cell line; U937, human monocytic cells from histiocytic lymphoma; VLDL, very low-density lipoprotein.

cultivated in Dulbecco's modified Eagle's medium high-glucose medium (PAA) supplemented with 10% FBS and antibiotics according to the supplier's instructions. All cell lines were routinely tested for mycoplasma contamination using the mycoplasma detection kit PP-401 (Jena Bioscience, Jena, Germany) based on a PCR reaction. Cells were incubated with various compounds in serum-free medium with concentrations and for times as indicated in the figures.

Isolation of Mouse Peritoneal Macrophages. Male C57BL/6J mice at the age of 27–34 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). All animal procedures were approved by the Animal Care Committee of the federal state Thuringia (Germany). Mouse peritoneal macrophages were isolated as previously described (Zhang et al., 2008). Peritoneal macrophages were obtained by peritoneal lavage. For this, mice were anesthetized with isoflurane (DeltaSelect, Dreireich, Germany) and sacrificed with CO_2. The abdomen was soaked with 70% ethanol and opened, and the peritoneal cavity was washed twice with 5 ml Hank's balanced salt solution containing 500 μM EDTA. The liquid that includes peritoneal macrophages was extracted with a syringe and collected in an ice-cold tube. The suspension was centrifuged at 300*g* for 10 minutes at 4°C; the pellet was resuspended in 2 ml RPMI 1640 medium supplemented with antibiotics and 10% FBS, and cells were plated in 24-well plates. Cells were incubated for 2 hours at 37°C in a humidified air atmosphere with 5% CO_2 and were then washed five times with medium to remove nonadherent cells (Kim et al., 2005). After resting the cells for another 24 hours in serum containing RPMI 1640 at 37°C, the medium was replaced by serum-free RPMI 1640, and macrophages were cultured in the presence or absence of compounds for the times and concentrations indicated.

Isolation and Maturation of Peripheral Blood Mononuclear Cells. Mononuclear cells were isolated from buffy coats obtained from peripheral blood of anonymized healthy male and/or female volunteers. Buffy coat blood was diluted 1:1 with PBS (PAA), layered onto Lymphocyte Separation Medium (LSM) 1077 (1.077 g/ml; ratio 1:1; PAA), and centrifuged at 1200*g* for 20 minutes at 20°C (Gruen et al., 2004). The peripheral blood mononuclear cell (PBMC)-containing interphase was collected, washed three times with PBS, and resuspended in RPMI 1640 containing 10% FBS. Cells were plated in 25-cm^2 flasks in RPMI 1640 medium including supplementations. After 2 hours at 37°C in an air atmosphere containing 5% CO_2, adherent cells were washed twice with serum-free medium and were differentiated into macrophages for 8 days in RPMI 1640 supplemented with antibiotics and 20% human serum. PBMC-derived macrophages were then stimulated as indicated in the figures.

RNA Isolation and cDNA Synthesis. Preparation of samples was conducted according to established protocols (Schnoor et al., 2009). Total RNA was prepared from cell lysates using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, including an on-column DNAse I digestion (Qiagen) as previously reported (Stolle et al., 2007). Adequate RNA quality was assessed by agarose gel electrophoresis, and RNA was quantified photometrically. RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) was used for reverse transcription of mRNA into cDNA as previously described (Stolle et al., 2005; Maeß et al., 2014). For each cDNA synthesis, 5 μg total RNA and 0.5 μg oligo (dT) primers were used. Finally, cDNA samples were diluted 10-fold and stored at −30°C prior to PCR analyses.

Quantitative Real-Time Reverse-Transcription PCR. Primer pairs (Supplemental Tables 1 and 2) for quantitative real-time reverse-transcription PCR (RT-qPCR) were designed using PrimerExpress software version 2.0.0 (Applied Biosystems, Weiterstadt, Germany) and were purchased from Invitrogen (Karlsruhe, Germany). Expression analyses by means of RT-qPCR were performed with QuantiTect SYBR Green PCR Kit (Qiagen) on a LightCycler 480 II instrument (Roche Diagnostics, Mannheim, Germany) as previously reported (Maess et al., 2010; Maeß et al., 2014). PCR runs consisted of a preincubation at 95°C for 15 minutes and 40 cycles of a two-step PCR, comprising a denaturing phase at 94°C for 15 seconds and a combined annealing and extension phase at 60°C for 30 seconds. Following PCR, a melting curve was recorded to estimate purity of the PCR product. PCR results were analyzed using the LightCycler software release version 1.5.0.39 (Roche Diagnostics). The fit point algorithm of the LightCycler software was used to calculate C_t values. Fold changes were calculated using Microsoft Excel 2007. RPL37A and Ppib were used as reference genes for human and murine cell lines, respectively (Maess et al., 2010, 2011). Samples were prepared in biologic replicates, and analyses were performed as technical duplicates as indicated.

Human PPAR Signaling 384 StellARray qPCR Arrays. For profiling the regulation of gene expression by GW9662, the Human PPAR Signaling 384 StellARray qPCR Arrays (Lonza, Basel, Switzerland) were used. Samples obtained from five independent experiments were pooled in equal proportions, and RT-qPCR analyses were performed with QuantiTect SYBR Green PCR Kit (Qiagen) on a Roche LightCycler 480 II instrument as previously outlined (Maess et al., 2010), with slight modifications according to Lonza's 384 StellARray qPCR Arrays manual. PCR runs consisted of a step for dissolving primers at 50°C for 2 minutes, a preincubation step at 95°C for 15 minutes, and 40 cycles of a two-step PCR composed of a denaturing phase at 94°C for 15 seconds and a combined annealing and extension phase at 60°C for 30 seconds. Following PCR cycles, melting curves were recorded to validate specificity of the PCR. PCR results were analyzed using the LightCycler software release version 1.5.0.39, and obtained raw data were imported to Lonza Global Pattern Recognition (GPR) Data Analysis Tool for calculation of fold changes. HSP90AA1 (cytosolic heat shock protein 90 kDa α, class A, member 1), HMOX1 (heme oxygenase 1), SLC22A5 (solute carrier family 22 (organic cation/carnitine transporter) member 5), HRAS (Harvey rat sarcoma viral oncogene homolog), NR2C1 (nuclear receptor subfamily 2, group C, member 1), MECR (mitochondrial *trans*-2-enoyl-CoA reductase), MED14 (mediator complex subunit 14), ELOVL3 (elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3), and UBC (ubiquitin C) were identified as stably expressed by GPR Data Analysis Tool, and the geometric mean of these reference genes was used for normalization as recommended by the GPR Data Analysis Tool and following the instructions of the manufacturer.

Data Retrieval. Regulatory signaling pathways and biologic functions were assigned to genes whose expression was measured using Human PPAR Signaling 384 StellARray qPCR arrays as illustrated in Supplemental Table 3. For this assignment, National Center for Biotechnology Information, Kyoto Encyclopedia of Genes and Genomes, Reactome, and ARIADNE Genomic databases were systematically searched; information for assignment was collected and filtered manually. Gene names and their synonyms were retrieved from these databases. Genes were finally categorized manually according to their regulation of expression using a cutoff of 1.5 into "upregulated", "downregulated", "unchanged", and "not expressed/detected" and their role in metabolic and regulatory pathways.

Western Blot Analysis. Preparation of samples and Western blotting were conducted as previously reported (Wallert et al., 2015), but the transfer buffer contained 0.25 M Tris, 1.92 M glycine, 1% SDS, and 20% methanol (pH 8.3). Cells were harvested using a nondenaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) containing 1% protease inhibitor (Abcam, Cambridge, UK) and were processed for Western blotting. The antibody against PLIN2 (mouse anti-adipophilin clone AP125) was purchased from Progen (Heidelberg, Germany) and against α-tubulin (mouse anti-α-tubulin clone B-5-1-2) from Sigma-Aldrich. Secondary antibody (rabbit anti-mouse labeled with horseradish peroxidase) was from DAKO (Hamburg, Germany). For enhancing chemoluminescence signals for PLIN2, SignalBoost Immunreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used. For detection, Pierce ECL Western Blotting Substrate and CL-XPosure Films (Thermo Fisher Scientific, Rockford, IL) were applied. Blots were analyzed densitometrically using ImageJ software version 1.4.3.67 (National Institutes of Health, Bethesda, MD).

Fluorescence Microscopy. Cells were cultured on coverslips as described above and incubated with compounds as indicated in the figures. After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS and processed according to established protocols (Buers et al., 2009). The primary antibody against PLIN2 (Progen mouse anti-adipophilin clone AP125) was diluted 1:500, and the secondary antibody (Alexa Fluor 555 goat anti-mouse; Invitrogen) was used in a dilution of 1:200. Nuclei were stained using Hoechst Dye 33258 (Invitrogen) in a 1:10 dilution. Confocal images were acquired with a Zeiss LSM 510 confocal laser-scanning microscope; files were processed using Zeiss LSM Image Browser software version 3.1 (Carl Zeiss AG, Oberkochen, Germany), and fluorescence intensity was quantified using ImageJ software version 1.4.3.67.

Measurement of Cellular Triglyceride and Cholesterol Content. Mature THP-1 macrophages were incubated in the absence or presence of 10 μM GW9662 for 24 and 48 hours. Cells were harvested with 5% Triton X-100 (Sigma-Aldrich) in H_2O and prepared for enzymatic triglyceride and cholesterol analysis as follows. The cell suspension was sonicated three times on ice (electronic Sonopuls GM70; Bandelin, Berlin, Germany). An aliquot was taken for protein quantification using BCA Protein Assay (Thermo Scientific). Remaining samples were heated to 80°C for 10 minutes, incubated under slight shaking at that temperature for another 5 minutes, and rested at room temperature for cooling down. This process was repeated twice before samples were centrifuged. For analyzing triglyceride and cholesterol content, CHOD-PAP Kit from Roche Diagnostics and Triglyceride Assay Kit (Cayman Chemical Company) were applied according to manufacturer's instructions. Absorption was measured at 544 nm on a FLUOstar OPTIMA instrument (software version 2.10 R2; BMG Labtech, Ortenberg, Germany). Total cholesterol and triglyceride levels were normalized to intracellular protein amount.

Measurement of Lipogenesis by ^{14}C-Acetate Incorporation in Macrophages. For investigating the influence of GW9662 on lipid synthesis, mature THP-1 macrophages seeded into a 12-well-plate were incubated with 10 μM GW9662 or solvent for 20 hours and with 0.5 μCi/ml ^{14}C-labeled sodium acetate (Hartmann Analytic, Braunschweig, Germany) (specific activity of 57 mCi/mmol) for another 4 hours in serum-free RPMI 1640 medium containing 50 μM β-mercaptoethanol, 100 ng/ml PMA, 0.25% free fatty acid-free bovine serum albumin (Sigma-Aldrich), 500 pM biotin (Sigma-Aldrich), and 50 μM L-carnitin (Sigma-Aldrich). As negative control, cells were stimulated with 50–100 μM C75 (Sigma-Aldrich) for 6 hours to inhibit fatty acid synthesis. All samples were performed and analyzed in duplicates. After incubation, cells were washed three times with ice-cold HEPES/Ca^{2+} buffer (pH 7.4) and harvested using 0.2 ml 50 mM Tris buffer (pH 7.5) (AppliChem, Darmstadt, Germany). After adding 0.5 ml methanol (AppliChem) and 0.25 ml chloroform (Sigma-Aldrich) for lipid extraction, samples were thoroughly mixed and incubated for 15 minutes at room temperature. Then, 0.25 ml chloroform and 0.25 ml 0.1 M potassium chloride (Sigma-Aldrich) were added. To enhance extraction of free fatty acids, the pH of samples was lowered by adding 2 μl 1 M citric acid (Sigma-Aldrich). Samples were incubated for 5 minutes at room temperature and centrifuged for 5 minutes at 400*g* for phase separation. After transferring the lower phase, chloroform was vaporized, and lipids were solubilized with 0.5 ml 1% Triton X-100. After adding scintillation cocktail (Carl Roth), radioactivity was measured using a Liquid Scintillation Counter LSC Wallac 1410 (Pharmacia-Wallac, Turku, Finland), and the amount of incorporated ^{14}C-acetate was determined. Protein content of cell lysates was analyzed using the Lowry method, and ^{14}C-acetate incorporation was normalized to protein content.

Flow Cytometric Analysis of Apoptosis and Necrosis. To investigate whether THP-1 macrophages were undergoing apoptosis and necrosis in response to GW9662, cells were incubated with increasing doses of GW9662 as indicated in Supplemental Fig. 1 and stained with annexin V and 7-aminoactinomycin D (BD Biosciences, Heidelberg, Germany). In brief, after detaching cells with Accutase I (PAA), cells were washed twice with ice-cold PBS and incubated in binding-buffer containing annexin V and 7-aminoactinomycin D for 15 minutes in the dark. Samples were then diluted with binding buffer. Positive cells were analyzed flow cytometrically using a Beckman Coulter EPICS XL-MCL (Krefeld, Germany) and quantified using WinMDI software version 2.8 (Scripps Research Institute, La Jolla, CA).

Transfection. THP-1 monocytes were differentiated for 24 hours using 100 ng/ml PMA in the presence of 50 μM β-mercaptoethanol in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Premature THP-1 cells were detached using Accutase I. Transfection was performed using Lonza's Human Monocyte Nucleofector Kit. Here, cells were resuspended in Nucleofector solution and transferred to a Nucleofector cuvette together with 0.25 μg silencer select siRNA (either s10880 PPARA, s10883 PPARD, s10888 PPARG, or Silencer Select Negative Control No. 2 siRNA; Life Technologies, Darmstadt, Germany). Nucleofection was carried out using program Y-001 in a Nucleofector 2b device. Next, cells were transferred to Lymphocyte Growth Medium-3 (Lonza) supplemented with 1% (v/v) nonessential amino acids (Lonza), 1% (v/v) sodium pyruvate (Sigma-Aldrich), and 1% human serum (Sigma-Aldrich) for 6 hours allowing reattachment. Subsequently, medium was replaced by Lymphocyte Growth Medium-3 supplemented with 1% (v/v) nonessential amino acids, 1% (v/v) sodium pyruvate, and 0.1% human serum. Cells were incubated 72 hours after transfection with GW9662 or vehicle for an additional 24 hours and subsequently harvested for RT-qPCR analysis as described. Transfection efficiency was determined after the 24-hour incubation period by RT-qPCR (Supplemental Fig. 14).

Data Presentation and Statistical Analyses. Data are presented as means ± S.E.M. by circles. Data of the respective individual independent experiments are represented by squares to provide information on variability of the data. Experiments were performed at least three times. In the case of high variability in the results, additional biologic replicates were performed to generate a meaningful result. Information on the times an experiment was performed are given in the respective figure legends, and the group sizes are always equal within the experiment and resemble the times the experiment was performed.

RT-qPCR data were analyzed based on the $2^{-\Delta\Delta ct}$ method, with controls set to one in each independent experiment. Presentation of individual data points (squares) was thus omitted for controls in RT-qPCR experiments.

Statistical analyses aim at the description of differences in the mean effect of treatments in individual experiments and were performed using OriginLab's OriginPro 9.1G. Appropriate statistical tests were chosen based on the experimental design and are indicated in the respective figure legends. $P < 0.05$ was considered statistically significant.

Results

While screening for potential PPARγ agonist activity of selected natural compounds, we intended to use PLIN2, a known PPARγ target gene (Gupta et al., 2001; Hodgkinson and Ye, 2003), as a readout. Blocking experiments using the widely used selective PPARγ antagonist GW9662 would confirm that regulation of PLIN2 mRNA expression by a compound of interest is mediated via PPARγ. Surprisingly, GW9662 exhibited unexpected effects on the expression of PLIN2 and other PPARγ target genes. Instead of blocking the expression of PLIN2 in THP-1 macrophages, the expression was induced by GW9662 at concentrations and under conditions widely used in studies published so far (Lea et al., 2004; Kourtidis et al., 2009). Therefore, we decided to investigate this phenomenon more systematically.

GW9662 Does Not Block Upregulation of PLIN2 Expression by Rosiglitazone and 15dPGJ2. Before starting to investigate the PPARγ participation in the signaling of the natural compounds of our interest, we conducted

a proof-of-concept, i.e., the combination of well-known PPARγ agonists with GW9662 and the use of known PPARγ target genes as readout. PLIN2 is a member of the family of perilipin, adipophilin, and TIP47 proteins and is one of the best investigated proteins involved in lipid storage in macrophages (Bickel et al., 2009). It has been shown to be regulated by PPARγ in macrophages (Hodgkinson and Ye, 2003). The scavenger receptor CD36 is pivotal in promoting macrophage foam cell formation (Nicholson and Hajjar, 2004) and is also a well-known PPARγ target gene (Chawla et al., 2001; Moore et al., 2001). Consequently, expression of PLIN2 and CD36 is induced by PPARγ ligands, such as rosiglitazone and 15dPGJ2 (Chawla et al., 2001; Hodgkinson and Ye, 2003). The confirmed PPARγ antagonist GW9662 (Leesnitzer et al., 2002; Seimandi et al., 2005) should block this induction. We therefore investigated the effect of GW9662 on rosiglitazone- and 15dPGJ2-induced expression of PLIN2 and CD36.

THP-1 macrophages were pretreated with 10 μM GW9662 in serum-free medium for 1 hour before incubating the cells with synthetic or natural PPARγ agonists rosiglitazone and 15dPGJ2 for another 24 hours. After harvesting the cells, total RNA was isolated and transcribed into cDNA for measuring relative mRNA expression levels of PLIN2 and CD36 using RT-qPCR. As expected, both 5 μM rosiglitazone and 5 μM 15dPGJ2 significantly induced the expression of PLIN2 mRNA to the 2.0-fold ($P < 0.05$) and to the 7.7-fold ($P < 0.001$), respectively (Fig. 1, top). Similarly, as shown in Fig. 1 (middle), expression of CD36 was significantly increased to the 3.5-fold ($P < 0.01$) and to the 7.8-fold ($P < 0.001$), respectively. Expression of the reference gene RPL37A was not affected and was used for normalization (Fig. 1, bottom). Surprisingly, treatment of THP-1 macrophages with 10 μM GW9662 significantly increased expression of PLIN2 and CD36 by more than the 2.1-fold ($P < 0.05$ and $P < 0.01$, respectively; Fig. 1, top and middle). Further data shown in Fig. 1 (top and middle) illustrate that pretreatment with 10 μM GW9662 abolished neither the effect of rosiglitazone nor of 15dPGJ2. Instead of repressing PPARγ ligand-induced expression of PLIN2 and CD36, GW9662 pretreatment significantly enhanced the effect of rosiglitazone (Fig. 1A) but did not alter induction by 15dPGJ2 (Fig. 1B). Interestingly, the synergistic effects were not limited to rosiglitazone but also observable for troglitazone. Troglitazone increased PLIN2 and CD36 gene expression like rosiglitazone and 15dPGJ2, and GW9662 did not block the effect (Supplemental Fig. 2). Pretreatment of THP-1 macrophages with GW9662 as well as incubating the cells in combination with agonist and antagonist at the same time for 24 hours also led to augmented

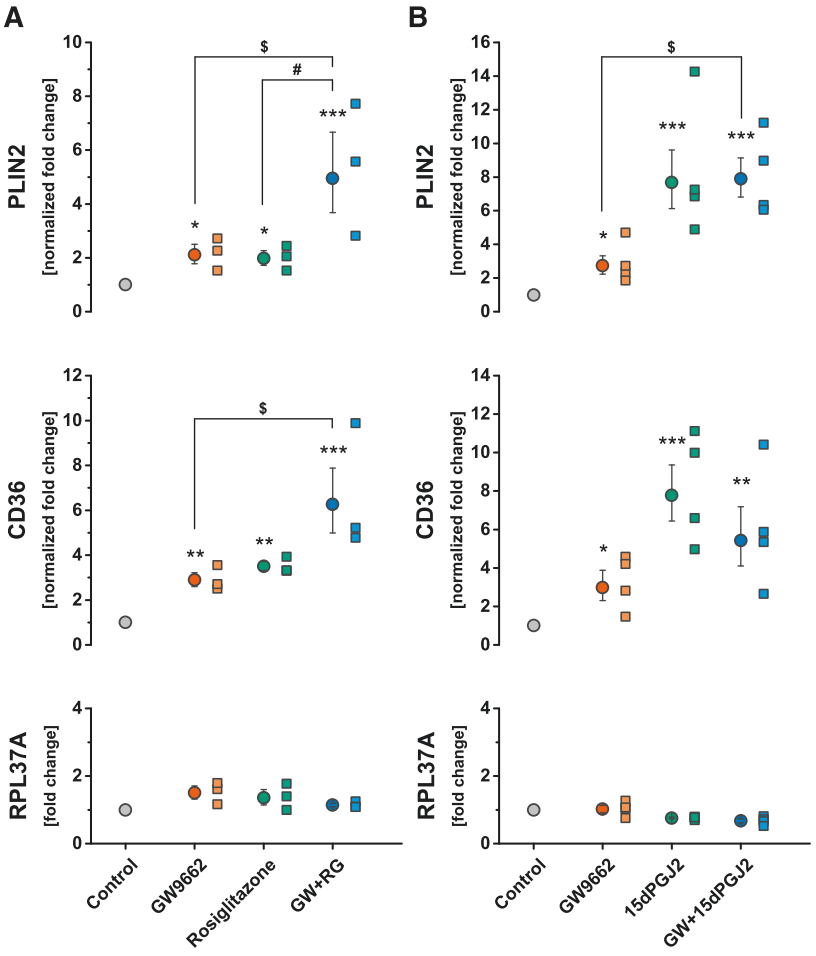

Fig. 1. GW9662 exhibits PPARγ agonist–like activity and fails to block PPARγ agonists in human THP-1 macrophages. (A) Mature THP-1 cells were either treated with 10 μM GW9662 (GW) or 5 μM rosiglitazone (RG) alone or were pretreated with 10 μM GW9662 for 1 hour and then incubated with 5 μM rosiglitazone for another 24 hours (GW+RG). (B) THP-1 macrophages were either treated with 10 μM GW9662 (GW) or 5 μM 15dPGJ2 alone or were pretreated with 10 μM GW9662 for 1 hour and then incubated with 5 μM 15dPGJ2 for another 24 hours (GW+15dPGJ2). Expression levels of PLIN2, CD36, and the reference gene RPL37A were assessed by RT-qPCR. Expression of the reference gene remained unchanged in both experimental setups and was used for normalization. GW9662 stimulated expression of PLIN2 and CD36 significantly. In combination with the PPARγ ligand rosiglitazone, GW9662 showed additive effects instead of blocking activity. Circles represent the mean of normalized fold changes of three (A) or four (B) independent biologic experiments. Error bars display calculated maximum and minimum expression levels based on the S.E.M. of ΔΔct values. Squares represent data of independent biologic experiments to visualize variability of the data. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; all vs. control (0 μM GW9662); $^{\#}P < 0.05$ vs. PPARγ ligand only; $^{\$}P < 0.05$ vs. GW9662 only. P values were calculated using repeated-measures ANOVA with Tukey's post hoc test.

levels of PLIN2 mRNA (Supplemental Fig. 3). Having identified the unexpected property of GW9662 to stimulate the expression of known PPARγ target genes, we aimed at the characterization of this effect. This is of interest, as GW9662 is widely used in scientific studies that do not necessarily resemble our initial setup.

Concentration-Dependent Activation of PLIN2 Expression by GW9662. Concentration dependency was investigated to obtain further insights into the dynamics of the regulation of PLIN2 expression by GW9662. THP-1 cells were therefore treated with different concentrations of GW9662 for 24 hours, as indicated in Supplemental Fig. 4. Stimulation with a concentration of 1 μM GW9662 already significantly induced PLIN2 mRNA expression in THP-1 macrophages to the 2.2-fold compared with controls ($P < 0.001$). This effect was augmented further with increasing concentrations up to the 3.2-fold ($P < 0.001$ at 5–50 μM for 24 hours). These findings indicate that upregulation of PPARγ target genes is a robust effect in different experimental setups. However, we used GW9662 at a concentration of 10 μM for further experiments because this concentration revealed reproducible results and is a commonly used concentration in published scientific studies (Lea et al., 2004; Kourtidis et al., 2009). As GW9662 reliably induces PPARγ target genes and is thus not usable for blocking experiments, we were interested in the PPARγ antagonist 2-chloro-5-nitro-N-4-pyridinyl-benzamide (T0070907) (Lee et al., 2002) with inverse agonist properties (Lee et al., 2002; Brust et al., 2018) as an alternative. However, incubation with 1 μM T0070907 for 24 hours induced PLIN2 mRNA expression in THP-1 macrophages to the 1.4-fold compared with controls, and this induction increased with higher concentrations up to the 2.5-fold, as shown in Supplemental Fig. 5. Thus, T0070907 has similar effects as GW9662 on PLIN2 gene expression and is neither an alternative for GW9662 nor a useful tool to further characterize GW9662's effects in our setup.

PLIN2 mRNA Expression Is Induced by GW9662 in Human and Mouse Macrophages. Because GW9662 is often used to confirm PPARγ-mediated effects, and no comparable effects to our observations have been reported (to the best of our knowledge), we considered a THP-1 cell–specific "artifact" as possible explanation. Thus, we examined PLIN2 and CD36 mRNA expression in primary macrophages and in other macrophage cell lines of human and murine origin in response to GW9662. For these studies, we incubated different human macrophage cells (THP-1, U937, and primary macrophages) as well as different mouse macrophages (RAW264.7, J774A.1, and primary peritoneal macrophages) with 10 μM GW9662 for 24 hours in serum-free medium and compared expression of PLIN2/Plin2 and CD36/Cd36 with controls cultured in serum-free medium in the absence of GW9662. Except for RAW264.7 cells, GW9662 induced expression of PLIN2/Plin2 mRNA in every cell type investigated at least to the 1.9-fold, as shown in Fig. 2 (top; $P < 0.05$ for THP-1, U937, and J774.A1). In contrast, the effect of GW9662 on CD36/Cd36 mRNA expression was more inconsistent. Though expression of CD36/Cd36 mRNA tended to be increased in U937 cells and was significantly increased in THP-1 macrophages as well as

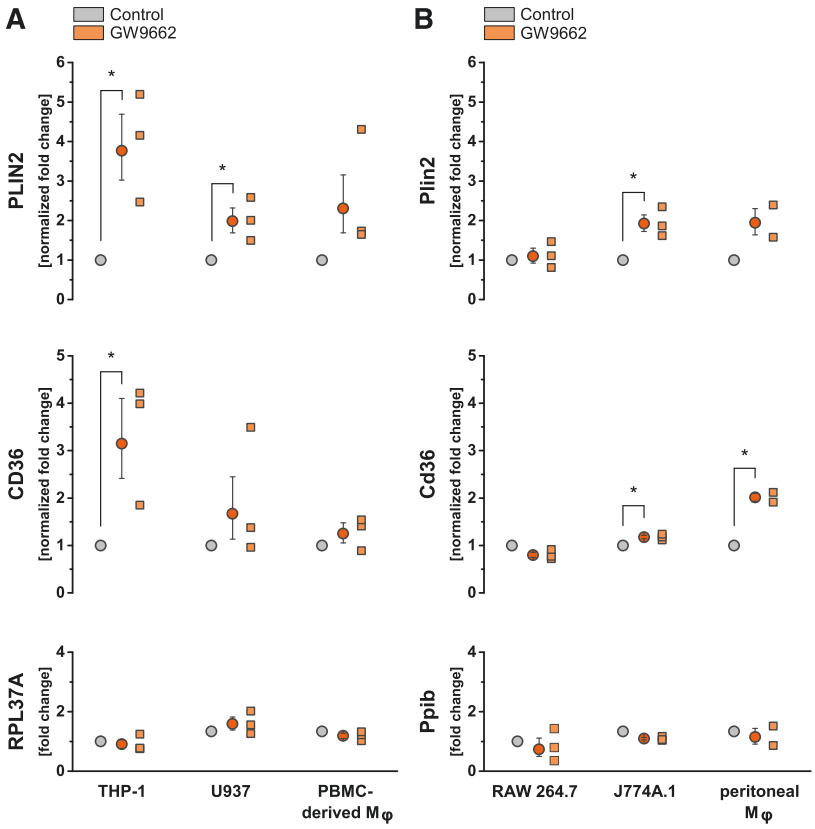

Fig. 2. GW9662 induces expression of PLIN2/Plin2 and CD36/Cd36 in different human and murine macrophage cell models. Human (A) and murine (B) macrophages of different origin were incubated with 10 μM GW9662 for 24 hours. Relative mRNA levels were measured by RT-qPCR and compared with control cells cultured in absence of GW9662. Gene expression levels of human cells were normalized to RPL37A; expression levels of murine cells were normalized to Ppib. Circles represent means of fold changes of three independent biologic replicates (except for peritoneal macrophages, for which independent experiments were performed twice). Squares represent the mean of two technical replicates of the biologic replicates. Error bars display calculated maximum and minimum expression levels based on the S.E.M. of ΔΔct values. *$P <$ 0.05 vs. corresponding control. P values were calculated using two-tailed paired t test.

in peritoneal macrophages and J774A.1 to the 3.2-fold ($P < 0.05$; Fig. 2A, middle), to the 2.0-fold ($P < 0.05$; Fig. 2B, middle), and to the 1.18-fold ($P < 0.05$), respectively, expression of CD36/Cd36 in human PBMCs and murine RAW264.7 was not affected. Expression of the reference genes RPL37A and Ppib remained unchanged in all experiments (Fig. 2, bottom). Consequently, the effect of GW9662 is not restricted to THP-1 cells but is also observed in other human and murine macrophage-like cells as well as in primary human macrophages and mouse peritoneal macrophages.

GW9662 Induces PLIN2 mRNA Consistently and Comparable to Synthetic and Natural PPARγ Agonists. To confirm that our observations do not depend on lot or supplier, GW9662 from two different distributors and in two lots of a single renowned distributor were tested. Consistently, PLIN2 mRNA expression was induced in mature THP-1 macrophages after treatment with 10 μM GW9662 for 24 hours in all cases. However, fold changes varied between experiments performed with GW9662 from different lots between about three- and sixfold (Supplemental Fig. 17). Most strikingly, in some experiments, the ability of GW9662 to induce PLIN2 mRNA and protein levels was comparable to that of the PPARγ agonists rosiglitazone and 15dPGJ2 (Fig. 3). In the case of CD36, GW9662 was almost as potent as 15dPGJ2 to induce CD36 mRNA expression but was less potent than rosiglitazone (Fig. 3A). However, as mentioned above, results for the impact of GW9662 on CD36 expression were inconsistent.

PLIN2 Protein Levels in THP-1 Macrophages Are Elevated by GW9662. Given the reliable induction of PLIN2 gene expression by GW9662 in different setups and different cell lines, we were interested whether this effect is mirrored on the protein level. This would point to further, likely more complex, changes in cellular functions by GW9662. Western blot analyses confirmed indeed that PLIN2 protein levels were significantly increased in THP-1 macrophages to about the 1.9-fold ($P < 0.05$) after 24 hours and to about the 1.4-fold ($P < 0.05$) after 48 hours of treatment with 10 μM GW9662 compared with the respective controls (Fig. 4, A and B). For immunoblotting, PLIN2 was evaluated densitometrically and normalized to α-tubulin. The expression of α-tubulin was not affected by GW9662 (Fig. 4A). Immunofluorescence staining further supports our notion that GW9662 significantly induced PLIN2 protein in THP-1 macrophages compared with nontreated controls ($P < 0.01$; Fig. 4, C and D). As with the effect of T0070907 on gene expression of PLIN2, we were interested if the alternative PPARγ antagonist exerts comparable effects to GW9662 on the protein level. Western blot

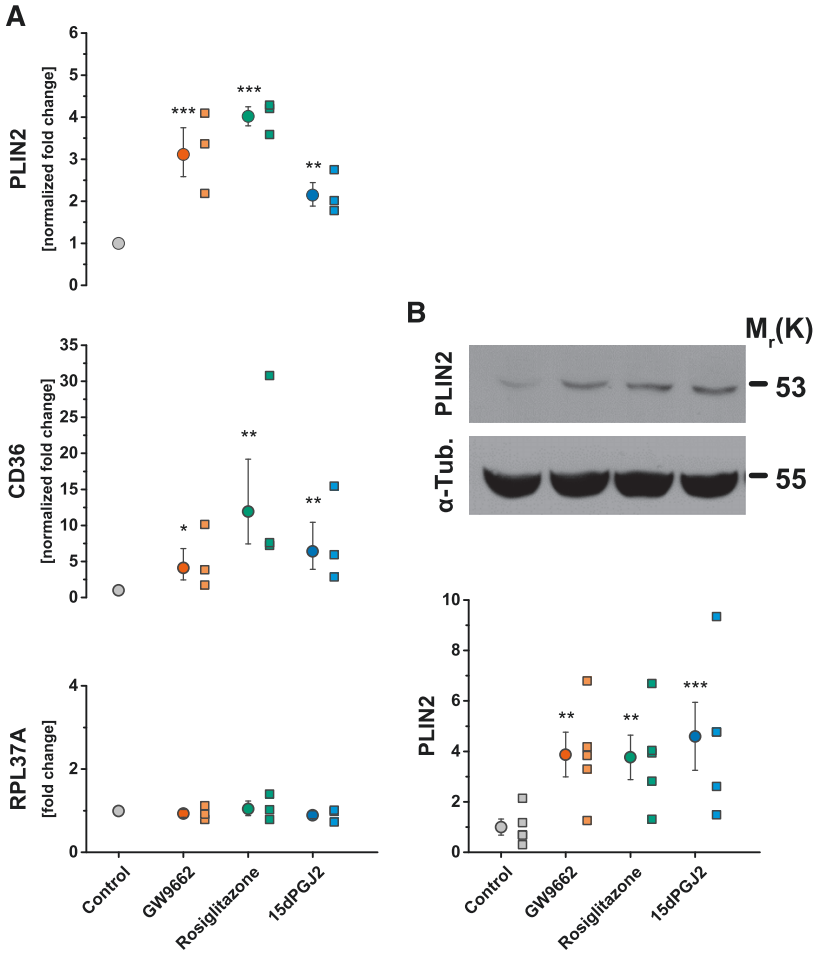

Fig. 3. GW9662 induces PLIN2 expression comparable to the PPARγ agonists rosiglitazone and 15dPGJ2. Mature THP-1 macrophages were treated with either vehicle (control), 10 μM GW9662 (GW), 5 μM rosiglitazone (RG), or 5 μM 15dPGJ2 for 24 hours. (A) PLIN2 and CD36 mRNA expression levels were assessed by RT-qPCR and normalized to the expression of RPL37A. Circles represent means of fold changes of three independent biologic replicates. Squares represent the mean of two technical replicates of the biologic replicates. Error bars display calculated maximum and minimum expression levels based on the S.E.M. of ΔΔct values. (B) Top: representative Western blot. Bottom: relative protein levels of PLIN2 as estimated by densitometric analysis of Western blots. Circles represent means of fold changes of five independent experiments. Squares represent means of two technical replicates of the biologic replicates relative to the mean of the control to visualize variability of the data. α-Tubulin (α-Tub.) was used as loading and cell viability control and was used for normalization. Data are shown as mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control. P values were calculated using repeated-measures ANOVA with Dunnett's post hoc test vs. control as reference.

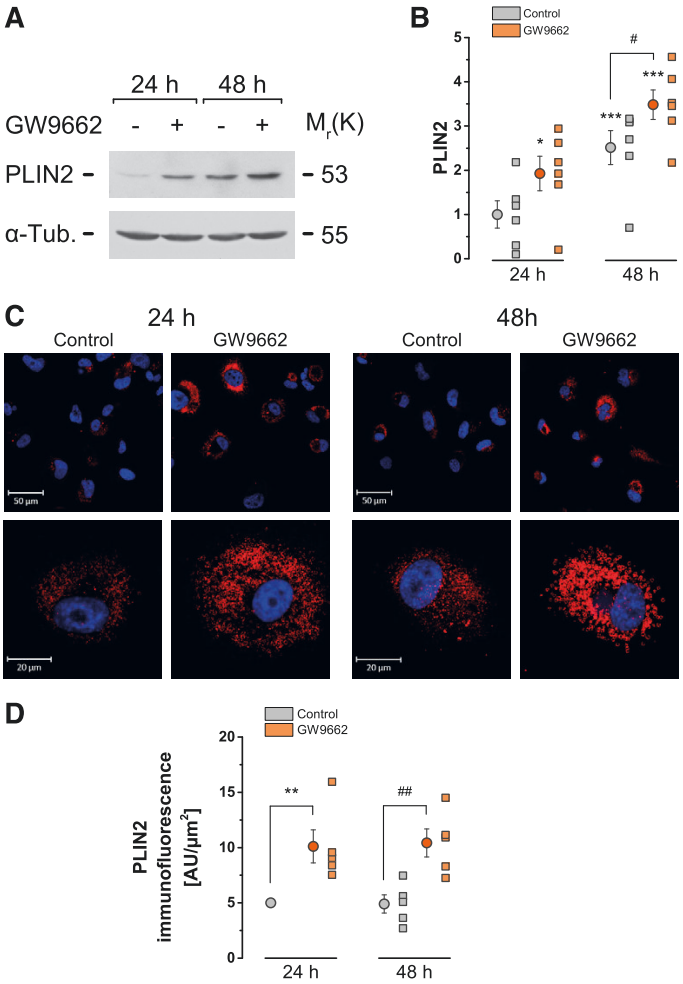

Fig. 4. GW9662 augments PLIN2 protein expression in mature THP-1 macrophages. PMA-matured THP-1 macrophages were cultured in presence or absence of 10 μM GW9662 for 24 and 48 hours. PLIN2 protein levels were assessed by Western blot analysis and immunofluorescence. (A) Representative Western blot of PLIN2 protein levels. α-Tubulin (α-Tub.) was used as loading and cell viability control. (B) Relative densitometric quantification of Western blot results. Circles represent means of fold changes of five independent experiments. Squares represent independent biologic replicates relative to the mean of the control to visualize variability of the data. (C) Immunofluorescence staining of PLIN2 and Hoechst Dye 33258 staining of nuclei. Representative cells are shown at magnifications as indicated. (D) Relative intensities of immunofluorescence signals per area shown as arbitrary units (AU/μm^2) × 10^4. Circles represent data of five independent experiments shown as mean ± S.E.M. Squares represent independent biologic replicates relative to the control to visualize variability of the data. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 24-hour control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. 48-hour control. *P* values were calculated using repeated-measures ANOVA with Tukey's post hoc test.

analyses confirmed that PLIN2 protein levels were slightly increased in THP-1 macrophages after 24 and 48 hours of treatment with 10 μM T0070907 (Supplemental Fig. 6). Thus, T0070907 apparently exerts less unwanted effects on the protein level and might be a usable alternative to GW9662 in some experimental setups.

GW9662 Affects Expression of Several Other PPAR Target Genes. Because we demonstrated that GW9662 unexpectedly induces the expression of PLIN2 and CD36 mRNA (as well as PLIN2 protein), we were interested in whether and how GW9662 affects the expression of other PPAR target genes. For this purpose, we used Lonza Human PPAR Signaling 384 StellARray qPCR arrays. These arrays allow the measurement of the expression of 384 genes that are either regulated by the PPAR subtypes (PPARα, PPARβ/δ, and PPARγ) themselves, are PPAR-interacting partners, or serve as controls (HsGenomic and Hs18s) and reference genes (nine most stably expressed genes).

For profiling analyses, THP-1 macrophages were treated for 24 hours either with solvent only as control or 10 μM GW9662. Samples obtained from five independent experiments were pooled in equal proportions, and relative changes in expression were measured using RT-qPCR. Results were normalized to the nine most stably expressed reference genes as selected by the Lonza Global Pattern Recognition algorithm. Genes were grouped according to their regulation into "upregulated," "downregulated," and "unchanged" (Supplemental Table 4). For this, a fold change of 1.5 was set as the cutoff as recommended by the supplier. Genes that were not detected are listed separately as "not expressed/not detectable" (Supplemental Table 3). As shown in Supplemental Fig. 7A, expression of most of the genes (47.4%) remained unchanged by GW9662, whereas expression was upregulated for 26.6% and downregulated for 16.9% of the genes. For 9.1% of the genes, no expression was detectable.

As illustrated in Supplemental Fig. 7B, most of the genes with expression altered by GW9662 in THP-1 macrophages are involved in lipid and glucose metabolism, immune and inflammatory response, and signal transduction and transcription. In more detail (Supplemental Fig. 8), genes affected by GW9662 are involved in: 1) glucose transport, such as sorbin and SH3 domain containing 1, protein phosphatase 2α, and insulin receptor; 2) in glycolysis, such as pyruvate dehydrogenase kinase 4; 3) lipid uptake, such as CD36, oxidized low-density lipoprotein receptor 1, and low-density lipoprotein receptor; 4) lipid transport, such as fatty acid transport

members 1 and 2 of the solute carrier family 27, and FABP2 to FABP4; 5) lipid storage, such as PLIN2; and 6) lipogenesis, such as stearoyl-CoA desaturase, acetyl-coenzyme A carboxylase α, and members 3–5 of the acyl-CoA synthetase long-chain family. Furthermore, proinflammatory genes, such as cytosolic phospholipase A_2, interleukin 1β, prostaglandin E receptor 2, and inducible nitric oxide synthase 2, are also induced by GW9662.

The results of the array nicely show that the effects of GW9662 go far beyond the initially observed regulation of PLIN2 and CD36. Interestingly, these genes are members of the most affected functional category, the lipid metabolism. Taken together, GW9662 influences genes in all classic PPAR-related metabolic pathways (lipid metabolism, glucose metabolism, immunity, and inflammation) (Clark, 2002; Wang, 2010). This indicates that use of GW9662 affects cellular metabolism in different ways, and thus applicability of GW9662 should be carefully evaluated in different experimental setups.

GW9662 Induces Triglyceride Accumulation in THP-1 Macrophages. According to the expression profiling, GW9662 regulates a variety of genes implicated in lipid metabolism. We were therefore interested in if subsequent functional consequences occur. The choice of an appropriate readout was based on our finding that PLIN2 is regulated at the protein level by GW9662. PLIN2 has been shown to be involved in the import and storage of cholesterol and fatty acids (Paul et al., 2008). Together with the observation that more lipid droplet-like structures appear in GW9662-treated THP-1 macrophages compared with controls, we decided to investigate whether GW9662 induces the accumulation of lipids, particularly triglycerides. Therefore, we analyzed the intracellular content of triglycerides and total cholesterol. In good agreement with our findings reported above, 10 μM GW9662 increased triglyceride levels in THP-1 macrophage cells by 27% compared with control after 24 hours ($P < 0.01$). However, after 48 hours, no significant effect compared with the respective control was observed (Fig. 5, left). In contrast, the total cellular cholesterol content was not altered by GW9662 after 24 or 48 hours (Fig. 5, right).

GW9662 Raises Lipogenesis in THP-1 Macrophages. The expression profiling revealed that several genes implicated in lipogenesis are upregulated by GW9662. Therefore, we analyzed lipogenesis in THP-1 macrophages to gain deeper insight into the molecular causes for the increased intracellular total triglyceride content. Mature macrophages were incubated with 10 μM GW9662 for 24 hours and with ^{14}C-acetate for 4 hours. We found a 34% increased incorporation of ^{14}C-acetate ($P < 0.001$) compared with controls cultured in the absence of GW9662 (Fig. 6). As a negative control, C75, an inhibitor of fatty acid synthesis, was used at concentrations of 50 or 100 μM for 6 hours. C75 inhibited ^{14}C-acetate incorporation to approximately 70% of control (Supplemental Fig. 15). The findings are in good agreement with the observed increased intracellular triglyceride content by GW9662 (Fig. 5) and the results obtained from mRNA expression analyses using Lonza Human PPAR Signaling 384 StellARray qPCR arrays (Supplemental Table 3 and Supplemental Figs. 7–9). Taken together, GW9662 treatment leads to substantial changes in macrophage lipid metabolism, as shown by alterations in gene regulation leading to higher levels of lipid synthesis and storage in consequence.

PPARδ Mediates the Effects of GW9662 on PLIN2 and CD36 Expression in THP-1 Macrophages. The treatment of THP-1 macrophages with GW9662 leads to substantial changes in cellular metabolism, particularly in lipid metabolism. Thus, the underlying mechanism of action is of special interest to assess the applicability of GW9662 in certain experimental setups. Previous studies using GW9662 revealed that this compound is not entirely specific for PPARγ (Leesnitzer et al., 2002). Hence, the interaction of GW9662 with other PPAR subtypes or nuclear receptors is a possible reason for the unexpected effects reported here. To reveal a possible role of the distinct PPAR subtypes in the observed regulation of PLIN2 and CD36, siRNA was used to knock down each PPAR subtype in THP-1 macrophages. For this purpose, THP-1 monocytes were differentiated for 24 hours using PMA. Resulting THP-1 macrophages were transfected with either negative control siRNA or siRNA for each PPAR subtype and transfection efficiency was verified (Supplemental Fig. 14). The macrophages were allowed to recover for 72 hours and were then incubated with 10 μM GW9662 or vehicle for 24 hours. The expression of PLIN2 and CD36 was induced by GW9662 in all transfected cells. However, the extent of the induction of PLIN2 and CD36 expression by GW9662 was significantly lowered in the PPARδ-depleted cells. PLIN2 was merely induced to the 2.5-fold compared with the respective control in PPARδ-depleted cells, whereas an increase to the 4.7-fold in cells transfected with negative control siRNA or with PPARα siRNA and an increase to the 4.2-fold over the respective control was observed in PPARγ-depleted cells (Fig. 7). Similarly, CD36 expression was induced only to the 1.8-fold by GW9662 in PPARδ-depleted cells but to the 2.7-, 2.8-, and 3.7-fold in

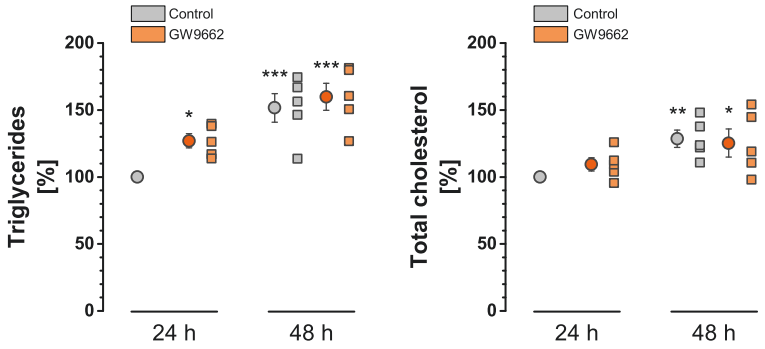

Fig. 5. GW9662 promotes intracellular accumulation of triglycerides but not of total cholesterol. Differentiated THP-1 cells were treated with 10 μM GW9662 in lipid- and serum-free medium for 24 and 48 hours. Cells cultured in absence of GW9662 served as controls. Triglyceride or total cholesterol levels in untreated 24-hour controls were arbitrarily set to 100%. Circles represent mean ± S.E.M. of five independent experiments. Squares represent independent biologic replicates relative to the control to visualize variability of the data. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. In both cases, P values are provided for GW9662 vs. control after 24 hours. P values were calculated using repeated-measures ANOVA with Tukey's post hoc test.

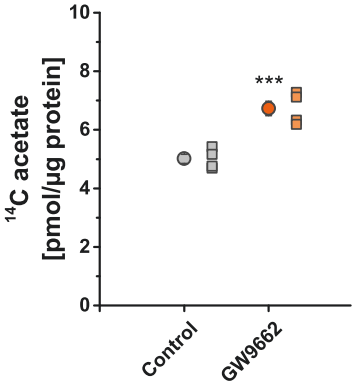

Fig. 6. GW9662 stimulates lipogenesis in THP-1 macrophages. Mature THP-1 macrophages were treated with 10 μM GW9662 for 24 hours in serum-free medium. After 20 hours, ^{14}C-acetate was added for the next 4 hours. Samples were processed, and ^{14}C-radioactivity was measured by scintillation. Incorporated ^{14}C-acetate was normalized to cellular total protein content. Circles represent mean ± S.E.M. of four independent experiments. Squares represent independent biologic replicates relative to the control to visualize variability of the data. ***$P < 0.001$. The P value is provided for GW9662 vs. control, calculated using two-tailed paired t test.

control cells transfected with negative control siRNA, PPARα, or PPARγ knockdown, respectively (Fig. 7). Thus, PPARδ knockdown significantly impairs the effect of GW9662 on PLIN2 ($P < 0.05$) and CD36 ($P < 0.05$; $P < 0.0565$ for negative control siRNA) expression in THP-1 macrophages.

The knockdown experiments provide evidence that PPARδ is involved in the effects of GW9662 on PLIN2 and CD36 expression. However, PPARδ knockdown did not completely abolish the induction of target genes by GW9662. Thus, there might be remaining activity of PPARδ or further regulatory proteins that might be affected by GW9662. However, to verify the contribution of PPARδ to the effects of GW9662, a chemical antagonist was used to block PPARδ activation. Mature THP-1 macrophages were therefore preincubated with 1 μM of the PPARδ antagonist GSK3787 (Shearer et al., 2010) for 1 hour, and GW9662 was added for an additional 24 hours. Furthermore, cells were treated with GSK3787 or GW9662 alone for 24 hours. The PPARδ antagonist GSK3787 itself induced PLIN2 expression to the 1.5-fold. As expected, GW9662 induced PLIN2 expression to the threefold. Preincubation with GSK3787 completely abolished the effect of GW9662, as the induction to the 1.4-fold over control shows (Fig. 8). Similarly, GSK3787 induced CD36 expression to the 1.8-fold, whereas GW9662 induced the expression to the 1.4-fold; in combination, merely an induction to the 1.5-fold was found (Fig. 8). Taken together, we provide convincing evidence that the unexpected effects of GW9662 on the expression of the PPAR target genes PLIN2 and CD36 are mediated at least in part by the ability of GW9662 to directly or indirectly activate PPARδ.

Discussion

We identified PPARδ as mediator of the unexpected effects of the well-known PPARγ antagonist GW9662 on lipid metabolism in human and murine macrophages. The off-target action of GW9662 was confirmed in pharmacological antagonist experiments as well as in studies with siRNA-mediated knockdown of PPARδ. The awareness of the PPARδ-activating properties of GW9662 requires a reinterpretation of results obtained using GW9662 in previous studies.

Initially, we used GW9662 with the intention to show the reported regulatory function of PPARγ on PLIN2 (Buechler et al., 2001; Fan et al., 2009) and CD36 (Chawla et al., 2001; Moore et al., 2001; Tontonoz et al., 1998) expression in macrophages. Surprisingly, GW9662 induced the expression of these genes comparable to synthetic (rosiglitazone) and natural (15dPGJ2) PPARγ agonists (Figs. 1 and 3). Moreover,

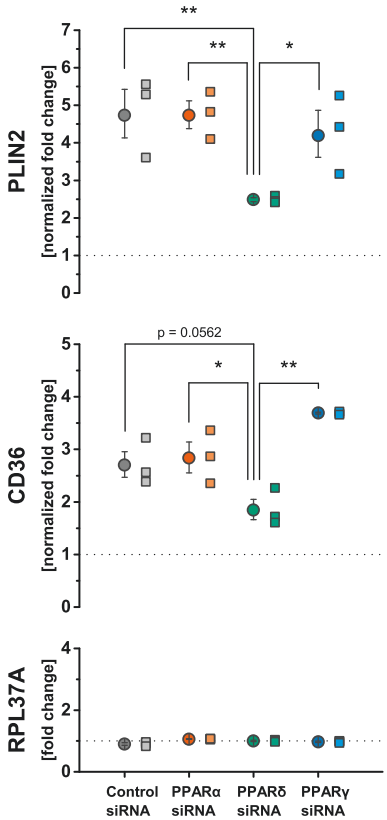

Fig. 7. siRNA-mediated PPARδ knockdown diminishes PLIN2 and CD36 induction by GW9662 in THP-1 macrophages. THP-1 monocytes were differentiated with PMA for 24 hours. Premature THP-1 cells were transfected with siRNA for the indicated PPAR subtypes or an appropriate negative control siRNA. The cells could recover for 72 hours. Then, cells were either treated with 10 μM GW9662 or vehicle (control) for 24 hours. Expression levels of PLIN2, CD36, and the reference gene RPL37A were assessed by RT-qPCR. Expression of the reference gene remained unchanged in the experimental setup and was used for normalization. Data are presented as fold change of treatment (GW9662) vs. the respective control (vehicle) in cells transfected with the indicated siRNAs. Vehicle controls were set to 1 and are represented by the dotted line. GW9662 induced the expression of PLIN2 and CD36 compared with the respective control, irrespective of the targeted PPAR subtype. The extent of PLIN2 and CD36 induction compared with the respective control is significantly lower in cells with PPARδ knockdown than with PPARα or PPARγ knockdown or the negative control siRNA. Circles represent the mean of normalized fold changes of three independent biologic experiments. Error bars display calculated maximum and minimum expression levels based on the S.E.M. of ΔΔct values. Squares represent the mean of two technical replicates of the biologic replicates. *$P < 0.05$; **$P < 0.01$; GW9662 treatment vs. GW9662 treatment and PPARδ knockdown. P values were calculated using one-way ANOVA with Tukey's post hoc test.

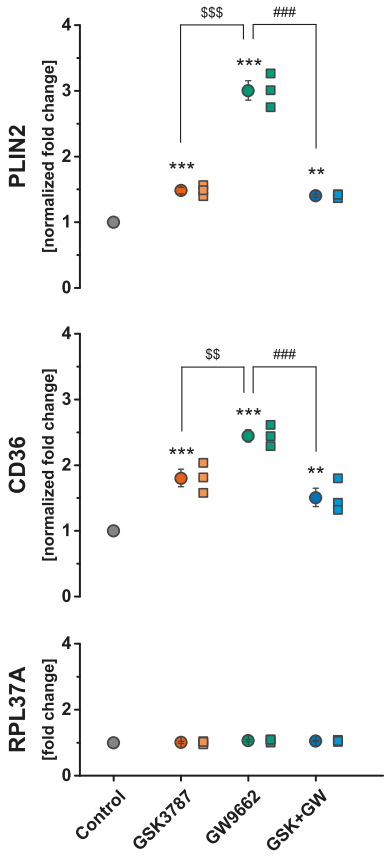

Fig. 8. PPARδ antagonist GSK3787 blocks PLIN2 and CD36 induction by GW9662 in THP-1 macrophages. Mature THP-1 cells were either treated with 10 μM GW9662 or 1 μM GSK3787 without pretreatment for 24 hours or pretreated with GSK3787 for 1 hour and then incubated with 10 μM GW9662 (GSK+GW) for another 24 hours. Expression levels of PLIN2, CD36, and the reference gene RPL37A were assessed by RT-qPCR. Expression of the reference gene remained unchanged in the experimental setup and was used for normalization. Treatment with GSK3787 or GW9662 alone significantly induced the expression of PLIN2 and CD36. In combination, GSK3787 blocks the induction of PLIN2 and CD36 by GW9662. Circles represent the mean of normalized fold changes of three independent biologic experiments. Error bars display calculated maximum and minimum expression levels based on the S.E.M. of ΔΔct values. Squares represent the mean of two technical replicates of the biologic replicates. $^{**}P < 0.01$; $^{***}P < 0.001$; all vs. control (0 μM GSK3787/GW9662); $^{\#\#\#}P < 0.001$; GSK+GW vs. GW9662 only; $^{\$\$}P < 0.01$; $^{\$\$\$}P < 0.001$; GW vs. GSK3787. *P* values were calculated using repeated-measures ANOVA with Tukey's post hoc test.

the combination of GW9662 with rosiglitazone even enhanced target gene expression (Fig. 1A), indicating a rather unlikely PPARγ-activating property of GW9662 or the activation of one or more PPARγ-independent signaling pathways. Therefore, GW9662 is not usable to elucidate PPARγ-mediated effects in macrophages. This observation leads to the question about the causes for the unexpected, even opposite, action of the accepted and frequently used PPARγ antagonist GW9662 in macrophages.

To address this question, known properties of the PPAR family were taken into consideration. The affinity of the PPAR subtypes for ligands is thought to be mainly determined by the size of the ligand-binding pocket (Bugge and Mandrup, 2010). Consequently, small molecules like GW9662 potentially bind to all PPAR subtypes. Furthermore, the DNA-binding domains of the PPAR subtypes show a high structural and sequence homology (Escher and Wahli, 2000); thus, most PPAR target genes are regulated by all three subtypes. Based on this, we performed combinatory experiments with synthetic PPARα and PPARδ antagonists. The PPARδ antagonist GSK3787 completely abolished the induction of PLIN2 and CD36 gene expression by GW9662 (Fig. 8), whereas the PPARα antagonist GW6471 did not (Supplemental Fig. 10). Moreover, we verified the contribution of PPARδ but not PPARα or PPARγ to the effects of GW9662 via siRNA-mediated knockdown of the PPAR subtypes (Fig. 7). These findings are in line with studies reporting the contribution of PPARδ to PLIN2 (Chawla et al., 2003; Fan et al., 2009) and CD36 (Li et al., 2004; Bojic et al., 2012) expression.

The proposed ability of GW9662 to bind to all PPAR subtypes (vide supra) has indeed been shown. Although GW9662 was identified as a potent antagonist of PPARγ, it also covalently modifies PPARα and PPARδ at higher concentrations (Leesnitzer et al., 2002). Notwithstanding the differences in the affinity of GW9662, the effects observed in this study are mediated by PPARδ. This is likely reasoned by the abundant expression of PPARδ, a hallmark of THP-1 cell differentiation (Vosper et al., 2001) (Supplemental Fig. 16). The resulting predominance of PPARδ in THP-1–derived macrophages (as well as in the other macrophage cell lines in this study; Supplemental Table 6) likely favors the effects of GW9662 via PPARδ over PPARγ and PPARα. This assumption is supported by the fact that differential tissue distribution is one main factor of subtype-specific effects of PPARs (Bugge and Mandrup, 2010). Concerning subtype distribution in macrophages, no effect of GW9662 treatment was observed (Supplemental Fig. 11). The induction of PPAR target genes by GW9662 as well as the blocking by a PPARδ antagonist suggests that GW9662 acts predominantly as a PPARδ agonist in macrophages. Contradictory to this, GW9662 has initially been characterized as an antagonist of PPARδ using an assay based on a fusion protein containing the ligand binding domain of PPARδ and the galactose-responsive transcription factor 4 (Leesnitzer et al., 2002) and was later confirmed with a similar approach (Leesnitzer et al., 2002; Seimandi et al., 2005). Interestingly, the authors of the initial study were not able to confirm the PPARδ antagonism in an assay based on the full-length receptor and a luciferase reporter. Here, even an activation of PPARδ was observed (Leesnitzer et al., 2002), indicating that GW9662 is indeed able to activate gene expression via PPARδ in "native" cellular systems like THP-1 macrophages. A recent report supports the notion of different effects in artificial and "native" systems (Brust et al., 2018). Though GW9662 shows no effect in a cell-based full-length PPARγ luciferase assay, the expression of the PPARγ target genes FABP4 and CD36 tends to be increased by GW9662 in "native" adipocyte-like cells (Brust et al., 2018). In line with these findings, GW9662 acted as a reliable inducer of PLIN2 gene expression even in low concentrations (1 μM) in our hands (Supplemental Fig. 4). Summarizing the above delineated results, we propose that GW9662 directly activates PPARδ in macrophages but do not prove this interaction experimentally. Thus, based on our data, we cannot completely rule out that GW9662 induces a signaling cascade that finally leads to PPARδ activation. In this context, the increased production of endogenous PPARδ

ligands via the cyclooxygenase (COX) pathway (Gupta et al., 2000) is unlikely, as GW9662 does not affect gene expression of COX1 and COX2 (Supplemental Fig. 12B). However, the elucidation of a potential signaling pathway indirectly activating PPARδ goes beyond the scope of this single work.

Notwithstanding that the mechanism of PPARδ activation by GW9662 is not fully elucidated, our data convincingly demonstrate that GW9662 causes notable effects in macrophage cell models and may thus provoke erroneous interpretation of experimental data (especially with respect to the contribution of PPARγ). Administration of GW9662 to macrophages leads to remarkable changes in the expression of PPAR target genes. About 44% of the target genes included in our profiling were regulated. This profiling analysis allows the attribution of these genes to PPAR subtypes (Supplemental Table 4). However, an unambiguous assignment is not possible. The expression of PPAR target genes is undoubtedly dependent on the differential expression of the PPAR subtypes in cells as well as the "setting" of the target sites like chromatin modification and the combination of other transcription factors, which is highly cell-type specific (Nielsen et al., 2006).

For this reason, the expression profiling data should be interpreted as PPAR-mediated effects in general rather than subtype-specific effects. Given the identification of PPARδ as mediator of PLIN2 and CD36 regulation, most of the genes in the array are likely also regulated via activation of PPARδ by GW9662. Although the predominance of PPARδ in THP-1 macrophages supports this assumption, we cannot give final proof. However, there are substantial changes in PPAR target gene expression induced by GW9662. These genes are involved in immunity and inflammation, signal transduction and transcription, and glucose metabolism and, particularly, lipid metabolism (Supplemental Table 5; Supplemental Fig. 7). More than half of the genes implicated in lipid metabolism included in the array were regulated by GW9662. In addition to PLIN2 (lipid storage) and CD36 (lipid import), other genes involved in the import, transport, storage, and export of lipids as well as β-oxidation and lipogenesis are affected. Most of the upregulated genes can be assigned to lipid import, lipid transport, and lipogenesis (Supplemental Fig. 8). Consequently, accumulation of fatty acids, and thus their storage form triglycerides, in the cell caused by GW9662 can be expected. In particular, the upregulation of acetyl-coenzyme A carboxylase-α as the rate-limiting enzyme in fatty acid synthesis (Tong and Harwood, 2006) is noteworthy. This enzyme catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is not only a substrate for fatty acid biosynthesis but is also known to suppress β-oxidation (Tong and Harwood, 2006). Thus, synthesis and storage of fatty acids is more likely than degradation. Indeed, we have shown that GW9662 enhances lipogenesis from ^{14}C-acetate in THP-1 macrophages (Fig. 6). The increased lipogenesis necessitates the storage (or export) of the resulting free fatty acids to prevent potential harmful lipotoxic effects. Macrophages treated with GW9662 showed an augmented triglyceride content, suggesting that enhanced lipogenesis indeed causes fatty acid synthesis and storage in the form of triglycerides (Fig. 5). This finding is in line with earlier reports from breast cancer cells. GW9662 causes triglyceride accumulation in T47D cells (Lea et al., 2004) as well as BT474 and MCF-7 cells (Kourtidis et al., 2009). In the latter two cell lines, total fat and triglyceride stores were increased by GW9662 treatment. The higher lipid levels in BT474 cells are primarily caused by an increase in palmitic and stearic acid (Kourtidis et al., 2009). This indicates a higher rate of fatty acid synthesis, which is in line with our observations in the THP-1 cell model. Furthermore, GW9662 downregulates hormone-sensitive lipase and patatin-like phospholipase domain containing 2 in BT474 cells, the major enzymes in intracellular triglyceride breakdown (Kourtidis et al., 2009). However, patatin-like phospholipase domain containing 2 remained unaffected, whereas hormone-sensitive lipase was upregulated in our expression profiling (Supplemental Table 3) but was downregulated in confirmatory RT-qPCR experiments (Supplemental Fig. 12A) in GW9662-treated THP-1 cells. Taken together, though augmented lipid import can be excluded (serum-free conditions), the reduced triglyceride breakdown might also contribute to triglyceride accumulation, but the main cause is likely the increase in lipogenesis by GW9662.

Given that GW9662 regulates PLIN2 and CD36 via PPARδ, the observed effects on lipogenesis and triglyceride accumulation in response to GW9662 (Figs. 5 and 6) might be a global consequence of the activation of PPARδ. In contrast to this assumption, PPARδ is known for the stimulation of fatty acid oxidation in different tissues such as adipose tissue and skeletal muscles (Reilly and Lee, 2008). However, the role of PPARδ in lipid metabolism of macrophages is insufficiently characterized, and results of studies on this topic are inconsistent. PPARδ apparently exerts different effects on lipid metabolism and thus is not invariably a stimulator of lipid catabolism. For example, very low-density lipoprotein (VLDL) even stimulates triglyceride accumulation via activation of PPARδ in macrophages (Chawla et al., 2003; Bojic et al., 2012). Interestingly, pretreatment with PPARδ agonists attenuates VLDL-induced triglyceride accumulation by indirect inhibition of lipoprotein lipase (Bojic et al., 2012). In addition, fatty acid import via CD36 is upregulated (Li et al., 2004; Bojic et al., 2012), and β-oxidation of fatty acids is stimulated (Lee et al., 2006; Bojic et al., 2012) via PPARδ. Collectively, these mechanisms result in lower triglyceride accumulation in cells treated with PPARδ agonists than the THP-1 cells that are faced with VLDL alone (Bojic et al., 2012). However, the main factor might be the inhibition of VLDL triglyceride hydrolysis and not the β-oxidation of fatty acids; thus, lowering triglyceride accumulation in this setup would not be in contrast to our findings. Interestingly, both VLDL and PPARδ agonists as well as their combination induce expression of PLIN2 (Chawla et al., 2003; Bojic et al., 2012), which is known to stimulate triglyceride storage and synthesis in macrophages and inhibits β-oxidation (Larigauderie et al., 2006), contrasting the abovementioned results. Thus, PPARδ possibly acts as a switch; moderate activation of PPARδ (e.g., by VLDL or a PPARδ agonist) might favor lipid storage, whereas excessive PPARδ activation could shift the metabolism to lipid catabolism, likely to prevent lipid overload. Furthermore, PPARδ selectively responds to different "stimuli" of lipid metabolism; VLDL reliably activates PPARδ (Chawla et al., 2003; Lee et al., 2006; Bojic et al., 2012), whereas high-density lipoprotein and low-density lipoprotein do not (Chawla et al., 2003). The relationship of oxidized low-density lipoprotein and PPARδ is not clear (Vosper et al., 2001; Chawla et al., 2003; Li et al., 2004). As GW9662 induces PLIN2 and CD36 only

moderately and no additional stimuli were used, we expect moderate activation of PPARδ. This might lead to a "lipid storage" scenario in THP-1 macrophages in our hands. As we did not use any lipoproteins as stimulus, the studies of Vosper et al. (2001) on PPARδ and lipid accumulation in macrophages most accurately resemble our experimental setup. In line with our findings, the treatment with a PPARδ agonist upregulated the expression of genes involved in lipid metabolism, including CD36 and PLIN2, in this study. Consequently, the PPARδ activation promotes lipid accumulation in THP-1 macrophages in the presence of serum (Vosper et al., 2001). Taken together, the sparse data available on PPARδ and lipid accumulation in macrophages are in agreement with our finding that GW9662 promotes lipid accumulation via activation of PPARδ and consequent induction of genes involved in lipid metabolism, such as PLIN2 and CD36.

Conclusion

The widely used PPARγ antagonist GW9662 is unsuitable for the investigation of PPARγ signaling in human and murine macrophages because of its prominent off-target effects, likely occurring via PPARδ activation. Moreover, GW9662 itself induces expression of target genes that have been linked to PPARγ, such as PLIN2 and CD36, rather than blocking it. The induction of several additional genes by GW9662 leads to notable alterations in cellular metabolism, especially lipid metabolism, manifested as augmented lipogenesis and triglyceride accumulation. Thus, data obtained with GW9662 might lead to misinterpretation, particularly in macrophages and regarding lipid metabolism. Based on our findings, PPAR subtype expression of the used cell line should be considered when GW9662 is applied. Furthermore, the structurally related antagonist or inverse agonist T0070907 (Lee et al., 2002; Hughes et al., 2014; Brust et al., 2018) might be a useful alternative depending on the experimental setup (Supplemental Fig. 13), as we observed similar effects to GW9662 on RNA but not necessarily on the protein level. However, siRNA-mediated knockdown of PPARγ or PPARγ-deficient cells should be used in addition to PPARγ antagonists to confirm PPARγ-dependent regulatory effects. Considering the relevance of PPARs as therapeutic targets, the collection of reliable data is essential to draw appropriate conclusions. In this regard, our work will help to accurately interpret existing studies with GW9662 and to better design future studies.

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Authorship Contributions

Participated in research design: Schubert, Becher, Heller, Grün, Lorkowski.

Conducted experiments: Schubert, Becher, Wallert, Maeß, Große.

Contributed new reagents or analytic tools: Rennert, Mosig, Heller, Grün, Lorkowski.

Performed data analysis: Schubert, Becher, Wallert, Maeß, Abhari, Rennert, Große, Heller.

Wrote or contributed to the writing of the manuscript: Schubert, Becher, Mosig, Grün, Lorkowski.

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The vitamin E long-chain metabolite α-T-13'-COOH is a reliable suppressor of CCL2 / MCP-1 and modulates regulatory mechanisms of MAPK and NFκB signaling and the inflammatory response of macrophages

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Abbreviations:

α-T	α-Tocopherol ((2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro-2H-chromen-6-ol)
α-T-13'-COOH	α-13'-carboxychromanol 13-((2R)-6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid
Acod1	Aconitate decarboxylase 1 (Synonym: Immune-responsive gene 1 (Irg1))
CCL2	C-C motif chemokine ligand 2 (Synonym: monocyte chemotactic protein 1 (MCP-1))
cDNA	Complementary deoxyribonucleic acid
Cdkn1a	Cyclin dependent kinase inhibitor 1A (Synonym: p21 or $p21^{WAF1/Cip1}$)
C_t	Threshold cycle
CVD	Cardiovascular disease
Cxcl11	C-X-C motif chemokine 11
DHMEQ	Dehydroxymethylepoxyquinomicin (2-Hydroxy-N-((1S,2S,6S)-2-hydroxy-5-oxo-7-oxabicyclo[4.1.0]hept-3-en-3-yl)benzamide)
DMEM	Dulbecco's modified eagle's medium
Dusp	Dual specificity phosphatase (Synonym: Mitogen-activated protein kinase phosphatase 1 (MKP-1))
ECL	Enhanced chemiluminescence
ERK	Extracellular-signal regulated kinase
ELISA	Enzyme-linked immunosorbent assay
Hif1α	Hypoxia-inducible factor 1-alpha
Hmox1	Heme oxygenase 1
IκB	Nuclear factor of kappa light polypeptide gene enhancer B-cells

	inhibitor, alpha
IL	Interleukin
Irak3	Interleukin 1 receptor associated kinase 3
	(Synonym: Irak-m)
JNK	c-Jun n-terminal kinase
JSH-23	NFκB Activation Inhibitor II
	(4-Methyl-N^1-(3-phenylpropyl)benzene-1,2-diamine)
LCM	Long-chain metabolite (of vitamin E)
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS2	Nitric oxide synthase 2, inducible (Synonym: iNOS)
p38	p38 mitogen-activated protein kinase
PD98059	Dual specificity mitogen-activated protein kinase kinase 1 inhibitor (2'-Amino-3'-methoxyflavone)
PTGS2	Prostaglandin-endoperoxide synthase 2
	(Synonym: Cyclooxygenase-2 (COX-2))
PVDF	Polyvinylidene fluoride
RAW264.7	*Mus musculus* macrophages, abelson murine leukemia virus transformed
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SB203580	p38 mitogen-activated protein kinase inhibitor (4-(4'-Fluorophenyl)-2-(4'-methylsulfinylphenyl)-5- (4'-pyridyl)-imidazole)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Standard error of mean
SP600125	Inhibitor of c-Jun n-terminal kinase (Anthra[1,9-cd]pyrazol-6(2H)-one,1,9-pyrazoloanthrone)
TNF	Tumor necrosis factor (Synonym: tumor necrosis factor alpha (TNFα))
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3 (Synonym: A20)

Abstract

Scope: The long-chain metabolites of vitamin E (LCM) are proposed as the active regulatory metabolites of vitamin E. The anti-inflammatory LCM provide an explanatory approach for the inconsistent effects of Vitamin E on inflammatory-driven diseases. We examined the modulation of cytokine expression and release from macrophages, a fundamental process in many diseases, to gain insights into the anti-inflammatory mechanisms of the LCM α-T-13'-COOH.

Methods and results: Suppressed gene expression of *Ccl2, TNFα,* and *IL6* in response to LPS by 24 h pre-treatment with α-T-13'-COOH in RAW264.7 macrophages was revealed using RT-qPCR. Further, suppressed secretion of IL1β and CCL2 was found in this setup using flow cytometry. In contrast, 1 h pre-treatment suppressed only CCL2. Consequent gene expression analysis within 24 h of α-T-13'-COOH treatment revealed the induction of MAPK and NFκB negative feedback regulators including the 'master regulators' *Dusp1/Mkp1* and *Tnfaip3/A20*. Approaches with immunoblots and chemical antagonists suggest a feedback induction via activation of ERK / p38 and NFκB pathways.

Conclusions: CCL2 is reliably suppressed by α-T-13'-COOH in macrophages, implicating new putative roles in related diseases like cardiovascular and immunological disorders. The indirect suppression of MAPK and NFκB pathways appears as considerable process in the anti-inflammatory mechanism of α-T-13'-COOH.

Introduction

The scientific community is preparing for the journey into the next 100 years of vitamin E research. The past almost 100 years of research on the enigmatic substance 'X' [1] revealed a structurally close group of lipid-soluble compounds henceforth called 'Vitamin E'.[2,3] The property of the most important representative, α-tocopherol (α-T), to serve as unique cure for 'Ataxia with isolated vitamin E deficiency' underpins its status as vitamin in human.[4] Insufficient plasma levels of α-T are further related to stunting of growth, anemia and increased infection.[5] Despite this, roles in cognitive impairments, age-related diseases, cancer and cardiovascular disease (CVD) were ascribed to Vitamin E.[6] While positive effects in the restoration of an impaired immune function are well-documented, data on a protective effect of Vitamin E on CVD is not convincing. Human intervention studies on this issue lead to contrasting results.[7–9] The pitfalls in trying to predict Vitamin E's effects are likely due to complex mechanisms of action going far beyond its prominent function as antioxidant.[10,6,11]

The identification of the potentially physiologically relevant Vitamin E long-chain metabolites (LCM) in human blood adds up to this complexity.[12] These metabolites are hypothesized to represent a naturally metabolically activated form of vitamin E.[13] This notion is based on the known metabolic activation of other lipid-soluble vitamins[13], as well as repeatedly reported more potent or distinct effects of the LCM from their precursors.[12,14–16] Intriguingly, effects of LCM have frequently been shown in immune cells and with respect to inflammatory activities. Recently reported *in-vivo* accumulation of LCMs in immune cells points to the potential of LCMs to influence (patho-) physiological processes related to the cardiovascular system and immune function.[17] Interestingly, this coincides on one hand with the striking role of vitamin E in modulation of immune function[18,19] and with its controversial role in CVD prevention on the other hand.[7–9]

This work focusses on the LCM α-T-13'-COOH as human physiology leads to the preferred retention of α-T, while the β-, γ-, and δ-forms are primarily transformed and excreted.[20] The LCM α-T-13'-COOH is formed in two steps from its precursor α-T: via 1) ω-hydroxylation to the

intermediate alcohol α-T-OH and 2) a subsequent ω-oxidation.[20] The carboxychromanol LCM can be expected as most relevant in human and was repeatedly shown to be more potent than its precursors α-T and α-T-OH.[12,14–16] As indicated above, LCM show a high potential to modulate inflammatory processes, especially in immune cells. So, α-T-13'-COOH was convincingly demonstrated to accumulate at sites of inflammation in mice, especially in immune cells, and suppress peritoneal inflammation and reduce hyper-reactivity in an asthma model.[17] These effects are ascribed to α-T-13'-COOH's repeatedly reported action as inhibitor of 5-lipoxygenase.[17,21,22] Beside this, further anti-inflammatory effects were reported: α-T-13'-COOH was shown to modulate the gene expression of cytokines, the expression of cyclooxygenase-2 (PTGS2) and nitric oxide synthase 2 (NOS2/iNOS) and the release of nitric oxide and certain prostaglandins in lipopolysaccharide (LPS)-stimulated murine macrophages.[16,23]

Albeit these anti-inflammatory effects in macrophages are described, the underlying molecular mechanisms are not completely understood. The aim of this study was therefore to elucidate the molecular reaction of macrophages to α-T-13'-COOH leading to the reported modulation of the LPS-response. Particular focus was on the modulation of cytokine expression since sparse data was reported before.[16] In the present report we characterize the cellular response of murine macrophages to α-T-13'-COOH with focus on mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling and how α-T-13'-COOH affects cytokine expression and secretion. Further, we discovered in the course of this work that α-T-13'-COOH suppresses C-C motif chemokine ligand 2 (CCL2) reliably and uniquely among the observed cytokines and hence examined this effect more detailed.

Materials and Methods

Chemicals

If not indicated otherwise, chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Thermo Fisher Scientific (Schwerte, Germany; includes Fermentas, Applied Biosystems, Invitrogen and Life Technologies), or Merck Millipore (Darmstadt, Germany). All chemicals were used as received from the supplier.

Cell Culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA, USA) were cultivated as described earlier.[24] In brief, cells were cultivated in a mixture of FBS-supplemented high glucose DMEM (2/3; Sigma-Aldrich) and used culture medium (1/3) and split thrice a week. Cells (2 x 10^6 per 75 cm^2-flask) were seeded in the same mixture and treated 24 h later in serum-free DMEM as indicated in the figure legends. Cells were harvested as described below.

RNA isolation and cDNA synthesis

Total RNA was isolated from cell lysates (obtained by scratching in provided buffer) using Qiagen RNeasy Mini kit including on-column DNase I digestion (Qiagen, Hilden, Germany) and cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis Kit (Fermentas) as described previously.[25]

Quantitative real-time RT-PCR (RT-qPCR)

Primer pairs (Supplementary Table S1) for RT-qPCR were designed using PrimerExpress software version 2.0.0 (Applied Biosystems) or PrimerBlast[26] and were purchased from Invitrogen. RT-qPCR was performed as outlined in detail previously[25]. In brief, target amplification was measured using SYBR Green on a LightCycler480 II instrument (Roche Diagnostics, Mannheim, Germany). C_t values were calculated using the LightCycler software (version 1.5.0.39) and further data analysis was conducted using Microsoft Excel. *Ppib* was used as reference gene for normalization.

Determination of cytokine levels

Supernatants were collected 24 h after addition of 100 ng/ml LPS to cells and debris was removed by centrifugation (400 x g, 5 min, 4 °C). Samples were stored at -80 °C until measurement. Levels of CCL2, IL1-α, IL1-β and TNFα were analyzed using BioLegend's (BioLegend Gmbh, Koblenz, Germany) LEGENDplex™ system according to manufacturer's instructions on an Attune NxT Acoustic Focusing Cytometer (Life Technologies) using Attune Software (version 2.4). Concentrations in pg/ml were calculated using LEGENDplex™ Data Analysis Software (version 8.0) and data was further processed using Microsoft Excel. Levels of IL6 were determined by an in-house made ELISA.

Immunoblot

Cells were harvested using a buffer with pH 6.8 containing 62.5 mM Tris-HCl, 2 % (w/v) SDS, 50 mM DTT, 0.01 % (w/v) bromophenol blue and 10 % glycerol. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (VWR, Darmstadt, Germany). The transfer buffer with pH 8.3 contained 0.25 M Tris, 1.92 M glycine, 0.1 % (w/v) SDS and 20 % methanol. SignalBoost™ Immunoreaction Enhancer kit (Calbiochem, Darmstadt, Germany) was used to incubate with anti-phospho-ERK (ab47339), anti-phospho-p38 (ab178867), anti-ERK (ab17942) and anti-p38 antibody (ab32142; all Abcam, Cambridge, UK). Tris Buffered Saline (pH 7.4, 200 mM Tris, 1.37 M NaCl) with 0.5 % (w/v) dry milk was used for anti-α-Tubulin antibody (clone B-5-1-2, Sigma-Aldrich) incubation. Appropriate horseradish-labeled secondary antibodies (DAKO, Hamburg, Germany) and Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) were used for detection.

Semi-Synthesis of α-T-13'-COOH and test for endotoxin contamination

The precursor substance garcinoic acid was isolated from *garcinia kola* nuts[27] and semi-synthesis of the LCM α-T-13'-COOH from garcinoic acid was conducted as described before.[14] Purity was confirmed by high-performance liquid chromatography and endotoxin-

contamination was excluded using the GenScript ToxinSensor™ Kit according to manufacturer's instructions.

Statistics

Detailed information on the graphical presentation and statistics applied on the individual experiments are delineated in the respective figure legends. Analysis of variance (ANOVA) as well as post-hoc tests were performed using OriginLab's Origin 2016 and t-Tests were calculated using Microsoft Excel. Appropriate statistical tests were chosen based on the experimental design and hypothesis.

Results and Discussion

Inflammatory processes are underlying virtually all major diseases and macrophages are considered playing a key role in inflammatory and immune responses.[28] Since cytokines are essential to macrophage behavior and moreover in the overall regulation of inflammation and immune functions, they represent a highly important research object and target for treatment strategies.[29] Albeit the immunomodulatory and anti-inflammatory capacity of α-T-13'-COOH was repeatedly shown[16,17,21,22], data on the modulation of cytokine expression and release from macrophages is sparse and the underlying regulatory processes remain elusive.[16] We here characterize the modulation of cytokine expression and secretion by α-T-13'-COOH in response to LPS and further provide evidence that the key inflammatory pathways via MAPK and NFκB are feedback regulated in response to α-T-13'-COOH.

The LCM α-T-13'-COOH differentially modulates the inflammatory response of macrophages to LPS and turns out to reliably suppress CCL2

We earlier reported that pre-treatment for 24 h with α-T-13'-COOH followed by a simultaneous incubation with α-T-13'-COOH and LPS leads to an alteration in the cytokine gene expression of RAW264.7 murine macrophages.[16] As first step in the characterization of the underlying mechanism we split the incubation regime[16] and compared a long pre-treatment period (24 h) with a short period (1 h) before sole addition of LPS for 24 h. This experimental setup revealed that the 24 h pre-treatment period leads to a significant suppression of the gene expression of *Ccl2, Tnf* and *IL-6* (Figure 1A). Pre-treatment for 1 h, by contrast, does not lead to a suppression of *Tnf* and *IL6.* Surprisingly, *Ccl2* gene expression is also significantly suppressed with 1 h of pre-incubation (Figure 1B). Using the same incubation regimes, we examined the cumulated cytokine secretion within the 24 h period of LPS treatment. Pre-treatment for 24 h lead to a significant suppression of CCL2 and IL1-β release and a diminished secretion of IL1-α in tendency. The release of TNFα and IL6 is not significantly altered but highly variable and in the case of TNFα even augmented (Figure 1C). With the exception of CCL2, which nicely follows the found suppression of gene expression, the gene expression and protein secretion

after 24 h of LPS is not congruent for the examined cytokines (Figs. 1A + 1C). This is no surprising finding: although cytokine expression is tightly regulated at the transcriptional level, complex post-translational mechanisms regulate their secretion.[30] As with the gene expression in 1 h pre-treatment, we found a contrasting result when examining the cytokine release with 1 h pre-treatment. Here, the secretion of IL1-α and IL6 are significantly augmented, while the release of IL1-β and TNFα in response to LPS are not altered. In strong contrast to this, CCL2 release is also suppressed under these conditions (Figure 1D).

In sum, the 24 h pre-treatment with α-T-13'-COOH leads to a rather anti-inflammatory effect since the expression of the pro-inflammatory genes examined is suppressed or unaltered. Further, the secretion of the pro-inflammatory cytokines CCL2, IL1-β and IL1-α is suppressed in response to LPS. However, TNFα excretion is in tendency augmented, yet highly variable in our hands. The release of IL6, a context-specifically pro- or anti-inflammatory cytokine[31], is also variable in 24 h pre-treatment condition. In contrast, IL6 is significantly elevated when α-T-13'-COOH is applied 1 h before LPS and so is also the pro-inflammatory cytokine IL1-α. This leads us to conclude that macrophages adapt within 24 h to α-T-13'-COOH and this process renders them more tolerant to LPS-treatment. Such mechanisms have repeatedly been described and were termed 'endotoxin (cross-) tolerance'.[32] Short pre-treatment with α-T-13'-COOH, in contrast, prevents this adaption. Notwithstanding this, the ability of α-T-13'-COOH to suppress CCL2 gene expression and protein secretion in both conditions is striking. This points to a unique regulatory mechanism among the here examined cytokines.

24 h pre-treatment with α-T-13'-COOH suppresses cytokine expression already in the early and also in the late course of the LPS-response

Given that the duration of pre-incubation with α-T-13'-COOH differently affects the response of macrophages to LPS, we sought to identify the time-point this effect evolves in the course of the LPS response. Cells were thus pre-treated with α-T-13'-COOH for 1 h or 24 h and harvested either at the time-point of LPS addition (0 h) or 1 h, 3 h, 6 h and 24 h later, respectively, and examined for the gene expression of the cytokines. Interestingly, after 24 h of pre-treatment, gene expression of *Ccl2, Tnf* and *IL6* is already below the level of the vehicle

controls (time-point 0 h). This suppression is further found at all observed time-points during the LPS challenge (Figure 2A). The interleukins 1α and 1β are differently regulated, i.e. the gene expression is in tendency suppressed at 1 h, 3 h, and 6 h but not at 24 h after LPS addition and not at time-point 0 h (Figure 2A). Accordingly, α-T-13'-COOH seems to induce a cellular state characterized by a suppression of the basal expression of *Ccl2, Tnf* and *IL6,* while *IL1a* and *IL1b* are not susceptible to this effect. These suppressive regulatory mechanisms seem to further take effect when LPS is applied. In support of this notion, a suppression of the cytokine gene expression is not found after 1 h pre-treatment with α-T-13'-COOH (Figure 2B). In fact, the expression of *IL1a, IL1b, Tnf* and *IL6* is in tendency elevated. This tendency is preserved over the whole time-course of LPS treatment. In conclusion, the suppressive mechanisms could not form within 1 h and consequently the LPS effect is not suppressed. The short pre-treatment with α-T-13'-COOH rather elevates the gene expression of cytokines, which is in part reflected by the significantly elevated secretion of IL1-α and IL6 protein (Figure 1D). This points to an augmentation of LPS signaling pathways by α-T-13'-COOH, classically the MAPK and NFκB pathways.[33] *Ccl2* gene expression is obviously regulated differently. Here, the expression at time-point 0 h is not changed (on average, yet variable) but is quickly suppressed in the course of LPS treatment by pre-treatment with α-T-13'-COOH. A tendency is observed already after 1 h and significant effects are found after 3 h, 6 h and 24 h (Figure 2B). In conclusion, *Ccl2* is either differently regulated by α-T-13'-COOH among the observed cytokines or is more sensitive to the proposed negative regulatory feedback of LPS signaling pathways than the other cytokines.

Taken together, 24 h pre-treatment with α-T-13'-COOH leads to a suppression of cytokine gene expression already at early stages of the time course, while 1 h pre-incubation in tendency elevates the expression (Figure 2C). The suppression of *IL1a* and *IL1b* gene expression during the LPS response in sum might explain the reduced protein secretion observed with 24 h pre-treatment with α-T-13'-COOH (Figure 1C). In contrast, the augmented *IL1a* gene expression by 1 h pre-treatment might account for the higher protein secretion

observed (Figure 1D). However, several mechanisms regulating IL1 secretion have been described[30] and α-T-13'-COOH might also affect these.

Comparison of the time-courses of *Ccl2* gene expression with the other cytokines in Figure 2C nicely depicts the unique regulation among the examined cytokines. While 24 h pre-treatment leads to a suppressed LPS response in all observed cytokines (circles), only *Ccl2* expression is gradually suppressed over time with 1 h pre-incubation (squares) and finally both treatments show the same level of about 70 % suppression at 24 h. Hence, α-T-13'-COOH represents a reliable suppressor of *Ccl2* gene expression and release (Figure 1). Given that α-T-13'-COOH suppresses *Ccl2* both, after a long period and a short period (and then very fast) of application, we propose that α-T-13'-COOH either 1) activates a repressor of *Ccl2* gene transcription acting quickly but also sustained over 48 h, or 2) induces a fast and sustained negative feedback mechanism, either very specifically or very potently acting on *Ccl2* gene expression or 3) that both proposed mechanisms are involved in our findings. However, the regulation of *Ccl2* gene transcription was early shown to be regulated in a complex manner involving the phosphorylation, translocation and assembly of transcription factor complexes[34]. The elucidation of the exact mechanism of gene suppression in response to α-T-13'-COOH is beyond the scope of this work.

The LCM α-T-13'-COOH induces an adaption process in macrophages characterized by the up-regulation of negative feedback regulators of MAPK and NFκB signaling

Given that the 24 h pre-treatment, but not the 1 h pre-treatment leads to a broader suppression of cytokine gene expression, we concluded that a cellular response to α-T-13'-COOH within these 24 h is essential for this effect. Such adaption and reprogramming processes called 'endotoxin tolerance' have been reported for different compounds.[35] These mechanisms are mostly characterized by a stimulation of MAPK and NFκB signaling and provoke a negative feedback mechanism ultimately leading to a suppressed reaction to LPS.[35] The latter is essentially what we found for α-T-13'-COOH. Consequently, we examined the gene expression of a range of relevant feedback mediators[35–38] in time-courses of α-T-13'-COOH

treatment. As mentioned before, regulation of *Ccl2* is exceptional in our hands, which lead us to also examine the time-course of its gene expression. Further, the central mediators of endotoxin tolerance, MAPK and NFκB have also been repeatedly connected to the expression of CCL2, and might thus account for our observations.[39–41]

In line with the proposed 'endotoxin tolerance-like' mechanism, we observed an induction of the gene expression of several known mediators in response to α-T-13'-COOH. Interestingly, we also found a time-course of *Ccl2* gene expression that strongly suggests a negative feedback regulation. *Ccl2* expression is quickly induced after 1 h to about the 16-fold of the level at 0 h of α-T-13'-COOH treatment and also quickly counterbalanced below the initial level after 6 h, 10 h and 24 h (Figure 3A). This strongly suggests that α-T-13'-COOH activates a positive regulatory pathway of *Ccl2* gene expression within minutes, provoking a negative feedback mechanism that is effective already after a few hours. Despite this specific effect on *Ccl2*, we found cellular reactions to α-T-13'-COOH implying global consequences. The gene expression of the most important negative feedback regulators of MAPK (in response to LPS) in RAW264.7, *Dusp1, Dusp2* and *Dusp16*,[42] is induced in response to α-T-13'-COOH. Here, the effects on *Dusp1* and *Dusp16* are striking, since they are significantly induced already after 1 h (about 3-fold and 2-fold, respectively) and stay elevated until 24 h (about 15-fold and 4-fold, respectively). *Dusp2* shows a delayed reaction with induction after 3 h and is quickly counterbalanced and below the initial level after 10 h and 24 h (Figure 3B). Moreover, the gene expression of the classical negative NFκB regulators nuclear factor of kappa light polypeptide gene enhancer B-cells inhibitor (IκB), IKBα, IKBβ and IKBε,[43] as well as the 'key negative regulator' *Tnfaip3* (also known as A20)[44] are induced short time after α-T-13'-COOH treatment (Figure 2C). However, effect sizes are small for *Nfkbia* and *Nfkbie* (about 2-fold to 3-fold induction) and the expression in the late phase of the time-course is highly variable. *Nfkbib*, by contrast, shows a sustained elevation over the initial level from 3 h until 24 h (about 3-fold). Remarkably, *Tnfaip3* gene expression shows a negative feedback pattern in response to α-T-13'-COOH with a significant induction already after 1 h until 6 h and a peak at 3 h (5.3-fold) after treatment followed by a counterbalancing after 10 h until 24 h.

We further included the atypical IKBζ in our measurements since it was shown to be critical for CCL2 secretion from macrophages[45] although it contrasts the other genes with its main function as transcriptional activator of NFκB target genes.[43] However, our results are in strong contrast to the reported finding that *Nfkbiz* mRNA and *Ccl2* mRNA levels are tightly and positively correlated in RAW264.7 macrophages.[45] In our hands, *Nfkbiz* is already significantly induced after 1 h (about 5-fold) and we detect high levels of *Nfkbiz* transcripts at every time-point until 24 h (about 8-fold) compared to the time-point of α-T-13'-COOH addition (Figure 3C). While *Ccl2* mRNA is up-regulated at early time-points (1 h and 3 h), it is counterbalanced already after 6 h and stays suppressed. Thus, the transcript levels from 6 h on are completely contrasting the *Nfkbiz* levels. In conclusion, IkBζ is unlikely to account for the regulation of Ccl2 gene expression in response to α-T-13'-COOH in RAW264.7 in our hands.

To sum up, α-T-13'-COOH induces a negative feedback response to MAPK and NFκB signaling at the level of gene expression. We observe a quick and sustained induction of the MAPK feedback regulators *Dusp16* and *Dusp1*. It is of note that the latter is regarded as the most important counter-regulator of MAPK signaling in macrophages.[46] Further, the NFκB target genes *Nfkbiz* and *Tnfaip3* which are classified as early primary response genes[47,48] (in response to LPS and thus NFκB[49]) are significantly induced within 1 h after α-T-13'-COOH treatment. This strongly suggests that α-T-13'-COOH directly signals via NFκB. In support of this, *Ccl2*, considered as late primary response gene, is also significantly induced after 1 h. Moreover, further genes implicated in the buildup of macrophage endotoxin tolerance are induced in response to α-T-13'-COOH. *Hmox1, Acod1/Irg1* (a further primary response gene) and *Tnfaip3,* proposed to build an axis to establish tolerance in RAW264.7[37], are all significantly induced (Supplementary Figure 1 and Figure 3C). Further, *Cdkn1a/p21* induction and subsequent suppression of *Cxcl11* have been implicated in tolerance.[38] Both are induced by α-T-13'-COOH and *Cxcl11* (a further primary response gene) shows a negative feedback regulation comparable to *Ccl2* (Supplementary Figure S1). Despite the strong induction of *Tnfaip3,* we observe diverging reactions of the gene expression of further negative regulators of the toll-like receptor 4 pathway. While *Hif1α* is induced by α-T-13'-COOH, the regulation of

the negative 'master regulator' *Irak-3*[50] is in tendency suppressed (Supplementary Figure S1). Taken together, α-T-13'-COOH induces a cellular response that is in large part comparable to the reported endotoxin tolerance mechanisms but shows a distinct pattern. We conclude that the cellular adaption mechanism to α-T-13'-COOH is likely mediated by MAPK pathways as well as NFκB pathways.

The LCM α-T-13'-COOH induces the phosphorylation of ERK1/2 and p38

The observed fast and sustained induction of the negative MAPK regulators *Dusp1* and *Dusp16* genes lead us to conclude that MAPKs take part in the induction of the adaption in response to α-T-13'-COOH. To test this hypothesis, we examined the phosphorylation of ERK1, ERK2 and p38 proteins within 30 min after α-T-13'-COOH treatment. In support of our hypothesis, we found that ERK1 and ERK2 phosphorylation is significantly induced about 3-fold after 30 min compared to the untreated controls (Figure 4A + 4B). The same was found for the phosphorylation of p38 in response to α-T-13'-COOH. Significant effects occurred 20 min and 30 min (2.5-fold) after addition of α-T-13'-COOH (Figure 4C) and a significant elevation over the level of the vehicle control was observed at all time-points for all proteins (Figure 4D). These findings indicate that the phosphorylation of ERK1/2 and p38 at least in part contribute to the adaption process α-T-13'-COOH provokes in RAW264.7. Further, ERK and p38 have repeatedly been connected to the expression of *Ccl2* in RAW264.7 and are thus likely to contribute to the observed induction of the gene expression in the early time-course.[51–53]

ERK is involved in the induction of *Dusps* and the regulation of *Ccl2* in response to α-T-13'-COOH

Having found that α-T-13'-COOH induces ERK1/2 and p38 phosphorylation and the gene expression of the main DUSPs in RAW264.7, we sought to verify a connection. Suppression of ERK phosphorylation by PD98059[54] should suppress or prevent the induction of the *Dusp* gene expression in response to α-T-13'-COOH. We pre-treated the cells with PD98059 for 1 h and examined the gene expression at 0 h (addition of α-T-13'-COOH), 3 h (the time point of stable induction of all three *Dusps*) and 24 h. Indeed, *Dusp1* induction in response to α-T-13'-

COOH is significantly suppressed by more than 50 % (5.5-fold vs. 13.2-fold induction) at 3 h by PD98059 pre-treatment (Figure 5B) and is also in tendency suppressed at 24 h (7.8-fold vs. 11.8-fold induction). *Dusp2* expression is also significantly suppressed by PD98059 pre-treatment after 3 h and 24 h in comparison to α-T-13'-COOH alone (Figure 5). However, the reduction by about 50 % with the inhibitor alone at 0 h indicates that *Dusp2* expression is in large part dependent on ERK-phosphorylation in RAW264.7. Still, the induction of gene expression in combination at 3 h suggests that *Dusp2* is in part regulated by ERK-phosphorylation and in part by other mechanisms induced by α-T-13'-COOH. In contrast, while reduced to about 75 % at 0 h by PD98059 treatment and thus likely also ERK-dependent, *Dusp16* induction by α-T-13'-COOH is not affected by pre-treatment with the inhibitor. Hence, *Dusp16* induction by α-T-13'-COOH is independent of ERK-phosphorylation. To sum up, *Dusps* are differently regulated by multiple pathways as suggested before [42], however, we provide strong evidence that the induction of ERK-phosphorylation by α-T-13'-COOH plays a role in the feedback via *Dusp1* and *Dusp2*. Albeit α-T-13'-COOH also induces p38 phosphorylation (Figure 4), inhibition of p38 by SB203580 pre-treatment did not suppress *Dusp1* induction. The same was found with application of SP600125 c-Jun n-terminal kinase (JNK) inhibitor (Supplementary Figure 2). As *Dusp1* represents the most sensitive feedback gene, we conclude, that p38 phosphorylation is not the driving force, and JNK is likely not involved in the MAPK feedback mechanism induced by α-T-13'-COOH.

Since *Ccl2* expression has also been reported to be connected to ERK in RAW264.7 cells [51–53], we further examined *Ccl2* in this setup. Inhibition of ERK-phosphorylation significantly lowers the levels of *Ccl2* after 3 h (Figure 5B), and in tendency also after 24 h (Figure 5C), in combination with α-T-13'-COOH compared to α-T-13'-COOH alone (Figure 5). In conclusion, *Ccl2* gene expression is in part dependent on ERK-phosphorylation in RAW264.7 and inhibition of ERK suppresses the inductive effect of α-T-13'-COOH at early time points and further augments the suppression at 24 h. This provides evidence that a negative feedback leading to the dephosphorylation of ERK via DUSPs[55] in response to α-T-13'-COOH may account for the suppressive effect of α-T-13'-COOH in the late time-course. The proposed

mechanism is supported by the above delineated induction of *Dusps*, the main MAPK negative feedback regulators.

Induction of endogenous NFκB suppressors and Ccl2 gene expression by α-T-13'-COOH is NFκB-dependent

Given that α-T-13'-COOH quickly induces several negative regulatory feedback genes and primary LPS response genes in RAW264.7 macrophages (Figure 3C), we wanted to prove that this response is due to an activation of NFκB signaling. In order to obtain further evidence for NFκB involvement, we used the translocation and transcriptional activity inhibitor JSH-23[56] (Figure 6) and the translocation inhibitor DHMEQ[57] (Supplementary Figure 3) in combinatorial experiments with α-T-13'-COOH. Again, we examined the expression 3 h after α-T-13'-COOH treatment, where all target genes are induced (Figure 6A), and 24 h after α-T-13'-COOH as the late time-point. After 1 h of pre-treatment with JSH-23 and 3 h of α-T-13'-COOH treatment (Figure 6B), we found that the induction of *Nfkbie*, *Nfkbiz* and *Tnfaip3* gene expression by α-T-13'-COOH is significantly blocked. Blocking is also observed for *Nfkbia* albeit not statistically significant. Further, *Nfkbia*, *Nfkbie* and *Tnfaip3* are suppressed by JSH-23 alone, indicating that these genes are under basal control of NFκB. Feedback control pushes the gene expression level below that of vehicle control in all examined genes at 24 h, except for *Nfkbiz* (Figs. 6A and 6C, dark green circles). The hypothesis that these genes are prone to negative feedback control by α-T-13'-COOH is thus indirectly supported by the suppression of all genes at 24 h by NFκB inhibition (JSH-23) alone. *Nfkbiz*, however, is still elevated at 24 h and also suppressed by JSH-23, although not statistically significant (Figure 6C). The same is found in combination with DHMEQ (Supplementary Figure 3), indicating that nuclear translocation of NFκB is an important process in the induction of these genes by α-T-13'-COOH. Taken together, the combinatorial experiments provide further evidence that α-T-13'-COOH treatment induces a feedback loop via NFκB in RAW264.7 mouse macrophages.

Ccl2 has repeatedly been shown to be regulated by NFκB.[39,40] In order to elucidate NFκB participation in *Ccl2* regulation in RAW264.7, we examined the gene expression in the above

delineated experimental setup (Figure 6). Gene expression at 3 h shows that JSH-23 strongly suppresses *Ccl2* to about 40 % ($p < 0.001$) of the vehicle level indicating that the *Ccl2* gene is under control of NFκB in RAW264.7. Further, combinatorial application of α-T-13'-COOH is not able to relieve this suppression, providing evidence that α-T-13'-COOH's effect on *Ccl2* is NFκB-dependent. In sum, JSH-23 significantly suppresses the gene expression of *Ccl2* in combination compared to α-T-13'-COOH alone (Figure 6B). At 24 h however, no significant differences between α-T-13'-COOH, JSH-23 and the combination are detectable (Figure 6C). Thus, the proposed negative feedback regulator(s) induced by α-T-13'-COOH are as potent as the NFκB inhibitor JSH-23 at that time.

In conclusion, *Ccl2* is strongly suppressed by JSH-23 and thus prone to NFκB inhibition. Combined with the induction of several negative regulators of NFκB by α-T-13'-COOH, we conclude that the feedback regulation in general, and on the *Ccl2* gene in particular, is likely in large parts mediated by negative NFκB regulators in RAW264.7 macrophages.

Concluding remarks

Our data show that an adaptive response to α-T-13'-COOH is fundamental for the suppressing effect on LPS-induced cytokine secretion in mouse macrophages. However, CCL2 suppression without the adaption process as well as suppressive effects by repeated application of α-T-13'-COOH[16] suggest that this is not the only mechanism of action. Thus, multiple contacts of immune cells with the LCM, as expectable *in-vivo*, can likely further suppress pro-inflammatory mechanisms. The initial adaption to α-T-13'-COOH may still be of relevance since immune cells undergo a turnover.[29] We show that α-T-13'-COOH quickly induces the phosphorylation of ERK and p38 and indirectly show activation of NFκB signaling in macrophages, both leading to a cellular reprogramming at the level of gene expression. This response is characterized by the fast induction of several negative mediators of MAPK and NFκB signaling. Desensitization of MAPK and NFκB pathways is a hallmark of the suppression of pro-inflammatory action[32,35] (of immune cells), which is in turn crucial to suppress the underlying pathological mechanisms of most major diseases including CVD and respiratory disease. With the reliable suppression of Ccl2 gene expression and secretion, we identified an important effect of α-T-13'-COOH in this study. CCL2 has been shown to play a role in the progression of immunological disorders, CVD, lung diseases and cancer.[29,41,58–60] We thus provide a new facet in the interpretation of the known effects of α-T (vitamin E) and α-T-13'-COOH as the putative regulatory metabolite[13] in these diseases.[6–9,17–19] This study therefore provides new aspects that will help to clarify the open questions in vitamin E research in the next 100 years.

Supplementary information

Supplementary Table S1: Primers used for RT-qPCR

Supplementary Figure S1: The LCM α-T-13'-COOH induces several markers of endotoxin (cross-) tolerance

Supplementary Figure S2: Inhibition of p38 and JNK pathway does not significantly influence induction of *Dusp1* by α-T-13'-COOH

Supplementary Figure S3: The NFkB translocation inhibitor DHMEQ suppresses *Tnfaip3* and *Nfkbiz* induction by α-T-13'-COOH

Figures

Figure 1: α-T-13'-COOH is a potent suppressor of CCL2 expression and secretion and alters the inflammatory response of RAW264.7 macrophages to LPS. RAW264.7 macrophages were pre-treated with 5 µM α-T-13'-COOH or vehicle for either 24 h (A + C) or 1 h (B + D) before addition of 100 ng/ml LPS for 24 h. Expression levels of the indicated mRNAs were assessed by RT-q-PCR (A + B) 24 h after LPS addition. Gene expression analyses show that 24 h pre-treatment with α-T-13'-COOH leads to significant suppression of *Ccl2*, *Tnf* and *IL6* genes but not the *IL1a* and *IL1b* gene expression in response to LPS after 24 h (A). Pre-treatment for 1 h with α-T-13'-COOH potently suppresses *Ccl2* gene expression but not *IL1a*, *IL1b*, *Tnf* and *IL6* expression in response to LPS after 24 h (B). The release of inflammatory mediators in the supernatant was either assessed by ELISA (IL6) or flow cytometry (all others). Pre-treatment with α-T-13'-COOH for 24 h leads to a significant suppression of CCL2 and IL1-β while IL1-α is suppressed in tendency, *Tnf* is induced in tendency and IL6 secretion is not altered in response to 24 h LPS (C). Pre-treatment for 1 h with α-T-13'-COOH potently suppresses CCL2 release, IL1-β and TNFα secretion are not altered and the release of IL1-α and IL6 is significantly induced after 24 h of LPS treatment (D). Circles represent the mean of normalized fold changes of four (A + B) or five (C + D) independent biological experiments. Error bars display calculated maximum and minimum expression levels based on the SEM of ΔΔct values (A + B) or SEM (C + D) respectively. Data are shown relative to the level of the respective vehicle control, represented by the dotted horizontal line. Squares represent data of independent biological experiments to visualize variability of the data. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; all *vs.* respective vehicle control. *p*-values were calculated using a paired two-sided t-test.

Figure 2A: Pre-treatment for 24 h with α-T-13'-COOH attenuates inflammatory marker gene expression in response to LPS challenge. RAW264.7 macrophages were pre-treated with 5 µM α-T-13'-COOH or vehicle for 24 h before addition of 100 ng/ml LPS for the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. LPS-treatment induces the expression of all

examined genes over time. The expression of *Ccl2* and *Tnf* is significantly lower and is in tendency lower in case of *IL6* already at the time-point of LPS addition (0 h). For these genes, the expression stays significantly below the level of the vehicle control for the observed period of LPS expression. This indicates, that the response to LPS is diminished in 24 h pre-treated cells. The suppressive effect on *IL1a* and *IL1b* gene expression induction is not as marked as for the other observed genes. Data is shown as $\log_2$ fold change (log2FC) for better visualization of low and high expression levels. Circles represent the mean of normalized fold changes of four independent biological experiments. Error bars display the SEM of log2FC values. Data are shown relative to the level of the vehicle control at 0 h, represented by the dotted horizontal line. Squares represent data of independent biological experiments to visualize variability of the data. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; all *vs.* respective vehicle control at the same time-point. *p*-values were calculated using a paired one-sided t-test to test the hypothesis of lower expression vs. vehicle.

Figure 2B: Pre-treatment for 1 h with α-T-13'-COOH significantly attenuates the gene expression of Ccl2 but not of other inflammatory markers in response to LPS. RAW264.7 macrophages were pre-treated with 5 µM α-T-13'-COOH or vehicle for 1 h before addition of 100 ng/ml LPS for the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. LPS-treatment induces the expression of all examined genes over time. The expression of *Ccl2* is significantly attenuated after 3, 6 and 24 h of LPS treatment when cells were pre-treated for 1 h with α-T-13'-COOH with respect to 1 h vehicle pre-treatment. All other examined inflammatory markers show no significant difference in their expression in response to LPS with respect to the pre-treatment. Pre-treatment with α-T-13'-COOH slightly elevates the expression of *IL1a*, *IL1b*, *Tnf* and *IL6* in tendency. Data is shown as $\log_2$ fold change for better visualization of low and high expression levels. Circles represent the mean of normalized fold changes of four independent biological experiments. Error bars display the SEM of log2FC values. Data are shown relative to the level of the vehicle control at 0 h, represented by the dotted horizontal line. Squares represent data of independent biological experiments to visualize variability of the data. *, $p <$

0.05; **, $p < 0.01$; ***, $p < 0.001$; all *vs.* respective vehicle control at the same time-point. *p*-values were calculated using a paired one-sided t-test to test the hypothesis of lower expression vs. vehicle.

Figure 2C: Pre-treatment for 24 h with α-T-13'-COOH is needed to render cells more tolerant to LPS challenge with the exception of Ccl2 expression. RAW264.7 macrophages were pre-treated with 5 µM α-T-13'-COOH or vehicle for 1 h or 24 h before addition of 100 ng/ml LPS for the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. Data from 24 h pre-treatment experiments (Figure 2A) and 1 h pre-treatment experiments (Figure 2B) are shown as $\log_2$ fold change relative to their respective vehicle control at the indicated time-points to visualize the effect of pre-treatment conditions. 24 h pre-treatment leads to lower expression of all examined genes (circles) while 1 h pre-treatment leads to a slightly higher expression in tendency (squares) with respect to the vehicle controls (dotted horizontal line) with the exception of *Ccl2*. *Ccl2* expression in response to LPS is attenuated by α-T-13'-COOH irrespective of the pre-treatment conditions in RAW264.7 macrophages. Large circles and squares represent the mean of normalized fold changes of four independent biological experiments. Error bars display the SEM of $\log_2$FC values. Small circles and squares represent data of independent biological experiments to visualize variability of the data. No statistical analyses were conducted.

Figure 3: α-T-13'-COOH leads to sustained induction of gene expression of various negative feedback regulators of MAPK and NFκB signaling. RAW264.7 macrophages were treated with 5 µM α-T-13'-COOH and gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. The untreated control is represented as 0 h. Ccl2 expression is significantly induced after 1 h and quickly counterbalanced, leading to a significant suppression after 24 h and in tendency after 6 h and 10 h, suggesting a negative feedback regulation (A). The main *Dusps* in RAW264.7 are induced by α-T-13'-COOH. *Dusp1* and *Dusp16* show a sustained induction already after 1 h after α-T-13'-COOH treatment until

24 h. *Dusp2* is elevated 3 h after α-T-13'-COOH treatment, followed by a fast counterbalancing (B). Negative regulators of NFκB signaling are induced by α-T-13'-COOH in RAW264.7 macrophages. *Nfkbia* and *Nfkbie* genes show a slight induction at early time-points and a highly variable expression at late time-points. *Nfkbib* and *Nfkbiz* are quickly elevated and stay induced over the 24 h of treatment. *Tnfaip3* is induced at early time points followed by a counterbalancing after 10 and 24 h (C). Circles represent the mean of normalized fold changes of three independent biological experiments. Error bars display the SEM of log_2FC values. Data are shown relative to the level of the untreated control at 0 h. Squares represent data of independent biological experiments to visualize variability of the data. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; all *vs.* untreated control at 0 h. *p*-values were calculated using repeated measures ANOVA with Dunnett's post-hoc test vs. untreated control (0 h) set as reference.

Figure 4: Phosphorylation of the MAPKs ERK and p38 is induced within 30 minutes by α-T-13'-COOH. RAW264.7 macrophages were treated with 5 µM α-T-13'-COOH and MAPK phosphorylation was examined at the indicated time-points. MAPK phosphorylation was assessed by immunoblot. Signals of phosphorylated protein were corrected for signals of basal protein and the loading / viability control α-Tubulin (α-Tub.). Representative immunoblots are shown in the panels below the graphs. Graphs in A, B and C show the fold change of vehicle-treated samples (squares) and α-T-13'-COOH-treated samples (circles) relative to the mean of two untreated controls (triangle) set as 1. ERK1 and ERK2 phosphorylation is quickly induced and reaches significance after 30 min (A and B, respectively). Phosphorylation of p38 is already induced after 10 minutes and is significantly induced after 20 and 30 min (C). Panel D shows the level of phosphorylation for vehicle controls and α-T-13'-COOH separately for each time-point and each protein. Treatment with α-T-13'-COOH significantly elevates the level of protein phosphorylation compared to vehicle controls at all time-points (D). Small circles and squares represent data of five (A + B) or four (C) independent biological replicates to visualize variability of the data. Large circles and squares represent the mean ± SEM. Dotted lines in panel D are intended to visualize tendency in each biological replicate. **, $p < 0.01$; ***, $p < 0.001$; all *vs.* untreated control at 0 min. *p*-values were calculated using repeated measures

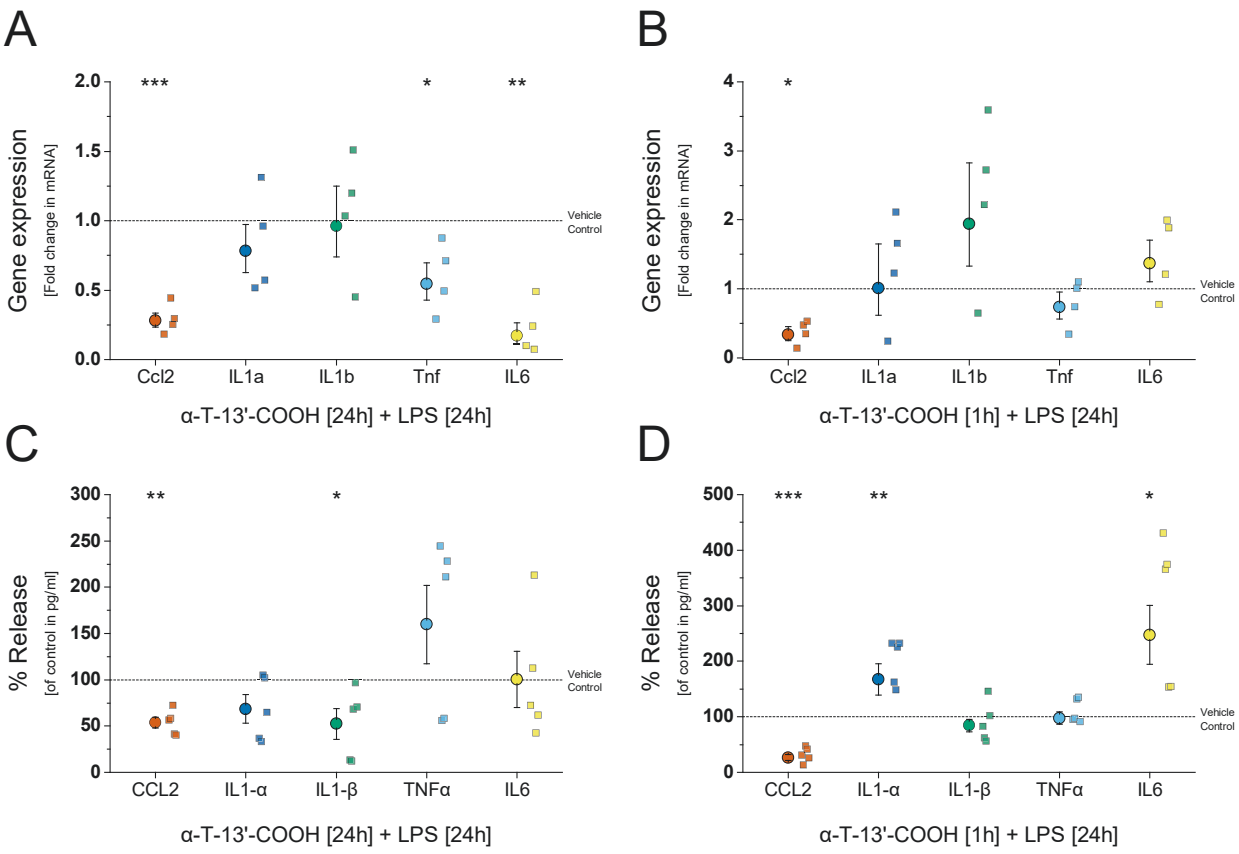
ANOVA with Dunnett's post-hoc test, applied on α-T-13'-COOH-treated samples and untreated control (0 h) set as reference (A + B + C). *p*-values were calculated using a paired one-sided t-test to test for significantly higher levels in α-T-13'-COOH-treated samples (D).

Figure 5: ERK is involved in the regulation of Ccl2 gene expression and induction of a negative MAPK feedback loop via *Dusps* by α-T-13'-COOH. RAW264.7 macrophages were treated with vehicle or 5 µM α-T-13'-COOH or pre-treated with 10 µM ERK-inhibitor PD98059 for 1 h and 5 µM α-T-13'-COOH was added at time-point 0 h. Gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. Fold changes are calculated vs. respective vehicle controls. Panel A shows the impact of ERK inhibition by 1 h pre-treatment with PD98059 on regulation of gene expression of *Ccl2*, *Dusp1*, *Dusp2* and *Dusp16* by α-T-13'-COOH at early (3 h) and late (24 h) time-points. Expression of *Dusp2* and *Dusp16* is already attenuated after 1 h of pre-incubation, indicating a role of ERK in the basal expression of these genes (A). ERK inhibition leads to significantly lower expression levels of *Ccl2*, *Dusp1* and *Dusp2* after 3 h of treatment compared to α-T-13'-COOH alone, *Dusp16* expression is not affected (B). Cells pre-treated with PD98059 show lower levels of Ccl2 and *Dusp1* in tendency and significantly lower levels of *Dusp2* compared to α-T-13'-COOH treatment alone. Gene expression of *Dusp16* is again not affected (C). Circles represent the mean of normalized fold changes of five independent biological experiments. Error bars display the SEM of $\log_2$FC values. Data are shown relative to the level of the vehicle control at the indicated time-point. Squares represent data of independent biological experiments to visualize variability of the data. *, $p < 0.05$; **, $p < 0.01$; pre-treatment with PD98059 vs. α-T-13'-COOH alone. *p*-values were calculated using a two-sided paired t-test.

Figure 6: NFκB is involved in the regulation of *Ccl2* gene expression and induction of a negative NFκB feedback loop by α-T-13'-COOH. RAW264.7 macrophages were treated with vehicle or 5 µM α-T-13'-COOH and gene expression was examined at 0 h, 3 h and 24 h (A). RAW264.7 macrophages were treated with vehicle, 5µM α-T-13'-COOH or 10 µM NFκB inhibitor JSH-23 or pre-treated with 10 µM JSH-23 for 1 h and 5 µM α-T-13'-COOH was added

at time-point 0 h (B + C). Gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. Fold changes are calculated vs. vehicle controls. Panel A shows time-courses of the gene expression of *Ccl2* and various negative NFκB signaling regulators in response to α-T-13'-COOH treatment. All examined genes or elevated at 3 h whereas a suppression is found after 24 h except for *Nfkbib* and *Nfkbiz* (A). Inhibition of NFκB signaling suppresses *Ccl2* gene expression, indicating a regulatory involvement of NFκB in *Ccl2* expression in RAW264.7 macrophages. Induction of the negative NFκB regulators *Nfkbia*, *Nfkbie*, *Nfkbiz* and *Tnfaip3* by α-T-13'-COOH is prevented by JSH-23 pre-treatment, providing evidence for an induction of a negative feedback loop via NFκB by α-T-13'-COOH (B). After 24 h, feedback induction is counterbalanced and expression levels are below vehicle controls, except for *Nfkbiz*. No significant differences between the treatments are observed after 24 h. *Nfkbiz* gene expression induction by α-T-13'-COOH is in tendency suppressed by JSH-23 after 24 h (C). Circles represent the mean of normalized fold changes of five (A), four (B) or three (C) independent biological experiments. Error bars display the calculated RQmin/RQmax based on the SEM of ΔΔct values. Data are shown relative to the level of the vehicle control at the indicated time-point. Squares represent data of independent biological experiments to visualize variability of the data. ***, $p < 0.001$, pre-treatment with JSH-23 vs. α-T-13'-COOH alone. *p*-values were calculated using a repeated measures ANOVA with Tukey's post-hoc-test.

Figure 1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CCL2
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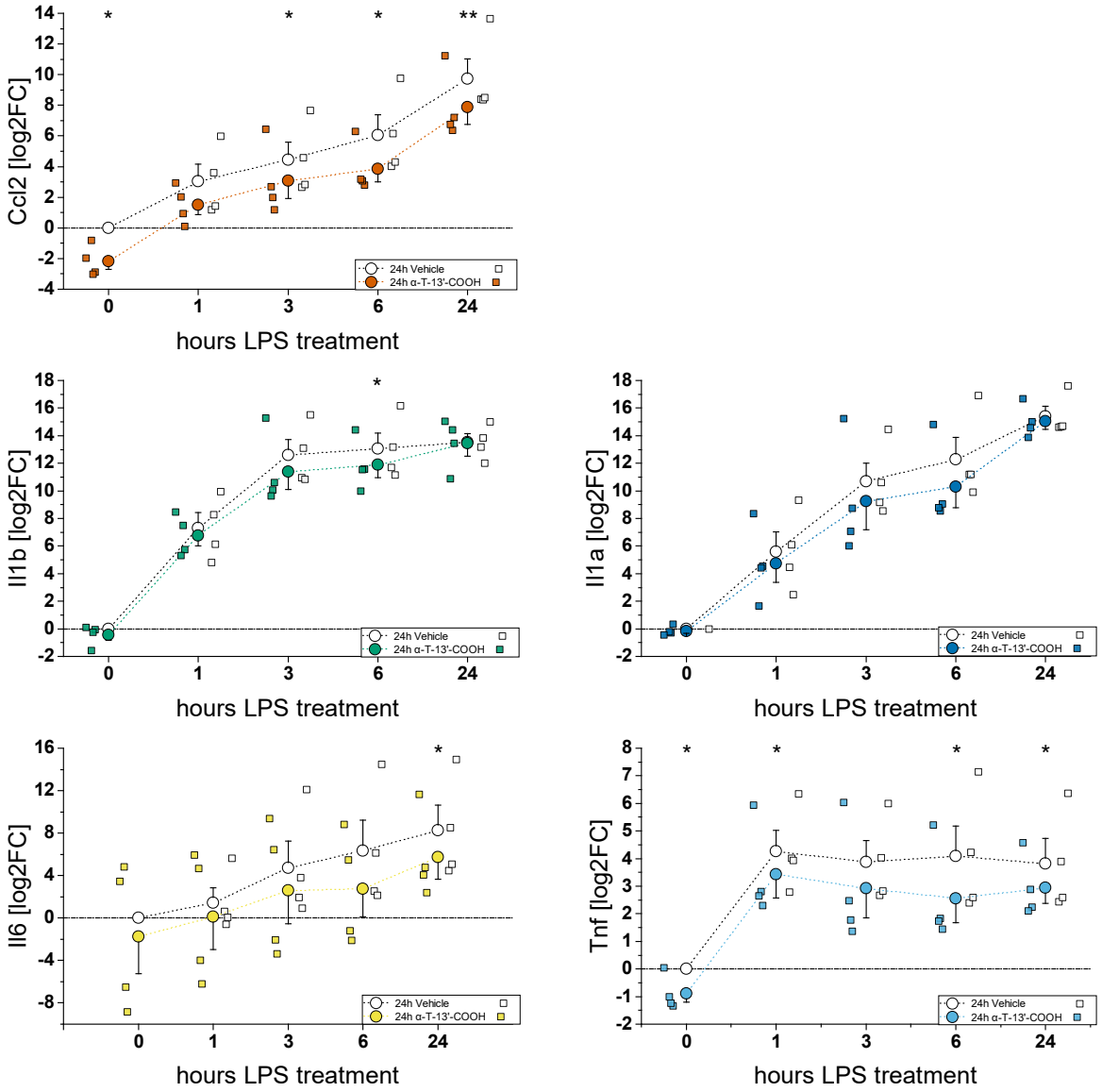
Figure 2A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hours LPS treatment
24h Vehicle
24h α-T-13'-COOH
Il6 [log2FC]
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Figure 2B

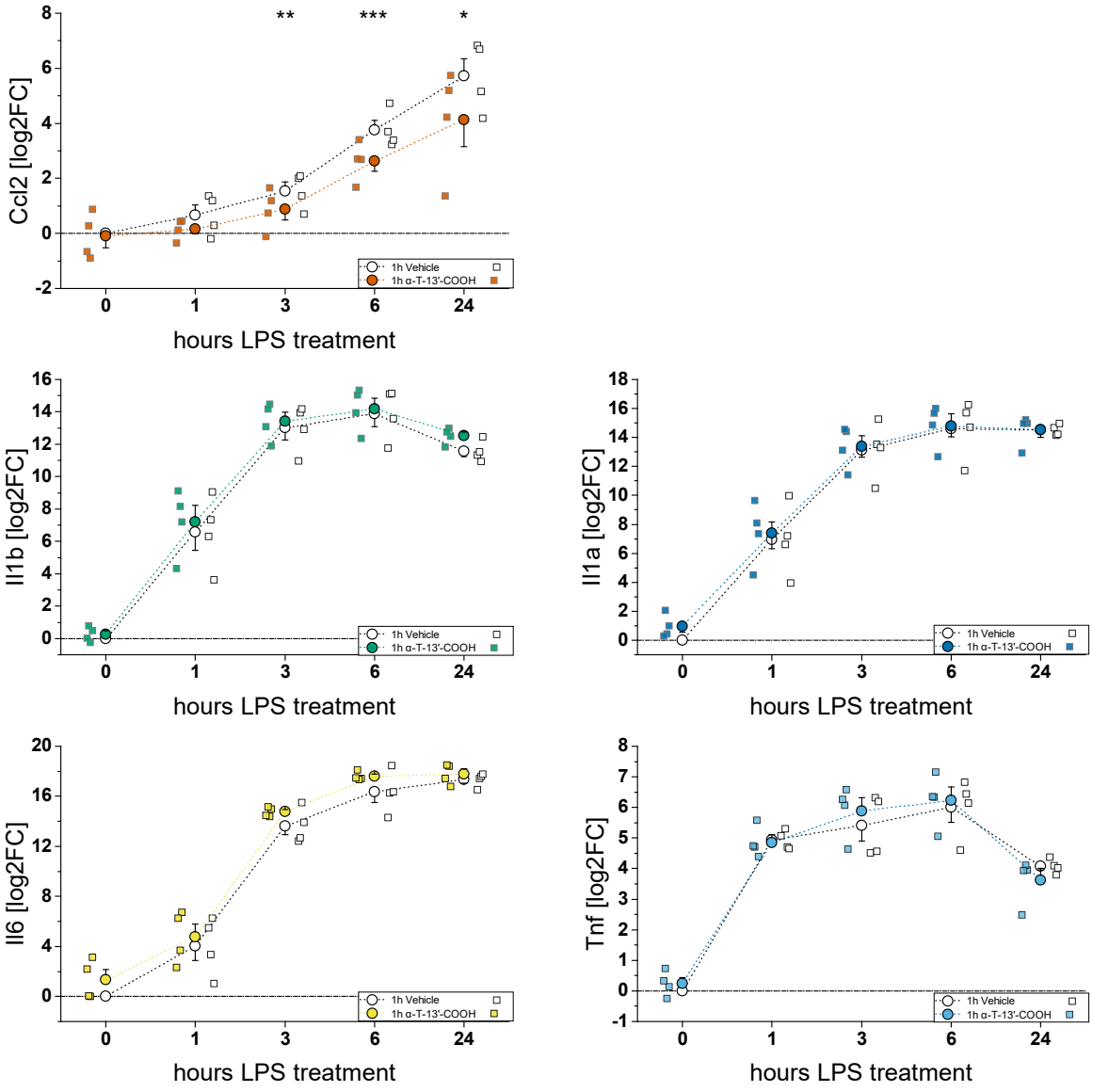
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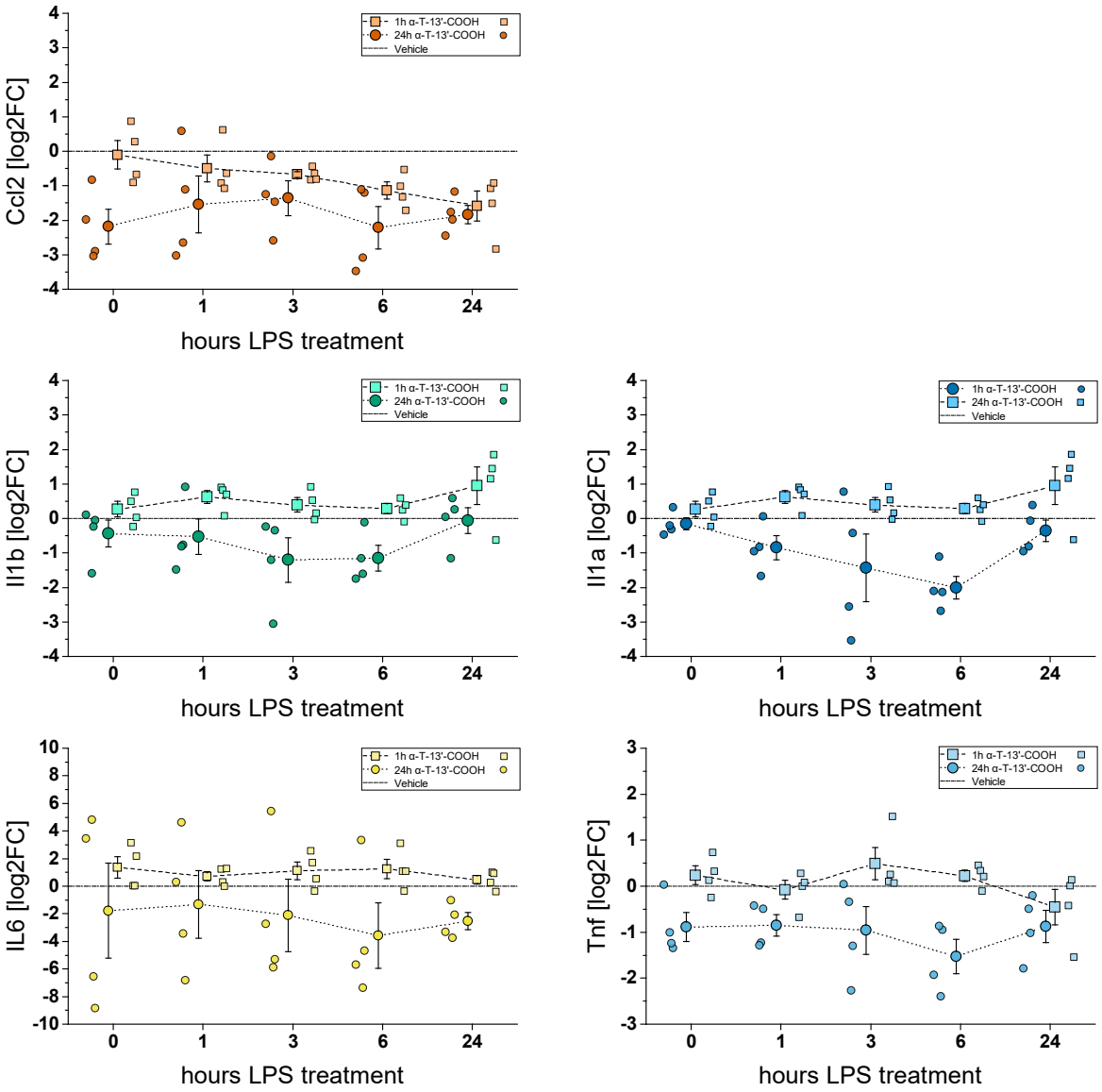
Figure 2C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Tnf [log2FC]
hours LPS treatment
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Figure 3

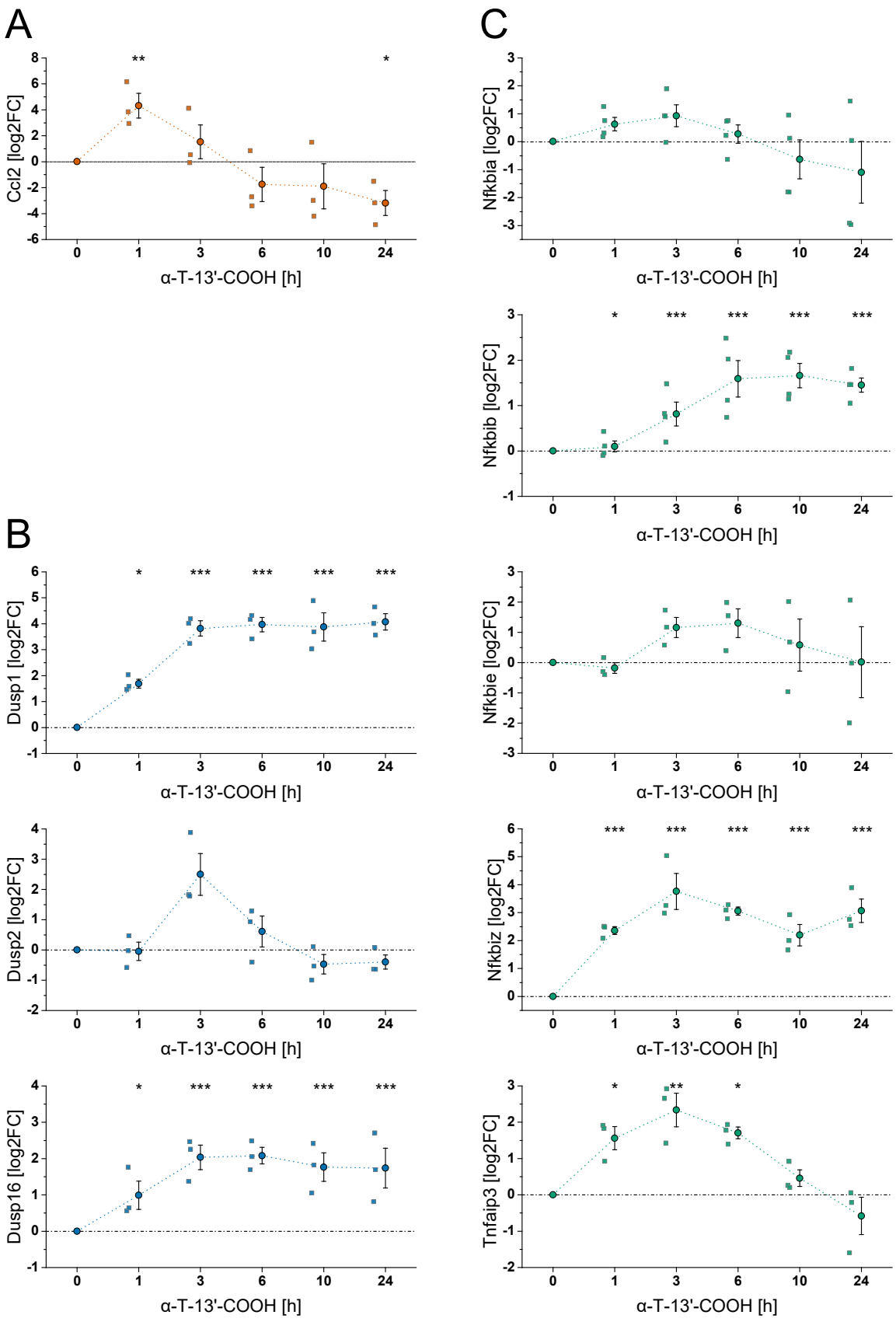
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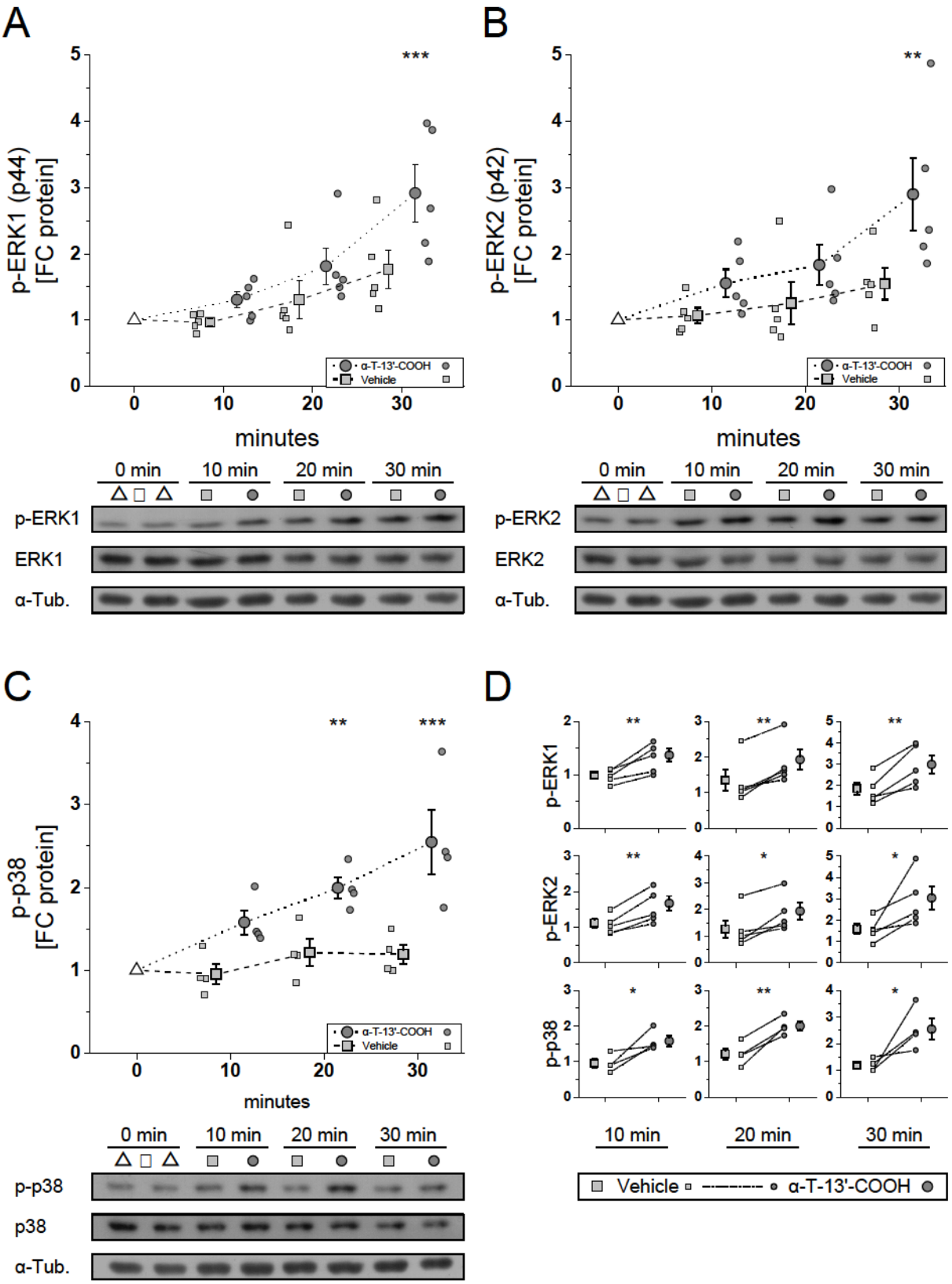
Figure 4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20 min
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p38
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p-ERK2
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α-T-13'-COOH

Figure 5

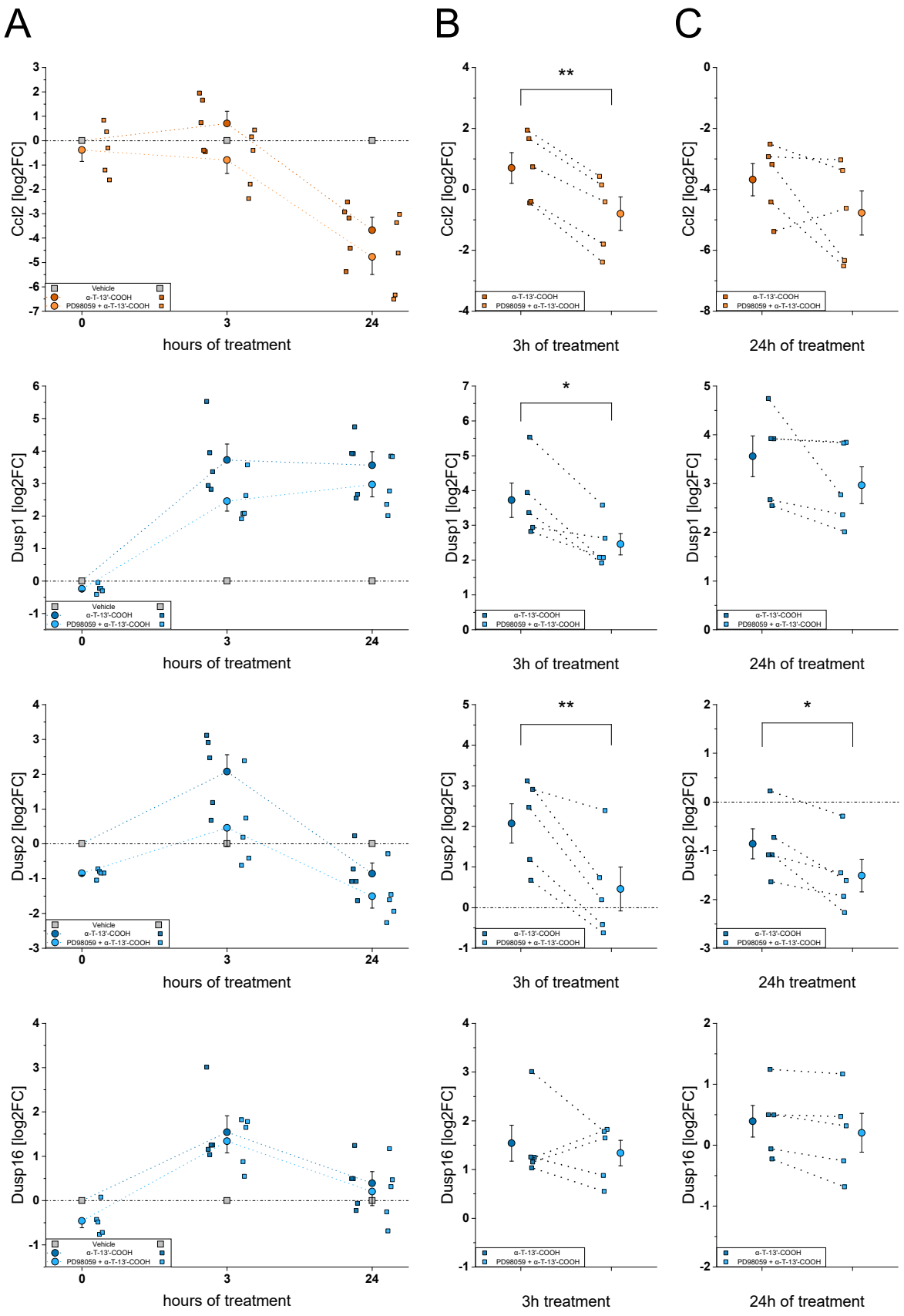
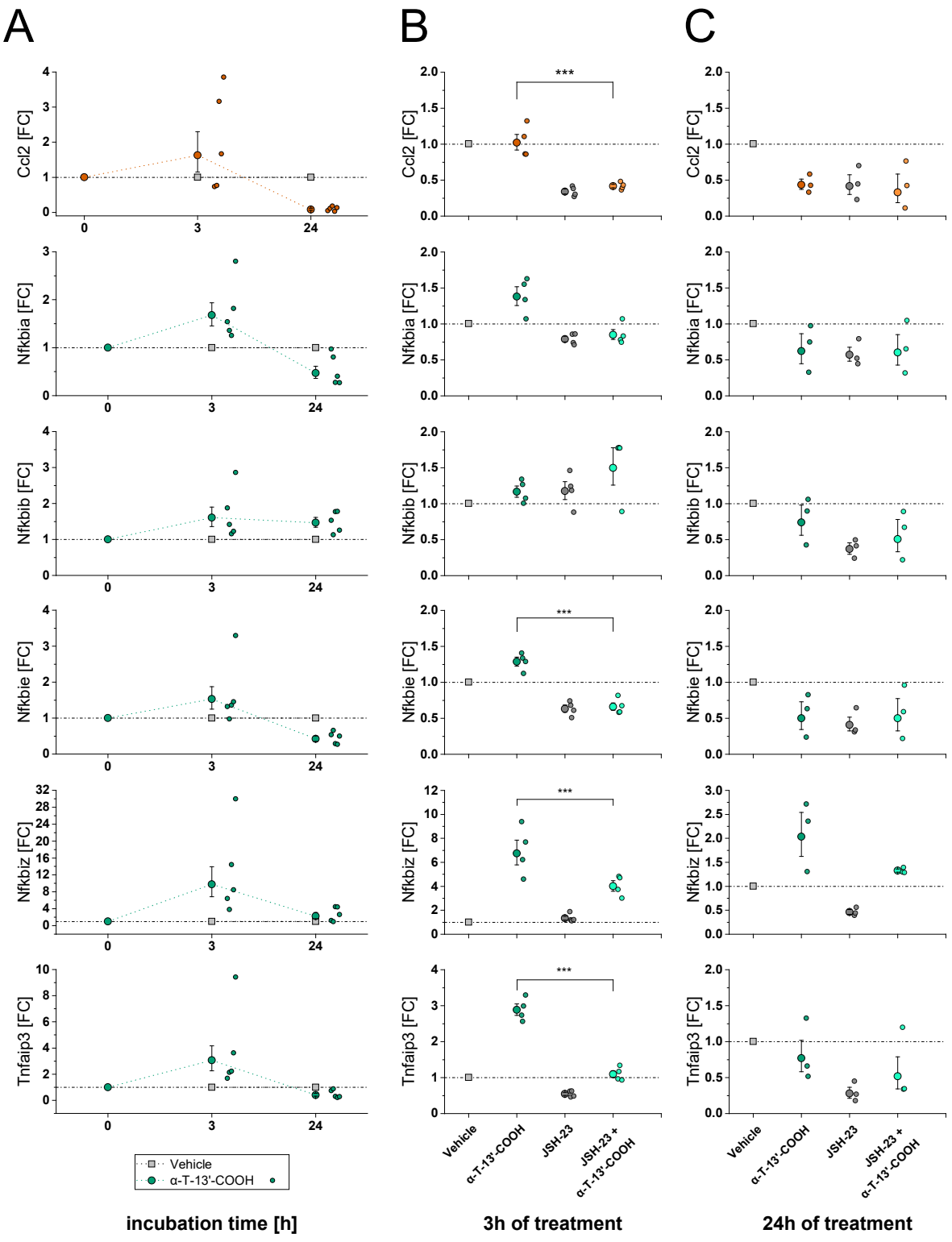

A
B
C
Ccl2 [log2FC]
Dusp1 [log2FC]
Dusp2 [log2FC]
Dusp16 [log2FC]
hours of treatment
3h of treatment
24h of treatment
24h treatment
3h treatment
Vehicle
α-T-13'-COOH
PD98059 + α-T-13'-COOH
**
*

Figure 6


A
B
C
Ccl2 [FC]
Nfkbia [FC]
Nfkbib [FC]
Nfkbie [FC]
Nfkbiz [FC]
Tnfaip3 [FC]
***
Vehicle
α-T-13'-COOH
JSH-23
JSH-23 + α-T-13'-COOH
incubation time [h]
3h of treatment
24h of treatment

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Author contributions:

Participated in research design: M.S., S.L.

Conducted experiments: M.S.

Contributed new reagents or analytic tools: S.L.

Performed data analysis: M.S.

Wrote or contributed to the writing of the manuscript: M.S., S.K., M.B., S.L.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary information to

The vitamin E long-chain metabolite α-T-13'-COOH is a reliable suppressor of CCL2 / MCP-1 and modulates regulatory mechanisms of MAPK and NFκB signaling and the inflammatory response of macrophages

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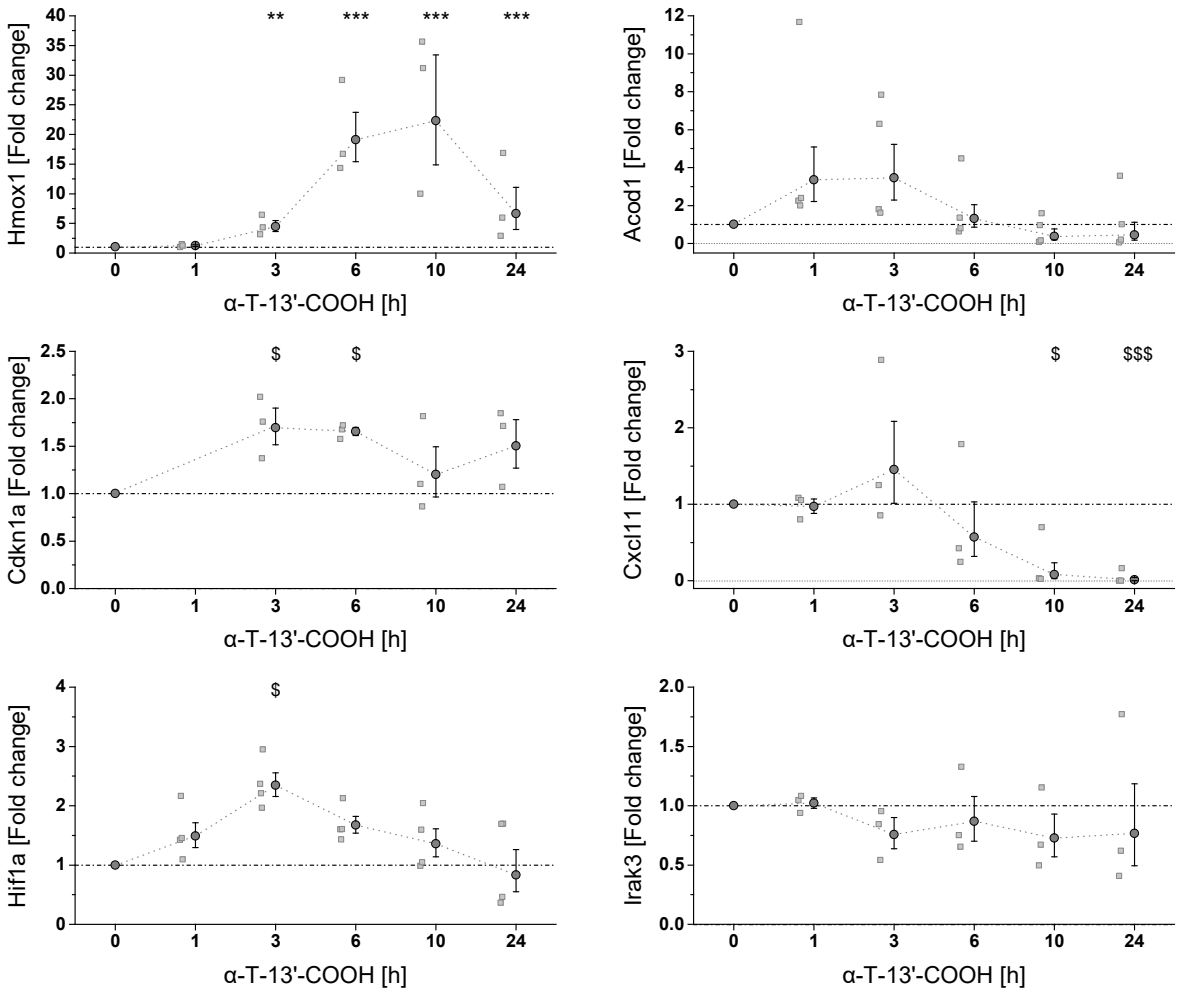
Supplementary Table S1: Primers used for RT-qPCR.

Gene	mRNA name	GenBank accession no.	Primer		Amplicon size [bp]
Ppib	*Mus musculus* peptidylprolyl isomerase B	NM_011149.2	**FW** **RE**	AAACAGCAAGTTCCATCGTGTCAT GAAGCGCTCACCATAGATGCTCT	103
Ccl2 (Mcp-1)	*Mus musculus* chemokine (C-C motif) ligand 2	NM_011333.3	**FW** **RE**	GGCTGGAGAGCTACAAGAGGATC CAGCACAGACCTCTCTCTTGAGC	90
Il1a	*Mus musculus* interleukin 1 alpha	NM_010554.4	**FW** **RE**	CGCTCAAGGAGAAGACCAGC GAGGTCGGTCTCACTACCTGT	82
Il1b	*Mus musculus* interleukin 1 beta	NM_008361.3	**FW** **RE**	TGAAGTTGACGGACCCCAAA CAGCCACAATGAGTGATACTGCC	140
Tnf (Tnfa)	*Mus musculus* tumor necrosis factor (α)	NM_013693	**FW** **RE**	AGAAACACAAGATGCTGGGACAGT CCTTTGCAGAACTCAGGAATGG	46
Il6	*Mus musculus* interleukin 6	NM_031168.1	**FW** **RE**	TCAATTCCAGAAACCGCTATGAA GGAAGGCCGTGGTTGTCAC	94
Dusp1	*Mus musculus* dual specificity phosphatase 1	NM_013642.3	**FW** **RE**	GACAACCACAAGGCAGACATC AATGAACAAACACTCTCCCTCCA	94
Dusp2	*Mus musculus* dual specificity phosphatase 2	NM_010090.2	**FW** **RE**	GGGGCCGAAAATAGCAACTC AGCTGCCCAAGTACAGGTAG	91
Dusp16	*Mus musculus* dual specificity phosphatase 16	NM_001048054.2	**FW** **RE**	CCTGTTGCCCATGTAACCCT CAGTGTTCCATCCCTGGCTT	121
Nfkbia	*Mus musculus* nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	NM 010907.2	**FW** **RE**	CCAGCATCTCCACTCCGTC TGGATAGAGGCTAGGTGCAGA	72

Supplementary table S1 (continued)

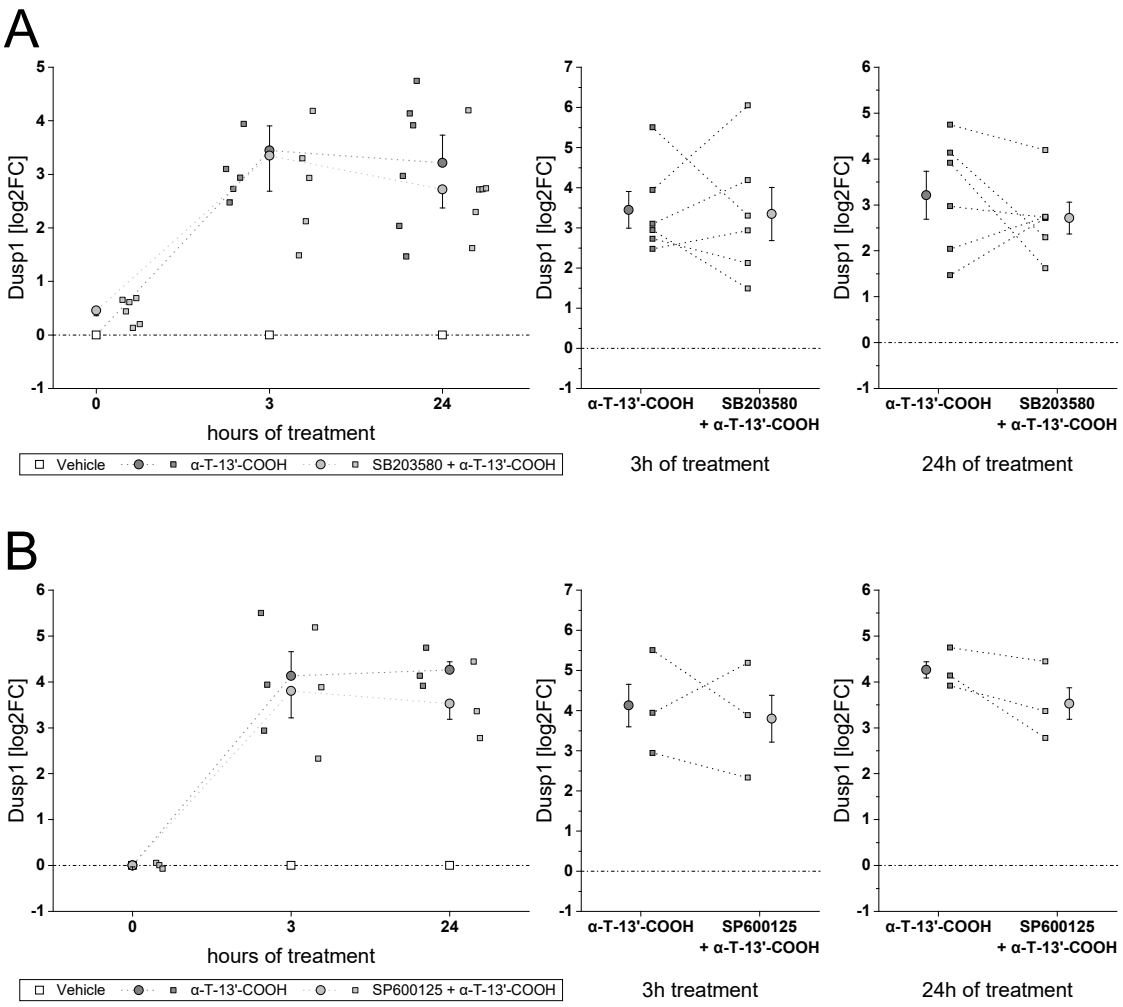
Nfkbib	*Mus musculus* nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta	NM 100908.5	**FW** GCGACAGTGACAGTGACAAC **RE** AAGAGGTTTGGATGCCGGG	110
Nfkbie	*Mus musculus* nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, epsilon	NM 001304956.1	**FW** ATGAGCAAACTGACCCGAGG **RE** AGTAAGCTTGCCTACCGCTG	102
Nfkbiz	*Mus musculus* nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	NM 001159395.1	**FW** TAGGGACGGAGATGGTGACA **RE** TGCAAGGACATACGAGAGCG	76
Tnfaip3 (A20)	*Mus musculus* tumor necrosis factor, alpha-induced protein 3	NM_009397.3	**FW** CAGCACTTTGTACCCCTGGT **RE** ACCTACCCCGGTCTCTGTTA	85
Hmox1	*Mus musculus* heme oxygenase 1	NM_010442.2	**FW** ACAGAAGAGGCTAAGACCGCCTT **RE** TCTGGTCTTTGTGTTCCTCTGTCAG	88
Acod1 (Irg1)	*Mus musculus* aconitate decarboxylase 1	NM_008392.1	**FW** ACTCCTGAGCCAGTTACCCT **RE** GGTGGTTCACTTTCAAGCCG	94
Cdkn1a (p21)	*Mus musculus* cyclin-dependent kinase inhibitor 1A (P21)	NM_001111099.2	**FW** TCTGGTGTCTGAGCGGCC **RE** TCTGCGCTTGGAGTGATAGAAAT	105
Cxcl11	*Mus musculus* chemokine (C-X-C motif) ligand 11	NM_019494.1	**FW** AACAGGAAGGTCACAGCCATAG **RE** GCCCCTGTTTGAACATAAGGAAG	84
Hif1a	*Mus musculus* hypoxia inducible factor 1, alpha subunit	NM_001313920.1	**FW** GAAGAACTTTTGGGCCGCTC **RE** TCCACCTCTTTTGGCAAGCA	138
Irak3 (Irak-m)	*Mus musculus* interleukin-1 receptor-associated kinase 3	NM_028679.4	**FW** AAAACAAGACGATCGGCGAC **RE** GGGGAATGGAAGCTCGTGAT	131

Melting temperatures of primers are about 60°C. Primers of a pair are located in different exons. Primers are designed to lead to the same amplicon from all known coding transcript variants of the target gene.

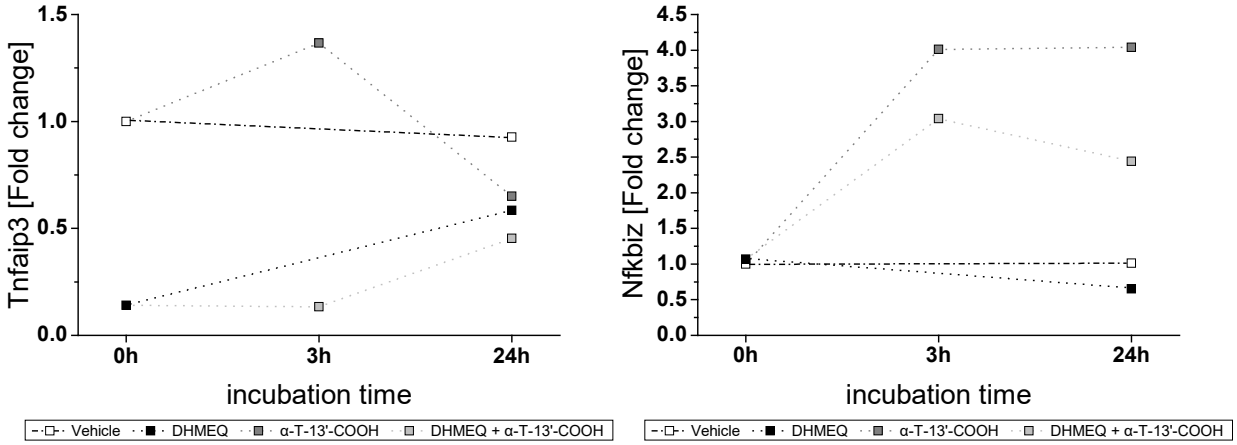


Supplementary Figure S1: The LCM α-T-13'-COOH induces several markers of endotoxin (cross-) tolerance. RAW264.7 macrophages were treated with 5 µM α-T-13'-COOH and gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene (*Ppib*) expression. The untreated control is represented as 0 h. The *Hmox1* gene shows a sustained, pronounced and significant induction in response to α-T-13'-COOH from 3 h until 24 h. Time-courses of *Acod1* (*Irg1*), *Hif1a* and *Cxcl11* reveal a feedback control pattern with induction at early time-points (1 h and 3 h) and a counterbalance at late time points, leading to a marked suppression of *Acod1* and *Cxcl11* at 24 h. *Cdkn1a* (*p21*) is induced by about 1.5-fold throughout the 24 h time-course. *Irak3* shows a variable response with a tendency of a weak suppression at late time-points. Circles represent the mean of normalized fold changes of three or four independent biological experiments. Squares represent data of independent biological experiments to visualize variability of the data. Error bars represent the calculated RQmin/RQmax. **, $p < 0.01$; ***, $p < 0.001$; all *vs.* untreated control at 0h. *p*-values were calculated using repeated measures ANOVA with

Dunnett's post-hoc test vs. untreated control (0h) set as reference. $ indicate significance level of Dunnett's test but no significant repeated measures ANOVA.



Supplementary Figure S2: Inhibition of p38 and JNK pathway does not significantly influence induction of *Dusp1* by α-T-13'-COOH. RAW264.7 macrophages were treated with vehicle or 5 µM α-T-13'-COOH or pre-treated with 10 µM p38 inhibitor SB203580 or vehicle for 1h and 5 µM α-T-13'-COOH was added at time-point 0 h. Gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene expression. Fold changes are calculated vs. respective vehicle controls. The p38 inhibitor SB203580 was used in combination with α-T-13'-COOH to examine the involvement in *Dusp1* induction (A). *Dusp1* induction is neither blocked by pre-treatment with SB203580 at 3 h (middle panel) nor at 24 h (right panel). Interestingly, SB203580 slightly but reproducibly increases *Dusp1* expression after 1 h of application (left panel). The JNK inhibitor SP600125 was used in combination with α-T-13'-COOH to examine the involvement in *Dusp1* induction (A). *Dusp1* induction is neither blocked by pre-treatment with SP600125 at 3 h (middle panel) nor at 24 h (right panel). Performed t-tests on the data depicted in the middle panels and right panels revealed no significant differences.



Supplementary Figure S3: The NFκB translocation inhibitor DHMEQ suppresses *Tnfaip3* and *Nfkbiz* induction by α-T-13'-COOH. RAW264.7 macrophages were pre-treated with 20 μM DHMEQ or vehicle for 1h and 5 μM α-T-13'-COOH or vehicle was added at time-point 0h. Gene expression was examined at the indicated time-points. Expression levels of the indicated mRNAs were assessed by RT-qPCR and normalized for reference gene (*Ppib*) expression. Fold changes are calculated vs. vehicle control at 0 h. *Tnfaip3* gene expression (left panel) is strongly suppressed after 1 h of DHMEQ treatment. This suppression cannot be relieved by α-T-13'-COOH incubation at 3 h albeit α-T-13'-COOH itself induces Tnfaip3 expression at 3 h. At 24 h, *Tnfaip3* is suppressed with all treatments, indicating that a negative NFκB regulation is involved in the effect of α-T-13'-COOH. *Nfkbiz* (right panel) is not suppressed by DHMEQ alone (0 h), suggesting that basal transcription is not dependent on NFκB translocation. The induction of *Nfkbiz* by α-T-13'-COOH is suppressed by DHMEQ at 3 h and 24 h, which is in line with the results from JSH-23 experiments. Taken together, induction of *Nfkbiz* by α-T-13'-COOH is at least in part dependent on the induction of NFκB translocation and transcriptional activity. One biological experiment is shown.

MANUSCRIPTS

antioxidants

MDPI

Review

Long-Chain Metabolites of Vitamin E: Metabolic Activation as a General Concept for Lipid-Soluble Vitamins?

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Abstract: Vitamins E, A, D and K comprise the class of lipid-soluble vitamins. For vitamins A and D, a metabolic conversion of precursors to active metabolites has already been described. During the metabolism of vitamin E, the long-chain metabolites (LCMs) 13′-hydroxychromanol (13′-OH) and 13′-carboxychromanol (13′-COOH) are formed by oxidative modification of the side-chain. The occurrence of these metabolites in human serum indicates a physiological relevance. Indeed, effects of the LCMs on lipid metabolism, apoptosis, proliferation and inflammatory actions as well as tocopherol and xenobiotic metabolism have been shown. Interestingly, there are several parallels between the actions of the LCMs of vitamin E and the active metabolites of vitamin A and D. The recent findings that the LCMs exert effects different from that of their precursors support their putative role as regulatory metabolites. Hence, it could be proposed that the mode of action of the LCMs might be mediated by a mechanism similar to vitamin A and D metabolites. If the physiological relevance and this concept of action of the LCMs can be confirmed, a general concept of activation of lipid-soluble vitamins via their metabolites might be deduced.

Keywords: vitamin E; long-chain metabolites of vitamin E; 13′-hydroxychromanol (13′-OH); 13′-carboxychromanol (13′-COOH); vitamin E metabolism; biological activity

1. The Biological Significance of Vitamin E

The term vitamin E comprises eight lipophilic molecules, which can be classified as tocopherols (TOHs) and tocotrienols (T3). Both classes share two common features: (i) the phytyl-like side chain, which is bound to (ii) the chroman ring system. A saturated side chain characterizes the TOHs, while the T3s carry three double bonds in this substructure. Further, the methylation pattern of the chroman ring determines the classification as α-, β-, γ- or δ-TOH or T3, respectively. Vitamin E is found in oils, nuts, germs, seeds and a variety of other plant products. The naturally found vitamin E forms exist either in *RRR*-configuration (TOHs) or in *R*-configuration (T3s), whereas only synthetically produced forms contain a mixture of the different possible stereoisomers [1].

Vitamin E was discovered in 1922 as vital factor for the fertility of rats, indicating its essentiality for animal and human health, and was therefore classified as a vitamin [2]. Nevertheless, the benefits of vitamin E for human health are still a contentious issue. However, several disease conditions, such as anemia, erythrocyte rupture and neuronal degeneration, as well as muscle degeneration, are linked to vitamin E deficiency or malabsorption (extensively reviewed in [3]). Further, vitamin E was shown in human intervention trials to slow down the progression of age-related neurodegenerative pathologies such as Alzheimer's disease, maybe due to its antioxidative properties [4,5]. Vitamin E is also an essential factor for the development of the central nervous system and cognitive functions of the embryo [6,7]. Next, vitamin E may play a supportive role in the prevention of neural tube defects in humans along with folic acid [8,9]. Initially, the effects of vitamin E were only attributed to its antioxidant properties, however more recent work unveiled non-antioxidant regulatory effects. There is growing evidence that vitamin E modulates gene expression and enzyme activities and interferes with signaling cascades independent of its capacity as an antioxidant [10]. Over time, several functions of vitamin E, such as suppression of inflammatory mediators, reactive oxygen species, and adhesion molecules, the induction of scavenger receptors, and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF_kB) (reviewed in [11]) were revealed. Based on these observations, it was concluded that vitamin E likely plays a role in several inflammatory but also other diseases. However, further research is required, as the results obtained from clinical trials with TOHs are inconsistent with respect to beneficial effects on the development of chronic diseases such as cancer and cardiovascular diseases [12].

2. Absorption and Distribution of Vitamin E

Like for all macro- and micronutrients, intestinal absorption is the limiting factor for the bioavailability of vitamin E in humans. As a fat-soluble vitamin, intestinal absorption, hepatic metabolism and cellular uptake of vitamin E follows that of other lipophilic molecules [13]. The absorption rate of vitamin E varies between 20% and 80% [13,14], and is thus generally lower than for vitamins A and D [15,16]. Differences in the rates of absorption of vitamin E and the other fat-soluble vitamins may result also from the parallel intake of additional food ingredients. For example, retinoic acid [17], plant sterols [18], eicosapentaenoic acid [14], alcohol (chronic consumption) [14], and dietary fiber [19] are natural food components that may compete with the absorption of vitamin E. In addition, it has been shown that the supplied form of vitamin E, either as a free molecule or coupled to other compounds like acetate, is also crucial for its bioavailability [20].

For optimal absorption, fat must be consumed along with the ingested vitamin E. This is a general requirement for all types of fat-soluble vitamins and is therefore also applicable for vitamins A, D and K [16,21]. The absorption of triacylglycerides and esterified fat-soluble molecules starts with enzymatic processing in the stomach by the action of gastric lipases [15]. The following digestion of dietary lipids appears in the intestinal lumen by the action of various enzymes, including pancreatic lipase, carboxyl esterase and phospholipase A_2 [22]. Since most of the vitamin E in the human diet is not esterified, lipolytic degradation is scarce [14]. In contrast, the human diet contains significantly more esterified vitamin A and D, mostly in the form of retinyl-esters and vitamin D_3 oleate, which can be hydrolyzed by the above mentioned enzymes [16,21]. A key step of the intestinal absorption of fat-soluble vitamins is the emulsification, i.e., the incorporation into micelles formed with phospholipids and bile acids. Under normal conditions, bile salts facilitate the absorption of all three vitamins, but especially the vitamin D forms differ in their dependency for bile salt availability, i.e., vitamin D_3 absorption is more dependent on the presence of bile salts than 25-hydroxyvitamin D (OHD) [23]. After emulsification, vitamin E is taken up into the intestinal enterocytes by passive diffusion or receptor-mediated transport via scavenger receptor class B type 1 (SRB1) [24], or Niemann–Pick C1-like protein 1 [25], which is also involved in the uptake of the vitamins A, D and K as well as cholesterol [16,26,27]. Since no specific plasma transport protein for α-TOH is known, the subsequent transport of vitamin E in blood follows largely that of cholesterol [25], meaning that under normal

physiological conditions, α-TOH is transported via chylomicrons. This transport is independent of the type of stereoisomer [28,29]. In addition, retinol, unconverted pro-retinoid carotenoids (β-carotene), non-pro-retinoid carotenoids (lycopene), vitamin D_3 and phylloquinone (representing the main dietary form of vitamin K) are also incorporated into chylomicrons [16,21,30]. After entering the circulation, chylomicrons undergo a process of remodeling that involves primarily the hydrolysis of triglycerides by lipoprotein lipase, resulting in the formation of chylomicron remnants [25]. Vitamins E, A, D and K are not affected by hydrolysis and remain in the lipoprotein particle for further transport to the liver [31]. The different forms of vitamin E are discriminated in the liver by the α-tocopherol transfer protein (α-TTP), which promotes the incorporation of 2*R*- or *RRR*-α-TOH into very low-density lipoproteins (VLDL) [32,33], whereas other forms and stereoisomers are either metabolized or secreted into bile [34]. Besides α-TTP, the TOH-associated protein and the TOH-binding protein are known mediators of the intracellular transport of vitamin E. Interestingly, α-TOH secretion from the liver is apparently not necessarily dependent on VLDL assembly and secretion, thus oxysterol-binding proteins [35] and ATP-binding cassette transporter A1 (ABCA1) [36] have been suggested to contribute to the release from the liver. Furthermore, ABCA1 mediates the efflux of vitamin E in the intestine, macrophages, and fibroblasts [36], and multidrug resistance P-glycoprotein has been identified as a transporter for the excretion of α-TOH via bile [37]. After the release of vitamin E-carrying VLDL into blood circulation and action of lipoprotein lipase as well as hepatic lipase, receptors such as SRB1, low-density lipoprotein (LDL) receptor as well as LDL receptor-related protein mediate the uptake of vitamin E into peripheral tissues and the liver [31,38].

3. Metabolism of Vitamin E

The metabolism of vitamin E is primarily localized in the liver (Figure 1) (reviewed in [39]), whereas extrahepatic pathways have been also suggested [40,41]. The degradation processes of hepatic metabolism remain poorly understood, but the initial mechanisms are generally accepted, i.e., all vitamers are degraded to vitamer-specific physiological metabolites with an intact chromanol ring and a shortened side-chain. Interestingly, accumulation of vitamin E to toxic levels is prevented by increased metabolism in response to higher vitamin E levels. Due to the preferential binding to α-TTP, α-TOH is the prevalent form of vitamin E in humans. It is speculated that α-TTP protects the α-form from degradation, thus leading to the accumulation of α-TOH. With the lower affinities of the other vitamin E forms to α-TTP taken into consideration, γ- and δ-forms are likely catabolized faster [42]. Despite of the different catabolic rates, all forms of vitamin E follow the same metabolic route, as confirmed by the detection of the respective end products of hepatic metabolism, α-, β-, γ-, and δ-carboxyethylhydroxychromanol (CEHC) [43,44]. However, the rate of catabolism is different for the vitamin E forms, possibly due to distinct affinities to key enzymes [42,45]. The chroman ring is not modified during catabolism (the catabolic end products are still classified as α-, β-, γ- and δ-forms); it is rather the aliphatic side chain where modifications are introduced. Metabolism of T3 follows the same principle, albeit further enzymes such as 2,4 dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase (necessary for the metabolism of unsaturated fatty acids) are likely required for the degradation of the unsaturated side chain [46].

The catabolism of the vitamin E molecule takes place in different cell compartments: endoplasmic reticulum, peroxisomes, and mitochondria. However, the mechanism of metabolite transfer between the compartments is not well understood and requires further investigation. The initial step at the endoplasmic reticulum leads to the formation of 13′-hydroxychromanol (13′-OH) metabolites via ω-hydroxylation by cytochrome P450 (CYP) 4F2 or CYP3A4, respectively [45,47]. The following ω-oxidation, which is probably mediated by alcohol and aldehyde dehydrogenases (an aldehyde intermediate is formed), results in 13′-carboxychromanol (COOH) metabolites. In general, the resulting metabolites with carboxy function are degraded like branched-chain fatty acids. Hence, the side chain is shortened by β-oxidation, and the formed propionyl-CoA or acetyl-CoA is eliminated. The intermediate-chain metabolites 11′-COOH and 9′-COOH are formed in peroxisomes during

the first two cycles of β-oxidation. Three additional cycles of β-oxidation are carried out in the mitochondria, resulting in the short-chain metabolites (SCMs) 7′-COOH and 5′-COOH as well as the end-product CEHC or 3′-COOH. Moreover, conjugation of the metabolites takes place during metabolism, resulting predominantly in sulfated and glucuronidated metabolites. However, glycine-, glycine–glucuronide-, and taurine-modified metabolites of vitamin E have also been identified [48].

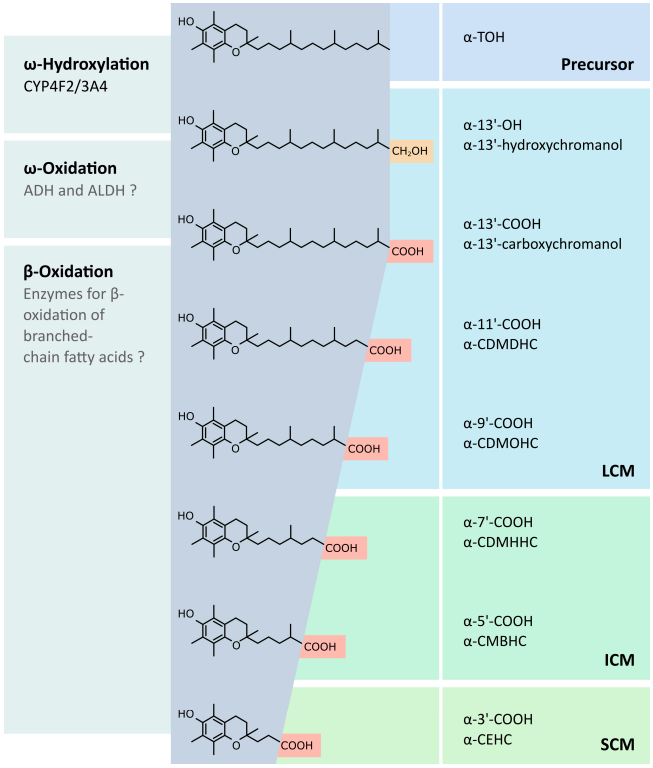

Figure 1. Metabolism of vitamin E. The metabolism of vitamin E is initiated by a terminal ω-hydroxylation of the side-chain via CYP4F2 and CYP3A4. The resulting hydroxychromanol is further modified by ω-oxidation, resulting in the formation of carboxychromanol, possibly by alcohol and aldehyde dehydrogenases. As a consequence, the metabolite can be subjected to β-oxidation. Five cycles of β-oxidation lead to the formation of the short-chain metabolite CEHC. However, this review focuses on the LCMs 13′-OH and 13′-COOH as these molecules have been synthesized in sufficient amounts for in vitro and in vivo investigations. The following abbreviations are used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CDMDHC, carboxydimethyldecylhydroxychromanol; CDMOHC, carboxymethyloctylhydroxychromanol; CDMHHC, carboxymethylhexylhydroxychromanol; CMBHC, carboxymethylbutylhydroxychromanol; CEHC, carboxyethylhydroxychromanol.

The conjugated SCMs are more hydrophilic and thus mainly found in glucuronidated form in human urine [44]. In contrast, the long-chain metabolites (LCMs) and their metabolic precursors are secreted via bile into the intestine and the metabolites in fecal samples are not conjugated. The fecal route is considered as the major pathway of vitamin E excretion [12,49].

Like vitamin E, other fat-soluble vitamins, such as the vitamins A (i), D (ii) and K (iii) are also metabolized in the human body:

(i). Under physiological conditions, retinyl esters (in the intestinal lumen) and carotenoids (in enterocytes) are converted into retinol before or during their intestinal absorption, respectively. Inside the enterocytes, retinol is re-esterified by lecithin-retinol acyl transferase or acyl-CoA:retinol-acyltransferase and packed into chylomicrons for transport. The retinyl esters are transferred to the liver and stored in hepatic parenchymal and non-parenchymal cells.

Vitamin A is mobilized from liver stores by the retinol-binding protein, a specific transporter allowing the transport of retinol in blood circulation [50]. These results suggest that vitamin A has an active (retinol) and a storage form (retinyl ester). In addition, the oxidation of retinol leads to the formation of retinal, another active form of vitamin A, which is primarily bound to opsins in the photoreceptors of the retina [51]. More current research indicates that all-*trans* retinoic acid (ATRA), 9-*cis*-RA, and all-*trans*-4-oxo-RA are the vitamin A metabolites with the highest biological activity. These active vitamin A metabolites serve as ligands for nuclear receptors, called retinoic acid receptors (RARs) [52] and retinoid receptors (RXRs) [53], which act as ligand-activated transcription factors controlling the expression of their respective target genes. Therefore, hepatic retinol is transferred to extrahepatic tissues and metabolized to retinoic acid by different enzymatic systems. LAMPEN and co-workers found that ATRA is also formed in the small intestine via direct oxidation of vitamin A. Based on this result, they hypothesized that biologically active retinoids are formed in the gastrointestinal tract and act as retinoid-receptor ligands controlling various processes in the intestinal mucosa via RAR [53].

(ii). The human metabolism of vitamin D is primarily located in liver and kidney. Metabolism of vitamin D_2 and D_3 starts with the formation of 25-OHD, the major circulating vitamin D metabolite, by vitamin D-25 hydroxylase. Afterwards, 25-OHD is transferred to the kidney and further catabolized by 25-OHD-1α-hydroxylase to 1,25-dihydroxyvitamin $D_{2/3}$. These molecules serve as ligands for the vitamin D receptor (VDR), a transcription factor expressed in various tissues. Vitamin D receptor binds to specific regions in the promoter regions of genes, the so-called vitamin D responsive elements, thus controlling the expression of respective target genes. Therefore, 1,25-dihydroxyvitamin D is the active metabolic form of vitamin D [54,55].

(iii). Phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) are summarized by the term vitamin K. Phylloquinone is synthesized in plants, while menaquinone is derived from animal and bacterial origins [30,56]. Both compounds share a 2-methyl-1,4-naphthoquinone structure, called menadione, and a side chain at the 3′-position. The side chain of phylloquinone is composed of three isopentyl units and one isopentenyl unit, while the side chain of menaquinone contains a variable number of only isopentenyl units (2–13) [30]. The metabolism of vitamin K is localized in the liver and has not been studied in detail so far [57]. Nevertheless, the metabolic pathway of phylloquinone and menaquinone degradation likely follows that of vitamin E. Hence, the degradation starts with an initial ω-oxidation, which is mediated by CYP. While the ω-oxidation of vitamin E is catalyzed primarily by CYP4F2, CYP3A4 has been described as the possible mediator for the ω-oxidation of vitamin K. Next, the following degradation of the side chain of vitamin K occurs via β-oxidation [30,56,58]. A 5-carbon carboxylic acid metabolite termed K acid 2 has been identified as the end-product of either phylloquinone or menaquinone metabolism and is excreted via urine and bile [30,58]. In addition to their metabolic degradation, it has been suggested that phylloquinones could also be converted to menaquinones [59,60]. For this, phylloquinone is likely transformed to the intermediate menadione by removing its side chain, which is subsequently replaced by a newly synthesized isopentenyl side chain to form menaquinone [30]. While menaquinone is considered as the physiologically active form of vitamin K in humans [56], almost nothing is known about a possible biological activity of the vitamin K metabolites. Further studies are needed to unravel whether vitamin K must be included into the general concept of a metabolic pre-activation of lipid-soluble vitamins.

Although the metabolisms of vitamin A and D differ in location and the involved enzymatic systems, the formation of active metabolites seems to be a key element of both metabolic pathways, i.e., both vitamins mediate their gene regulatory effects by metabolic pre-activation. Therefore, the discovery of vitamin E metabolism in animals and humans and the emerging evidence for important biological functions of vitamin E metabolites could indicate a general metabolic activation mechanism of fat-soluble vitamins in the human body.

In Vivo Verification of Systemic LCM Availability

Since the discovery of vitamin E by EVANS and BISHOP in 1922 [2], α-TOH has been accounted as an antioxidant capable to scavenge reactive oxygen species, and decreased α-TOH levels have been associated with several diseases including different types of cancer, cardiovascular diseases and diabetes [61]. It took 80 years since AZZI and co-workers set up the hypothesis for an additional gene regulatory role of α-TOH in the human body [62]. In addition, the discovery of vitamin E metabolism in animals and humans and the emerging evidence for important biological functions of the vitamin E metabolites [63,64], suggested that the TOHs may gain biological activity after metabolism (as confirmed for vitamin A and D). This prompted studies that investigated also the putative functions of the LCMs of TOH. In 2014, Wallert and co-workers showed the occurrence of α-13′-COOH in human serum, which has been confirmed later by others [65,66]. For these studies, serum obtained from a healthy, middle-aged (39 years), non-smoking male, who received a balanced diet with no additional vitamin E supplementation was used for the detection of α-13′-COOH via liquid chromatography coupled mass spectrometry [63]. The analyses revealed for the first time that α-TOH metabolites are transferred into blood circulation following metabolism of α-TOH in the liver. Furthermore, cell experiments showed that α-13′-OH and α-13′-COOH are more potent regulators of gene expression than their metabolic precursor α-TOH [63]. Taken together, the results of Wallert et al. provided the first evidence that the LCMs are an active form of their metabolic precursor [63], promoting regulatory effects in peripheral tissues of the human body. However, while the role of vitamin E as a lipophilic antioxidant in vitro is widely accepted, the relevance in vivo is still a matter of debate [67–69].

4. Biological Activity

Not much is known about the biological activity of the LCMs. However, the publications on this topic published during the last ten years can be categorized by the biological effects of the LCMs as follows: (i) anti-inflammatory actions [64,70–75]; (ii) anti-carcinogenic effects [72,76,77]; (iii) regulation of cellular lipid homeostasis [63,64]; (iv) interaction with pharmaceuticals [78]; and (v) regulation of their own metabolism [79] (Figure 2).

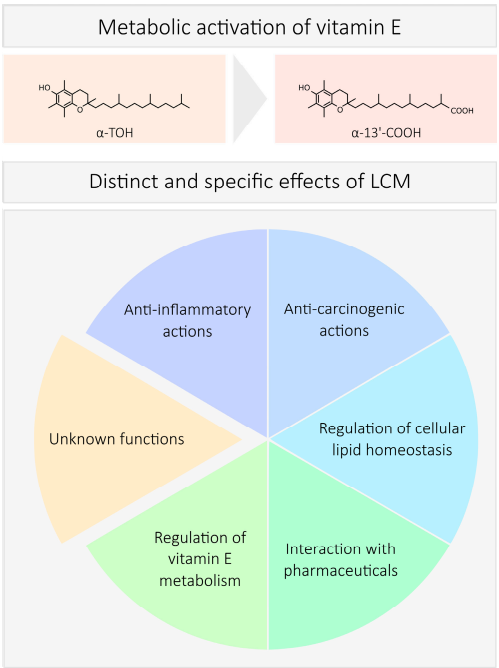

Figure 2. Reported biological functions of the LCMs of vitamin E.

4.1. Anti-Inflammatory Actions

Investigations on anti-inflammatory actions often focus on the regulation of pro-inflammatory enzymes, such as inducible cyclooxygenase 2 (COX2) [70–72,74], inducible nitric oxide synthase (iNOS or nitric oxide synthase, NOS2) [64,71,74,75], or 5-lipoxygenase (5-LO) [72,73], as well as mediators such as chemokines or cytokines. For this purpose, cells were treated with the LCMs and challenged with a pro-inflammatory stimulus or alternatively, isolated enzymes were used. Several LCMs (α-, γ-, δ-13′-COOH; δ-9′-COOH; α-13′-OH) have been tested and reduced the stimulus-induced expression (mRNA or protein) or enzyme activity. In general, 13′-COOH are more potent than the shorter LCMs and the conjugation of LCMs with sulfate abrogates their anti-inflammatory effects [64,70].

Jiang et al. gained first hints on the anti-inflammatory actions of LCMs [70]. A549 cells, which are capable of metabolizing vitamin E, were incubated with TOHs and an inhibition of the arachidonic acid-stimulated COX activity was reported. When the metabolism of vitamin E was suppressed by sesamin, the effects were less pronounced, indicating the involvement of the LCMs as regulatory molecules. For further experiments, the LCMs were extracted from the cell culture medium and their inhibitory capacity on COX activity was tested (half maximal inhibitory concentration (IC_{50}): δ-13′-COOH: 4 μM; δ-9′-COOH: 6 μM). The impact of conjugation was tested, and the sulfate LCM conjugates were unable to exert anti-inflammatory effects. In 2016, a comparison of the different types of LCMs was performed, and the LCMs showed similar effects regardless of their origin (isolated from cell culture medium or semisynthetic isolation from *Garcinia kola*) [72]. In RAW264.7 macrophages, the anti-inflammatory action on lipopolysaccharide (LPS)-stimulated COX2 mRNA and protein expression, as well as prostaglandin (PG) release was reported for α-13′-OH [71] and α-13′-COOH [74].

The regulation of iNos by the LCMs was studied in RAW264.7 macrophages [64,71,74,75]. The LPS-stimulated iNos mRNA and protein expression as well as release of nitric oxide were reduced by the LCMs tested (α- and δ-13′-OH, α- and δ-13′-COOH) [64]. The inhibitory effect of the LCMs was highly dependent on the structure of the LCMs. The 13′-COOH were more effective than the 13′-OH, while the substitution of the chromanol ring system (α- vs. δ-LCMs) had no influence.

The inhibition of ionophore-induced leukotriene release (leukotriene B_4) in HL-60 cells and neutrophils was reported with IC_{50} values of 4–7 μM [73]. Furthermore, the activity of isolated 5-LO was inhibited by δ-13′-COOH with IC_{50} values of 0.5–1 μM, which is more effective than the synthetic 5-LO inhibitor zileuton (IC_{50}: 3–5 μM) [73]. The inhibition of 5-LO activity by δ-13′-COOH was also confirmed by Jang et al. [72]. An overview of the known anti-inflammatory actions of the different LCMs of vitamin E studied so far is provided in Table 1.

Table 1. Overview of anti-inflammatory actions of the LCMs of vitamin E.

Targets	Cells	Effects	Substances	Refs.
COX2	A549 cells	Reduced activity in arachidonic acid-pre-induced cells	γ-13′-COOH δ-13′-COOH δ-9′-COOH	[70] [70,72] [70]
	Isolated enzyme	Inhibition of activity	δ-13′-COOH δ-9′-COOH	[70]
	RAW264.7	Inhibition of LPS-stimulated mRNA and protein expression, as well as reduced PG release	α-13′-OH α-13′-COOH	[71] [74]
iNos	RAW264.7	Inhibition of LPS-stimulated mRNA and protein expression, as well as reduced release of nitric oxide	α-13′-OH α-13′-COOH δ-13′-OH δ-13′-COOH	[64,71,74,75]
5-LO	Isolated enzyme HL-60 neutrophils	Inhibition of activity Reduced activity and LT release in pre-induced cells	δ-13′-COOH δ-13′-COOH	[72,73] [73]

PG, prostaglandin; LT, leukotriene.

The metabolites of vitamin K have also been shown to exert anti-inflammatory functions. First experiments were carried out with a synthetic 7-carbon carboxylic acid vitamin K metabolite (2-methyl, 3-(2′methyl)-hexanoic acid-1,4-naphthoquinone; K acid 1), which was a more effective

inhibitor of LPS-induced IL-6 release from fibroblast than the precursors phylloquinone and menaquinon-4 [80]. In LPS-challenged MG63 osteoblasts the 7-carbon carboxylic acid metabolite as well as the 5-carbon carboxylic acid metabolite (K acid 2) attenuated the expression of IL-6 [81]. Later, the long-chain metabolites of vitamin K (10 to 20-carbon carboxylic acid metabolites) were also synthesized and examined for their anti-inflammatory activity. In LPS-challenged mouse macrophages, these compounds reduced the induction of gene-expression of the inflammatory markers IL-1β, IL-6 and TNFα [82]. However, K acid 1 and K acid 2 were also effective in this study; and it is not possible to estimate, which vitamin K metabolite (either long-chain or short-chain) is the most effective [82]. Interestingly, the minor 7-carbon carboxylic acid metabolite was more effective in MG63 osteoblasts than the 5-carbon carboxylic acid metabolite, and a replacement of the carboxy function by a methyl group made the two metabolites less effective [81]. This is in line with findings for the LCMs of vitamin E. Here, the carboxy metabolite is more effective than the respective TOH precursor with respect to the anti-inflammatory actions (vide supra). However, the in vivo relevance of the regulatory activities of the vitamin K metabolites is a matter of debate, as they increase with vitamin K intake in urine [83], but have not yet been found in human blood or other tissues to the best of our knowledge.

4.2. Cancerogenesis and Chemoprevention

The metabolites of vitamin E were investigated with respect to putative anti-cancerogenic, i.e., anti-proliferative and pro-apoptotic, properties in several studies. First experiments revealed that the SCMs inhibit cell proliferation in different cell lines [84,85]. Interestingly, the metabolites as well as the precursor molecules showed different efficiencies, depending on the methylation pattern of the chroman ring and also on the cell type tested [84,85]. Based on the anti-proliferative effects of the SCMs, the interest in the effects of the LCMs aroused. Hence, Birringer et al. investigated the effects of the LCMs α-13′-COOH and δ-13′-COOH as well as α-13′-OH and δ-13′-OH on the proliferation of the human hepatocyte carcinoma cell line HepG2 [77]. Interestingly, both 13′-COOH metabolites effectively caused cell growth arrest, but the hydroxy metabolites did not exhibit anti-proliferative effects. Thus, the introduction of the carboxy group during TOH metabolism renders the molecule active with respect to cell growth arrest. This is supported by the finding that the metabolic precursors, i.e., TOHs, did not affect proliferation of HepG2 cells [77]. As mentioned above, the methylation of the chroman ring alters the efficiency of the molecules. With an effective concentration of 6.5 μM in HepG2 cells regarding the effects on cell growth, the δ-metabolite is more effective than its α-counterpart with 13.5 μM [77]. At first glance, contradictory results were reported for human prostate cancer cells. Here, not only δ-13′-COOH inhibited cell proliferation, but also the hydroxy metabolite α-13′-OH. The LCMs as well as the tested SCMs α-CEHC and γ-CEHC inhibited the proliferation by about 60% in a concentration of 10 μM [76]. Hence, the efficiency of the hydroxy metabolite is likely dependent on the cell type. It is possible that the differences in TOH metabolism in different cell types lead to divergent effects. Interestingly, even differences between different cancer and non-cancer cell lines have been described. The proliferation of the colon cancer cell lines HCT-116 and HT-29 was inhibited by δ-13′-COOH, with IC_{50} values of 8.9 μM and 8.6 μM, respectively [72]. While 10 μM of the LCMs reduce the viability of the cancer cells by around 60%, normal colon epithelial cells showed a reduction of 10–20% at this concentration. Comparable effects were found for the δ-T3 LCM δ-T3-13′-COOH (δ-garcinoic acid), which reduced the viability of the colon cancer cells by about 75%, but the viability of normal colon cells merely by 10–20% [72].

The actions of the vitamin E metabolites are comparable to that of the metabolites of vitamin D and vitamin A. The active vitamin D metabolite $1,25(OH)_2D_3$ has been shown to modulate differentiation and proliferation of colon cancer cells and prostate cancer cells [86]. However, $1,25(OH)_2D_3$ led to an arrest of most cells that express a functional vitamin D receptor in G0/G1 phase [87]. The actions are mediated by interference with several regulatory proteins, such as epidermal growth factor receptor (EGFR), insulin-like growth factors (IGFs), p21, p27 as well as cyclins and cyclin-dependent kinases (CDKs) [87]. The retinoids are also known for their modulation of the cell cycle. In several cancer cell

lines, retinoic acid (RA) led to a cell cycle arrest in the G0/G1 phase via direct or indirect modulation of cyclins, CDKs and cell-cycle inhibitors [88]. Interestingly, TOHs and TOH SCMs have also been linked to cyclins and CDKs. In the human prostate cancer cell line PC3, γ-TOH as well as γ-CEHC led to a strong decrease in cyclin D1 protein expression. In line with this observation, CDK4 and p27 expression are reduced, albeit less pronounced [85]. Moreover, α-TOH and α-CEHC are ineffective with respect to anti-proliferative actions as well as suppression of cyclin D1 and CDK4 [85]. However, to date, no data is available on the action of the vitamin E LCMs on cell cycle regulators, although strong anti-proliferative effects have been shown for this class of metabolites.

More detailed investigations were carried out on the pro-apoptotic effects of the vitamin E LCMs. Birringer et al. found a significant induction of apoptosis in HepG2 cells treated with 20 μM of α-13′-COOH, δ-13′-COOH or δ-13′-OH [77]. The LCMs induced the cleavage of caspases 3, 7 and 9, and in line with this, the cleavage of the downstream mediator poly-ADP ribose polymerase-1 (PARP-1). Again, the 13′-COOH were more effective in caspase-cleavage and apoptosis induction than the hydroxy metabolite [77]. Moreover, induction of mitochondrial apoptosis by the LCMs was identified as the process leading to apoptosis. This process is accompanied by the formation of reactive oxygen species (ROS). Birringer et al. observed a significant increase in ROS production in cells treated with α- and δ-13′-COOH but not with the hydroxy metabolites and the TOHs [77]. The augmented ROS production was not only measured intracellularly but also intramitochondrial, hence providing evidence for mitochondrial-derived apoptosis. Alterations in the mitochondrial membrane potential supported this finding. Treatment with 20 μM of the LCMs led to a significant reduction of the mitochondrial membrane potential. Interestingly, in this particular case, the α-metabolite was more potent than the δ-metabolites with 60% reduction vs. 20% reduction [77]. The pro-apoptotic actions of the δ-LCMs of vitamin E were confirmed in colon cancer cells [72]. Early and late apoptosis were induced by δ-13′-COOH and δ-T3-13′-COOH. The activation of caspase-9 and cleavage of PARP found by Birringer et al. [77] were confirmed in colon cancer cells [72]. Moreover, an induction of the autophagy marker microtubule-associated protein 1A/1B-light chain 3 (LC3)-II was found. Jang et al. assumed that alterations in sphingolipid metabolism caused by the carboxy-LCMs are the reason for the induction of apoptosis. Indeed, both δ-13′-COOH and δ-T3-13′-COOH increased total ceramides, dihydroceramides and dihydrosphingosines, while all measured sphingomyelins were decreased. Inhibition of sphingosine biosynthesis revealed that LC3-II expression but not PARP-cleavage is modulated by the LCMs via alterations in sphingolipid metabolism [72].

Taken together, there are several similarities between the metabolites of vitamins A, D and E with respect to anti-cancerogenic properties. Data on anti-cancerogenic effects of vitamin K metabolites, however, are sparse. Merely synthetic carboxylic derivatives of menaquinone with different side-chain lengths have been studied [89]. The biologically most abundant 5-carbon carboxylic acid metabolite (K acid 2) was not included in this study and the 7-carbon carboxylic acid metabolite (K acid 1) was the structure with the shortest side-chain. Interestingly, the growth-suppressing effect on hepatocellular carcinoma cells increased with the length of the side chain of the carboxy derivatives, except for the full-length metabolite, which was as effective as the 7-carbon carboxylic acid metabolite. Conversely, menaquinone itself was completely ineffective, showing nicely that the introduction of a carboxy function activates the compound. Blocking of the effects with chemical antagonists suggested that the derivatives act through caspase/transglutaminase-related signaling [89]. The above mentioned disruption of mitochondrial function by the LCMs of vitamin E has also been described for the metabolites of vitamin A [90], and induction of apoptosis by $1,25(OH)_2D_3$ via mitochondrial pathways (e.g., via B-cell lymphoma (BCL)-2 and BCL-xL) in breast, colon and prostate cancer cells are also known [87]. Based on their anti-proliferative and pro-differentiation actions but also due to the induction of cell death, retinoids are used for treating certain types of cancer [91]. Vitamin A metabolites were successfully used in the treatment of acute promyelocytic leukemia (ATRA and 13-*cis*-RA, 13*c*RA), squamous cell skin cancer and neuroblastoma (13*c*RA), lung cancer (ATRA) and Kaposi's sarcoma (9-*cis*-RA, 9*c*RA). Beneficial effects of retinoids in cancer prevention have also been observed.

These properties can be explained by the targeting of regulators of cell cycle progression by retinoids. The expression of the CDK inhibitors p21 and p27 is regulated by ATRA via RARβ2 upregulation, and retinoic acid has been shown to stimulate the degradation of cyclin D1, leading to a suppression of CDK activity [91]. Interestingly, TOHs as well as SCMs of vitamin E modulate cyclins, CDKs and CDK inhibitors [85]. Albeit the LCMs of vitamin E efficiently suppress proliferation, the identification of effects on regulators of cell cycle progression is pending. However, given that 'decreased proliferation is one of the best biomarkers of a cancer preventive effect' [91], vitamin E and its metabolites are promising compounds for cancer prevention.

4.3. Cellular Lipid Homeostasis

To date, the effects of the LCMs of vitamin E on cellular lipid homeostasis have not been investigated extensively. However, the regulation of key metabolic pathways in foam cell development of macrophages by the LCMs were of particular interest in a study by Wallert et al. [63]. Here, the regulation of the expression of the cluster of differentiation 36 (CD36), the uptake of oxidized low density lipoprotein (oxLDL), phagocytosis and the intracellular storage of lipids were investigated [63]. For this, the monocytic THP-1 cell line, which can be differentiated to macrophage-like cells, was used. In differentiated macrophages, the LCMs α-13′-OH and α-13′-COOH induced the expression of CD36 mRNA and consequently CD36 protein levels. In contrast, the precursor α-TOH exerted opposite effects on CD36 mRNA and protein. Whereas α-TOH reduced the expression of CD36 at a concentration of 100 μM, the α-LCMs induced the expression of CD36 in concentrations of 5 and 10 μM, respectively [63]. Thus, the α-LCMs not only act in a different way than their precursors, but appeared to be also significantly more potent. Interestingly, similar effects were described for the lipid soluble vitamin A. Langmann et al. found that the precursor β-carotene is less effective in inducing expression of CD36 than its metabolites ATRA and 9*c*RA in human monocytes and macrophages [92]. The authors stated that the metabolites 9*c*RA and ATRA displayed high biological activity [92], while the precursors retinol and β-carotene were only marginally metabolized, an observation that parallels the characteristics of the LCMs of vitamin E with respect to their reported serum concentrations [63,93]. The effects of vitamin A metabolites are better characterized than that of the LCMs of vitamin E. It was repeatedly shown that the metabolites of vitamin A regulate CD36 expression in macrophage cell models. The metabolite 9*c*RA induced CD36 mRNA [94,95] and protein expression [95] in human THP-1 macrophages. ATRA increases expression of CD36 mRNA in THP-1 cells [96] and CD36 protein in THP-1 and HL60 macrophages [96,97]. The induction of CD36 expression by ATRA and 9*c*RA has been confirmed in primary human monocytes and macrophages [92,96] to show the physiological relevance in non-cancer cells. With the same intention, it was also shown that the LCMs of vitamin E induced CD36 expression in peripheral blood mononuclear cell (PBMC)-derived primary human macrophages [63].

The scavenger receptor CD36 mediates the uptake of the modified lipoprotein oxLDL [98], a process that in turn stimulates CD36 expression [99]. Given the induction of the expression of CD36 by the LCMs of vitamin E under basal conditions (vide supra), a further stimulation by oxLDL treatment could be expected. As the uptake of oxLDL is a hallmark of macrophage foam cell formation, Wallert et al. examined whether preincubation of THP-1 macrophages with the LCMs of vitamin E affects the oxLDL-induced expression of CD36 [63]. As expected, CD36 expression was induced by oxLDL treatment. Pre-treatment with α-TOH suppressed the induction by oxLDL. In contrast, the pre-incubation with the LCMs augmented the induction of CD36 expression by oxLDL. These findings resemble the reaction of the cells in the absence of oxLDL to α-TOH and its LCMs. Given the higher CD36 expression in the presence of the LCMs, the uptake of oxLDL should in turn be induced in LCM-treated macrophages. However, pre-incubation of the macrophages with the LCMs for 24 h led to decreased oxLDL uptake. Incubation with both, α-13′-OH or α-13′-COOH, decreased the uptake by about 20%. This effect was again confirmed in PBMC-derived macrophages. Here, oxLDL uptake was decreased by α-13′-OH pre-treatment by 24% and by α-13′-COOH pre-treatment

by 20%, respectively [63]. The LCMs of vitamin E thus exerted unexpected effects on oxLDL uptake. As mentioned before, vitamin A metabolites also caused increased CD36 expression, but the metabolite 9*c*RA induced the binding and uptake of oxLDL in THP-1 macrophages as expected [94]. Generally, an activation of RXR leads to an augmented association of oxLDL to THP-1 macrophages [100]. However, 9*c*RA also promoted the degradation of oxLDL and the cholesterol efflux via ATP binding cassette transporters, thus leading to a net depletion of cholesterol esters. Triglyceride levels were apparently not affected, neither by oxLDL treatment nor combination with 9*c*RA [94]. In contrast, in the study of Wallert et al. on the LCMs of vitamin E, oxLDL treatment of the macrophages led to an increase of neutral lipids in the cells. Preincubation with the LCMs diminished the oxLDL-induced neutral lipid accumulation [63]. However, the contradictory results on the effects of the LCMs on CD36 expression and oxLDL uptake required an alternative explanation how the LCMs decrease oxLDL uptake. Thus, Wallert et al. focused on phagocytosis as an alternative uptake mechanism for oxLDL [101]. Indeed, treatment of the macrophages with α-13′-OH led to an inhibition of phagocytotic activity of 16% and with α-13′-COOH of 41%, respectively [63]. Hence, the inhibition of phagocytosis by the LCMs might explain the discrepancy between their effects on CD36 expression and oxLDL uptake in this study.

Taken together, the metabolites of vitamin E and vitamin A induce the expression of CD36 in macrophages. However, their effects on oxLDL uptake are different. While the vitamin A metabolite 9*c*RA induces oxLDL uptake, the LCMs of vitamin E reduce it. In contrast to vitamin A and vitamin E metabolites, the metabolite of vitamin D, 1,25$(OH)_2D_3$ has been shown to reduce the expression of CD36 mRNA and protein in oxLDL-treated macrophages obtained from diabetic subjects. Concomitantly, oxLDL and cholesterol uptake are decreased [102,103]. Hence, the vitamin D metabolite as well as the vitamin E LCMs suppress macrophage foam cell formation and may thus exert positive effects in the context of atherosclerosis prevention.

4.4. Interaction with Pharmaceuticals

The interaction of the vitamin E LCMs with pharmaceuticals was tested by analyzing the regulation of P-glycoprotein (P-gp). P-gp regulates, inter alia, the intracellular concentration of pharmaceuticals and its expression is regulated by various transcription factors, including heat shock transcription factor 1, nuclear factor Y and the pregnane X receptor (PXR) [104,105].

Several vitamin E forms and their metabolites (α-TOH, α-T3, α-13′-COOH, α-CEHC, γ-TOH, γ-T3, γ-CEHC and plastochromanol-8) were used and the regulation of P-gp expression was analyzed in human epithelial-like colon LS180 cells [78]. Only α-13′-COOH and γ-T3 induced P-gp expression and α-T3, α-13′-COOH as well as γ-T3 induced the activity of PXR in a reporter gene assay. In case of vitamin E supplementation, an interaction with the metabolic handling of pharmaceuticals might be possible.

4.5. Regulation of LCM Formation

The regulatory processes, which modulate the metabolism of vitamin E, are largely unknown. In this context, two key issues are important: (i) Apart from CYP4F2 and CYP3A4, the full set of enzymes involved in the first steps of the catabolism of vitamin E remains to be identified, and (ii) the mechanisms by which vitamin E metabolism is regulated have not yet been sufficiently unraveled. However, the upregulation of CYP4F2 protein expression by α-13′-OH in human HepG2 liver cells was reported recently [79], pointing to a positive regulatory feedback loop. If this concept holds true, the enhancement of metabolism by products would be a new facet for the fat-soluble vitamins, as the metabolism of vitamin A and D is mainly regulated negatively by their metabolic products [54,106].

The aldehyde- and alcohol-dehydrogenases have been suggested to be responsible for the ω-oxidation steps and the enzymes for branched-chain fatty acids might catalyze the subsequent β-oxidation [107]. Following the identification of the specific set of enzymes required for vitamin E

metabolism, a major aim will be the characterization of the regulatory factors, which modulate the metabolism of vitamin E.

5. Structure-Specific Effects

To get deeper insights into the specificity of the regulatory effects of the LCMs of vitamin E, a structure-activity study was conducted [64]. For this purpose, substances were used that represent specific substructures of the LCMs or their precursors. The chromanol ring system was mirrored by the SCM α-CEHC and the modified side-chain was represented by the branched-chain fatty acid pristanic acid. Furthermore, the α- and δ-forms of 13′-OH and 13′-COOH were used to study the influence of the side-chain modification. Overall, the application of α- and δ-forms of LCMs and their precursors (α-TOH, α-13′-OH, α-13′-COOH, δ-TOH, δ-13′-OH, δ-13′-COOH) should clarify the importance of the substitution of the ring-system. The regulation of CD36 and iNos by the test compounds was similar for all of the LCMs, but neither the precursors nor their substructures were able to cause the same effects on the expression of the target genes as the LCMs. The substitution of the chromanol ring system had no influence (α- and δ-forms), while the modification of the side-chain (oxidation of TOH to 13′-OH and 13′-COOH) was highly relevant for the effects. Overall, the 13′-COOH was most potent in this study. Based on these specific regulations the existence of specific regulatory molecular pathways for the LCMs has been suggested.

6. Receptors of Vitamin Metabolites

As indicated above, the lipid-soluble vitamins A and D need a conversion to their active metabolites to exert their effects. These metabolites are either bound intracellularly and transferred to the receptor or directly bind the receptor. The receptors for the vitamin A metabolites, RARs and RXRs, were identified in the late 1980s [108–111]. Evidence for binding proteins for the active vitamin D metabolite 1,25$(OH)_2D_3$ was already provided in the 1970's [112,113]; however, cloning of the human vitamin D receptor also succeeded in the late 1980's [114]. In contrast, no specific receptor for vitamin E and/or its metabolites has been identified yet. Interestingly, the metabolites of vitamin A and D act through nuclear receptors. This class of transcription factors can roughly be divided into more specific and rather unspecific members. The vitamin D receptor can be categorized as a more specific receptor, as it is activated by its endogenous ligand 1,25$(OH)_2D_3$ already at sub-nanomolar concentrations [115,116]. This feature is also shared by steroid hormone receptors (estrogen receptor, androgen receptor, ergosterone receptor, cortisol receptor), the thyroid hormone receptor and RARs. The RARs specifically bind ATRA, and also 9*c*RA with lower affinity [117]. The specificity of the nuclear receptors is mainly determined by the structure of the ligand binding pocket. Specific receptors have a relatively small ligand binding pocket, which allows only a limited number of molecules to interact. In contrast, the so-called adopted orphan receptors have a larger ligand binding pocket, allowing the activation of the receptor by a larger number of ligands [115]. Members of this group are the liver X receptors (LXRs), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs) and RXRs. The latter have been shown to bind the vitamin A metabolite 9*c*RA [118]. However, it is not entirely accepted that 9*c*RA represents the endogenous ligand for RXR [119]. Nonetheless, the example of 9*c*RA opens the possibility that vitamin metabolites act through highly specific receptors but also through rather unspecific ones.

Following the concept that the LCMs of vitamin E represent biologically active metabolites similar to 1,25$(OH)_2D_3$, ATRA and 9*c*RA, these molecules might also exert their effects through nuclear receptors. Indeed, Podszun et al. reported an activation of PXR by α-13′-COOH in the human colon adenocarcinoma cell line LS180 [78] (for detailed information, the reader is referred to the section 'Interaction with pharmaceuticals'). Interestingly, α-T3 and γ-T3 were also able to activate PXR, while α-TOH and γ-TOH as well as the SCMs α-CEHC and γ-CEHC failed to activate PXR [78]. These findings confirm earlier findings in HepG2 cells only in part. In HepG2 cells transfected with PXR and a CAT (chloramphenicol acetyltransferase) reporter gene, α-T3 and γ-T3 efficiently

activated PXR-mediated gene transcription, but α-TOH, γ-TOH and δ-TOH were also able to induce the expression of the reporter gene via PXR [120]. In contrast, the SCMs α-CEHC and α-CMBHC were not able to activate PXR in this study and the LCMs were not tested [120]. Taken together, the T3s reliably activate PXR but the effects of the TOHs need further investigation. Possibly, LS180 and HepG2 metabolize TOH with different efficiency, in turn determining the amounts of LCMs formed as PXR-activating metabolites. Hence, the observed effects of TOHs in HepG2 might be explained by the intracellular formation of the LCMs. However, further investigations on the cell-type specific metabolism of TOH are needed to confirm this hypothesis. Further, with PXR a rather unspecific nuclear receptor is identified for TOHs and their LCMs. As a general sensor for toxic compounds and xenobiotics, PXR has a large ligand binding cavity, which allows the binding of a wide range of ligands [121]. Thus, it is not surprising that PXR has been described as a receptor of vitamin K [122,123], and it has been reported that several menaquinone derivatives activate PXR [124]. Unfortunately, the biologically occurring carboxy derivatives were not included in this study. Hence, merely speculations about the activity based on structure-function-relationships are possible. A reporter gene assay revealed that a terminal phenyl group enhances the activity of the derivatives, while a terminal hydroxy group diminished it compared to the unmodified menaquinone [124]. In conclusion, a more hydrophobic side chain leads to an increased activity on PXR. Hence, the natural metabolic products in humans bearing a terminal carboxy group are likely less potent with respect to the activation of PXR. However, this concept is in contrast to the findings for vitamin E. The TOH precursors are unable to activate PXR, while the LCM α-13′-COOH activates it [78]. Hence, further studies are needed to clarify whether vitamin K metabolites are physiological ligands for the rather unspecific nuclear receptor PXR, like their metabolic precursor menaquinone and the LCMs of vitamin E.

Given that RXR as a receptor for the vitamin A metabolite 9*c*RA is also rather unspecific, it might be possible that the LCMs of vitamin E also act through PXR. However, it is questionable whether all of the reported biological effects of the LCMs, i.e., anti-inflammatory actions, anti-cancerogenic features, and effects on cellular lipid homeostasis (please refer to the respective sections here) can be ascribed to PXR activation. Hence, further investigations aiming at the identification and characterization of receptors for the LCMs of vitamin E LCMs are highly required. Strategies for the identification of further receptors or a receptor specific for the LCMs of vitamin E might be the use of target fishing approaches, gene expression arrays, knockdown/knockout studies, as well as reporter gene assays and ligand binding studies.

7. Conclusions

With the detection of the LCMs of vitamin E in human serum, an important hint for the possible action of these metabolites as signaling molecules was provided. Several studies reinforced this hypothesis by the characterization of the biological effects of the LCMs, as summarized in Figure 2. Interestingly, the LCMs act more potent and in part even contrary to their metabolic precursors. Some of the controversial effects reported for vitamin E might be therefore explained by the action of the LCMs. The evidence of circulating α-LCM in human blood (nanomolar concentrations) provides a new perspective in vitamin E research [63]. Therefore, the LCMs must be seriously considered to correctly interpret the effects of vitamin E in humans, beside the better studied TOHs and T3s. So far, only a few studies have focused on this class of compounds. However, based on our current knowledge and our studies in progress, we speculate that the LCMs comprise a new class of regulatory molecules. These molecules can exert effects that are different from their metabolic precursors, complicating the interpretation of studies on the effects of vitamin E in vivo. Nevertheless, the LCMs share properties with their precursors but also exert unique or even adverse effects. It is evident that the LCMs and their precursors act in the same manner with respect to the modulation of COX2 and 5-LOX activity, but it is of note that the LCMs are significantly more potent than their precursors. Furthermore, the LCM can act in areas where the TOHs are virtually not effective. A prime example is the regulation of COX2 expression. Hence, the LCMs may indeed play a role in mediating some of the effects of vitamin E

in the human body although blood concentrations are significantly lower than those of TOH. So far, blood concentrations are the only valid value for the systemic distribution of the LCMs of vitamin E in the human body. However, based on preliminary data of unpublished in vitro and in vivo studies of our group, we can hypothesize that the LCMs of vitamin E may also accumulate in different parts of the human body, where they reach concentrations higher than in blood. Further studies are required to study this issue in more detail and to differentiate between physiologic (at low concentrations) and pharmacologic (at high concentrations) actions of the LCMs.

To sum up, the LCMs could be regarded as the metabolically activated forms of vitamin E. This is in line with the metabolic activation of the other lipid-soluble vitamins A and D. Consequently, the concept of metabolic activation established for vitamin A and D could now be extended to vitamin E. Thus, a general concept for the biological activity and modes of action of the lipid-soluble vitamins could be defined.

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Chapter 6
Bioactivity of Vitamin E Long-Chain Metabolites

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Keywords Vitamin E · Long-chain metabolites of vitamin E · 13′-hydroxychromanol (13′-OH) 13′-carboxychromanol (13′-COOH) · Vitamin E metabolism · Biological activity

Key Points

- Metabolic activation of vitamin E precursors by hepatic catabolism
- Unknown distribution mechanisms and storage of LCMs (i.e., occurrence in organs and tissues)
- Detection of both α-LCMs in complex biological matrices
- Involvement of the LCMs in various regulatory processes
- Evidence for a general concept of metabolic activation for fat-soluble vitamins

Abbreviations

Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
APCI	Atmospheric pressure chemical ionization
α-TTP	α-Tocopherol transfer protein
ABCA1	ATP-binding cassette transporter A1
13′-COOH	Carboxychromanol

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CEHC	Carboxyethylhydroxychromanol
CD36	Cluster of differentiation 36
CYP	Cytochrome P450
ECD	Electrochemical detector
ESI	Electrospray ionization
FID	Flame ionization detector
FLD	Fluorescence detector
GC	Gas chromatography
HPLC	High-pressure liquid chromatography
13′-OH	Hydroxychromanol
ICM	Intermediate-chain metabolite
LCM	Long-chain metabolite
LDL	Low-density lipoprotein
LDLR	LDL receptor
LRP	LDL receptor-related protein
MS	Mass spectrometry
LC3	Microtubule-associated protein 1 light chain 3
PBMC	Peripheral blood mononuclear cell
P-gp	P-Glycoprotein
PARP	Poly-ADP ribose polymerase
Q-TOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
SR-B1	Scavenger receptor B1
SCM	Short-chain metabolite
SPE	Solid phase extraction
TOH	Tocopherol
T3	Tocotrienol
UV-Vis	Ultraviolet visible spectroscopy
VLDL	Very low-density lipoprotein

Formation of Vitamin E Long-Chain Metabolites

The metabolism of vitamin E is initiated by an ω-hydroxylation via cytochrome P450 (CYP) 4F2/3A4, resulting in the formation of 13′-hydroxychromanols (13′-OH). The subsequent ω-oxidation forms 13′-carboxychromanols (13′-COOH). Subsequent cycles of β-oxidation shorten the side chain and finally result in water-soluble carboxyethylhydroxychromanols (CEHCs o r 3 ′-COOH). A m ore detailed description is here provided in the chapter "Vitamin E metabolism." The knowledge about the regulatory processes of vitamin E metabolism is sparse, since the responsible enzymes are largely unknown (except CYP4F2 and CYP3A4) or have not yet been experimentally confirmed. Torquato et al. provided first hints by the observation of the upregulation of CYP4F2 protein by α-13′-OH in human HepG2 liver cells, indicating a positive regulatory feedback loop [1]. Whether this holds true for the other long-chain metabolite (LCM) forms, i.e., β-, γ-, or δ-13′-OH, or even for other metabolites (e.g., α-13′-COOH), is subject of further investigations. The enzymes that are responsible for the ω- and β-oxidation have not yet been experimentally validated, but Mustacich et al. described the organelles that are responsible for the metabolism of vitamin E and suggested the respective enzyme classes [2]. Based on this work, the identification of the aldehyde and alcohol dehydrogenases that are responsible for the ω-oxidation of 13′-OH is possible. Furthermore, the enzymes that are responsible for the degradation of branched-chain fatty acids have been suggested to degrade also the side chain

of vitamin E. However, this also requires validation in future studies. The successful identification of the respective enzymes is a vital starting point for unraveling regulatory mechanisms of vitamin E metabolism.

Distribution of Vitamin E Long-Chain Metabolites in the Human Body

Hepatic Formation and Transport of Vitamin E Long-Chain Metabolites

Storage and metabolism of vitamin E are strictly balanced in healthy humans to ensure constant and sufficient supply. Therefore, hepatic metabolism of vitamin E is regulated by a physiological feedback loop to avoid excessive accumulation of vitamin E and to bridge periods of vitamin E deficiency. All ingested vitamin E forms are first transferred via chylomicrons to the liver, but α-tocopherol (TOH) is selectively bound by the α-tocopherol transfer protein (α-TTP) [3] to direct it either for release or metabolism in different cellular compartments, the endoplasmic reticulum, the peroxisomes, and the mitochondria (for details, the reader is referred to the chapter "Vitamin E metabolism"). Hence, the intermediate metabolites of vitamin E must be transported between these compartments during their metabolic degradation. But, no experimental data is available whether this inter-compartment transport occurs by passive diffusion or is conducted by active transport via specific proteins. However, non-metabolized vitamin E is released from the liver, and the contribution of very low-density lipoproteins (VLDL) [4], oxysterol-binding proteins [5], and the ATP-binding cassette transporter A1 (ABCA1) [6] to this process has been discussed. The chemical structure of the α-13′-long-chain metabolites (α-13′-LCMs) and α-TOH is similar, except for the terminal oxidation of the aliphatic side chain. Therefore, the lipophilic nature of these molecules may be similar, and their transport via VLDL is conceivable but needs to be verified in future studies. In addition, the intracellular transport of the LCMs may occur via specific LCM-binding and LCM-transport proteins or via binding and transport proteins jointly used for α-TOH (e.g., α-TTP [3], tocopherol-associated protein [7]) or fatty acids (e.g., fatty acid-binding proteins [8]), since α-TOH follows the general transport pathway of lipids [9]. However, whether the transport of the LCMs is realized by specific (i.e., binding and transport proteins) or unspecific (i.e., via lipoproteins) mechanisms remains unclear.

Extrahepatic Transport and Storage

Only limited information about the transport of the LCMs in blood and their bioavailability in extrahepatic tissues is yet available. α-13′-OH [10] and α-13′-COOH [11] have been found in human serum, and increased serum concentrations after supplementation with 1000 IU α-TOH/d were observed [10, 11]. It might be possible that their extrahepatic transport in blood occurs via lipoproteins as it has been described for α-TOH [12]. Vitamin E in general is packed into chylomicrons after intestinal absorption and later transferred to other lipoproteins like HDL via phospholipid transfer protein, or it remains in chylomicron remnants. After the hepatic uptake, discrimination for α-TOH and resecretion into the blood via VLDL take place. According to the known fate of VLDL, α-TOH also occurs in LDL and HDL particles [13]. However, further studies are needed to clarify the involvement of lipoproteins or specific binding proteins in the distribution of the LCMs. The cholesteryl ester transfer protein, another member of the protein family of serum lipid transfer proteins, is also discussed to contribute to vitamin E transport and metabolism [14] and may also be involved in the transport of the LCMs. Until today, no specific plasma transport proteins for α-TOH [12, 15] nor the LCMs have been described.

It has been shown that normal vitamin E plasma levels of 25 μM can be increased about threefold by vitamin E supplementation, whereas α-13′-COOH serum levels increased even about eightfold. Preliminary studies in mice showed that α-13′-COOH reaches its highest concentrations in plasma 6 h after injection (unpublished data). Based on the increase of the LCMs after supplementation and the fast rise of plasma concentration and clearance from blood, LCMs might reflect the current nutritional status with respect to vitamin E. However, this hypothesis needs confirmation under non- supplemented normal conditions. As adipose tissue and skeleton muscles have been identified as long-term storage depots for vitamin E and the LCMs exhibit structural similarities to their respective vitamin E precursors, similar storage characteristics are conceivable [16, 17]. Hence, continuous release of the LCMs from these depots is likely. Nevertheless, a reliable analysis of tissue distribution and tissue-specific concentration of the LCMs is needed to draw conclusions about their bioavailability, stability, overall physiological relevance, and relevance in single organs. Thereby, the potential of these molecules for the therapeutic treatment of organ-related diseases such as nonalcoholic fatty liver disease should be studied. Next, investigations of putative regulatory feedback loops for the uptake of the LCMs are needed. Beside the major storage organs and tissues known for α-TOH (i.e., fat and muscles), the accumulation of α-TOH and its corresponding metabolites has also been shown for the kidney and small intestine [18]. Further, the analysis of the concentrations of the LCMs in the brain, since vitamin E deficiency is known to cause cognitive dysfunctions [19], as well as the heart, where the concentration of α-TOH increases after supplementation [20], is of interest. In addition, α-TTP is expressed in the placenta, indicating the importance of α-TOH for preventing fetus resorption [15]. However, to date there is no data available on the contribution of α-TTP to the tissue distribution of the LCMs. Crucial for the binding specificity of α-TTP is the substitution of the chroman ring system of possible ligands as well as the *R*-configuration at the 2′-position [3]. The phytyl side chain is very flexible, thus barely contributing to the specificity [21]. As the α-LCMs differ only in the terminal modification of the phytyl-like side chain from α-TOH, they may be likely bound by α-TTP with good affinity. Supporting this assumption, α-TOH analogues with terminal side chain modifications, like nitrobenoxadiazyl (NBD)-α-TOH and anthroyloxy (AO)-α-TOH, bind α-TTP and are in use to investigate functions of α-TPP [22, 23]. Hence, terminal modification of the α-TOH side chain does not prevent binding to α-TTP. However, the final confirmation of the binding of the α-LCMs to α-TTP is pending. Apart from human plasma or serum [11, 24], the concentrations of the LCMs in different organs or tissues have not been investigated. Given that only 1% of total body TOH is located in the blood [25], most of the vitamin E is stored in other parts of the body. To date, only serum concentrations of the LCMs are known, and it is possible that other tissues display higher concentrations of the LCMs than the blood.

Intercellular Transport and Intracellular Distribution

In addition to information about the accumulation of the LCMs in organs, investigations of uptake mechanisms for the LCMs into cells and cellular compartments will provide valuable information about intracellular distribution patterns. Initial experiments in human skin fibroblasts, human THP-1 macrophages, and human HepG2 liver cells revealed similar uptake kinetics for the LCMs (unpublished data), but further experiments are needed to characterize the transport mechanisms. As indicated above, the transport via lipoproteins, as known for α-TOH, has not yet been shown for the LCMs. The data on LCM uptake from in vitro experiments indicate for an independent transport mechanism but do not exclude lipoprotein-specific mechanisms. Hence, internalization of particles carrying the LCMs might involve among others the LDL receptor (LDLR) or the LDL receptor-related protein (LRP), as known for α-TOH [13]. In addition, the scavenger receptor B1 (SR-B1) and ABCA1 are of particular importance within the distribution pathways of vitamin E [14]. SR-B1 mediates the uptake of vitamin E into the intestine and in peripheral tissue, whereas LDLR and LRP

mediate the uptake into the liver. The efflux of vitamin E via ABCA1 takes place in the intestine and the liver. In addition, ABCA1 regulates the cellular efflux of vitamin E in macrophages and fibroblasts [6]. Based on its lipophilic nature, vitamin E and thus most likely also the corresponding LCMs are present in the endoplasmic reticulum as well as the peroxisomes [26]. Besides α-TTP, further intracellular binding proteins, such as the tocopherol-associated protein and the tocopherol-binding protein, are known to be involved in intracellular trafficking of α-TOH [27]. Further, the multidrug resistance transporter P-glycoprotein (P-gp) has been identified to mediate α-TOH transport across the plasma membrane and its secretion into bile [28]. All members of this protein family bind α-TOH with lower affinity than α-TTP [13]; however, their relevance as transporters of the LCMs remains unclear. Further experiments are needed to clarify the importance of α-TOH-related binding proteins, such as afamin [29], as well as the uptake mechanisms and kinetics that are responsible for the cellular and tissue distribution of the LCMs and hence their regulatory effectiveness.

Excretion

The metabolism of vitamin E finally forms the hydrophilic end-product α-CEHC, which is excreted via urine or secreted into the circulatory system [18]. In contrast, free α-13′-OH has been found to be excreted with feces [30], as shown earlier for γ-TOH and α-TOH [31]. Therefore, the bioavailability-to-excretion ratio of the LCMs needs to be investigated in further studies. A useful experimental tool to observe and analyze the formation of metabolites in humans is to use deuterium-labeled α-TOH [32]. As shown by Freiser et al., the γ-LCMs are mainly conjugated with sulfates [33] and glucuronides in plasma and are likely excreted. However, biological activity is only possible if the LCMs are available in unconjugated form [34]. To gain deeper insights into the pharmacokinetics of vitamin E, in particular into the formation of the metabolites as well as their bioavailability and distribution in tissues and excretion, robust and reliable analytical tools are indispensable.

Analytical Approaches for Vitamin E Metabolites

In the early days of vitamin E research, simple chromatographic methods were used to separate the different forms of vitamin E from other lipids and lipophilic molecules such as triglycerides and phospholipids. Thin-layer chromatography was one of the early approaches to detect vitamin E in biological samples [35]. Next, the development of gas chromatography (GC) and high-pressure liquid chromatography (HPLC) approaches allowed for better separation of the different vitamers [36, 37]. By coupling of either GC or HPLC to high-sensitive mass spectrometry (MS), vitamin E forms could be detected even in nanomolar concentrations within different matrices [38, 39]. Next, the discovery of vitamin E metabolism in animals and humans and emerging evidence for important biological functions of vitamin E metabolites made it necessary to enhance the existing analytical procedures for vitamin E [40]. All vitamin E forms undergo enzymatic modification (sulfation and glucuronidation) during their metabolic degradation, complicating their detection in biological samples. Thus, only a few research groups were able to establish valid methods for the determination of vitamin E metabolites in human matrices (reviewed in [40]), and so far only a few detected the LCMs in human blood [10, 11, 24, 41].

The different physicochemical properties of the vitamin E metabolites are major criteria for the development of appropriate analytic strategies. Short-chain metabolites (SCMs) and LCMs were found as sulfates, glucuronides, and glucosides or as unconjugated carboxychromanols in different matrices [40]. Therefore, the quantitative extraction of these metabolites from biological samples requires enzymatic deconjugation using sulfatase and β-glucuronidase or chemical hydrolysis.

Table 6.1 Subset of analyzable metabolites in biological samples with their corresponding preparation and detection methods

Deconjugation	Extraction	Chromatography	Sample	Metabolite	Ref.
–	LL	GC-MS	Cell culture supernatant (HepG2)	γ-7′,9′,11′,13′-COOH γ-13′-OH	[43]
S	LL	HPLC-FLD LC-MS (ESI(−))	A549 cells	γ-9′,11′13′-COOH γ-13′-OH γ-9′S,11′S,13′S-COOH δ-9′,11′,13′-COOH δ-9′S,11′S,13′S-COOH	[44]
			Rat plasma (200 µl)	γ-9′S,11′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
			Rat liver (homogenate of the entire organ)	γ-9′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
–	–	HPLC-UV-Vis	Cell culture supernatant (HepG2)	α-5′,13′-COOH α-13′-OH	[48]
S + G	LL	HPLC-ECD LC-MS (ESI(−))	Human serum (20 µl)	ICM, SCM, TOH	[49]
			Human urine (20 µl)	ICM, SCM	
			Human feces (10 mg)	α-3′,5′,13′-COOH γ-3′,11′-COOH δ-3′,11′-COOH α-TOH, γ-TOH, δ-TOH α-T3, γ-T3	
S + G	LL	Q-TOF-LC-MS (ESI(+))	Human serum (500 µl)	α-13′-COOH	[11]
–	LL	HPLC-ECD GC-MS	Human serum (1 ml)	α-13′-OH	[10]
n.i.	LL	LC-MS/MS (APCI)	Human serum (1 ml)	α-13′-COOH α-13′-OH	
S + G	LL	LC-MS/MS (ESI(+))	Human plasma or serum (500 µl)	α-3′,13′-COOH γ-3′-COOH α-13′-OH	[24]
			Human plasma or serum (100 µl)	α-TOH, γ-TOH	

Abbreviations used are the following: *n.i.* no information, *LL* liquid-liquid, *GC* gas chromatography, *HPLC* high-pressure liquid chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *FLD* fluorescence detector, *S* sulfatase, *G* β-glucuronidase, *ESI* electrospray ionization, *APCI* atmospheric pressure chemical ionization, *SCM* short-chain metabolite, *ICM* intermediate-chain metabolite, *T3* tocotrienol, *TOH* tocopherol, *OH* hydroxychromanol, *COOH* carboxychromanol

The SCMs have been measured in urine, plasma, feces, cell extracts, and other biological fluids (e.g., bile), with analytical strategies adopted to their chemical characteristics, including water solubility and chemical conjugation. In contrast, the LCMs exhibit a more lipophilic nature and thus occur in feces, cells, tissues, and blood but not in urine. Hence, analytical strategies for the LCMs focus on their lipophilic properties and their occurrence as sulfated derivatives (especially the carboxychromanols) [40]. A brief overview of a subset of analyzable metabolites and the corresponding methods for preparation and detection is provided in Table 6.1.

Specifications of Long-Chain Metabolites

The vitamin E metabolites with a side chain length between 13 and 9 carbon units are summarized as LCMs, with 13′-OH and 13′-COOH being the first metabolites formed from their metabolic precursors, the TOHs or tocotrienols (T3s) [42]. The first analytical approach for the detection of γ-13′-OH,

γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH has been published in 2002 by Sontag and Parker, who used a GC-coupled MS-based approach for the determination of these metabolites in the culture supernatant of HepG2 liver cells that have been incubated with *RRR*-γ-TOH [43]. This finding was confirmed by Jiang and coworkers who detected γ- and δ-LCMs in the cell culture supernatant of human lung epithelial A549 cells. Especially the carboxychromanols were detected in their sulfated form, indicating that acid metabolites are preferred for this type of chemical modification [44]. The sulfate modification can be removed by enzymatic deconjugation, leading to facilitated detection and improved analytical recovery of the acid metabolites [41]. Hence, Jiang and coworkers detected for the first time the γ-LCMs in complex matrices, such as rat liver and plasma [44]. At about the same time when Sontag and Parker published their first analytical approach for the detection of the γ-LCMs, Azzi and coworkers discovered gene regulatory actions of α-TOH, a finding that was also confirmed for the corresponding LCMs [45, 46]. Further, recent insights into the metabolism of vitamin E showed that *RRR*-α-TOH is the preferred form for hepatic uptake, rendering α-TOH as the most relevant vitamin E form with biological activity in humans [42, 47]. Based on these findings, the focus of analytical interest switched from the γ- to the α-TOH LCMs. In 2010, Birringer and coworkers detected for the first time α-13′-OH and α-13′-COOH in the culture supernatant of HepG2 liver cells enriched with *RRR*-α-TOH [48].

In the same year, Mustacich and coworkers used a LC-MS technique to analyze α-13′-OH in rat liver microsomes, presenting the first determination of an α-LCM in a complex matrix [2]. Further, the first determination of the α-LCMs in humans was done by the group of Zhao et al., who detected α-13′-COOH in human feces [49]. In 2014, 12 years after Azzi and coworkers published their hypothesis outlining a gene regulatory role for α-TOH, Wallert et al. found α-13′-COOH in human serum, indicating a systemic relevance in humans. In this study, 500 µl serum of a healthy, middle-aged (39 years), nonsmoking male, who received a balanced diet with no additional supplementation of vitamin E, was used for the detection of α-13′-COOH via quadrupole time-of-flight (Q-TOF) LC-MS [11]. This study provided first evidence that the α-LCMs are transferred into blood circulation after α-TOH has been metabolized in the liver. Further, Wallert and coworkers showed in a cell model that α-13′-OH and α-13′-COOH are more potent regulators of gene expression than their metabolic precursor [11]. Taken together the results of Wallert et al. indicate that the LCMs are a more active form than their precursor molecules that might promote regulatory effects in peripheral tissues of the human body. Only 1 year later, α-13′-OH was also found in human serum using a GC-MS approach [10]. Based on these two analytical approaches, current analytical research is focused on the development of analytical strategies enabling the simultaneous determination of all LCMs and their respective metabolic precursors. The first attempt was made in 2016, when Torquato et al. used LC-MS/MS combined with atmospheric pressure chemical ionization (APCI) (−) for the detection of α-13′-OH and α-13′-COOH in the same analytical session [41]. This analytical approach has been confirmed by Giusepponi and coworkers by using LC-MS/MS with a different type of ionization (electrospray ionization (ESI) (+)) [24]. In 2012, Bardowell and coworkers were able to determine 12′-OH and 11′-OH in the feces of mice fed a γ-TOH-enriched diet [50]. The detection of these metabolites in feces provided evidence for ω-1 and ω-2 hydroxylation activity and that 12′-OH cannot undergo oxidation followed by side chain truncation. Therefore, this metabolite is excreted via bile and can be found in the feces of mice and humans [50].

SamplePreparation

In the early days of vitamin E research, solid extraction of lipid fractions from biological matrices was the only challenge in a preparation procedure. With the growing knowledge about chemical properties, metabolic pathways, and tissue distribution of vitamin E metabolites, the challenges got more difficult and complex. Today, issues such as conjugation, oxidation, chemical differences between the single compounds, and even their appearance in various matrices must be considered. While the LCMs can be detected in feces, cells, tissues, and blood, the SMCs are mostly found in biological

fluids, such as bile, urine, and plasma, but also in feces, isolated cells, and solid tissues [40]. To increase the recovery of vitamin E metabolites, cell and solid tissue samples must be homogenized, and complex lipids need to be hydrolyzed before lipid extraction. During these processes, antioxidants like butylated hydroxytoluene, ascorbic acid, and pyrogallol can be added to avoid autoxidation of the metabolites [11, 51].

The use of analytical standards is essential to assess metabolite recovery during workup and analysis. Therefore, authentic compounds or stable isotope-labeled synthetic analogues need to be added at the beginning of the sample preparation. In the case of the 13′-OH and 13′-COOH LCMs, no analytical standards are commercially available, and the molecules must be synthesized or semi-synthesized from natural compounds [34, 48, 52]. Therefore, Wallert and coworkers used garcinoic acid, a natural compound occurring in the nuts of the African plant *Garcinia kola* (reviewed in [53]) for the semi-synthesis of α-13′-COOH and α-13′-OH as well as δ-13′-COOH and δ-13′-OH [11].

Another important step of the preparation procedure is the enzymatic deconjugation of the vitamin E metabolites. The LCMs as well as the SCMs mostly appear as sulfated or glucuronidated conjugates in biological samples [11, 51]. These conjugates are a result of the chemical modification during the hepatic metabolism and can lower the recovery of their corresponding metabolites. Freiser and coworkers reported that especially the acid forms of the LCMs are conjugated, with a predominance of the sulfate conjugates [54]. This mismatch between sulfation and glucuronidation has been confirmed in various studies, indicating that enzymatic sulfation could be the predominant phase II reaction in vitamin E metabolism [55–57]. The enzymatic hydrolysis of conjugated metabolites with β-glucuronidase and sulfatase appeared as a reliable method for the workup of biological fluids or tissues in different publications [11, 51]. Wallert and coworkers incubated 500 µl human serum for 30 min (at 34 °C) with a combination of 1500 IU β-glucuronidase and 26 IU/ml sulfatase for enzymatic deconjugation. This procedure led to a higher recovery of the unconjugated acid LCMs, enabling the first determination of α-13′-COOH in human serum [11]. In addition, the application of methanolic HCl for the deconjugation of CEHCs in urine samples appeared to be more efficient than enzymatic hydrolysis [56]. Beneath the strategy of enzymatic deconjugation, Pope and coworkers tried to analyze the conjugates directly using MS (ESI) to avoid artificial production of Simon metabolites by deconjugation steps. This method showed promising results for CEHC but has to be improved further for the general determination of vitamin E metabolites [58].

In most analytical studies on vitamin E, liquid-liquid extraction was used for the purification of the metabolites and their metabolic precursors from different matrices [10, 11, 24, 44, 49]. Wallert and coworkers performed liquid-liquid extraction with a mixture of hexane and dichloromethane (ratio 5:2) containing 1% butylated hydroxytoluene. The serum samples were mixed with solvent for 1 min at room temperature and were then centrifuged (2000 × g, 15 min, 10 °C) to achieve the separation of organic and inorganic layers. The upper organic phase was collected in glass tubes, dried under N_2, and resuspended in 50 µl methanol [11]. Solid-phase extraction (SPE) is another way to extract vitamin E metabolites from biological matrices. Yang et al. isolated γ-13′-OH, γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH from cell culture medium of A549 cells with a C_{18}-SPE cartridge, using acetic acid for metabolite elution [59]. Next, Wallert and coworkers are currently working on a SPE-based method for the extraction of the LCMs from human blood (unpublished data). Here, only 100 µl plasma will be used for metabolite extraction, providing a significant advantage compared to the liquid-liquid extraction-based alternatives, which require 500 µl plasma.

Detection of Vitamin E Long-Chain Metabolites

LC-/HPLC Analysis

HPLC-based analysis of vitamin E metabolites is performed with either normal phase or reverse phase columns coupled to electrochemical (ECD), fluorescence (FLD), UV-Vis, or evaporative light scattering detectors, with ECD being the most sensitive for vitamin E determination. Therefore,

HPLC-ECD has been used by Zhao and coworkers to detect TOH metabolites in human feces [49]. Fluorescence detection has lower sensitivity than ECD, and the response for some of the physiological metabolites is too low for applications involving human samples. However, FLDs have been also used for the determination of TOHs, T3s, and their corresponding metabolites in cell culture supernatants [44], rat plasma and liver [44, 54], and fetal bovine serum [57]. LC-MS/MS is the most widely used technique for the determination of the LCMs, providing an accurate quantitative analysis of these compounds in various biological matrices [24, 41, 57, 60]. Hence, LC-MS/MS enabled the first simultaneous detection of α-13′-OH and α-13′-COOH in human serum [1]. Further, Jiang et al. showed that negative polarity (ESI(−)) LC-MS/MS can be also used to quantify conjugated and unconjugated vitamin E metabolites in rodent blood [57].

GC Analysis

GC-based methods for the analysis of vitamin E are either coupled to flame ionization detectors (FID) or MS detectors. In contrast to HPLC-based methods, GC-based analysis of vitamin E metabolites requires an additional derivatization step for TOH and carboxychromanols [43, 51]. The purified extracts are heated and silylated with N-methyl-N-trimethyl-silyltrifluoroacetamide (MSTFA) o r N,O-(bis-trimethylsilyl) trifluoroacetamide (BSTFA) to accomplish derivatization [61]. This a dditional procedure is required for the detection of TOH and its metabolites by GC-based separation and detection. Traditionally, FID is the most often used detector in GC, due to its high response to organic molecules, but nowadays this detection technique is more and more replaced by MS, allowing a more sensitive detection of vitamin E metabolites. Therefore, GC-MS-based methods have been used for metabolite detection in cell culture supernatants [43, 44], the simultaneous detection of α-TOH and its oxidation product α-tocopherolquinone in human blood [61], and the first determination of α-13′-OH in human serum [10]. Further, GC-MS procedures were also used to investigate Simon's metabolites and α-CEHC in plasma and urine of animals and humans [62, 63], as well as for the analysis of α- and γ-TOH with their corresponding metabolites in human plasma [51].

Features of Long-Chain Metabolite Analysis in Human Blood

In recent years, the LCMs have emerged as a new class of signaling molecules with possible relevance for the regulation of physiological functions. This change of direction in vitamin E research resulted in the development of new analytical methods to assess the LCMs in human matrices. Unfortunately, the determination of these compounds, especially in blood, appeared to be very difficult. The first detection of the α-LCMs in human serum was in 2014 by Wallert and coworkers. This group detected α-13′-COOH, but not its metabolic precursor α-13′-OH, in the serum of a healthy volunteer, receiving 1000 IU of *RRR*-α-TOH/day over 1 week. Before the measurement, the LCMs undergo enzymatic deconjugation with a mixture of sulfatase and β-glucuronidase and were extracted with hexane and dichloromethane. Based on the results of Wallert et al., the LCMs seem to appear in low nanomolar concentrations in human serum, indicating that detection sensitivity could be a major problem for metabolite analysis in future studies [11]. Only 1 year later, Ciffolilli et al. determined α-13′-OH in the same serum sample with a GC-MS-based method. Again, the applied method could not be used to detect also the second α-LCM (α-13′-COOH) [10]. To overcome these drawbacks, Torquato and coworkers tried to optimize the proposed methods by using LC-MS/MS. First, APCI and ESI sources were compared in positive and negative acquisition mode for the simultaneous determination of the TOHs and the LCMs with APCI (−) providing the best signal intensity for the α-LCMs [41]. As a result of the optimized protocol, Torquato and coworkers were able to detect α-13′-OH and α-13′-COOH simultaneously in one serum sample [41]. Only 1 year later, Giusepponi and colleagues obtained the same results using an ESI (+) source [24]. Interestingly, both groups were for the first time able to separate α-13′-OH and α-13′-COOH from several unknown compounds with identical

masses. An accurate mass investigation performed by Giusepponi et al. identified these unknown compounds as possible structural isomers of α-13′-OH and α-13′-COOH. If this holds true, the blood concentrations of α-13′-OH and α-13′-COOH would be detected as a bulk parameter, comprised of up to three different isomers [24, 41].

Regulatory Actions of Vitamin E Long-Chain Metabolites

Anti-inflammatoryActions

For studies on the anti-inflammatory actions of the LCMs, (i) cells were treated with the respective LCM in conjunction with a pro-inflammatory stimulus, or (ii) isolated enzymes were used to study the influence of the LCMs on their activity. Several LCMs (α-, γ-, δ-13′-COOH; δ-9′-COOH; α-13′-OH) affected the inflammatory response, i.e., expression (mRNA or protein) or the activity of various pro-inflammatory enzymes, including cyclooxygenase 2 (COX2) [10, 34, 64, 65], inducible nitric oxide synthase (iNOS) [10, 65–67], or 5-lipoxygenase (5-LO) [64, 68], as well as inflammatory mediators such as chemokines and cytokines. In general, the 13′-COOH metabolites are more potent than the shorter LCMs, and the conjugation of the LCMs with sulfate abrogates their anti-inflammatory actions [34, 67].

The first study on the anti-inflammatory actions of the LCMs was carried out in 2008 by Jiang and coworkers in human adenocarcinomic alveolar basal epithelial cells. This cell line is capable to metabolize vitamin E and showed an inhibition of the arachidonic acid-stimulated COX activity after treatment with TOH [34]. The inhibitory effect was less effective after pre-treatment with sesamin, a known suppressor of the metabolism of vitamin E, indicating an involvement of the LCMs as regulatory substances. In addition, the LCMs were extracted from the cell culture supernatant of the A549 cells to confirm their inhibitory capacity on COX activity (IC_{50}: δ-13′-COOH: 4 μM; δ-9′-COOH: 6 μM). The same experiments were performed with the sulfated LCM conjugates, which did not exert anti-inflammatory effects, indicating that only unconjugated LCMs can act as anti-inflammatory compounds [34]. Anti-inflammatory actions on lipopolysaccharide (LPS)-stimulated COX2 mRNA and protein expression as well as release of COX-derived prostaglandins PGE_2 for α-13′-OH [10] and PGE_2, PGD_2, and PGF_{2a} for α-13′-COOH [65] were also shown in murine RAW264.7 macrophage-like cells.

In addition, the α- and δ-LCMs (α- and δ-13′-OH, α- and δ-13′-COOH) mediated the inhibition of iNOS mRNA and protein expression, as well as release of NO in response to LPS in RAW264.7 macrophages [10, 65–67]. Interestingly, the observed inhibitory effects depended on the structure of the LCMs, with the 13′-COOH metabolites being more effective than the 13′-OH metabolites, while the substitution of the chroman ring (α- vs. δ-LCMs) had no detectable influence.

The inhibitory effects of the LCMs on 5-LO activity have been shown in (i) human promyelocytic HL60 leukemia cells, where the LCMs blocked the ionophore-induced release of leukotriene B_4, as well as (ii) on the isolated 5-LO, where δ-13′-COOH was more effective than zileuton, a synthetic antagonist for 5-LO [68]. The inhibition of 5-LO activity by δ-13′-COOH was also reported by Jang et al. [64].

Cellular Lipid Homeostasis

Until now, only a few aspects of lipid homeostasis have been studied regarding their modulation by the LCMs. Hence, merely these aspects can be discussed in the following. These include the regulation of cluster of differentiation 36 (CD36), uptake of oxidized LDL, phagocytosis, and the

intracellular storage of lipids. Taken together, these mechanisms represent key processes in macrophage foam cell formation, a significant hallmark of the pathogenesis of atherosclerosis [69]. The human monocytic THP-1 cell line, which can be differentiated to macrophage-like cells, was used to study the effects of the LCMs on foam cell formation by Wallert et al. in 2014 [11]. Under basal conditions (i.e., without the stimulation with oxidized LDL), the LCMs α-13′-OH and α-13′-COOH induced the expression of CD36 mRNA as well as CD36 protein. Interestingly, this result contrasts with the effects of the precursor α-TOH, which downregulated the expression of CD36 at a concentration of 100 μM in the THP-1 macrophage model. Thus, the metabolites likely function in a different mode than their natural precursors. In addition, the LCMs appear to be several times more effective than α-TOH, as they exert their effect on CD36 expression in concentrations as low as 5 and 10 μM for α-13′-OH and α-13′-COOH, respectively. To confirm these findings in a more physiological model, the LCMs were also applied to peripheral blood mononuclear cell (PBMC)-derived primary macrophages in this study. Here, the effects of both LCMs on CD36 protein expression were confirmed [11].

The scavenger receptor CD36 is a receptor binding oxidized LDL in macrophages and mediates the uptake of this modified lipoprotein [70]. In a feed-forward mechanism, oxidized LDL induces the expression of CD36, leading to an increased uptake of oxidized LDL [71]. This mechanism promotes foam cell formation; thus, Wallert et al. examined whether the LCMs interrupt or support oxidized LDL-induced CD36 expression. As expected, the incubation with oxidized LDL induced the expression of CD36 in human THP-1 macrophages [11]. The TOH precursor diminished the induction by oxidized LDL, resembling the findings under basal conditions. Likewise, the effect of the LCMs resembled the initial findings. The preincubation with the LCMs augmented the induction of CD36 protein expression by oxidized LDL significantly. In contrast, naïve LDL did not induce CD36 expression in THP-1 macrophages. In combination with naïve LDL, α-TOH downregulated CD36 protein expression, while the LCMs induced the expression. Given the augmented expression of CD36 by the LCMs, increased oxidized LDL uptake by LCM-treated macrophages can be expected. Interestingly, incubation of THP-1 macrophages with the LCMs for 24 h before challenging the cells with oxidized LDL leads to a decrease of about 20% in the uptake of oxidized LDL compared to untreated control cells. Again, PBMC-derived macrophages were treated in a similar fashion, and the findings were confirmed. The LCM α-13′-OH decreased the uptake by 24% and α-13′-COOH by 20%, respectively. In foam cell formation, the consequence of an increased uptake of oxidized LDL is an increase in intracellular lipid content. Thus, the THP-1 macrophage model reacted with an increase of intracellular neutral lipids in response to the incubation with oxidized LDL. Concomitant with the decreased uptake of oxidized LDL in response to the LCM preincubation, the neutral lipid content of THP-1 macrophages was not increased in cells treated with the LCMs and oxidized LDL in combination [11].

However, the induced expression of CD36 is contradictory to the observed inhibitory effects of the LCMs on the uptake of oxidized LDL. Consequently, the LCMs likely act through a distinct mechanism. Results by Wallert et al. suggest that phagocytosis, as a major uptake pathway for oxidized LDL [72], is also affected by the LCMs. Experiments with fluorescence-labeled microbeads revealed that the LCMs significantly decreased the phagocytic activity of THP-1 macrophages. Here, α-13′-COOH seems to be more potent than α-13′-OH, with 41% inhibition vs. 16% inhibition, respectively [11]. This finding is not perfectly in line with the equal inhibitory effect of the two LCMs on the uptake of oxidized LDL. However, the inhibition of phagocytosis by the LCMs provides a good explanation for the discrepancy between CD36 regulation and uptake of oxidized LDL.

Taken together, the LCMs modulate macrophage lipid metabolism on the level of lipid uptake and storage. Different pathways implicated in foam cell formation, a hallmark of atherosclerosis, are affected by the LCMs. In total, the treatment of macrophages with the LCMs leads to a reduced uptake of oxidized LDL and concomitantly reduced lipid accumulation, a desirable effect in terms of the prevention of atherosclerosis. However, the underlying molecular mechanisms are not fully understood. Thus, further studies on the modes of action of the LCMs are needed.

Cancerogenesisand Chemoprev ention

Antiproliferative Effects of Tocopherol Long-Chain Metabolites

Abnormal proliferation is a characteristic of cancer cells and represents a crucial element of cancer development and progression. Thus, cancer therapy is based in part on drugs that kill cells with high rates of proliferation and regeneration. However, such substances cause severe side effects as they also affect rapidly proliferating healthy tissues like the skin, hair, or parts of the gastrointestinal tract [73]. Hence, natural compounds with antiproliferative activities are regarded as beneficial in the prevention and treatment of cancers, as they generally exert less side effects. Several natural compounds have been identified that inhibit pathways contributing to cell proliferation. A mong them are the promising constituent of *Curcuma longa*, namely, curcumin, which has been shown to affect Wnt, NF-κB, and mTOR signaling inter alia and resveratrol, a constituent of grapes, with blocking activity on mitogen-activated protein kinases and tyrosine kinases inter alia [73]. The chemopreventive properties of curcumin [74] and resveratrol [75] have been found in several studies. Cancer-preventing properties have also been reported for TOHs and T3s. This effect can be attributed at least in part to the LCMs as outlined below.

First studies on the TOH metabolites in this context were carried out with a focus on the SCMs. Here, the SCMs exerted comparable effects to their precursors with respect to the inhibition of cell proliferation. Interestingly, the γ-forms appeared to be more potent in inhibiting cell proliferation than the α-forms, a finding that was earlier reported for the T3s [76]. Accordingly, it was found that γ-TOH as well as γ-CEHC reduced the proliferation of human PC3 prostate cancer cells in a concentration of 1 μM by about 30–40%. Almost maximal inhibition of cell proliferation, i.e., 70–80%, was obtained with 10 μM. However, α-CEHC and α-TOH inhibited cell growth by 40–45% in concentrations of 50 μM [77]. Interestingly, the antiproliferative effects seem to be cell type-dependent, as PC3 cells showed higher inhibition compared to human HTB-82 rhabdomyosarcoma cells and human endothelial vascular cells (HEVC) [77]. Given that the precursors and the SCMs exert antiproliferative effects, Birringer et al. were interested in the effects of the LCMs on cell proliferation. Therefore, α-13′-COOH and δ-13′-COOH as well as α-13′-OH and δ-13′-OH and their respective precursors were applied to HepG2 liver cells [48]. While the LCMs with carboxy function potently led to cell growth arrest, the hydroxy metabolites failed to exert antiproliferative effects. Again, the α-forms were less potent than their δ-counterparts. Neither the hydroxy metabolites nor the TOHs inhibited cell growth in the concentrations tested. Thus, the authors concluded that the carboxylation of the TOH side chain is essential for the antiproliferative effects of the LCMs [48]. However, in PC3 cells, not only α- and γ-CEHC impeded cell proliferation but also δ-13′-COOH and α-13′-OH. All compounds inhibited cell proliferation by about 60% at concentrations of 10 μM [52]. Thus, the effect of the hydroxy metabolites seems to be cell type-dependent. This might be explained by differences in the cellular metabolism of the TOHs and the TOH metabolites. Different responsiveness of cell types to vitamin E metabolites was also reported in colon cells [64]. The δ-13′-COOH metabolite reduced the proliferation of human HCT-116 colon carcinoma and human HT-29 colorectal adenocarcinoma cells with IC_{50} values of 8.9 μM and 8.6 μM, respectively, whereas the T3 metabolite δ-T3-13′-COOH (i.e., δ-garcinoic acid) was less potent with IC_{50} values of 16 μM and 17 μM. Interestingly, normal colon epithelial cells were less affected by the metabolites. While 10 μM of δ-13′-COOH suppressed cell viability of HCT-116 and HT-29 cells by around 60%, normal human colon epithelial cells showed reduction of merely 10–20%. In line with this, 20 μM of δ-T3-13′-COOH (δ-garcinoic acid) reduced the viability of the cancer cells by 70–80%, but the viability of normal colon cells was affected only by 10–20% [64].

Taken together, the precursor molecules, i.e., the TOHs and T3s, apparently exert antiproliferative effects depending on the methylation pattern of the chroman ring. The γ-forms of T3 and TOH have antiproliferative properties [76, 77], while the α- and δ-forms have not [48, 64]. It should be noticed that Jang et al. found no effects of γ-T3 in their setting [64]. However, the metabolic conversion leads to

LCMs and SCMs with antiproliferative properties, independent of the methylation pattern of the chroman ring. While the action of the hydroxy LCMs is controversial [48, 52] and likely depends on the cell type, the carboxy LCMs reliably affect the proliferation of different cancer cell lines [48, 52, 64]. Thus, a key determinant of the antiproliferative properties is likely the carboxy function, a notion that is further supported by the reported actions of the SCMs carrying a carboxy group (α- and γ-CEHC) [52, 77]. A promising finding with respect to anticancer properties of the LCMs is the resistance of normal colon cells to the LCMs, while the proliferation of colon cancer cells is strongly reduced [64]. If this effect is reproducible, vitamin E and its metabolites might be useful in cancer prevention and treatment.

Pro-apoptotic Effects of the Tocopherol Long-Chain Metabolites

Apoptosis is a coordinated cellular process, ultimately leading to programmed cell death. The balance of cell division and cell death is crucial for the homeostasis of organisms. Inappropriate rates of apoptosis are implicated in several pathological conditions, such as neurodegenerative diseases, autoimmune disorders, and cancers [78]. The rate of apoptosis is usually lower in cancer cells, leading to malignant cells, tumor metastasis, and resistance to anticancer drugs. Thus, apoptosis is part of the problem as well as a possible solution. Several therapeutic strategies based on the targeting of apoptosis pathways have been developed [78].

In addition to the antiproliferative effects, Birringer et al. also analyzed the apoptotic effects of the LCMs in HepG2 liver cells [48]. Flow cytometric analyses using annexin V staining revealed a significant induction of apoptosis, when HepG2 cells were treated with 20 μM α-13′-COOH, δ-13′-COOH, or δ-13′-OH. In line with this, α-13′-COOH and δ-13′-COOH strongly induced the cleavage of caspases 3, 7, and 9. The hydroxy LCM δ-13′-OH leads to an activation of the same caspases but less effectively. In contrast, α- and δ-TOH as well as α-13′-OH were not able to induce caspase cleavage. Accordingly, poly-ADP ribose polymerase (PARP)-1 cleavage as a downstream effect of caspase activation followed a similar pattern. The α- and δ-carboxy metabolites showed strong induction, while merely a slight effect for δ-13′-OH and no effect for α-13′-OH and the TOHs were observed. Further, mitochondrial apoptosis, a process accompanied by the increased production of reactive oxygen species (ROS), was examined. On that account, ROS production in the HepG2 cells in response to TOHs and their metabolites was analyzed. Here, in contrast to the precursors and the hydroxy metabolites, the carboxy metabolites significantly induced ROS formation. Not only intracellular but also intramitochondrial ROS levels were induced by the carboxychromanols. Again, the other substances tested did not induce ROS production. With these findings, evidence was provided for mitochondrial-derived apoptosis. Further, alterations in the mitochondrial membrane potential in TOH- and LCM-treated cells were found. Significant reductions in the mitochondrial membrane potential were observed for α-13′-COOH, δ-13′-COOH, and δ-13′-OH in concentrations of 20 μM. Here, α-13′-COOH was again more effective (60% reduction) than the δ-LCMs (20% reduction for both the hydroxy and the carboxy metabolites) [48].

Taken together, the carboxy LCMs reliably induce apoptosis in HepG2 cells, and evidence was provided that a pathway leading to mitochondrial apoptosis is involved in this effect. Interestingly, the authors have shown that δ-13′-OH is efficiently metabolized to δ-13′-COOH by HepG2 cells, while the conversion of α-13′-COOH to α-13′-OH is less effective [48]. This finding provides a nice explanation for the discrepancy in the effects of α-13′-OH and δ-13′-OH. The δ-metabolite leads to apoptosis through a rapid conversion to the pro-apoptotic carboxy metabolite, while the α-metabolite is slowly converted and thus unable to induce apoptosis. In conclusion, as shown for the antiproliferative actions of the LCMs, the carboxy function of the metabolite seems to be crucial for the observed pro-apoptotic effect.

The apoptotic actions of the long-chain vitamin E derivatives with carboxy function were confirmed in a study on colon cancer cells [64]. Here, δ-13′-COOH and δ-T3-13′-COOH (δ-garcinoic acid) induced early and late apoptosis. In line with the findings of Birringer et al., induction of

caspase-9 activation and PARP cleavage by the carboxy metabolites was found. Further, the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-II was increased by the treatment with the carboxy LCMs. Interestingly, the TOH metabolite was more effective than the T3 metabolite in the induction of apoptosis and autophagy. Based on previous findings on the metabolic precursors, Jang et al. examined whether an alteration in sphingolipid metabolism by the LCMs is causing the induction of apoptosis. It was found that δ-13′-COOH increased dose-dependently the total content of ceramides, dihydroceramides, and dihydrosphingosines. In contrast, the content of all sphingomyelins was decreased. Similar effects were observed for the T3 metabolite. Thus, both carboxy LCMs modulate sphingolipid metabolism when apoptosis and autophagy are induced. An inhibition of sphingosine biosynthesis by myriocin treatment partly inhibited the induction of LC3-II expression but not the induction of PARP cleavage by the metabolites. Hence, elevated levels of dihydroceramides and dihydrosphingosines likely contribute to LCM-induced autophagy [64].

The LCMs have been shown to induce apoptosis in different cell types. Interestingly, evidence for two different modes of action has been provided by the studies on the LCMs so far. Birringer et al. have shown the induction of mitochondrial apoptosis by the LCMs in HepG2 cells, while Jang et al. have reported that an altered sphingolipid metabolism contributes to LCM-induced apoptosis in colon carcinoma cells. Treatment strategies targeting apoptosis aim at different signaling pathways, including B-cell lymphoma proteins, p53, or caspases. Effects of the LCMs on caspases have been shown in both studies on LCM-induced apoptosis. However, based on these findings, the applicability of the LCMs for chemoprevention or inhibition of cancerogenesis can hardly be assessed. Further studies are required to confirm desired properties like specificity for malignant cancer cells or to unravel distinct apoptosis signaling pathways [78].

Interaction with Pharmaceuticals

The cellular uptake of molecules is tightly regulated by several mechanisms, one of which is the excretion of, for example, pharmaceuticals from the cells via exporter proteins, such as the multidrug resistance protein P-gp. P-gp is a well-known representative of these exporters [79]. A specific inhibition in tumor cells is helpful, when antitumor therapies are applied, since the activation of these exporters may lead to a reduced cellular net uptake and efficiency of the pharmaceuticals.

Podszun et al. studied the effects of vitamin E (α-TOH, α-T3, γ-TOH, and γ-T3) and their metabolites (α-13′-COOH, α-CEHC, γ-CEHC) as well as plastochromanol-8 on the expression of P-gp in LS 180 Dukes' type B colorectal adenocarcinoma cells and found an induction of the expression and activity for α-13′-COOH and γ-T3 [80]. Furthermore, pregnane X receptor activity was induced by α-T3, α-13′-COOH, and γ-T3, as assayed by a reporter gene assay. The authors summarized that an increased uptake of vitamin E via supplements could lead to interactions with pharmaceuticals due to an increased activity of the P-gp exporter.

Structure-SpecificEffects

A structure-function relationship study of the LCMs revealed a highly specific regulation of target genes by the LCMs (α-13′-OH, α-13′-COOH, δ-13′-OH, and δ-13′-COOH). Neither the precursors (α- and δ-TOH) nor their substructures (pristanic acid and α-CEHC) were able to cause the same effects on the expression of scavenger receptor CD36 or inducible nitric oxide synthase (iNos) as the 13′-hydroxy or 13′-carboxy LCMs. Furthermore, the regulation was almost independent of the substitution pattern of the chromanol ring system (α- vs. δ-LCMs) but dependent on the modification of the side chain (TOH vs. 13′-OH and 13′-COOH, respectively), with the 13′-COOH being most potent. Hence, this specific regulation might suggest the existence of receptor-specific pathways for the LCMs [67].

Conclusions and Outlook

With the demonstration of the occurrence of the LCMs in human serum, Wallert and coworkers provided evidence for their possible role as systemic signaling molecules [11]. This concept was supported by several studies, characterizing the involvement of the LCMs in the regulation of inflammatory processes, lipid metabolism, cancerogenesis, and chemoprevention as well as xenobiotic metabolism. Interestingly, the LCMs act more potent and in part even contrary to their precursors. Thus, some of the controversial effects found for vitamin E might be explained by the actions of the LCMs. Nevertheless, large parts of their mode of action are still unrevealed and need further characterization. Although the analysis of the LCMs made great progress over the last several years, especially the distribution of the LCMs in extrahepatic tissues beside human serum needs further investigation. Taken together, the LCMs could be regarded as the active forms of vitamin E, as it has already been shown for the metabolites of vitamin A and D (reviewed in [81]). If this concept of a general mechanism for metabolic activation of fat-soluble vitamins holds true, the LCMs of vitamin E could comprise a new class of signaling molecules in the human body. This concept sheds new light to the field of vitamin E research and may help for better understanding the complex mode of action of vitamin E as well as its function as a vitamer. A brief overview about the current knowledge on LCMs in the human body and issues for future investigations is provided in Fig. 6.1.

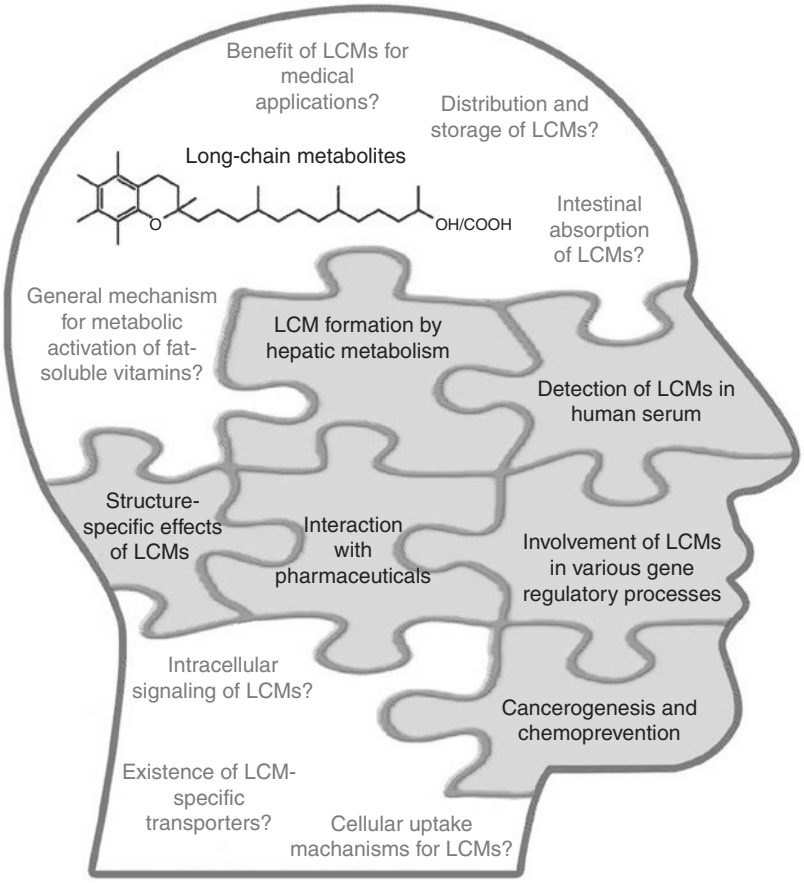

Fig. 6.1 Completing the puzzle of the activities of the LCMs in the human body

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Chapter 9

Garcinoic Acid: A Promising Bioactive Natural Product for Better Understanding the Physiological Functions of Tocopherol Metabolites

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Chapter Outline

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INTRODUCTION

Organisms produce bioactive natural products (secondary metabolites) as an adaption to their environment or as defense mediators. In contrast to primary metabolites such as protein, fat, and carbohydrates, they are not essential for growth, development, or reproduction [1,2]. Nevertheless, secondary metabolites are, like no other compounds, representatives for medical progress and have enormous importance for human health care. The use of natural products as medicines developed over generations and has been described throughout history in the form of folk medicine. The traditional African, Korean, Chinese, Islamic, and herbal medicines are the most important forms of historical folk medicine. Especially in Africa and Asia, 80% of the population still relies on traditional medicine for their primary health needs [3]. In these regions, fungi, plants, marine algae, or marine sponges are the most popular sources for bioactive natural products, but many of these compounds remain unexplored [2]. Nevertheless, plants are the dominant source of natural products in folk medicine. Plants have been well documented for their medicinal use for several thousands of years [4]. A well-known example is the plant *Alhagi maurorum*, which was used by the Romans for treating nasal polyps [5]. Plant-based traditional medicine was very important for primary health care over hundreds of years, but during the 18th century, the understanding of medicine changed. After Leeuwenhoek identified the first microorganism, enormous progress in the prevention of diseases was made. The knowledge associated with traditional medicine has promoted further investigations of compounds and extracts obtained from medicinal plants as potential medicines. This led to the isolation of many natural products from different sources.

One of the most famous examples is the antiinflammatory agent acetylsalicylic acid (aspirin) derived from the natural product salicin, which was isolated from the bark of the willow tree *Salix alba* [6,7]. During this

period, ethnopharmacological knowledge has been used for early drug discovery. Today, advances in analytical technologies improve the discovery of new bioactive natural products. These compounds have unique structural properties in comparison to products from standard combinatorial chemistry, making them the most promising source of lead structures for drug development [8,9].

A good example for the development from a medical plant used in traditional African medicine to a source of bioactive products for putative drugs is the African plant *Garcinia kola*. The parts of this plant contain many bioactive compounds, including the δ-tocotrienol (δ-T3) derivate garcinoic acid, which comprises an interesting molecule for functional studies. The aim of this review is to summarize the knowledge on this promising molecule and its use in research on vitamin E and its metabolites.

GARCINIA KOLA

G. kola or bitter kola is a dicotyledonous plant of the family *Clusiacea* (Fig. 9.1). It can be found in the rain forests of west and central Africa where it grows as a medium-sized tree with a height up to 12 m [10,11], but *G. kola* is also used for commercial farming, especially in Nigeria. The plant has reddish fruits containing two to four seeds. Both fruit components can be eaten [12]. *G. kola* plants bloom between December and January and their fruits mature from June to August [13]. From the botanical point of view, the fruits belong to the class of berries, but the seeds are often called *G. kola* nuts [14]. The nuts are dried and afterward available over the whole year, which gives them a small economical relevance [12]. Because of the bitter flavor of its seeds, the plant is colloquially called “bitter kola” or “bitter nut.” The locals also name it “Orogbo” (Yoruba), “Aku ilu” (Igbo), and “Namijin goro” (Hausa) [3].

Apart from its small economical relevance, *G. kola* is very important for African ethnomedicine. Approximately 60−80% of the African population depend on herbal cures for their primary health care [3]. In the traditional African medicine, each part of the *G. kola* plant is used for different medical applications. For example, the root is used for oral hygiene and the tree bark as an abstergent agent. The latex of the tree is put on fresh wounds to prevent septic inflammation and to support healing [15]. The nuts are used for treating bronchitis and infections of the pharynx and colic [10]. Furthermore, the nuts are also used as antivenom for people with suspected intoxication [16]. It is also speculated that *G. kola* nuts protect against the toxic effects of alcohol [17]. Because of their bitter flavor, the bitter nuts are also used as stimulants for inducing anorexia [15]. Furthermore, antimicrobial effects [18−20], antiviral effects [10], antiparasite effects [21], antidiabetic effects [22], and hepatoprotective effects [23] have been described.

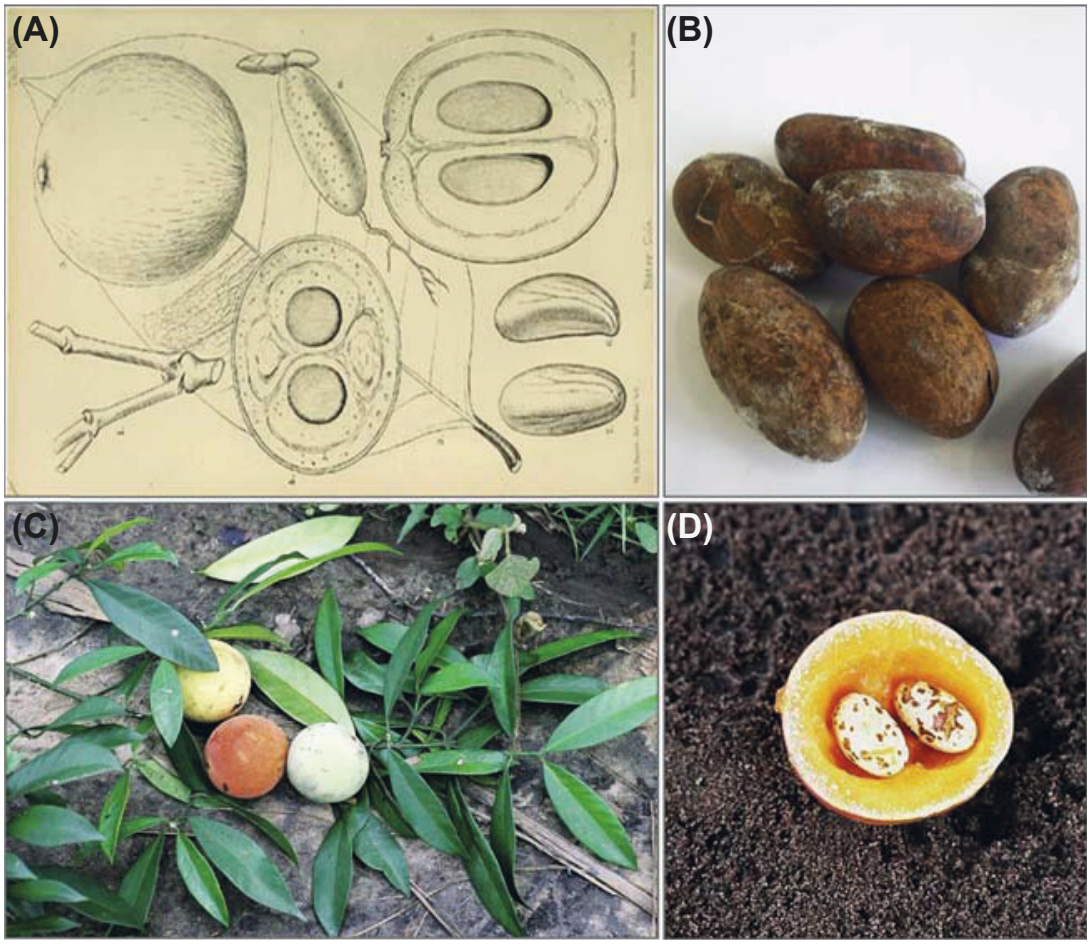

FIGURE 9.1 (A) Botanical illustration of the fruits of *Garcinia kola* E. Heckel *(Drawing by W.G. Smith published in 1875.)*. The fruits are shown completely and in cross section. Further, the seeds, colloquially called "bitter nut," can be seen. The illustration is entitled "bitter nut." (B) Photography of *G. kola* seeds. (C) Photograph of *G. kola* plant with fruits *(By courtesy of Paul Latham.)*. (D) Cross section of *G. kola* fruit, showing seeds *(By courtesy of Paul Latham.)*.

Bioactive Ingredients of the *Garcinia kola* Nut

The main components of *G. kola* nuts are carbohydrates, protein, fiber, fat, and water [3,12]. In contrast to the real kola nuts (*Cola nitida*), the bitter nuts do not contain caffeine [17], but they are a good source for calcium, potassium, sodium, and magnesium [3,24]. Furthermore, many other bioactive compounds, including tannins, saponins, alkaloids, and glycosides, have been isolated from *G. kola* nuts [3,13]. The nut also contains flavonoids and benzophenone derivatives such as kolaflavones and *Garcinia*-biflavones 1 (3,4,4,5,5,7,7-heptahydroxy-3,8-biflavanone) and 2 (3,4,4,5,5,5,7,7-hexahydroxy-3,8-biflavanone), which might be responsible for the observed antimicrobial effects of *G. kola* nuts [3]. Furthermore, two chromanols, garcinal and garcinoic acid, which have been described as strong antioxidants, have been isolated from *G. kola* seeds [25].

Biflavones and Benzophenone Derivatives

Most of the biochemical and physiological effects of the *G. kola* nut are attributed to its content of biflavones and benzophenone derivatives. One of the most investigated nut biflavones is kolaviron, a dimeric flavonoid (Fig. 9.2). In addition to its hepatoprotective effects [22] and its ability to lower blood cholesterol [26], antiinflammatory capacity has been shown for this compound in different animal models. For example, diabetic rats were supplemented with 100 mg/kg kolaviron for 6 weeks. The treatment with kolaviron resulted in a reduction of inflammatory processes, indicated by reduced serum concentrations of interleukin (IL)-1β and monocyte chemotactic protein 1 (MCP1) [27]. Similar results have been found in hepatic tissues of diabetic rats, where treatment with kolaviron reduced the amount of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor α (TNFα) [28]. Further studies investigated the effects of kolaviron on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) 2 expression in hepatic tissues of dimethylnitrosamine-treated rats. Dimethylnitrosamine is known as a hepatotoxin that enhances expression of iNOS and COX2 proteins as part of the proinflammatory response. After treatment with kolaviron, a significant reduction of dimethylnitrosamine-upregulated iNOS and COX2 expression was measured, indicating that kolaviron acts as an antiinflammatory factor. In addition, electrophoretic mobility shift assays showed that this effect may result from reduced formation of the transcription factors nuclear factor "kappa-light-chain-enhancer" of activated B cells (NFκB) and activator protein 1 (AP-1) [29]. Furthermore, interactions of kolaviron with several intracellular immune mediators, such as IL-1α, IL-1β, IL-18, and IL-33, have been observed in murine RAW264.7 macrophages. In this context, kolaviron has been shown to modulate expression and phosphorylation of proteins involved in NFκB, mitogen-activated protein kinase, AP-1, and protein kinase B (PKB/Akt) signaling, leading to an inhibition of the lipopolysaccharides (LPS[1])-induced immune response [30].

Garcinal

The isolation procedure from *G. kola* not only provides garcinoic acid and δ-T3[2], but also garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H,2--chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al] [25]. This structure is closely related to garcinoic acid; solely an aldehyde moiety terminates the side chain instead of a carboxylate moiety (Fig. 9.3).

1. Lipopolysaccharides are endotoxins composed of lipid and polysaccharide components found in gram-negative bacteria that provoke strong immune responses in eukaryotes.
2. Tocotrienols are composed of a chroman ring system and an unsaturated side chain; they constitute a subgroup of vitamin E (the reader is referred to the section "Vitamin E").

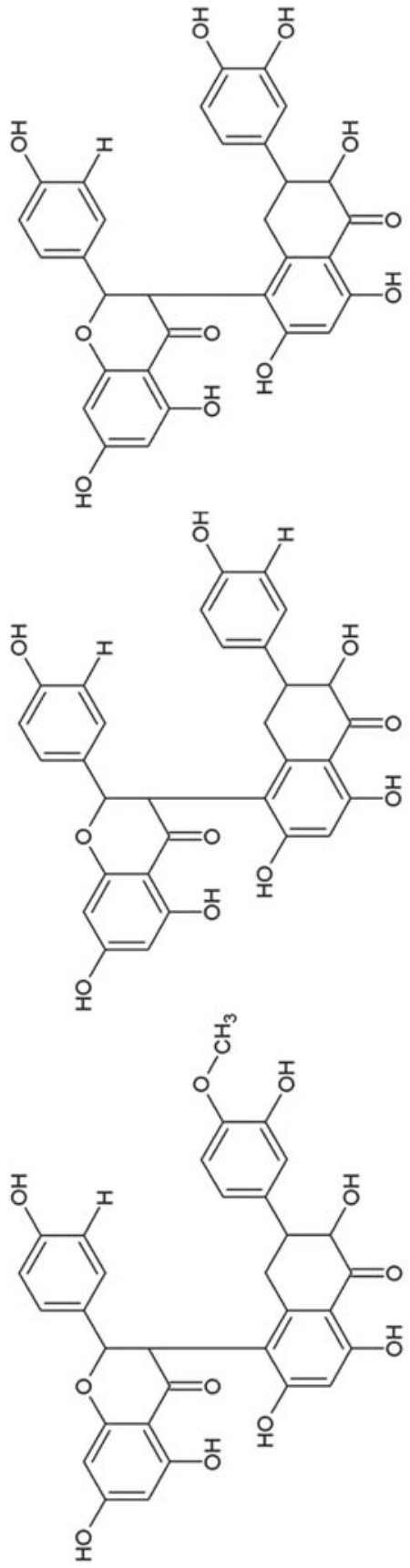

FIGURE 9.2 Chemical structures of biflavones of *Garcinia kola*. *Adapted from O.A. Adaramoye, V.O. Nwaneri, K.C. Anyanwu, E.O. Farombi, G.O. Emerole, Clin. Exp. Pharmacol. Physiol. 32 (2005) 40–46.*

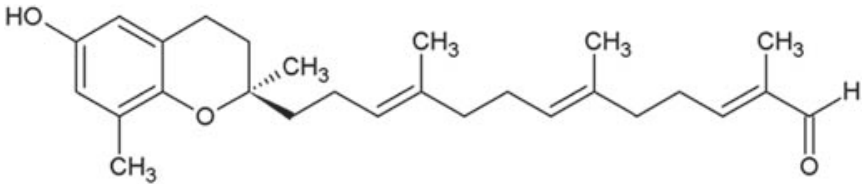

FIGURE 9.3 Chemical structure of garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H, 2-chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al]. *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619–1625.*

However, the metabolic pathways leading to the formation of garcinal in plants have not been elucidated. Based on the structural similarity to garcinoic acid, garcinal likely has a comparable bioactive potential. Nevertheless, the bioactive properties of garcinal are largely unknown. To the best of our knowledge, merely two works addressed the effects of the isolated compound. According to these, garcinal is 1.5 times more potent than α-tocopherol (α-TOH[3]) and has a similar antioxidative activity as garcinoic acid as well as δ-T3 [25]. Furthermore, replacing the terminal functional group of the side chain of garcinoic acid (or garcinal respectively) does not alter the antioxidative capacity [25]. These findings support the hypothesis that garcinoic acid and garcinal may have similar properties in biological systems. Although the health-promoting effects of extracts from *G. kola* (vide supra) are generally ascribed to the biflavones, garcinoic acid and garcinal should be taken into account. This became evident when different fractions of the crude extract were examined regarding their antioxidant and radical-scavenging activities. It turned out that the most potent fraction contained the *Garcinia* biflavone 1 and 2 but also garcinoic acid and garcinal [31]. Given the antioxidative potential of the isolated chromanols, garcinoic acid and garcinal likely contribute substantially to the effects of extracts from *G. kola*. Garcinal is therefore an interesting compound for functional studies due to its structural properties and for explaining the health-promoting effects of *G. kola*.

Garcinoic Acid

Garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol) is an interesting δ-T3 derivative and its occurrence in *G. kola* nuts was first described by Terashima and coworkers in 1997 [32]. A few years later, the same group published a method for the isolation of garcinoic acid from *G. kola* nuts [25]. However, *G. kola* nuts are not the only source of δ-tocotrienolic acid. The extraction of garcinoic acid from members of the *Clusiaceaen* plant family [33] and the development of a stereo-controlled synthesis [34] have been described. For an explicit description of the isolation and synthesis of garcinoic acid, the reader is

3. Tocopherols are characterized by a chroman ring system and a saturated side chain; they constitute a subclass of vitamin E (the reader is referred to the section "Vitamin E").

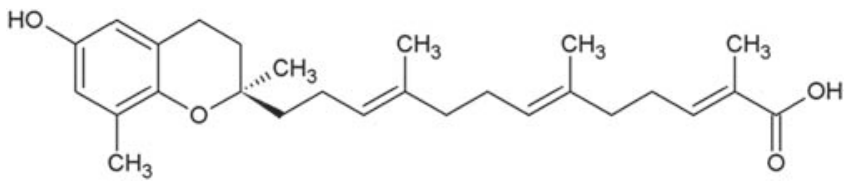

FIGURE 9.4 Chemical structure of garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol). *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619–1625.*

referred to the section "Synthesis of Vitamin E Long-Chain Metabolites." Garcinoic acid is in principle a metabolite of δ-T3 with the carboxylic group placed at the end of the aliphatic side chain (Fig. 9.4), which would be formed in humans in the liver after dietary intake of δ-T3. Thus, garcinoic acid shares structural similarities with δ-T3 [33].

Garcinoic acid shows many bioactive properties. The high antioxidant potential is probably one of the best investigated ones [25,31]. Furthermore, antiproliferative effects were shown in carcinoma cells by Mazzini et al. [33]. The acid also acts as a DNA polymerase β inhibitor, indicating that garcinoic acid is able to disturb base excision repair in tumor cells [34]. This finding supports the results of Mazzini and coworkers. For an explicit description of the bioactive properties of garcinoic acid, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites."

Because of its high content of bioactive components, the *G. kola* nut has great potential for pharmaceutical applications, which is reflected by a number of patents. In 1987, the first patent for a biflavone isolated from *G. kola* as an ingredient for the treatment of liver diseases was registered [35]. The natural product reduced hepatocyte damage in a galactosamine-treated rat model of acute hepatotoxicity and improved liver values in patients with hepatitis [35]. Furthermore, an extract containing a mixture of different biflavones (*Garcinia* biflavones 1, 1a, and 2 as well as kolaflavones) of *G. kola* is used as an antiglycation agent and is also registered in a patent [36]. This compound lowers the accumulation rate of advanced-glycation adducts in the human body; high concentrations of these adducts can damage cells and tissues [36]. The existing patents on bioactive compounds of *G. kola* for the use as pharmaceuticals provide evidence for the growing interest in this plant [35,36]. The role of *G. kola* as an important part of the African ethnomedicine evolved to an interesting source of natural compounds for modern drug development. Although only patents on biflavones have been registered to date, garcinoic acid is also a promising lead compound for future pharmaceuticals.

VITAMIN E

Vitamin E is naturally found in a variety of plant products, such as oils, nuts, germs, seeds, and in smaller quantities in vegetables and some fruits. Due to their lipophilic character, the several molecules summarized as "vitamin E"

are associated to fats in dietary sources. In fact, vitamin E is a hypernym for different molecules, which can be classified as TOH, T3, and a less consistent group of vitamin E–related structures (Fig. 9.5). The common feature of all molecules is the chroman ring and a covalently connected phytyl-like side chain, whose respective constitutions define the individual vitamin E forms. Characteristic for the TOH is their saturated side chain, whereas T3 carry three double bonds in this substructure. The methylation pattern of the chroman ring determines the classification as α-, β-, γ-, or δ-form of the TOH or T3, respectively. More precise, besides position 8, positions 5 and 7 are crucial: α means methylation at position 5, 7, and 8, β at position 5 and 8, γ at position 7 and 8, and δ solely at position 8 of the chroman ring. Natural forms of vitamin E exist in the RRR configuration (TOH) or the R configuration (T3), whereas synthetic vitamin E is a mixture of the different stereoisomers. Members of the group of the vitamin E–related structures can either be more similar to TOH, such as tocomonoenol or marine-derived TOH, or to T3, such as desmethyl-(P_{21})T3, desmethyl-(P_{25})T3, and plastochromanol-8 (Fig. 9.5).

Biological Significance of Vitamin E

Although it is controversially discussed how vitamin E benefits human health, it is an essential factor, as the classification as a vitamin shows. Vitamin E was discovered in 1922 as vital for the fertility of rats [36a], but is also essential for the maintenance of human health. Several disease states have been linked to vitamin E deficiency. A severe effect of inadequate vitamin E supply is anemia. Vitamin E is known for its strong antioxidative properties; if these are lost, erythrocytes are prone to rupture due to higher fragility of their cell membrane [37]. Based on this observation, erythrocyte hemolysis was used as a biomarker to set the recommended daily allowance of 15 mg per day for adults [37]. Not only erythrocytes, but also components of the nervous system are negatively affected by vitamin E deficiency. An isolated vitamin E deficiency, i.e., a deficiency not caused by fat malabsorption, characterizes "ataxia with vitamin E deficiency."[4] This disease is caused by defects in the gene encoding for the α-TOH transfer protein, namely *TTPA*, leading to an impaired ability to retain α-TOH and to depleted α-TOH plasma levels [38,39]. Likely due to the loss of antioxidant protection, nerve cells degenerate and neurological symptoms such as ataxia, dysarthria, hyporeflexia, and decreased vibration sense occur [40].

Vitamin E deficiency might also occur due to fat malabsorption, for example, caused by cystic fibrosis or some liver diseases as well as genetic

4. Ataxia with vitamin E deficiency is an autosomal recessive disorder characterized by markedly reduced plasma levels of vitamin E, ataxia (neurological symptom with a lack of voluntary coordination of muscle movements), spinocerebellar degeneration, and peripheral neuropathy that resembles Friedreich ataxia.

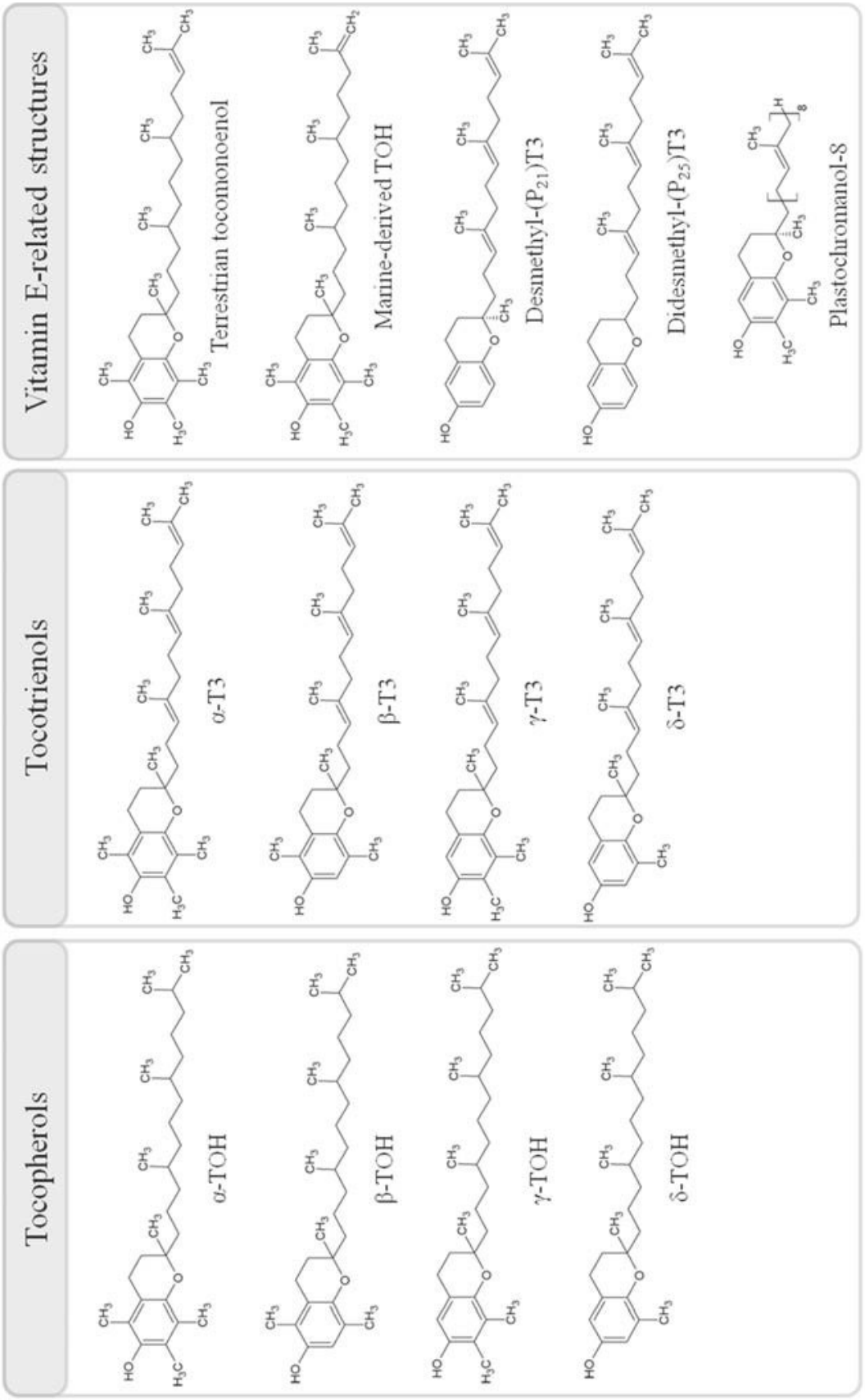

FIGURE 9.5 Chemical structures of vitamin E forms and vitamin E–related natural compounds.

defects, such as abetalipoproteinemia [41–43]. Further, the Marinesco-Sjögren syndrome and chylomicron retention disease likely cause vitamin E deficiency, as they are characterized by impaired chylomicron assembly or delivery [44,45]. Consequently, peripheral nerves die due to the lack of vitamin E, leading to spinocerebellar ataxia. Long-term vitamin E deficiency is further characterized by muscle degeneration. This process can ultimately lead to death if the heart muscle is affected [46]. Given its protective role on neurons, vitamin E was expected to prevent age-related neurodegenerative diseases such as Alzheimer disease. Indeed, vitamin E supplementation slowed down the progression of Alzheimer disease in some human intervention trials [47,48]. Supportive findings were also made in mice, where vitamin E deficiency caused axonal degeneration in brain areas important for memory and cognition [49]. Furthermore, impaired motor coordination and cognitive function was normalized by supplementation with vitamin E in vitamin E–depleted mice [50].

Vitamin E status seems to be important not only for the maintenance of neurons, but also for their development. Several animal studies suggest that the sufficient supply with vitamin E (of the mother) is critical for the development of the central nervous system and cognitive function of the offspring [51–53]. Furthermore, vitamin E along with folic acid may play a supportive role in the prevention of neural tube defects in human [54,55].

For a long time, the effects of vitamin E were attributed to its antioxidant properties (vide supra), but more recent work was dedicated to its non-antioxidant properties. Hence, it became evident that vitamin E modulates gene expression and enzyme activities and interferes with signaling cascades independent of its antioxidative capacity [56]. Examples for such functions are the suppression of inflammatory mediators, reactive oxygen species (ROS[5]), and adhesion molecules; the induction of scavenger receptor; and the activation of NFκB [57]. Given these (and further known) actions, vitamin E is most likely playing a role in several, but not only, inflammatory diseases (for more details, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). In addition, T3—another relevant form of vitamin E in our diet—are gaining more attention. Neuroprotective, anticancerogenic, antidiabetic, and cardioprotective effects have been suggested for this group of vitamin E [58]. However, further research is required, as the results obtained from clinical trials for TOH are inconsistent with respect to beneficial effects on chronic diseases such as cancer and cardiovascular diseases (CVD[6]) [59].

5. Reactive oxygen species are oxygen-containing molecules that are highly reactive, such as superoxides, peroxides, hydroxyl radicals, and singlet oxygen.
6. Cardiovascular diseases comprise disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, pulmonary embolism, and others.

Absorption, Transport, and Distribution of Vitamin E

Vitamin E comprises a class of lipophilic molecules and hence its intestinal uptake follows the pathway known for lipids. A key step is the lipid emulsification, i.e., the incorporation into micelles formed with the help of phospholipids and bile acids. The transfer into enterocytes of the intestine is carried out by passive diffusion, scavenger receptor class B type 1 (SRB1) [60], or Niemann-Pick C1-like protein 1 [61]. As there are no specific transport plasma proteins known for α-TOH [62], it is assumed that vitamin E transport in blood follows that of lipoproteins (reviewed in Ref. [61]). Here, key players in the uptake of vitamin E are SRB1 in peripheral tissue and low-density lipoprotein (LDL) receptor as well as LDL receptor−related protein in the liver [63,64]. Once in the liver, discrimination between the different forms of vitamin E occurs. Responsible for this process is the α-tocopherol transport protein (α-TTP), which promotes the incorporation of *2R*- or *RRR*-α-TOH into very-low-density lipoproteins (VLDL) [65,66], whereas other forms and stereoisomers are secreted into bile [67]. Besides α-TTP, the TOH-associated protein and the TOH-binding protein are known mediators of the intracellular transport of vitamin E. Interestingly, α-TOH secretion from the liver is apparently not dependent on VLDL assembly and secretion, thus oxysterol-binding proteins [68] and ATP-binding cassette transporter A1 (ABCA1) [69] have been suggested to contribute to the release from the liver. Furthermore, ABCA1 mediates the efflux of vitamin E in the intestine, macrophages, and fibroblasts [69], and multidrug resistance P glycoprotein has been identified as a transporter for the excretion of α-TOH via bile [70].

Metabolism of Vitamin E

The metabolism of vitamin E mainly takes place in the liver, whereas extrahepatic pathways have also been suggested [71,72]. Interestingly, rates of vitamin E metabolism increase with higher levels of the vitamin to prevent its accumulation to toxic levels. As indicated before, the preferred form of vitamin E in humans is α-TOH, which is due to the preferential binding of a specific hepatic protein, namely α-TTP. It has been hypothesized that α-TTP protects the α-form from metabolism, in turn leading to its enrichment. Given the lower affinities of the other vitamin E forms to α-TTP, their rate of catabolism is likely more pronounced [73]. In principle, metabolism of all forms of vitamin E follows the same route, which was confirmed by the detection of the respective end products of hepatic metabolism, α-, γ-, and δ-carboxyethyl-hydroxychromanol (CEHC) (Fig. 9.6) [74,75]. However, the catabolic rates depend on the vitamin E form, possibly due to different affinities to key enzymes [73,76]. The classification of the metabolic end product as α-, γ-, and δ-CEHC indicates that the chroman ring is not modified in this process; the aliphatic side chain is rather the substructure

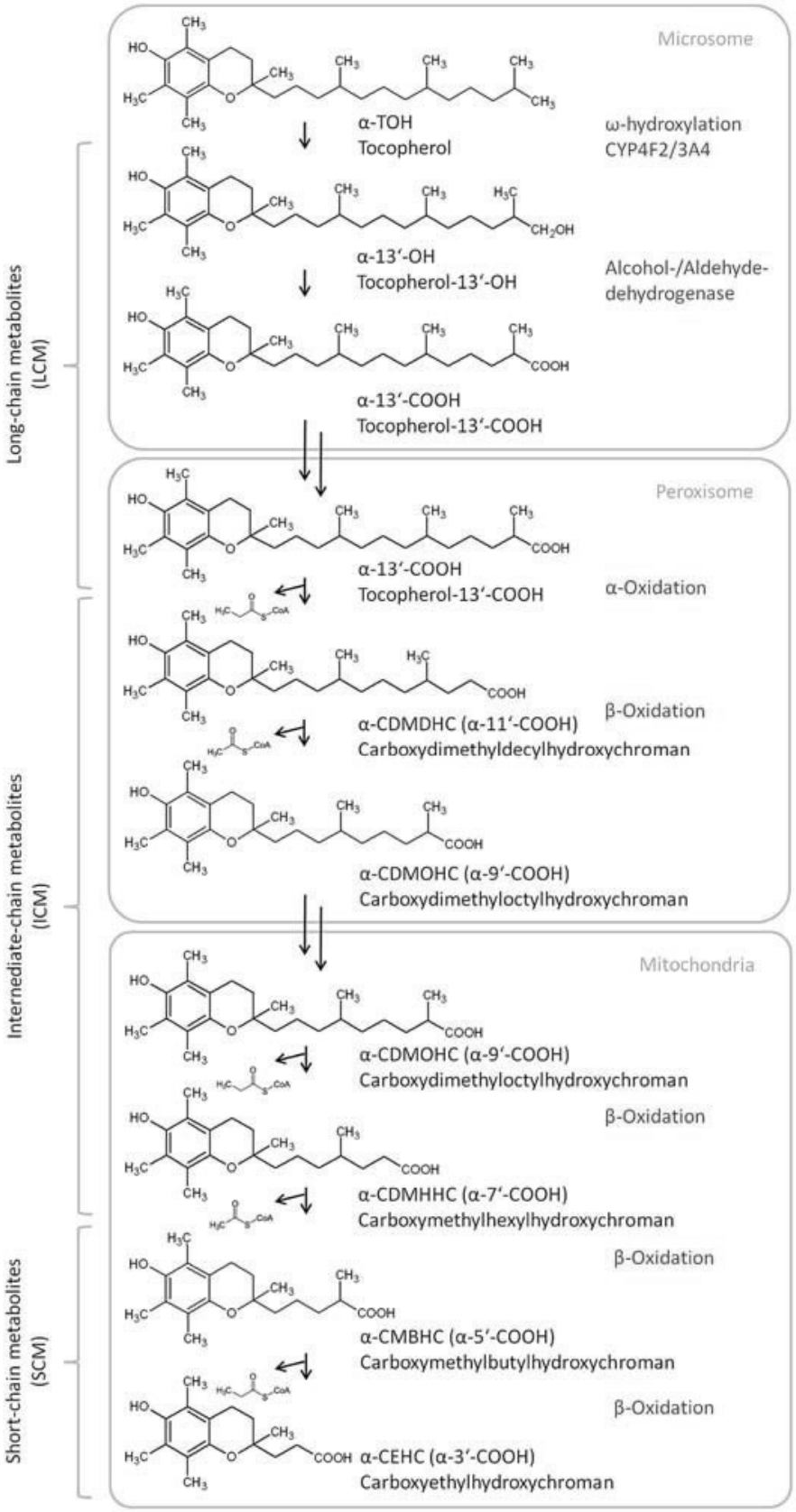

FIGURE 9.6 Principle hepatic metabolism of vitamin E. *Adapted from M. Birringer, P. Pfluger, D. Kluth, N. Landes, R. Brigelius-Flohé, J. Nutr. 132 (2002) 3113–3118.*

where modification takes place. The same applies to T3, whereas further enzymes such as 2,4-dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase (known from the metabolism of linoleic acid) are likely needed for metabolizing the unsaturated side chain [77].

Metabolism of vitamin E is therefore characterized by the shortening of the side chain *au fond*. Catabolism of the vitamin E molecule takes place in three cell compartments: the endoplasmic reticulum (microsomes), peroxisomes, and mitochondria. However, the transfer of the metabolites between the compartments is not yet understood. The initial step takes place at the endoplasmic reticulum and results in the formation of 13′-hydroxychromanol (13′-OH) metabolites via ω-hydroxylation by cytochrome P450 (CYP) 4F2 or CYP3A4, respectively [76,78]. Subsequent ω-oxidation by alcohol and aldehyde dehydrogenase (an aldehyde intermediate is formed) leads to 13′-COOH metabolites. Hence, the metabolites are handled like fatty acids and the side chain is shortened by β-oxidation, resulting in the elimination of propionyl-CoA or acetyl-CoA, respectively. The first two rounds take place in the peroxisome, leading to the intermediate-chain metabolites 11′-COOH and 9′-COOH, respectively. Three further rounds of β-oxidation are carried out in the mitochondria, forming the short-chain metabolites (SCM) 7′-COOH and 5′-COOH as well as the final product CEHC or 3′-COOH. During catabolism, the metabolites are modified simultaneously by conjugation, i.e., the metabolites are either sulfated or glucuronidated, but glycine-, glycine-glucuronide-, and taurine-modified metabolites have also been identified [79]. The more hydrophilic conjugated SCM are released via urine. In human urine, however, vitamin E is mainly found in conjugated form after glucuronidation [75,80–82]. The long-chain metabolites (LCM[7]) and their metabolic precursors are secreted via bile into the intestine. This fecal route is considered as the major way of excretion for vitamin E. In contrast to urine, the metabolites in fecal samples are not conjugated [80,83].

SYNTHESIS OF VITAMIN E LONG-CHAIN METABOLITES

The LCM can be obtained in vitro by incubation of cultured cells with the respective TOH precursors (the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). The culture supernatants of these cells can be used to investigate the cellular effects of the LCM or their action on isolated enzymes, as it has been already practiced by Jiang et al. [84]. However, this method is not feasible for all investigations, as the cells produce a mixture of carboxychromanols with different chain lengths, including SCM, as well as sulfated and nonconjugated metabolites. Furthermore, not all cell types exhibit the capability to metabolize all forms of TOH

7. The long-chain metabolites of vitamin E are the metabolites of tocopherols and tocotrienols with a side chain that is comprised of 13 carbon atoms.

[85,86]. A purification of defined metabolites is therefore needed if one is interested in investigating the specific effects of a single metabolite.

An alternative way to obtain pure metabolites is their chemical (semi) synthesis. The semisynthesis of α- and δ-13′-OH and the respective 13′-COOH metabolites has been established using the natural product garcinoic acid [33,87]. The first step in the entire process is the extraction (or synthesis) of garcinoic acid from appropriate sources, which is described in the following section. The subsequent synthesis of the α- and δ-LCM from garcinoic acid is outlined in another section.

Isolation of Garcinoic Acid

The isolation of garcinoic acid was first mentioned in 1984 by Franco Delle Monache and colleagues, who used *Clusia grandiflora* from Venezuela as source material [88]. In general, the family of *Clusiaceae* is the source of choice for isolating garcinoic acid. The *Clusiaceae* family is comprised of about 40 genera including about 1600 species, which are found in tropical regions worldwide [89–91]. Members of the family are sources of, inter alia, edible fruits, drugs, pigments, and dyes [90] and have therefore been used in traditional medicine in the regions of their occurrence [89]. So far, three genera of the *Clusiaceae* are known to contain garcinoic acid, namely *Tovomitopsis*, *Clusia*, and *Garcinia*. An overview of reported isolation procedures is provided in Table 9.1.

Tovomitopsis psychotriifolia, a plant from Costa Rica, has been shown to contain garcinoic acid in its leaves. In 1995, Setzer et al. extracted the compound from fresh chopped leaves using 80% aqueous ethanol with a subsequent isolation by liquid chromatography and thin-layer chromatography (TLC) using a 1:1 ethyl acetate/hexane mixture. Determination of the structure was carried out by nuclear magnetic resonance (NMR). Here, the detected structure was *trans*-δ-tocotrienolic acid, whereas Monache et al. mainly found the *cis*-isomer [92].

Among the *Clusia* genus, several members produce garcinoic acid. The trunk of Brazilian *Clusia obdeltifolia* contains a mixture of garcinoic acid in its *cis*- and *trans*-configuration. Extraction of the compounds from dried and powdered material was carried out by hexane with subsequent evaporation of the solvent [93]. Following fractionation with ethyl acetate/hexane and hexane/acetone on a silica column led to the isolation of garcinoic acid. Here, the *cis*-form was more prominent than the *trans*-form with an approximate ratio of 9 to 1, as determined by NMR [93]. The related plant *Clusia burlemarxii*, found in Brazil, also contains garcinoic acid in its leaves. The natural product was extracted from the dried and powdered material by maceration with 95% ethanol, concentration, mixing with 80% ethanol and subsequent treatment with ethyl acetate. Garcinoic acid was then purified by column chromatography over silica gel with mixtures of

TABLE 9.1 Overview of Procedures for Garcinoic Acid Isolation

		Method[a]					
Plant	**Source**	**Extraction**	**Separation Process**		**Input**	**Yield**	**Refs.**
Tovomitopsis psychotriifolia	Leaves	EtOH	LC, TLC	HEX/AcOH	0.16% of starting weight		[92]
Clusia obdeltifolia	Trunk	HEX	CC	**1.** EtAc/HEX **2.** HEX/ACE	6 kg	1.512 g	[93]
Clusia burlemarxii	Leaves	**1.** EtOH **2.** EtAc	CC	**1.** TCM/MeOH **2.** AcOH/MeOH	1.6 kg	5 mg	[89]
Clusia pernambucensis	Bark	EtAc	CC, TLC	**1.** cHEX/EtAc **2.** EtAc/MeOH	197 g	85.3 mg	[94]
			HPLC	H_2O/MeOH/ACN			
Garcinia kola	Seed	**1.** MeOH **2.** MeOH/TCM	CC	**1.** MeOH/TCM **2.** HEX/ACE	1 kg	3.8 g	[87]
Garcinia amplexicaulis	Bark	**1.** DCM **2.** MeOH	CPT	HEP/EtAc/ MeOH/H_2O	270 g	10 mg	[95]

ACE, acetone; *ACN*, acetonitrile; *AcOH*, acetate; *CC*, column chromatography; *cHEX*, cyclohexane; *CPT*, centrifugal partition chromatography; *DCM*, dichloromethane; *EtAc*, ethyl acetate; *EtOH*, ethanol; *HEP*, heptane; *HEX*, hexane; *HPLC*, high-performance liquid chromatography; *LC*, liquid chromatography; *MeOH*, methanol; *TCM*, chloroform; *TLC*, thin-layer chromatography.
[a]*For detailed information, the reader is referred to the text.*

chloroform and methanol in increasing polarity and in a second washing step with mixtures of ethyl acetate and methanol in increasing polarity. Again, the *cis*-isomer was more prominent [89]. A third member of the family, *Clusia pernambucensis* from Brazil, contains garcinoic acid in the bark [94]. The extract was obtained by maceration with ethyl acetate and subsequently fractionated by column chromatography with a cyclohexane/ethyl acetate gradient and sequentially an ethyl acetate/methanol gradient. After profiling with TLC, the appropriate fraction was purified by reverse-phase high-performance liquid chromatography (HPLC) using an isocratic 8:32:60 mixture of water, methanol, and acetonitrile. In addition to the *cis*-isomer of garcinoic acid, the related compounds δ-T3, δ-T3 alcohol, and δ-T3 methyl ester were obtained. However, in terms of quantity, garcinoic acid was substantially more abundant than the other compounds [94].

Members of the genus *Garcinia* are another valuable source of garcinoic acid. The isolation of garcinoic acid from seeds of *G. kola*, which originate from Nigeria, was first described by Terashima et al. in 1997 [25,96]. Based on this procedure, Birringer et al. developed a modified method [87]. Here, the mashed seeds were extracted with methanol, and after evaporation of the solvent, the extract was dissolved in a 95:5 mixture of methanol and chloroform. The crude extract was obtained by drying. For the isolation of garcinoic acid, the extract was again dissolved in 95:5 methanol/chloroform and applied to a silica gel column for purification. Further chromatographic separation on silica gel with a 65:35 mixture of hexane and acetone led to purified garcinoic acid, as characterized by NMR and mass spectroscopy (MS) [87]. A further member, *Garcinia amplexicaulis* from New Caledonia, contains garcinoic acid in the bark. Extraction of garcinoic acid from dried and grounded material was carried out with dichloromethane and subsequently methanol in a Soxhlet apparatus. The extract was further fractionated with a 2:1:2:1 mixture of heptane, ethyl acetate, methanol, and water using centrifugal partition chromatography. Garcinoic acid was subsequently isolated from the appropriate fraction by preparative HPLC using methanol and determined by NMR and MS [95].

Synthesis of Garcinoic Acid

With the first isolation and description of garcinoic acid (δ-*trans*-tocotrienolic acid) from *Clusia grandiflora*, the groundwork for approaches to synthesize this bioactive compound was laid. In 2005, David Maloney and Sidney Hecht reported a procedure to synthesize garcinoic acid (Fig. 9.7).

The basis for their stereo-controlled synthesis was to elaborately produce two molecules: alkyl iodide, (*S*)-1-iodo-5-(2,5-dimethoxy-3-methylphenyl)-3-methylpentan-3-ol (**4**), and vinyl iodide, (2*E*,6*E*,10*E*)-ethyl 11-iodo-2,6,10-trimethylundeca-2,6,10-trienoate (**5**). The alkyl iodide (**4**) was synthesized in two reaction steps from 4-(2,5-dimethoxy-3-methylphenyl)

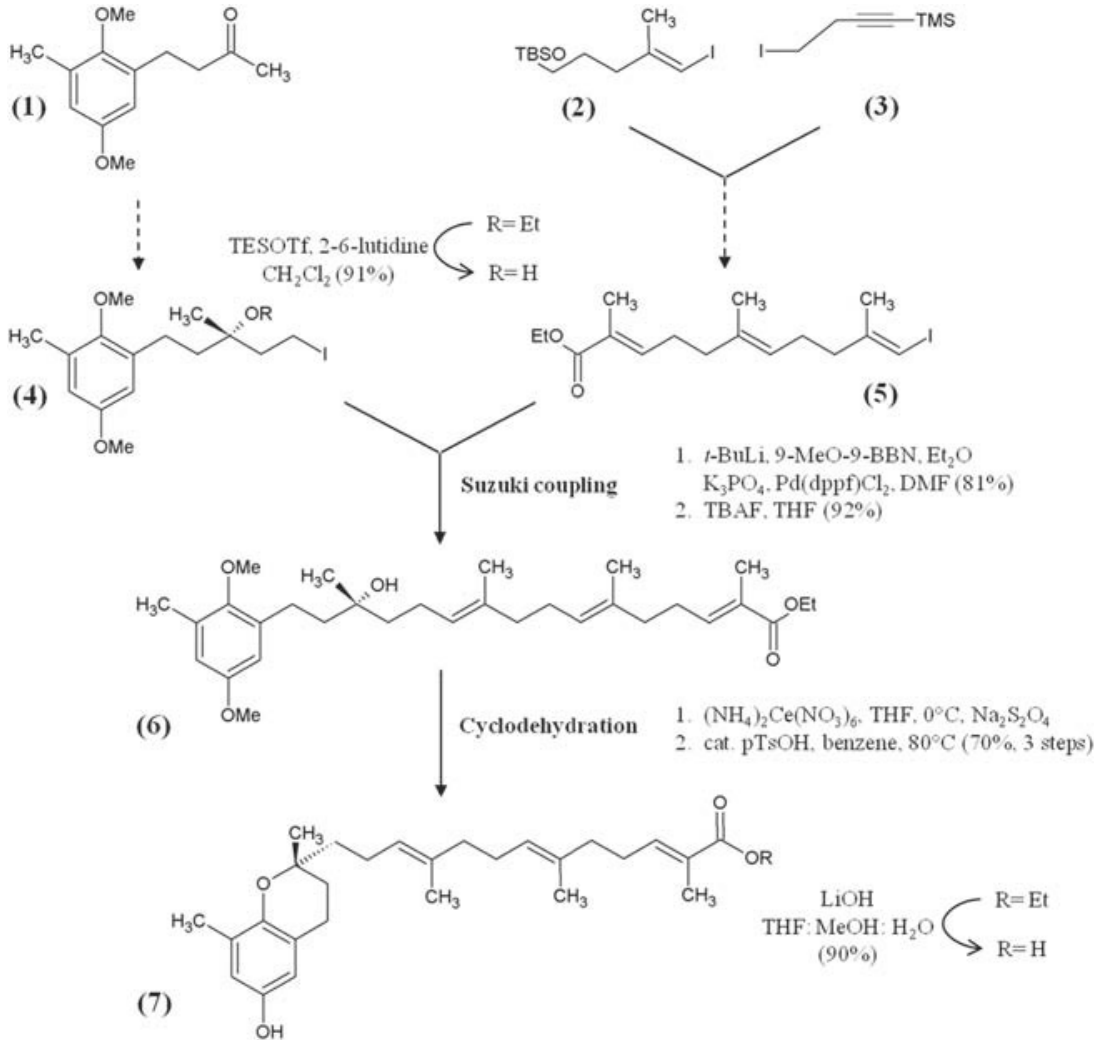

FIGURE 9.7 Stereo-controlled synthesis of garcinoic acid. *Adapted from D.J. Maloney, S.M. Hecht, Org. Lett. 7 (2005) 4297–4300.*

butan-2-one (**1**). The Negishi coupling of *tert*-butylsilyloxy-5-iodo-4-methylpent-4-ene (**2**) and 4-iodo-1-(trimethylsilyl)but-1-yne (**3**) yielded the vinyl iodide (**5**). Suzuki coupling of (**4**) and (**5**) gave the protected prenylated 1,4-benzoquinone, (2*E*,6*E*,10*E*,14*R*)-ethyl 14-hydroxy-16-(2,5-dimethoxy-3-methylphenyl)-2,6,10,14-tetramethylhexadeca-2,6,10-trienoate (**6**). The acid-catalyzed cyclodehydration followed by saponification leads to synthetic garcinoic acid (**7**) [34]. In principle, this synthesis route provides an alternative way to obtain the α- and δ-LCM, starting with synthetic garcinoic acid.

Semisynthesis of Long-Chain Metabolites From Garcinoic Acid

Garcinoic acid, either isolated from the various natural sources or chemically synthesized, can be used for the semisynthesis of α- and δ-LCM. Mazzini et al. reported the respective approach in 2009 [33]. A synthesis route leading to α-TOH was outlined, using the isolated compound from *G. kola* (obtained according to the procedure provided by Terashima et al. [25]) (Fig. 9.8).

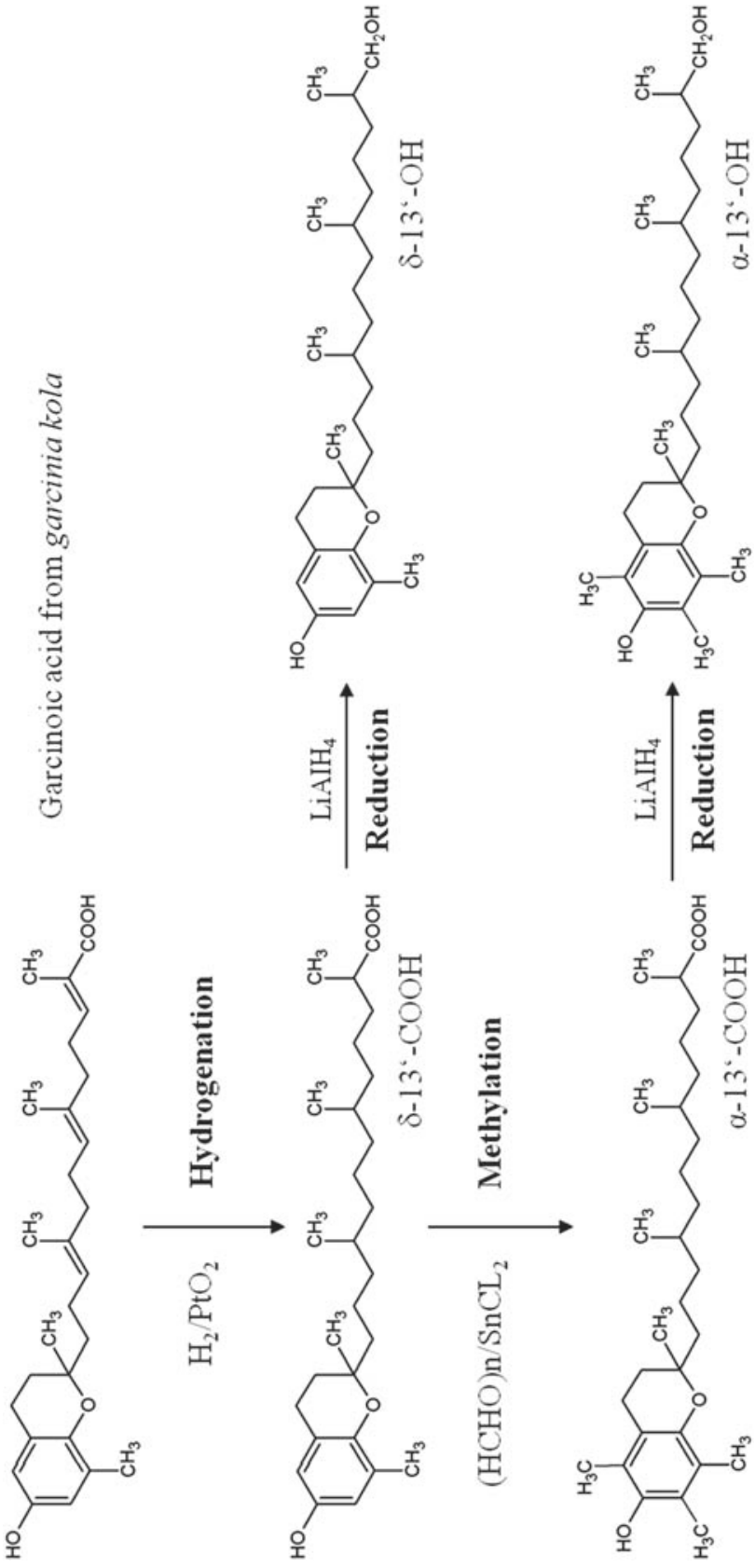

FIGURE 9.8 Semisynthesis of α-13′- and δ-13′-LCM of vitamin E from garcinoic acid. *Adapted from M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Free Radic. Biol. Med. 49 (2010) 1315–1322.*

Here, the unsaturated side chain of garcinoic acid is first hydrogenated in a platinum-catalyzed reaction to receive δ-13′-COOH. The corresponding α-LCM, α-13′-COOH, is obtained by permethylation of δ-13′-COOH, catalyzed by $SnCl_2$. A reduction with $LiAlH_4$ leads to α-13′-OH. To obtain α-TOH, the alcohol can be converted into a ditosylate derivative, and subsequently, the tosyl groups are removed by treatment with $LiAlH_4$ and heating in an aqueous basic solution (not shown). Finally, a hydroxy group resides at the chroman ring and the chain loses its functional moiety [33]. This synthesis route was reproduced by Birringer et al. later. Again, *G. kola*—derived garcinoic acid was used, but δ-13′-OH was derived by reduction of δ-13′-COOH with $LiAlH_4$, additionally. Hereby, the δ-LCM as well as the α-LCM can be obtained from garcinoic acid at sufficient purity for further usage in functional assays [87].

BIOACTIVITY OF GARCINOIC ACID, VITAMIN E, AND LONG-CHAIN METABOLITES

Several functions of vitamin E have been proposed until today. In the early days of vitamin E research, the focus was on the radical chain breaking and radical scavenging capacity of α-TOH, which is regarded as the most potent member of the vitamin E family in this respect [97]. However, Angelo Azzi was the first who provided evidence for further properties of α-TOH that are independent of its function as an antioxidant. He found that α-TOH regulates several cell functions via modulation of signal transduction, nuclear receptors, as well as gene and protein expression besides its function as a natural antioxidant [98,99]. T3 possess similar and sometimes even stronger biological activities than TOH; in particular, T3 show antioxidative, antiatherogenic, anticancer, antidiabetic, antiinflammatory, and neuroprotective properties [58,100]. Apart from the well-known functions of the different vitamin E forms, the bioactivity of their metabolites is not well understood.

Vitamin E metabolism has been studied intensively since the 1990s, but it took about a decade until the first groups were able to detect α-, γ-, and δ-13′-OH as well as the corresponding 13′-COOH metabolites in cell culture supernatants [76], in human liver cells [87], and also in human serum [101]. Current research on LCM is focused on their antiinflammatory properties. Investigations of different groups showed regulatory actions of the LCM on enzymes of the inflammatory cascade [102,103]. Further studies revealed antioxidative and cytotoxic effects [33,87], as well as regulatory properties in lipid metabolism [101]. Based on these studies, the LCM seem to have higher activity and modes of action different from those of the respective vitamin E forms. Garcinoic acid is a natural compound with high structural similarity to the LCM of δ-T3 (and identical to the 13-carbon side chain acid metabolite) [25], indicating that the bioactivity of this substance may be comparable to the LCM of TOH and T3. However, only a few studies on the biological actions of

garcinoic acid have been described so far. The acid exhibits high antioxidative potential [25,31] and antiproliferative effects [33]. However, almost nothing is known about its antiinflammatory or regulatory potential.

The following paragraphs provide an overview on the properties of garcinoic acid and the different LCM in comparison to their precursors.

Cytotoxicity

Recent animal studies on toxic effects of natural or nonnatural vitamin E forms and derivatives on reproduction and development revealed no toxic effects [104]. Physiological vitamin E intake can be increased up to 300 mg/day (mixture of TOH and T3, ~190 IU/day) without causing any complications [105,106]. No clear adverse effects have been described, even for short-term high-dose administration of vitamin E. However, persistent high-dose supplementation has been shown to interfere with blood clotting and is therewith associated to an increased risk of hemorrhagic stroke in animal studies [104]. In the past, TOH was considered to be a safe food additive [107], but an increase in total mortality after high-dose vitamin E intake was discussed during the last years [108]. However, excessive intake of vitamin E results in increased metabolite formation and excretion [109]. This could be a hint that the metabolites of vitamin E may cause noxious effects after a high-dose intake of vitamin E.

Cytotoxic Effects of Vitamin E

Reports on cytotoxic effects of vitamin E are inconsistent. There are considerable differences in the cytotoxicity of the different vitamin E forms. McCormick and coworkers investigated the cytotoxic potential of α-, γ-, and δ-TOH in RAW264.7 macrophages. Concentrations up to 60 μM γ-TOH and especially δ-TOH decreased cell viability by 50% and 90%, respectively, whereas α-TOH had no effect [110]. This has been confirmed in CEM/VLB100 and murine C6 glioma cells [111,112]. Experiments with δ-TOH in different cell types, such as MCF-7 cells, HepG2 cells, and fibroblasts, indicate that δ-TOH-triggered cytotoxicity may depend on the cell type. While δ-TOH incubation results in a massive reduction of viability in MCF-7 breast cancer cells and fibroblasts, no effect was observed for HepG2 liver cells [110]. The first hypothesis—the cell type-—dependent cytotoxicity due to different intracellular accumulation of TOH—was disproved [110]. Another concept implies that the degree of methylation of the chroman ring is important for cytotoxicity [110].

In comparison to TOH, T3 show diverse cytotoxic effects. In A549 and U87MG cells, δ-T3 exhibited the highest cytotoxicity followed by γ- and α-T3. Further, the cytotoxicity of T3 derivatives also depends on the cell type [113]. Moreover, cell viability was also reduced in HepG2 liver cells by 40 μM of δ-T3 or γ-T3 [114]. Thus, T3 are able to reduce cell viability in cell types where TOH have no effect. Taken together, the δ-forms of TOH and T3 seem to be the most

cytotoxic vitamin E forms. Moreover, lower concentrations of T3 are needed compared to TOH. TOH and T3 are also known to affect cell proliferation. Antiproliferative effects of all TOH forms have been observed in C6 glioma cells with concentrations higher than 50 μM. Here, α-TOH and γ-TOH were the most potent proliferation inhibitors [112]. The underlying mechanism is probably a block of the cell cycle via p27-mediated inhibition of the cyclin E/cyclin-dependent kinase 2 complex [115] and by increased p53 expression [116]. In particular γ-TOH and δ-TOH, but not α-TOH, affect these pathways [112].

Similar effects can be induced by T3. Because of their higher reactivity, antiproliferative effects of T3 have been studied in cancer cells to use T3 as therapeutic reagents. T3—δ-T3 more effectively than γ-T3—reduced cell proliferation in HL-60, A549, and U87MG cells by induction of apoptosis [113,117]. Thus, TOH (cell cycle arrest) and T3 (apoptosis) exert their anti-proliferative effects via different mechanisms.

Metabolites of Vitamin E

As mentioned before, high doses of vitamin E increase formation of metabolites and their excretion. Therefore, TOH and T3 metabolites might contribute to cytotoxic effects of vitamin E. Studies of Conte et al. in 2004 provided first impressions of CEHC-mediated cytotoxic effects in cancer cell lines. In this work, γ-TOH, γ-T3, and γ-CEHC inhibition of cell proliferation were compared to their respective α-homologues. It should be emphasized that the γ-forms of TOH and T3 have higher transformation rates to CEHC than the respective α-forms. This has been evaluated in PC3, LNCaP, and HepG2 cells [118]. γ-T3 and γ-CEHC are the most potent inhibitors of cancer cell proliferation. At 10 μM, both compounds reduced proliferation of PC3 cells by 70–82%, while their α-analogues were less effective [119]. Francesco Galli and coworkers presume that this effect is triggered by a block of cyclin D1, but further investigations are needed to prove this concept [119]. In conclusion, the SCM are as effective as their precursors in inhibiting cell growth, with γ-forms being most potent.

In contrast to SCM, LCM are widely uncharted. Based on earlier results of Galli et al. and Conte et al. indicating that carboxy-SCM exhibit pro-apoptotic properties, Birringer et al. discovered similar effects for the 13′-LCM [87,118,119]. In this study, HepG2 cells were incubated with α-13′-COOH and δ-13′-COOH and α-13′-OH and δ-13′-OH. The carboxy metabolites appeared to be potent inducers of cell death, while the hydroxy metabolites did not affect cell survival. Furthermore, the δ-forms have been more active than the α-forms. This is reflected by the EC_{50} values of the two substances: 6.5 μM for δ-13′-COOH and 13.5 μM for α-13′-COOH [87], in comparison to α-TOH ($EC_{50} > 100$ μM) and α-CEHC, which showed very low antiproliferative effects at concentrations >10 μM [119]. This finding is in line with the observation that α-13′-COOH and δ-13′-COOH significantly increased the

ratio of apoptosis of HepG2 cells, compared to their metabolic precursors α-TOH and δ-TOH [87]. The treatment of HepG2 cells with α-13′-COOH and δ-13′-COOH also caused increased expression of caspase-3, which is a key enzyme of apoptosis. While δ-13′-OH slightly increased caspase-3 expression, α-13′-OH, α-TOH, and δ-TOH did not [87].

To sum up, the LCM show effects on cell proliferation and cell viability similar to those of their metabolic precursors, but there are significant differences in their activity and the LCM act at much lower concentrations.

Garcinoic Acid

Based on its structural similarities to δ-13′-COOH, it is hypothesized that garcinoic acid has comparable antiproliferative and cytotoxic properties as other vitamin E analogues. To confirm this hypothesis and to get more information about the structural requirements for antiproliferative properties, Mazzini et al. [33] investigated cell proliferation in glioma C6 cells after incubation with garcinoic acid. The acid reduced growth of C6 cells by 50% at concentrations of 10 μM. This effect has also been observed for α-CEHC and δ-CEHC in this study, indicating that the length of the side chain has barely influence on the antiproliferative properties [33,119]. Nevertheless, δ-13′-COOH and α-13′-OH showed higher inhibitory effects on proliferation of C6 cells than α- and δ-CEHC. This indicates that the presence of the carboxyl or hydroxyl group of the vitamin E metabolites enhances antiproliferative effects [33,87]. Based on the limited data on the cytotoxicity of garcinoic acid, its properties seem to be comparable to the other vitamin E metabolites. We found that garcinoic acid showed cytotoxic effects in the RAW264.7 mouse macrophage model system in which we revealed EC_{50} concentrations of about 5.5 μM (unpublished data).

The cytotoxicity of natural compounds is of particular interest for cancer treatment. Several plant-derived anticancer agents are already in clinical use. In particular, taxanes, camptothecines, vinca alkaloids, and podophyllotoxins are worth mentioning [120]. The compounds exert different modes of action, but all have been shown to have antiproliferative effects on cancer cells [121−124]. This is also a characteristic of garcinoic acid, making it interesting for cancer research. Although the effects of garcinoic acid on cancer cells and the underlying mechanisms have still to be characterized, one promising property is already known: garcinoic acid inhibits DNA polymerase β with an IC_{50} of about 4 μM [34]. Compared to other natural DNA polymerase β inhibitors, garcinoic acid is one of the most potent ones (reviewed in Ref. [125]). Cells deficient in DNA polymerase β activity are hypersensitive to certain chemotherapeutic agents due to their impaired ability to repair induced DNA damage [126]. For this reason, the further characterization of the cytotoxic effects of garcinoic acid is of great interest. If garcinoic acid is able to induce DNA damage and simultaneously to suppress DNA damage repair mechanisms, it might be a

powerful agent for cancer treatment. However, the effects of garcinoic acid should first be well characterized in cellular systems before experiments in animal models or even clinical trials in humans can be conducted.

Antioxidative Properties

The antioxidative properties of the different vitamin E forms and metabolites have been extensively studied during the last decades, considering α-TOH as the most important antioxidant, mainly due to the protection against peroxidation of polyunsaturated fatty acids (PUFA[8]) in phospholipids of cellular membranes and plasma lipoproteins, a finding made at least in vitro [56,127]. Higher PUFA intake requires higher vitamin E supply to provide adequate antioxidative protection against lipid peroxidation. Unsaturated fatty acids tend to form radicals, which can be scavenged by the free hydroxyl group at the chroman ring of α-TOH; the reaction product is afterward excreted to bile as α-TOH hydroquinone [128]. All TOH and T3 forms exhibit antioxidative properties. Besides the free hydroxyl group, the mobility of the molecule in cellular membranes is a crucial factor [97,129]. The T3 have higher membrane mobility due to their unsaturated side chain. This should lead to an increase in their antioxidative capacity compared to the respective TOH forms. Yoshida and coworkers compared the effects of either TOH or T3 treatment on peroxyl radical scavenging, but no differences were detectable in membrane uptake or reactivity. However, another investigation on leptosome complexes revealed different results. In this experiment, α-T3 and α-TOH were integrated separately into synthetic membranes. Afterward, lipid peroxidation was induced in another part of the liposomal complex. It appeared that α-T3 was more potent in inhibiting peroxyl radical formation than its TOH equivalent. The more pronounced antioxidative potential of α-T3 seemed to be a result of its better intermembrane mobility, making α-T3 able to reach the radicals faster than α-TOH [130]. This observation has been confirmed by Serbinova and coworkers in rat liver microsomes [97]. However, there are also studies showing similar antioxidant activities of TOH and T3 [130,131].

In addition to membrane mobility, the number of methyl groups of the chroman ring increases the antioxidative capacity of TOH and T3. Despite this, the position of the methyl group in relation to the hydroxyl group at the chroman ring is important. For this reason, the α-forms have higher antioxidative potential than β-, γ-, and δ-derivatives. This has been shown for TOH and T3 in liposomal membranes. After induction of peroxyl radical–triggered lipid peroxidation, the α-derivatives were the most potent inhibitors of oxidative stress. The antioxidant activity decreased from α

8. Polyunsaturated fatty acids are a class of fatty acids characterized by more than one double bond; they are often essential for human nutrition.

through β to γ down to δ [130]. A further investigation in rat serum confirmed this observation [132]. Apart from these results, there are several in vitro studies indicating a reverse order of antioxidant efficiency with α-TOH being the least potent compound compared to δ- and γ-TOH [133,134]. In conclusion, TOH and T3 are highly potent antioxidants with a theoretically decreasing antioxidant activity from α- through β- to γ- and down to δ-forms. Furthermore, T3 seem to be more active than the respective TOH equivalents.

Due to the similarity of the chemical structure of garcinoic acid with T3, comparable antioxidant activities of these compounds can be expected. The antioxidative properties of this natural compound have been investigated in two independent studies. Okoko and coworkers used a methanolic extract from *G. kola* seeds for in vitro experiments. First, the extract was divided into five fractions by TLC. Afterward, the radical scavenging abilities of each fraction were compared to those of vitamin C. The fraction with the highest activity in hydroxyl radical scavenging was further investigated via HPLC analysis. Chromatographic fractioning and spectroscopic analysis revealed four compounds, including *Garcinia* biflavones GB1 and GB2, garcinal, and garcinoic acid [31]. The combination of these four compounds had a 40% higher antioxidative activity than vitamin C at a concentration of 0.5 μg/mL. Further investigations in U937 macrophage cells revealed inhibitory effects on nitric oxide formation [31]. However, Okoko and coworkers were not able to draw a conclusion whether a single compound or the combination of the four substances is responsible for the observed effects. The lack of compound-specific investigations is a crucial limitation of this study. In another investigation, the antioxidative potential of garcinoic acid has been compared to α-TOH using antioxidant activity assays. Terashima et al. found that the antioxidant activity of the natural product was 1.53 times that of α-TOH. This value was comparable to δ-T3 (1.47) and δ-TOH (1.53), molecules sharing high structural similarity to garcinoic acid [25]. Terashima and coworkers chemically modified garcinoic acid by shortening of the side chain. It appeared that the antioxidative activity was significantly affected by structural features, i.e., the shorter the side chain the higher the antioxidative potential. The garcinoic acid analogue with the shortest side chain had 18.7 times higher antioxidant activity than α-TOH [25]. To conclude, garcinoic acid seems to be one of the most potent antioxidative compounds in *G. kola* seeds with an antioxidant activity comparable to compounds such as δ-TOH and δ-T3.

The lack of in vivo studies with garcinoic acid makes predictions difficult whether the antioxidative capacity of garcinoic acid can contribute to drug development and disease treatment. Natural antioxidants in general are believed to have beneficial effects on different diseases. One of the best investigated groups of natural antioxidants are the polyphenols. Compounds such as quercetin, resveratrol, and curcumin are well-investigated members of this class of compounds that have almost similar antioxidative properties as garcinoic acid. All three substances are potent radical scavengers, especially

for hydroxyl radicals [135−137]. Furthermore, quercetin and curcumin have inhibitory effects on nitric oxide formation in different cell types [138,139]. In contrast to garcinoic acid, the use of these polyphenolic compounds for the treatment of diseases in which oxidative stress is involved has already been investigated in mouse models and humans. For example, natural antioxidants showed beneficial effects in nonalcoholic fatty liver disease (NAFLD[9]) and Alzheimer disease (reviewed in Refs. [140,141]). NAFLD is a metabolic disorder associated with high levels of free fatty acids and an increased cardiovascular and liver-related morbidity [142]. High oxidative and inflammatory damage in hepatocytes can also lead to nonalcoholic steatohepatitis (NASH[10]) [143]. Experiments in mice fed a Western diet showed that quercetin lowers oxidative stress in hepatocytes, which in turn leads to reduced liver steatosis [144]. In addition, resveratrol showed promising effects for NAFLD patients in a controlled clinical trial, mainly through lowering inflammatory markers and the reduction of oxidative stress [145]. Resveratrol was further used in studies on Alzheimer disease. Studies demonstrated the importance of neuroinflammation and oxidative stress in the pathogenesis of this disease. One of the most important factors contributing to the development of Alzheimer disease is β-amyloid, because of its ability to generate superoxide anions and α-carbon-centered radicals. The high ROS production caused by β-amyloid may lead to neuronal death [146,147]. Due to its antioxidant activity, resveratrol was used for the treatment of Alzheimer disease in rats, where the compound protected glioma cells from β-amyloid-triggered oxidative damage [148]. Furthermore, curcumin also protected neuronlike PC12 cells from β-amyloid toxicity and displayed neuroprotective effects larger than those of well-known antioxidants such as α-TOH [149]. Besides studies in cellular models, Lim and coworkers have also shown that dietary curcumin suppresses inflammation and oxidative damage in the brain of Tg2576 mice [150]. Furthermore, the epidemiological study by Ganguli and coworkers provides evidence that the Indian population, known for its curcumin-rich diet, shows reduced prevalence of Alzheimer disease compared to the US population [151].

Based on the fact that oxidative stress is a crucial factor for the development of both diseases and natural antioxidants have already shown promising effects on disease prevention, it can be hypothesized that the antioxidative properties of garcinoic acid bear potential for its use in drug development as well as disease prevention and treatment. The well-known effects of other

9. Nonalcoholic fatty liver disease is characterized by the accumulation of fat in the liver of people with no or low alcohol consumption that can lead to inflammation and scarring of the liver.
10. Nonalcoholic steatohepatitis is hallmarked by the accumulation of fat in the liver of people with no or low alcohol consumption accompanied by chronic inflammation, progressive scarring, and cirrhosis of the liver.

natural antioxidants in the prevention of NASH and Alzheimer disease are a promising starting point for in vivo experiments with garcinoic acid.

Apart from the vitamin E isoforms and garcinoic acid, almost nothing is known about the antioxidant activity of the 13′-LCM. Because of their high reactivity, the two LCM 13′-OH and 13′-COOH may act as prooxidants. To prove this hypothesis, Birringer et al. [87] investigated 13′-LCM-triggered ROS production. HepG2 cells were treated with α-13′-OH, δ-13′-OH, α-13′-COOH, and δ-13′-COOH. The corresponding TOH forms were used as controls. Generation of intracellular and mitochondrial ROS was measured via dichlorofluorescein assay [152]. Incubation with 10 μM α-13′-COOH or δ-13′-COOH increased intracellular ROS formation while α-13′-OH, δ-13′-OH, and both TOH forms showed no effect. Similar effects have been observed for mitochondrial ROS production. Here, α- and δ-13′-COOH increased mitochondrial ROS production by 30−50% while the other compounds had no effect. A decrease in mitochondrial ROS production was observed only for δ-TOH [87]. In conclusion, α-13′-COOH and δ-13′-COOH seem to have strong prooxidant potential while α-13′-OH and δ-13′-OH do not act as prooxidants. Due to the structural similarity to the α-13′-COOH and δ-13′-COOH, it can be expected that garcinoic acid exhibits a similar prooxidant potential, but this has to be confirmed experimentally. These observations differ from the results for the antioxidant effects of the different TOH and T3 forms. Particular attention should be paid to studies showing that α-TOH can possibly act as prooxidant [153,154].

Antiinflammatory Actions

Multiple cell types of the innate immune system and paracrine-acting as well as autocrine-acting mediators contribute to the complex process of inflammation. Here, the interplay of proinflammatory and antiinflammatory mediators is vital for the outcome of the inflammatory process, i.e., resolution or chronic inflammation. CVD and cancer, two of the leading causes of death worldwide, are inflammatory diseases, thus highlighting the importance of research for new antiinflammatory treatment approaches. Moreover, diseases of civilization, such as diabetes and obesity as well as asthma, rheumatoid arthritis, osteoporosis etc., have been linked to inflammation.

For this reason, the natural modulators of inflammation are of particular interest. Although several mediators of inflammation and underlying pathways have been identified, we here draw attention to the factors only, which have been investigated in the context of LCM and garcinoic acid.

Cyclooxygenases and Their Lipid Mediator Products

Eicosanoids comprise a group of lipid mediators involved in inflammation, which include prostaglandins, thromboxanes, leukotrienes (LT) and lipoxins. All eicosanoids are metabolically derived from arachidonic acid. Key enzymes

of the conversion of arachidonic acid to eicosanoids are COX1 and COX2 as well as lipoxygenases (LOX; the reader is referred to the section "Vitamin E and Lipoxygenases"). Arachidonic acid is released by action of phospholipases A_2 from phospholipids of the cell membrane. The bifunctional COX (cyclooxygenation and peroxidation function) forms prostaglandin G_2 from arachidonic acid by cyclization and addition of two molecules of oxygen and reduces it further to prostaglandin H_2. This endoperoxide serves as substrate for specific synthases and isomerases, which form prostaglandins of the E_2, F_2, D_2, and I_2 series as well as thromboxane A_2 [155].

While COX1 is constitutively expressed, COX2 can be induced by a variety of proinflammatory stimuli. Hence, COX2 is regarded as the more important source of eicosanoids during inflammation. All of the abovementioned prostaglandins are implicated in proinflammatory actions (reviewed in Ref. [156]).

Vitamin E Modulates Prostaglandin E_2 Release and Cyclooxygenase Activity

Tocopherol Inhibit Cyclooxygenase Activity

The release of prostaglandin E_2 (PGE_2) is widely used as a marker for the activity of COX. The effect of TOH on the release of PGE_2 has been studied in several cell types and settings. In BV-2 microglia cells the induction of PGE_2 by LPS could be attenuated by α-TOH dose-dependently. While 25 μM showed no effect, 50 μM diminished the effect significantly and 100 μM almost completely blocked the induction [157]. An interesting finding was made in human aortal endothelial cells: α-TOH induced the release of PGE_2 dose-dependently in concentrations above 10 μM. In contrast, COX activity, measured as conversion of exogenous arachidonic acid to PGE_2, was attenuated by α-TOH at 10 μM or higher. The authors postulated that α-TOH induces (1) the release of arachidonic acid from membrane phospholipids and (2) the expression of $cPLA_2$. The discrepancy in the abovementioned results is explained by a more relevant effect of α-TOH on substrate release (i.e., the release of arachidonic acid from membrane phospholipids) than on COX activity [158].

These findings implicate that the effects of TOH on PGE_2 release depend on the cell type. However, similar findings were made in macrophages. In peritoneal macrophages obtained from rats treated with 5 mg/day α-TOH (i.p.) for 6 days, the production of PGE_2 in response to different stimuli was diminished. Interestingly, macrophages from control animals showed a response similar to untreated control cells, when preincubated with α-TOH [159]. In a different approach with peritoneal macrophages the most effective reduction of PGE_2 production was observed with δ-TOH (1.25−12.5 μM) and α-TOH (12.5−150 μM). γ-TOH was less effective and β-TOH had no effect (up to 12.5 μM). Interestingly, all TOH forms reduced COX activity, measured

as the conversion of PGE_2 from exogenous arachidonic acid. Again, δ-TOH was most potent followed by β-, α-, and γ-TOH in descending order [160]. Thus, the substitution of the chroman ring seems to be important for the modulation of PGE_2 synthesis. However, it is possible that the different TOH forms act in different ways, either on substrate availability or on COX activity.

Are Tocotrienols the More Potent Vitamin E Form?

T3 have also been shown to be potent inhibitors of PGE_2 release. In malign mammary epithelial cells, PGE_2 release was reduced about 50% of controls by 3 μM γ-T3 [161]. Different effects were observed in mouse RAW264.7 macrophages stimulated with LPS to induce PGE_2 release and subsequently incubated with three different T3 forms at 10 μg/mL. While γ-T3 showed no effect, δ-T3 was the most potent inhibitor with about 55% reduction followed by a T3-rich fraction and α-T3. Surprisingly, α-TOH increased the effect of LPS induction [162]. In IL-1β-stimulated A549 lung epithelial cells, γ-T3 was as effective as δ-TOH in inhibiting release of PGE_2. The IC_{50} for both compounds were about 1−3 μM. γ-T3 was more potent than its γ-TOH counterpart (IC_{50} of 6−7 μM), while α-T3 exerted only weak inhibitory action (20% at 20 μM), and α- and β-TOH were completely ineffective below 50 μM [84]. The aforementioned results suggest that the T3 are more potent inhibitors of COX activity than their respective TOH forms. However, the substitution pattern of the chroman ring appears to be also a major determinant for the effectivity of the compound.

Tocopherol Metabolites Outclass Their Metabolic Precursors

While little is known about the bioactivity of TOH LCM in general, some studies focused on their effects on COX activity. We recently reported that α-13′-COOH is a potent COX-regulating metabolite. In mouse RAW264.7 macrophages, the upregulation of COX2 mRNA and protein by LPS and the subsequent increase in PGE_2 release was diminished by α-13′-COOH and α-TOH. Whereas α-TOH reduced PGE_2 production about 55%, α-13′-COOH abolished PGE_2 production almost completely. These findings are of particular significance as 100 μM α-TOH was less effective than 5 μM of α-13′-COOH. This underlines the higher effectivity of the LCM. In addition to PGE_2, the LPS-induced formation of further arachidonic acid−derived eicosanoids, namely prostaglandin D_2 and prostaglandin $F_{2\alpha}$, was blocked by α-13′-COOH. In contrast, α-TOH did not diminish the induction by LPS significantly [102]. In agreement with this, Jiang et al. reported no effect of 50 μM α-TOH on PGE_2 production in lung epithelial cells [84]. Compared to α-TOH, δ-TOH is more potent in inhibiting PGE_2 production (vide supra). In contrast to Wallert et al., Jiang et al. used no synthetic LCM, but cell culture medium collected from cells treated with TOH, containing the self-synthesized metabolites 9′-COOH, 11′-COOH, and 13′-COOH. An intact-cell assay with preinduced

COX and arachidonic acid as substrate revealed that the medium containing the δ-metabolites is superior to that with γ-metabolites in inhibiting COX activity. Unfortunately, the authors used a cell line that is unable to metabolize α-TOH to its respective carboxychromanols [85,86], resulting in no effect of α-TOH in this assay. δ-9′-COOH and δ-13′-COOH isolated from cell culture supernatants inhibited COX2 with IC_{50} of 6 or 4 μM, respectively. However δ-9′-COOH was unable to inhibit activity of purified COX1 and COX2 enzymes in concentrations <20 μM. In contrast, δ-13′-COOH was highly potent with an apparent IC_{50} of 5 μM for COX1 and 4 μM for COX2, which is comparable to ibuprofen. Only weak inhibition of both COX isoforms was shown for the SCM α-CMBHC (α-5′-COOH; $IC_{50} > 140$ μM) and γ-CEHC (γ-3′-COOH; $IC_{50} > 300$ μM) [84]. This finding indicates that LCM rather than SCM may be responsible for the antiinflammatory effects of TOH. This assumption is supported by the fact that A549 lung epithelial cells are not able to produce SCM [85,86]. Anyway, δ-SCM would be preferable for comparison, as the structure of the chroman ring likely influences the effectivity.

Garcinoic Acid: A New Player on the Court?

To date, no systematic investigation of the modulation of COX activity by garcinoic acid, the principal δ-13′-LCM of δ-T3, has been published. With respect to its structural similarities, garcinoic acid shares the chroman ring with δ-TOH, which has been shown to be the most potent TOH in this context [160]. In addition, δ-T3 is more effective in modulating COX activity than the other T3, which in turn can be considered more effective than TOH [84,162]. The unsaturated chain is a structural feature of garcinoic acid shared with T3. For this reason, we expect that garcinoic acid is more potent in modulating COX than TOH. As garcinoic acid carries a carboxylic acid moiety, one can compare it to the 13′-carboxychromanols generated from TOH. In particular, 13′-COOH have been shown to be substantially more effective in inhibiting COX activity than their metabolic precursors [84,102]. Based on these observations, garcinoic acid is likely more potent than TOH and comparable to (δ-)T3 or its LCM, respectively. However, experiments are required to confirm whether this hypothesis holds true.

Vitamin E and Cyclooxygenase Expression

While the effects of the different vitamin E forms on COX activity are evident, the underlying mechanisms are not yet fully resolved. A common way to decrease the activity of an enzyme—in addition to its inhibition—is its downregulation. As COX1 is constitutively expressed, no regulation is expected nor has been shown experimentally [158,161,162]. Divergent results have been obtained with respect to the influence of vitamin E on COX2 expression. In murine microglia cells, 50 μM α-TOH abolished LPS-induced gene expression and 100 μM moreover reduced protein synthesis of COX2,

likely via NFκB [157]. However, contradictory results were obtained in other studies. COX2 levels were reduced neither by 100 μM α-TOH in LPS-treated murine macrophages [102] nor by 60 μM α-TOH in IL-1β-stimulated human lung epithelial cells [158]. For the suggested molecular mode of action on COX activity, the reader is referred to the section "Tocopherol Inhibit Cyclooxygenase Activity."

In contrast to TOH that may exert posttranscriptional effects on COX activity, T3 have been shown to downregulate COX expression. In LPS-treated RAW264.7 macrophages, 10 μM of α-, γ-, and δ-T3 blocked COX2 expression while α-TOH did not [162]. In line with this, 10 μM of γ-T3 downregulated constitutive COX2 expression in human pancreatic cancer cells and 50 μM completely blocked the expression [163]. These findings are supported by further studies, characterizing γ-T3 [161,164] and δ-T3 [165] as highly efficient suppressors of COX2 expression. Interestingly, in both studies comparing the effects of T3 forms on COX2 expression, δ-T3 was the most potent one [162,165]. The higher ability of δ-T3 to diminish COX2 activity is in accordance with results for the different TOH forms. However, the ability to regulate COX2 expression seems to be a characteristic of T3.

Effect of Long-Chain Metabolites of Vitamin E on Cyclooxygenase 2 Expression

Based on the findings for TOH and T3, it can be assumed that α-TOH LCM are rather ineffective in regulating expression of COX2. Surprisingly, Wallert et al. reported significant blocking of LPS-induced expression of COX2 by α-13′-COOH in murine RAW264.7 macrophages: preincubation with 5 μM α-13′-COOH and subsequent coincubation with LPS significantly diminished the effect of LPS on COX2 expression at mRNA and protein levels. In contrast, 100 μM of α-TOH showed no significant effect [102]. These results show that the α-LCM act in a different fashion and at lower concentrations than their respective metabolic precursors. The underlying pathways have not been elucidated so far and remain to be investigated.

Effect of Garcinoic Acid on Cyclooxygenase 2 Expression

So far, no studies have been published that investigate the effects of garcinoic acid on COX2 expression. Due to the unsaturated chain, garcinoic acid is structurally comparable to δ-T3 but also shares similarities with α-13′-COOH. Considering this, it can be assumed that garcinoic acid may also interfere with the LPS-mediated upregulation of COX2. Preliminary results of our group indicate that garcinoic acid indeed has the potential to block the LPS-induced upregulation of COX2 mRNA as well as protein (unpublished data). However, this is merely a first hint and further experiments are needed. Nevertheless, garcinoic acid would not be the first compound isolated from plants for the treatment of inflammatory diseases in folk medicine. Well-known examples

are curcumin from *Curcuma longa*, capsaicin from *Capsicum* species, and epigallocatechin-3-gallate from *Camellia sinensis* [166]. All of these compounds have been shown to inhibit COX2 expression [167,168]. Especially *C. longa* has been used for centuries in Ayurvedic medicine to treat inter alia the inflammation-related diseases asthma, rheumatism, and diabetes [169]. Today, more than 50 completed clinical trials with curcumin display the interest in this valuable ingredient of *C. longa* in modern medicine. As with *C. longa*, *G. kola* is used in folk medicine to treat inflammation-related diseases (the reader is referred to the section "*Garcinia kola*"). Despite kolaviron, garcinoic acid has now been identified as an antiinflammatory active ingredient of *G. kola*. In principle, garcinoic acid is an interesting natural compound with antiinflammatory actions that should be further characterized. Possibly, the properties of kolaviron and garcinoic acid can be used jointly in the form of a *G. kola* nut extract to treat CVD, cancer, and other diseases of civilization.

Vitamin E and Lipoxygenases

Lipoxygenases and Their Lipid Mediators

LT are formed by LOX, a family of enzymes with four subclasses, namely 5-, 8-, 12-, and 15-LOX, which are classified according to the position at which these enzymes catalyze the dioxygenation of PUFA. The release of arachidonic from membrane phospholipids by $cPLA_2$ is crucial for LT synthesis. 5-LOX catalyzes the oxidation of arachidonic acid and thus the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which in turn is converted to LTA_4 by the same enzyme. LT A_4 is the precursor of LTB_4 or LTC_4, which are in turn the precursors of LTD_4 and LTE_4, respectively, formed by LTA_4 hydrolase and LTC_4 synthase, respectively [170].

Tocopherols Inhibit Lipoxygenase Activity

The first demonstration of 5-LOX inhibition by TOH was published in 1985 [171]. It was shown that α- and γ-TOH inhibit the conversion of arachidonic acid to 5-HPETE by 5-LOX from potato tubers. Interestingly, the inhibition was as efficient as with known 5-LOX inhibitors, such as nordihydroguaiaretic acid and butylated hydroxytoluene, and furthermore irreversible and noncompetitive with arachidonic acid [171]. LT B_4 is a major product of the 5-LOX pathway (vide supra) and is thus widely analyzed in activity and signaling studies. In 1999, Devaraj and Jialal noticed that preincubation of human peripheral blood mononuclear cells (PBMC) with α-TOH (but not β-TOH) impaired the release of IL-1β in response to LPS. Treatment of the cells with LTB_4 restored IL-1β release. By the use of 5-LOX inhibitors it was confirmed that 5-LOX mediates the effects of α-TOH. Furthermore, α-TOH diminished LTB_4 release [172]. A later study of the same group confirmed these results. PBMC isolated from α-TOH-supplemented healthy subjects showed impaired ability to produce TNFα in response to LPS compared to

cells obtained at baseline or after washout. Preincubation of LPS-stimulated PBMC with 50 or 100 μM α-TOH as well as 5-LOX inhibitors showed the same effect. The impaired TNFα release could be restored by LTB_4 [173]. In vitro experiments show that concentrations of 25 μM of α-TOH are not sufficient to inhibit release of TNFα. This raises the question whether a supplementation with 1200 IU/day (corresponds to about 800 mg/day) is sufficient to achieve the required plasma levels of α-TOH. It might be possible that the α-LCM mediate or contribute to the effects observed in vivo, but this remains to be shown experimentally.

Two human trials in hemodialysis patients support the abovementioned findings [174,175]. Patients under hemodialysis exhibit increased 5-LOX levels in their PBMC. In these studies, patients were subjected to α-TOH administration to improve oxidative stress markers [174]. Supplementation with α-TOH, 300 mg/day i.m., 600 mg/day orally [150], or via vitamin E-coated cuprammonium rayon membranes [151] for 4 weeks diminished LTB_4 release and 5-LOX activity. The expression of 5-LOX was not affected by the treatments [175,176].

Although there is evidence that TOH are capable of inhibiting 5-LOX activity and LTB_4 production, further research is required. The majority of studies were done on α-TOH, but the different vitamin E forms seem to act differently and there might be more potent forms [171−173]. Furthermore, α-TOH was administered in the mentioned human trials and the effects were attributed to the TOH itself, regardless of metabolic conversions.

Effects of Metabolites and Tocotrienol on Lipoxygenase Activity

Despite the observation that T3 inhibit 12-LOX activity (reviewed in Ref. [177]), little is known about T3 and their effects on LOX. In fact, just a single study addressed effects of T3 on 5-LOX. In this study, γ-T3 was compared to different TOH forms and δ-13′-COOH [103]. For this, HL60 cells were differentiated into neutrophils and eosinophils to induce 5-LOX expression. Activity of 5-LOX was subsequently stimulated by different concentrations of the calcium ionophore A23187 and measured as formation of LTB_4 and LTC_4. In cells incubated with 1 μM, α-TOH was less effective (IC_{50} = 60 and 40 μM, respectively) than its γ- and δ-counterparts (IC_{50} = 5 μM). Interestingly, γ-T3 was as effective as γ-TOH, and δ-13′-COOH was the most potent compound tested (IC_{50} = 4 μM). Strikingly, δ-13′-COOH inhibited formation of LTB_4 with an apparent IC_{50} of 7 μM, when cells were stimulated with 5 μM A23187, while none of the other vitamin E forms was able to inhibit 5-LOX activity in this setting with concentrations up to 50 μM. The superiority of δ-13′-COOH was confirmed in a cell-free assay with recombinant 5-LOX. Here, the LCM efficiently inhibited the activity of 5-LOX with an IC_{50} of 0.5−1 μM, while all the other vitamin E forms failed to inhibit 5-LOX with concentrations of up to 50 μM. The efficiency of the carboxychromanol is thus similar to that of zileuton, a specific inhibitor of the 5-LOX-activating protein. For this

reason, δ-13′-COOH is thought to inhibit 5-LOX directly. However, final evidence is pending. An alternative way by which δ-13′-COOH may modulate 5-LOX activity is by inhibiting the increase in intracellular Ca^{2+} levels in response to N-formylmethionine leucyl-phenylalanine or thapsigargin. In this respect, the metabolite was superior to its metabolic precursor δ-TOH, which failed to inhibit the induction in calcium influx [103].

Garcinoic Acid and Lipoxygenase Activity

No experimental data regarding garcinoic acid and LOX activity are currently available, but based on the observation that the structurally related LCM δ-13′-COOH is a potent inhibitor of 5-LOX activity, garcinoic acid may likely exert similar effects on this enzyme. The finding that γ-T3 is also able to inhibit 5-LOX activity with an efficiency comparable to γ-TOH further supports this hypothesis, because the unsaturated chain has obviously no effect on the inhibitory capacity. The same may likely be true for garcinoic acid, but this has to be confirmed experimentally. 5-LOX and its products are involved in many inflammation-related diseases, including CVD, cancer, osteoporosis, inflammatory bowel disease, rheumatoid arthritis, skin diseases, and bronchial asthma. The latter is the major 5-LOX-associated disease and zileuton, the only approved 5-LOX inhibitor so far, is available for treatment [178]. Nevertheless, zileuton has two major drawbacks, liver toxicity and a short half-life [179]. There is thus an urgent need to find new potent 5-LOX inhibitors. Many natural products have been identified as 5-LOX inhibitors (reviewed in Ref. [178]). However, most of them are not well characterized and far from use as drugs [178]. Flavocoxid, a mixture of the bioflavonoids baicalin from *Scutellaria baicalensis* and catechins from *Acacia catechu*, made it to a phase III trial but the problem with this natural 5-LOX inhibitor is the reported risk of acute liver injury [180]. Garcinoic acid could line up with the known natural 5-LOX inhibitors, with the potential advantage of modification of multiple inflammatory pathways simultaneously (the reader is referred to the respective chapters on COX). Furthermore, extracts of *G. kola* have hepatoprotective effects [23], so a nut extract might exert effects on 5-LOX without liver injury. Moreover, garcinoic acid could be hepatoprotective itself, as the related structures TOH and T3 have been reported to be beneficial for liver health repeatedly [181,182]. If garcinoic acid is indeed a 5-LOX inhibitor, its exact mechanism of action should be investigated to assess its clinical potential. The lack of 5-LOX inhibitors with satisfying properties shows the need of new sources for their development and garcinoic acid is a promising candidate.

Modulation of Lipid Homeostasis

In addition to inflammation, dysbalanced lipid homeostasis is a key factor for diseases such as atherosclerosis. A plethora of signaling pathways and cellular

processes are required to regulate lipid homeostasis, involving uptake, intracellular trafficking and storage, metabolism, as well as efflux of lipids. The following sections will only focus on that parts of lipid metabolism that have been linked to the LCM so far, namely, expression of the scavenger receptor cluster of differentiation 36 (CD36), uptake of oxidized LDL (oxLDL), and phagocytosis as well as intracellular lipid storage. These are essential elements of macrophage foam cell formation, which in turn is a key event in the pathogenesis of atherosclerosis.

Tocopherols and Macrophage Foam Cell Formation

Macrophage-derived foam cells contribute significantly to the pathogenesis of atherosclerosis. This cell type is therefore studied extensively with respect to its role in inflammation and lipid metabolism. CD36 is a scavenger receptor that significantly contributes to the uptake of oxLDL and is thus involved in the accumulation of cholesterol in intracellular lipid droplets, a hallmark of macrophage foam cells. Therefore, factors that modulate CD36 expression and the uptake of oxLDL are of particular interest. The ability of TOH to modulate the regulation of CD36 and the uptake of oxLDL as well as subsequent processes has been described in several studies: α-TOH is able to suppress the upregulation of CD36 during macrophage differentiation [183,184]. Furthermore, it blocks the upregulation of CD36 in response to oxLDL in THP-1[11] macrophages [185] and to modified LDL in PBMC-derived macrophages [183]. Moreover, the uptake of oxLDL can be decreased by α-TOH in several macrophage models [183−185]. The incubation with oxLDL causes a lipid accumulation in macrophages, which can be also prevented by α-TOH [185]. In line with this, the accumulation of cholesteryl esters in response to modified LDL is diminished in α-TOH-treated macrophages [183].

The regulatory effects of α-TOH on CD36 have been observed also in mice. Apolipoprotein E-knockout mice fed a diet supplemented with 100 mg/kg α-TOH per day for 8 weeks showed a reduced extent of atherosclerotic lesions as well as the expression of CD36 therein and serum concentrations of oxLDL than the respective control group [186]. Similar findings were obtained in LDL-receptor-knockout mice. Here, supplementation with α-TOH acetate and α-TOH (equivalent to 50 IU vitamin E per kilogram of diet, ad libitum) for 18 months resulted in a decrease in lesional and nonlesional expression of CD36 [187].

These findings are also supported by results from liver disease research. The HepG2 liver cell line shows decreased CD36 expression when treated with α-TOH [188]. Rats fed a diet enriched with 80 IU/kg diet (ad libitum) α-TOH

11. THP-1 cells are a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 monocytes can be differentiated into macrophages using phorbol 12-myristate 13-acetate.

acetate showed reduced hepatic CD36 mRNA levels compared to controls [189], and a comparable result was obtained with merely 6 mg/kg of the diet α-TOH combined with 11 mg/kg of the diet γ-TOH ad libitum [190]. A study with guinea pigs points to a posttranslational regulatory mechanism of α-TOH decreasing CD36 protein levels in the liver [191].

Effects of Long-Chain Metabolites and Garcinoic Acid on Macrophage Foam Cell Formation

A surprising result was obtained when we examined the effects of LCM on CD36 expression. In contrast to the downregulatory potential of 100 μM α-TOH, its LCM α-13′-OH and α-13′-COOH upregulated CD36 mRNA and protein in human THP-1 macrophages and human PBMC-derived macrophages obtained from healthy volunteers with as little as 10 and 5 μM, respectively. Generally, primary cells showed a slightly lower response. In addition, the increase in CD36 expression by oxLDL was attenuated by α-TOH and markedly augmented with the LCM [101].

Given the LCM-induced CD36 expression, an increase in oxLDL uptake is expected, but this was not the case. Treatment with LCM before addition of oxLDL led to decreased oxLDL uptake in THP-1 and PBMC-derived macrophages. In line with this, the accumulation of neutral lipids by oxLDL was attenuated in LCM-pretreated cells [101]. We found that garcinoic acid also induces the expression of CD36 in the nonproliferating THP-1 macrophage model. Here, the effectivity of garcinoic acid was comparable to that of the α- and δ-LCM (unpublished data).

Since the current state of knowledge on the regulation of lipid metabolism by garcinoic acid is based only on cellular models, it is difficult to draw conclusions whether these observed effects may have an influence on in vivo models. As mentioned before, garcinoic acid shows functions similar to other natural compounds such as resveratrol, especially with regard to its antioxidative properties. For this reason, resveratrol is preferred for deducing possible in vivo effects of garcinoic acid on lipid homeostasis. Independent experiments in THP-1 and 3T3-L1 cells showed that CD36 expression is upregulated by resveratrol [192,193]. Unfortunately the uptake of oxLDL has not been measured in these cell models. In addition to the mentioned in vitro studies, Chen and coworkers investigated the effect of resveratrol treatment on lipid homeostasis in skeletal muscles of rats fed a high-fat diet. After 8 weeks of high-fat feeding, the basal CD36 mRNA expression was increased in the intervention group in comparison to controls. The treatment with resveratrol led to a further induction of CD36 expression [194]. Based on this observation it was quite surprising that the enhanced expression of an important lipid importer did not lead to increased intracellular lipid accumulation, indicating that the induction of CD36 expression by resveratrol has no negative effect on in vivo lipid balance [194]. Because of the similarities between the properties of resveratrol and garcinoic acid, it could be hypothesized that a possible upregulation of CD36 expression by garcinoic acid will also have no negative

effects on lipid metabolism in vivo. However, further experiments are needed to prove this concept.

CONCLUSIONS AND PERSPECTIVES

With the evidence of circulating α-LCM in human blood, a new perspective in vitamin E research was presented. In addition to the well-studied TOH and the latterly more focused T3, their LCM must be taken into account to correctly interpret the effects of vitamin E in humans. We speculate that the LCM comprise a new class of regulatory molecules that complicate the interpretation of studies on the effects of vitamin E in vivo as these molecules exert effects that are different from their metabolic precursors. So far, only a few studies have focused on this class of compounds. However, the LCM seem to share properties with their precursors but to exert also unique or even adverse effects. It is evident that the LCM and precursors act in the same manner with respect to cytotoxicity and modulation of COX2 and 5-LOX activity but it is of note that the LCM are significantly more potent than their precursors in these cases. Hence, the LCM may indeed play a role in mediating these effects of vitamin E in the human body although the blood concentrations are significantly lower than those of TOH. In addition, the LCM exhibit different effects, like their prooxidative capacity reported by Birringer et al. [87]. This in turn is surprising, as vitamin E in general is well known for its antioxidative properties. Moreover, the LCM apparently upregulate CD36, while the downregulation of this receptor by TOH has been shown repeatedly. Furthermore, the LCM can act in areas where the TOH are virtually not effective. A prime example is the regulation of COX2 expression (for more information, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites").

The natural product garcinoic acid is structurally related to the α-LCM. However, little is known about its bioactivity (Fig. 9.9). Merely, its antioxidative and antiproliferative potential as well as its inhibition of DNA polymerase β have been examined. Due to the structural similarities to TOH, T3, and the LCM, many, yet unknown, effects of garcinoic acid can be expected, making garcinoic acid an interesting natural product for pharmacologic research itself. Although little is known on the effects of garcinoic acid, *G. kola* nuts have been reported as inter alia antidotal, antiinflammatory, antidiabetic, and hepatoprotective. It is likely that garcinoic acid contributes to these properties as it has strong antiinflammatory and antioxidative properties. First results support this hypothesis, as garcinoic acid has shown antiinflammatory actions via downregulation of COX2 expression. For this reason it will be interesting to see what effects garcinoic acid shows in different cell and animal models. If the proposed beneficial properties shown in Fig. 9.10 come true, garcinoic acid has to be tested in clinically relevant studies in animals and later on humans. This may lead to the transfer of knowledge from folk medicine to modern medicine to cure disease.

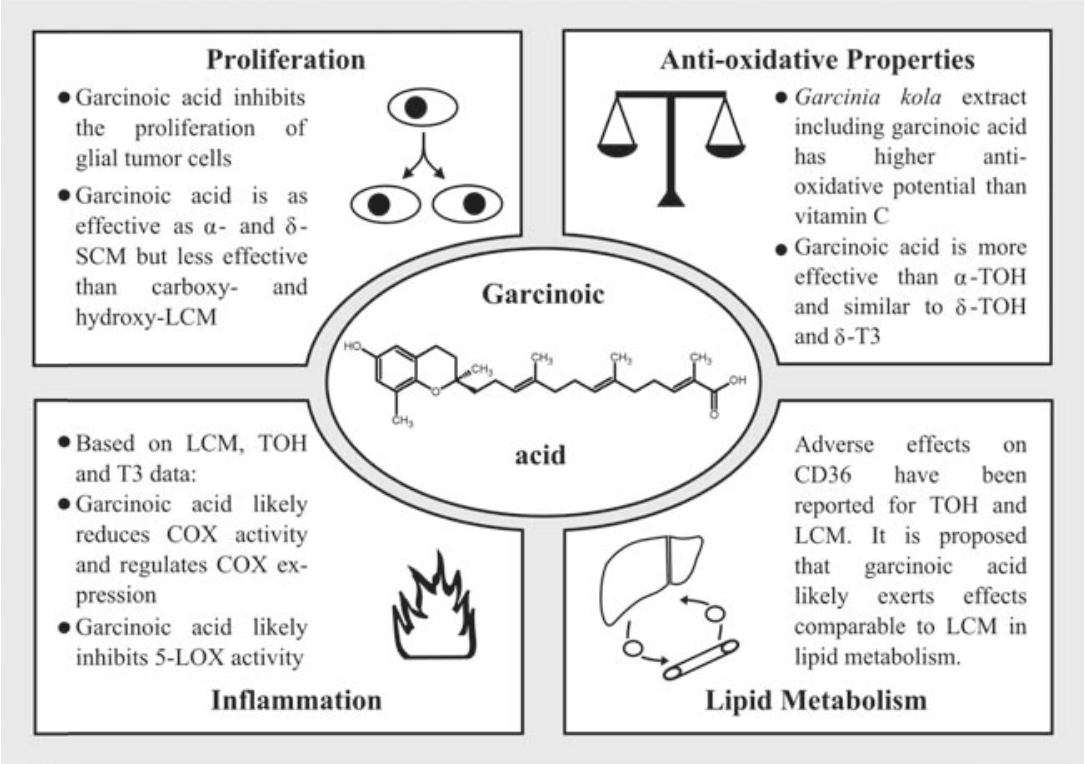

FIGURE 9.9 Known and proposed effects of garcinoic acid.

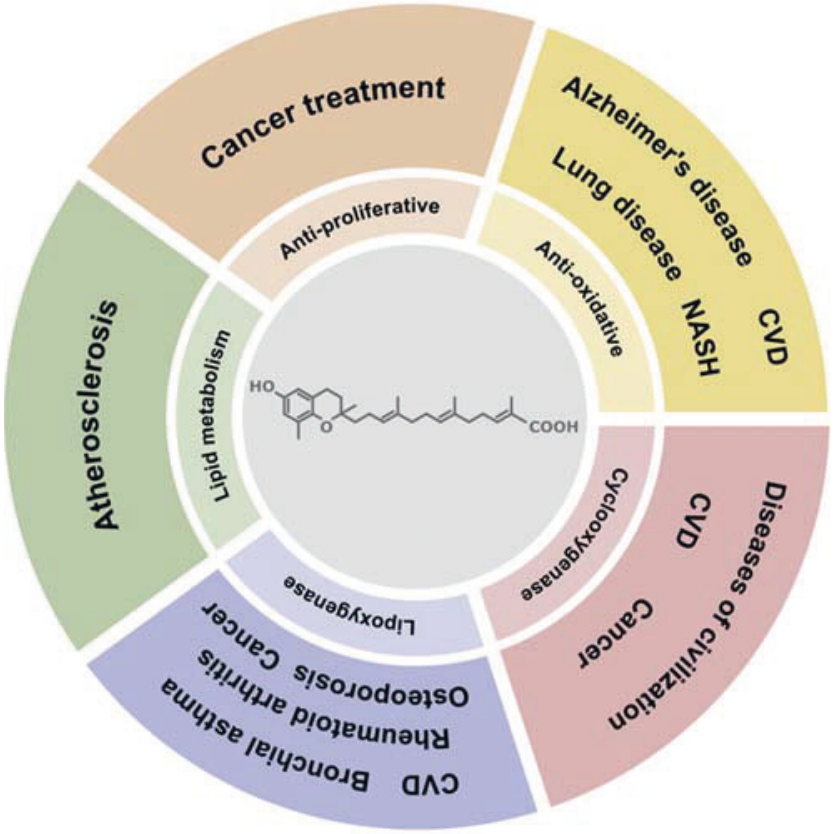

FIGURE 9.10 Proposed beneficial effects of garcinoic acid on human diseases. For detailed information, the reader is referred to the relating chapters. *CVD*, cardiovascular disease; *NASH*, nonalcoholic steatohepatitis.

In addition, garcinoic acid is a helpful substrate that can be reliably isolated from *G. kola* nuts in pure form for synthesizing the δ-LCM, namely δ-13′-OH and δ-13′-COOH, as well as the α-LCM, namely α-13′-OH and α-13′-COOH. The isolation of garcinoic acid from *G. kola* nuts is a simple yet effective method with a yield superior to other reported isolation procedures (the reader is referred to Table 9.1). Thus the procedure provides a reliable base for the synthesis of large amounts of LCM. Pure α- and δ-LCM can be simply and efficiently obtained with the semisynthesis route presented by Mazzini and Birringer. Taken together, this procedure is the most effective way to obtain sufficient amounts of the respective LCM of interest for cellular, animal, as well as human experiments. For this reason, the isolation of garcinoic acid allows the synthesis of the LCM in an elegant and efficient way and the investigation of physiological functions of the α- and δ-LCM in vitro as well as their pharmacological modes of action in vivo in appropriate animal disease models.

To unravel unknown effects and better understand known effects of the different vitamin E forms, as well as to elucidate the underlying regulatory mechanisms that likely involve the LCM, some central questions should be addressed. These include inter alia (1) Which proteins are involved in the uptake and intracellular trafficking of garcinoic acid and of the LCM? (2) Which cellular receptors, signaling proteins, or enzymes mediate the effects of garcinoic acid and of the LCM? (3) What are the regulatory mechanisms that mediate expression of genes in response to garcinoic acid and to the LCM? (4) Which molecular structures are responsible for the effects of garcinoic acid or of the LCM? (5) Do the different LCM differ in their effects and effectiveness? To answer these questions, systematic and comprehensive studies are required. The studies likely involve the identification of potential transporters, binding protein receptors for garcinoic acid, and the LCM. These studies should be complemented by profiling of the effects of garcinoic acid and LCM on gene expression and signaling pathways in different cell types as well as studies in animal models that will shed new light on the regulatory modes of action of the different vitamin E forms and their metabolites. To understand the structure—activity relationship, further structurally related compounds, such as synthetic derivatives of garcinoic acid or of the LCM or enantiomer-pure molecules as well as compounds that represent substructures of the molecule, i.e., the chroman ring or the side chain, should be studied. To sum up, the availability of the LCM as pure compounds provides new perspectives for vitamin E research that will likely contribute to a better understanding of the physiological function of vitamin E. In this respect, the natural product garcinoic acid is a very helpful tool that provides simple and efficient access to the pure α- and δ-LCM for functional studies.

ABBREVIATIONS

13′-COOH 13′-carboxychromanol
13′-OH 13′-hydroxychromanol

5-HPETE	5-hydroperoxyeicosatetraenoic acid
ABCA1	ATP binding cassette transporter A1
ACE	acetone
ACN	acetonitrile
AcOH	acetate
AP-1	activator protein 1
AVED	ataxia with vitamin E deficiency
CC	column chromatography
CD36	cluster of differentiation 36
CEHC	carboxyethyl-hydroxychromanol
cHEX	cyclohexane
CoA	coenzyme A
COX	cyclooxygenase
CPT	centrifugal partition chromatography
CVD	cardiovascular diseases
CYP	cytochrome P450
DCM	dichloromethane
EMSA	electrophoretic mobility shift assays
EtAc	ethyl acetate
HEP	heptane
HEX	hexane
HPLC	high-performance liquid chromatography
ICM	intermediate-chain metabolite(s)
IL	interleukin
iNOS	inducible nitric oxide synthase
LC	liquid chromatography
LCM	long-chain metabolite(s)
LDL	low-density lipoproteins
LOX	lipoxygenase
LPS	lipopolysaccharides
LT	leukotriene
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
MS	mass spectroscopy
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NFκB	nuclear factor “kappa-light-chain-enhancer” of activated B cells
NMR	nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like protein 1
oxLDL	oxidized LDL
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E_2
PKB	protein kinase B (Akt)
PKC	protein kinase C
PUFA	polyunsaturated fatty acid(s)
ROS	reactive oxygen species
SCM	short-chain metabolite(s)
SRB1	scavenger receptor class B type 1

T3	tocotrienol(s)
TCM	chloroform
TLC	thin-layer chromatography
TNFα	tumor necrosis factor α
TOH	tocopherol(s)
TTP	tocopherol transfer protein
VLDL	very-low-density lipoproteins

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Chapter 1

The Hepatic Fate of Vitamin E

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Abstract

Vitamin E is a lipophilic vitamin and thus is naturally occurring mainly in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols, tocotrienols, and "vitamin E-related structures." Vitamin E follows the same route in the body like other lipophilic substances. In brief, vitamin E is absorbed in the intestine, packaged into chylomicrons together with other lipophilic molecules, and distributed via lymph and blood in the body. As the liver is the central organ in lipoprotein metabolism, it is also essential for the uptake, distribution, metabolism, and storage of vitamin E. Based on the current knowledge on that field, the physiological, nonphysiological, and pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis, are described.

Keywords: vitamin E, liver, hepatic handling, vitamin E homeostasis, AVED

1. Introduction

Vitamin E is a lipophilic vitamin and thus naturally mainly occurring in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols (TOH), tocotrienols (T3), and "vitamin E-related structures". However, α-TOH is considered as the most important representative of vitamin E in humans as the central vitamin E metabolizing organ, the liver, discriminates for this form [1]. Notwithstanding the classification as vitamin, the way vitamin E exactly contributes to human health is controversially discussed. Vitamin

E deficiency has been linked to several disease states like ataxia with vitamin E deficiency (AVED) [2, 3] and Alzheimer's disease [4, 5], indicating a role in the preservation of human health. AVED has severe neurological consequences and is caused by a defect in the α-TOH transfer protein (α-TTP); the protein responsible for the discrimination of α-TOH from the other vitamin E forms in the liver [2, 3]. This emphasizes the role of the liver as a central organ in human vitamin E handling. The liver further distributes vitamin E in the body [6] and metabolizes excess vitamin E in order to form products for excretion [6] or presumably to produce activated metabolites of vitamin E as known for other lipophilic vitamins [7]. Given the crucial role of the liver for vitamin E handling, this review aims to summarize the knowledge on the physiological hepatic handling of vitamin E as well as on factors influencing hepatic handling of vitamin E.

2. Physiological hepatic handling of vitamin E

The liver is the central organ of vitamin E handling. While intestinal absorption efficiency is similar for all forms of vitamin E [8], the plasma concentrations of vitamin E forms differ a lot (e.g., 22.1 μM for α-TOH vs. 2.2 μM for γ-TOH [9]). The preference of α-TOH in the human body is mediated by several complex and interacting hepatic mechanisms.

2.1. Hepatocellular uptake of vitamin E

Vitamin E is absorbed in the intestine along with lipids (for details, see [8]) and is packed into lipoproteins. These are transported via lymph or blood toward the liver (via chylomicron remnants, low density lipoproteins (LDL), and high density lipoproteins (HDL) [10, 11]). Different mechanisms facilitate the cellular uptake of vitamin E: (i) via lipid transfer proteins or lipases, (ii) receptor-mediated lipoprotein endocytosis, and (iii) selective lipid uptake [12]. The degradation of chylomicrons to chylomicron remnants by lipoprotein lipase (LPL) seems to be highly important for vitamin E uptake in the liver; when lipolysis of triglyceride-rich chylomicrons by LPL is inhibited, the α-TOH uptake in the liver is diminished [13]. The phospholipid transfer protein (PLTP) mediates the exchange of phospholipids between lipoproteins [14] and is also able to bind α-TOH *in vitro* [15]. PLTP-null mice have lower hepatic levels of vitamin E than the wild-type mice [16]; hence, the transfer of vitamin E between the lipoproteins seems to be important for its effective hepatic uptake. The chylomicron remnants and LDL are taken up by the liver via endocytosis, mainly mediated through the LDL receptor (LDLR) or LDLR-related proteins [6, 17]. In addition, the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) is involved in hepatic vitamin E uptake; α-TOH binds to the N-terminal domain of NPC1L1, which mediates α-TOH uptake via endocytosis (mechanism similar to intestinal cholesterol uptake) [18]. The scavenger receptor B type I (SR-BI) is known to mediate the uptake of vitamin E in several tissues (e.g., intestine [19], epithelium [20], and hepatocytes [21]) by channeling the molecules into the cells (shown for cholesterol or triglycerides [22]). Furthermore, the scavenger receptor cluster of differentiation 36 (CD36) is likely involved in hepatic uptake of vitamin E [23].

2.2. Intracellular trafficking of vitamin E

Following its lipophilic nature, vitamin E is transported by intracellular carrier proteins [24]. The intestinally absorbed vitamin E is taken up via endocytosis [25] and follows endosomal fate. Here, the hepatic sorting of vitamin E forms starts as a specific protein, called α-TTP selectively recognizes and preferentially binds α-TOH, which is then extracted from endosomes and transported to the inner leaflet of the plasma membrane [26]. α-TTP is therefore considered to be a "gatekeeper", which discriminates non-α-TOH forms [27] and regulates the plasma concentrations of α-TOH [1]. The affinity of α-TTP to the different forms of vitamin E differs greatly: it is defined as 100% for α-TOH, whereas β-TOH has 38%, γ-TOH 9%, δ-TOH 2%, and α-tocotrienol (T3) 12% affinity to α-TTP [28]. The regular function of α-TTP is crucial, since missense mutations lead to the disruption of α-TOH distribution and the development of a severe degenerative disease, termed AVED [29]. The transfer of α-TOH from endosomes to the plasma membrane is a multi-step process. First, it is speculated whether the ATP-binding cassette transporter A1 (ABCA1) enriches the outer layer of endosomes with α-TOH [30]. The cholesterol transporter NPC1 may also be involved, as a genetic missense mutation of the *NPC1* gene leads to an accumulation of α-TOH in late endosomes [31]. Second, α-TTP extracts the α-TOH from endosomes, and third, α-TTP mediates its transport to the plasma membrane [24]. This process seems to depend on phosphatidylinositol phosphates (PIPs; preferentially $PI(4,5)P_2$ and $PI(3,4)P_2$) in the plasma membrane, as α-TTP binds to them, in turn targeting α-TOH to the plasma membrane and stimulating its release [32]. Chung et al. analyzed the localization of α-TTP depending on the cellular α-TOH concentration [33]. They found (i) perinuclear localization for α-TOH-depleted cells, (ii) a directional transport of α-TOH/α-TTP toward the plasma membrane, when depleted cells were pulsed with a low dose of α-TOH, and (iii) a homogenous cytosolic pattern under long-term and high-dose treatment of cells with α-TOH, which was suggested to be the picture of several α-TOH transport cycles [33]. Furthermore, the authors also postulated a bi-phasic concentration-dependent circulation of α-TTP: the $PI(4,5)P_2$ gradient (low in endosomes and high in plasma membrane) forces the α-TTP-mediated transport of α-TOH toward the plasma membrane, whereas the α-TOH gradient (low in plasma membrane and high in endosomes) triggers the recycling of α-TTP toward the endosomes [33]. It has been proposed that once α-TOH is incorporated into the plasma membrane, it is mediated toward the outer leaflet of the membrane by a flippase, maybe ABCA1, and is then available for the uptake via very low density lipoproteins (VLDL) [34]. For more details on the process, please see Section 2.5 "Release of vitamin E".

2.3. Intracellular storage of vitamin E

Intracellular storage of vitamin E is limited to the lipophilic sites of the cell, which are membranes and lipid droplets [33]. Not much is known about a specific localization of vitamin E accumulation in liver cells, apart from the observation that lysosomal membranes of rat livers seemed to have the highest concentration of all membranes [35–37]. However, it is known that one-third of the total body vitamin E is stored in the liver [38]. Within membranes, vitamin E is thought to stabilize the membrane bilayers due to colocalization with phosphatidylcholine [39] and cholesterol (leading to an association to lipid rafts) [40]. It was further hypothesized

that vitamin E also colocalizes with poly-unsaturated fatty acids (PUFAs) in nonraft domains in order to provide protection from lipid peroxidation [41]. Newly added α-TOH in cell culture enriches in the same organelles as the endogenous α-TOH pool [42]. Hereby, the subcellular content of α-TOH was directly proportional to the lipid content [43].

Our knowledge about the storage of vitamin E in lipid droplets is also limited. It was recently reported that newly endocytosed vitamin E was also found in lipid droplets, thus indicating endosome-lipid-droplet interactions [33].

2.4. Hepatic metabolism of vitamin E

The hepatic metabolism of vitamin E has not been fully characterized. However, the principle steps of vitamin E degradation, that is, the shortening of the side chain without the alteration of the chroman ring, are generally accepted. Hence, the metabolites are classified as α-, β-, γ-, and δ-metabolites according to their respective precursors.

In principle, TOHs and T3s are degraded like long branched chain fatty acids (TOH) or long unsaturated branched chain fatty acids (T3) via β-oxidation in peroxisomes. However, as TOHs and T3s do not bear a terminal carboxy function in their side chain, they are not susceptible to β-oxidation. Hence, the initial and rate-limiting step in vitamin E degradation is the introduction of a carboxy function to the ω-terminus of the side chain. This first step is carried out in the endoplasmic reticulum (ER) of liver cells [44]. Here, two representatives of the cytochrome P450 (CYP) protein family, namely, CYP4F2 [45] and CYP3A4 [46, 47], have been identified to catalyze the initial ω-hydroxylation step. The resulting 13′-hydroxychromanol (13′-OH) is then further metabolized via ω-oxidation, a step that most likely involves alcohol dehydrogenase and aldehyde dehydrogenase [44], leading to 13′-carboxychromanol (13′-COOH). The carboxylated side chain resembles a long branched chain fatty acid that is further degradable via β-oxidation. However, a transport mechanism for the carboxychromanol from the ER to the peroxisomes has not been identified so far. Nevertheless, two cycles of peroxisomal β-oxidation after the activation of α-13′-COOH to the respective CoA ester have been suggested [44], as the peroxisomal β-oxidation system has a higher affinity toward long branched chain fatty acids than the mitochondrial counterpart [48]. The proposed 11′- and 9′-COOH metabolites have indeed been identified in human and mouse samples [49] as well as in a hepatic cell line [45, 50]. Subsequently, three more cycles of β-oxidation are needed to form the final product of vitamin E degradation, namely, carboxyethyl hydroxychromanol (CEHC) or 3′-COOH. These steps, however, are assigned to mitochondrial β-oxidation, as CEHC has solely been found in the mitochondria of hepatic cells [44]. Again, the transport mechanisms of the long-chain metabolites (LCM) (13′- to 9′-COOHs) from peroxisomes to the mitochondria are not known. The respective products for each cycle of β-oxidation (7′-COOH, 5′-COOH, and 3′-COOH) have been identified in different human and murine tissues [49, 51–54] as well as the hepatic cell line HepG2 [45, 47, 51]. Taken together, the hepatic metabolism of vitamin E is characterized by a series of β-oxidation steps after an initial introduction of a carboxy moiety at the ω-terminus of the phytyl-like side chain. The metabolism likely takes place in different cell compartments depending on the enzymatic systems needed for the different degradation steps. However, a concept of vitamin E degradation exclusively in mitochondria cannot be excluded [44]. T3 degradation is believed to follow the same route as TOH degradation but requiring further steps due to the unsaturated side

chain. In line with this assumption is the identification of the respective unsaturated metabolites from 13′-carboxytrienol down to carboxymethylbutadienylhydroxychromanol (CMBenHC) in human and mouse samples [49]. According to these findings, the side chain of the T3 metabolites needs a saturation step before the shortening of the chain. Enzymes involved in the degradation of unsaturated fatty acids like 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase were suggested to contribute to the degradation of T3s [55].

2.5. Release of vitamin E

Following the nature of the lipoprotein metabolism, hepatic release of vitamin E is mostly realized via VLDL. Thus, this section will focus on the packaging of vitamin E into VLDL particles, notwithstanding that the mechanism is not well understood. However, hepatic transfer of vitamin E to HDL has also been suggested [56]. Since it was shown that the expression of α-TTP is crucial for the maintenance of plasma α-TOH levels [57, 58] and that the liver is controlling plasma α-TOH levels [59], hepatic α-TTP is likely involved in the incorporation of vitamin E into lipoproteins. This concept is supported by the observation that nascent VLDL particles are preferentially enriched with *RRR*-α-TOH after oral administration of vitamin E ([60, 61]. In contrast, in the liver, no preferential retention of *RRR*-α-TOH was found, indicating that α-TTP is not involved in the delivery of vitamin E to the liver, but in the release from the liver [62]. Hence, efforts have been made to identify the intracellular location of VLDL enrichment with α-TOH mediated by α-TTP [30]. According to the assembly of VLDL, either the rough ER or the Golgi apparatus were assumed. However, the action of α-TTP in these compartments was not confirmed as the nascent VLDL particles contained equal amounts of SRR and RRR α-TOH forms [30]. Further, the inhibition of ER/Golgi action in cells overexpressing α-TTP did not prevent α-TOH secretion [63]. In conclusion, α-TTP is necessary for the hepatic release of vitamin E, but the enrichment of VLDL with *RRR*-α-TOH occurs after exocytosis.

Based on this, the hypothesis of α-TOH uptake by VLDL directly from the plasma membrane was developed. This idea was inspired by the proposed mechanism of the incorporation of free cholesterol into nascent VLDL [64], that is, the spontaneous transfer from membranes to lipoproteins [65]. The hypothesis involves also the α-TTP-mediated trafficking of vitamin E from late endosomes (where vitamin E occurs after cellular uptake and large parts of α-TTP are located [66]) to the plasma membrane. This process might involve ABCA1, which has been shown to transport α-TOH [67] and could thus present vitamin E to α-TTP at the outer leaflet of the endosomal membrane. After the transport to the plasma membrane, a yet unidentified flippase is required to transfer α-TOH to the appropriate site of the membrane for uptake by nascent VLDL [30]. This hypothesis is supported by findings of Chung et al. [33], which provided a model of α-TTP-facilitated trafficking of vitamin E from endosomes to the plasma membrane (the reader is referred to Section 2.2 "Intracellular trafficking of vitamin E"). Taken together, the release of α-TOH from hepatocytes depends on vesicular transport [21, 31, 63, 68, 69], but is independent from ER or Golgi [63]. Hence, lipoproteins are not loaded with TOH during their intracellular assembly, but rather after exocytosis, a mechanism is required for the presentation of α-TOH at the plasma membrane. Evidence has been provided that the trafficking of α-TOH to the plasma membrane is realized via α-TTP which is located at recycling endosomes in hepatocytes [33]. However, the mechanism of

the loading of lipoproteins with α-TOH from the plasma membrane has not been elucidated yet, although the involvement of ABC transporters has been suggested [56, 67, 70]. However, ABC transporters are fueling HDL particles, which is in contrast to the assumption that the hepatic release of α-TOH is mediated via VLDL. In turn, two explanations have evolved: first, α-TOH translocates spontaneously from the membrane to VLDL like free cholesterol [65], and second, α-TOH is transported to HDL via ABCA1 and is then spontaneously transferred to VLDL [71]. However, both hypotheses need evaluation. A recent report on the self-assembly of α-TTP to form nanoparticles and transport vitamin E to tissues protected by endothelial barriers like the brain [34] opens another possible way for the distribution of vitamin E throughout the body starting from the liver.

3. Factors influencing hepatic handling of vitamin E

3.1. Effects of vitamin E

3.1.1. Intracellular handling of vitamin E

Key factors in the hepatic handling of vitamin E have been outlined in the previous sections. This section will focus on the action of vitamin E on its own intracellular handling. As indicated above, the key enzyme for the intracellular trafficking of vitamin E is α-TTP, and the rate-limiting enzymes in vitamin E metabolism are CYP4F2 and CYP3A4. Hence, we will here focus on the known actions of vitamin E on these key players.

The key protein of the hepatic handling of vitamin E is α-TTP, with its implications in cellular trafficking, metabolism, and release of vitamin E. Hence, several studies have been conducted to elucidate a possible feedback regulation of α-TTP in response to vitamin E intake, resulting in alterations of the metabolism or the distribution of the vitamin. In principle, research is focused on three levels of regulation: mRNA expression, protein expression, and stabilization of α-TTP protein. However, contradictory results from rodent models have been reported. Fechner et al. found that hepatic α-TTP mRNA expression was strongly induced in rats depleted from vitamin E for 5 weeks after the intake of a TOH-supplemented diet for 24 h [72]. However, rats fed a vitamin E-depleted diet, control diet, or vitamin E-enriched diet for 20 weeks showed upregulation of α-TTP mRNA when vitamin E is deprived, but a downregulation when vitamin E was repleted. Hepatic α-TTP protein levels were comparable for depletion and control, but lowest in rats fed the repleted diet [73]. A similar study reported no differences in hepatic α-TTP mRNA levels of rats fed either a control diet or a diet rich in or low in vitamin E. However, in contrast to the aforementioned study, downregulation of α-TTP protein was reported in the vitamin E-depleted group, while high vitamin E intake did not alter the levels compared to control [74]. The lack of an effect of a vitamin E deficient diet for 290 days on hepatic α-TTP mRNA levels was also reported in another rat model [75]. In line with this, subcutaneous injection of vitamin E for up to 18 days did not alter α-TTP protein levels in rats [76]. However, mice fed a diet rich in vitamin E showed 20% higher hepatic α-TTP protein levels than mice fed a low vitamin E diet [77]. Taken together, some studies

report elevated α-TTP levels due to a higher intake of vitamin E [72, 77], but some revealed no effect [74–76] or even lower levels [73]. Hence, further studies are needed to clarify the role of vitamin E in the regulation of α-TTP. In addition, an *in vitro* study suggested that vitamin E does not regulate α-TTP at the level of gene expression, but stabilizes α-TTP at the protein level upon binding and thus protects the protein from degradation, leading to higher α-TTP protein levels [78]. Reports on the hepatic mRNA levels might thus be of minor importance for the interpretation of the contribution of vitamin E to α-TTP action; however, the findings on α-TTP protein expression are also inconsistent.

The rate-limiting enzymes of vitamin E metabolism are CYP4F2 and CYP3A4. The latter was reported to be under transcriptional control of pregnane-X-receptor (PXR) [79, 80]. Hence, vitamin E might regulate its metabolism by binding to PXR and subsequent alteration of the expression of the enzymes involved in the first catabolic step. Indeed, studies using cells transfected with reporter genes provided evidence for an activation of PXR by different vitamin E structures (i.e., TOHs, T3s, and metabolites) [81, 82]. Interestingly, α-, δ-, and γ-TOH as well as α- and γ-T3 activated PXR in HepG2 liver cells transfected with human PXR and chloramphenicol acetyl transferase linked to two PXR responsive elements [81], while α- and γ-TOH as well as their metabolites α- and γ-CEHC did not in transfected colon carcinoma cells [82]. However, the LCM α-13′-COOH activated PXR in the latter cellular system and so did γ-T3 [82]. This finding implicates that the LCM of TOH are the responsible mediators of reported TOH actions via PXR. Hence, the findings in hepatic HepG2 cells [81] might be due to a higher catabolic rate of TOH and in turn the more efficient formation of the LCM than in colon cells. However, these findings were made in artificial cellular reporter systems and might not resemble the actual (hepatic) situation *in vivo*. Further, the specificity of PXR might depend on the species, as γ-T3 (the vitamin E form that activated PXR in both of the aforementioned studies) fails to bind murine PXR [83]. However, results obtained *in vivo* support the regulation of Cyp3a11 (the murine orthologue of CYP3A4) by vitamin E via PXR. Mice supplemented with α-TOH show elevated hepatic expression of Cyp3a11, while their PXR-deficient counterparts as well as mice with humanized PXR showed no upregulation of Cyp3a11 in response to α-TOH [84]. The same finding was made for Cyp4f13, the murine orthologue of CYP4F2, in this model [84]. These findings suggest that both enzymes are under the control of PXR and murine, but not human PXR is susceptible to α-TOH (or its metabolites as outlined above). Further studies reporting upregulation of hepatic Cyp3a in rodent models with α-TOH supplementation support this finding [76, 83, 85]. Interestingly, in these studies, γ-TOH and γ-T3 had no effect on Cyp3a expression [83, 85], supporting the suggested specificity of murine PXR for α-TOH. In line with this, γ-TOH did not alter the expression of Cyp4f13 in mice [85]. However, subcutaneous application of α-TOH in rats did not induce Cyp4F2 levels [76], which is in contrast to the above mentioned induction of Cyp4f13 in mice via PXR [84]. The reported induction of CYP4F2 activity in HepG2 cells by α-TOH further complicates the interpretation of the data on the effect of vitamin E on CYP4F2 [45]. Taken together, there is evidence for the regulation of CYP4F2 and CYP3A4 via PXR by vitamin E in the human liver. However, several aspects need further clarification, for instance, species and vitamin E isoform specificity of PXR, the regulation of CYP4F2 by vitamin E or the relevance of the α-LCM as true mediators of α-TOH effects via PXR.

3.1.2. Vitamin E intake

Several key enzymes determine the rate of vitamin E catabolism (the reader referred to Section 2.4 "Hepatic metabolism of vitamin E") and, as outlined in the previous section, there is evidence that vitamin E in general might regulate its own metabolism. However, there are differences in the ability to regulate the metabolism depending on structural properties of the vitamin E isomers (i.e., methylation of the chroman ring, saturation of the side-chain, and stereochemistry). In principle, high intake of vitamin E, independent from the isomer, leads to enhanced formation of the respective metabolites [49]. However, the catabolic rates of the different forms of vitamin E clearly differ: the γ-isoforms are more susceptible to metabolization than the α-isoforms. Subjects supplemented with γ-T3 and α-T3 (125 mg or 500 mg) showed four to six times higher urinary excretion of the catabolic end product γ-CEHC and an induction of α-CEHC only after high dose (500 mg), but not after low dose supplementation (125 mg) [86]. In line with this, equimolar supplementation with 50 mg of α- and γ-TOH leads to a twofold increase of plasma γ-CEHC, but no alterations in α-CEHC [87]. These data indicate that there might be a threshold for the intake of α-TOH and α-T3 (or plasma levels, respectively) that needs to be exceeded to accelerate catabolism of α-TOH and α-T3 to form α-CEHC, as suggested by Schuelke et al. [88]. Interestingly, already in 1985, Handelman et al. reported that high α-TOH levels in human plasma are related to low γ-TOH levels [89]. After supplementation of α-TOH, the plasma α-TOH levels were, as expected, twofold to fourfold higher, but the γ-TOH level decreased to between one-third and one-half of the initial level [89]. Hence, α-TOH intake seems to boost γ-TOH catabolism. Supporting data were generated in a rat model, where the combined supplementation of α- and γ-TOH leads to higher excretion of γ-CEHC than the supplementation of γ-TOH alone [90], as well as the reported stimulation of γ-TOH catabolism by α-TOH in HepG2 liver cells [91]. Although the underlying mechanisms are not fully unraveled, there is evidence that α-TOH induces the activity of enzymes involved in the metabolism of vitamin E, leading to the degradation of non-α-forms, while α-TOH remains protected (please refer to Section 3.1.1 "Intracellular handling of vitamin E").

3.2. Effects of other compounds

3.2.1. Intake of sesamin

Sesamin is a lignan, a group of natural compounds derived from vegetable sources, like sesame seeds [92]. Sesamin is known as a natural inhibitor of the metabolism of TOH [93–97]. The cell regulatory actions of sesamin have been initially investigated in *in vitro* models, where Parker et al. showed that sesamin acts as a selective inhibitor of CYP3A4, an initial enzyme of TOH metabolism [46]. In this study, the authors compared the inhibitory potential of sesamin on TOH metabolism in human HepG2 cells to the well-characterized CYP3A4 inhibitor ketoconazole. HepG2 cells were treated with one of the mentioned compounds in combination with either 25 μM α-TOH or 25 μM γ-TOH. Afterwards, the concentration of the corresponding CEHC was determined as a marker for TOH metabolism in cell culture media. It became apparent that ketoconazole (1 μM) and sesamin (1 μM) inhibited the formation of α- and γ-CEHC. This result provides evidence that sesamin is able to modulate TOH metabolism via the inhibition of

CYP3A4 [46]. In addition to the *in vitro* data, Uchida and coworkers investigated the inhibitory effects of sesamin on vitamin E metabolism in rats. Vitamin E-deficient rats (vitamin E free diet for 4 weeks) were treated with 50 mg/kg *RRR*-α-TOH alone or in combination with 200 g/kg sesame seeds [95]. Next, the concentration of α-TOH in different tissues as well as the urinary excretion of α-CEHC was measured. The urinary excretion of α-CEHC in the sesamin group was significantly lower compared to the α-TOH control group. Further, the combination of α-TOH and sesamin provoked a significant increase of hepatic α-TOH concentrations compared to α-TOH treated animals [95]. These observations have been confirmed in other animal studies [93, 94]. Beside the investigations in animal models, there are also a few results originating from studies in humans. In 2004, Frank and colleagues used muffins enriched with sesame oil (94 mg sesamin/muffin) or corn oil (control) to investigate the effect of a single dose sesamin application on urinary excretion of γ-CEHC as well as blood levels of γ-TOH in 10 healthy volunteers [97]. Both, control and intervention group, received the muffins together with a capsule containing deuterium-labeled γ-TOH (50 mg) in a crossover design. Blood and urine samples were collected over 72 hours after the application of the muffins and capsules. While the urinary excretion of γ-CEHC was significantly lowered, the sesamin treatment did not affect γ-TOH concentrations in blood compared to the corn oil control group [97]. Unfortunately, the study does not provide data on the elevation of the hepatic γ-TOH concentration in response to the reduced urinary excretion of γ-CEHC. Taken together, *in vitro* and *in vivo* studies provide evidence that the dietary intake of sesamin leads to an increase of the hepatic concentration of TOH via the inhibition of vitamin E metabolism, but further experiments are needed to characterize the interaction of sesamin and vitamin E metabolism in more detail.

3.2.2. Pharmacological activation or inhibition of CYP3A4

The pharmacological modification of the enzymatic activity of CYP3A4 represents an effective way to influence vitamin E homeostasis in the human body. Mechanistically, the direct or indirect interference of vitamin E metabolism is usually just a side effect of the pharmacological inhibition or induction of CYP3A4 by various chemical compounds. Thus, it is not surprising that the first evidence for the involvement of CYP3A4 in vitamin E metabolism was provided in an experimental subset using ketoconazole as a specific inhibitor for CYP3A4 [46, 98]. In HepG2 liver cells, different concentrations of ketoconazole (1 mmol/l or 0.25 mmol/l) inhibited the metabolic conversion of γ- and δ-TOH (25 μmol/l cell culture media) to γ- or δ-CEHC by almost 90% [46]. This finding has been confirmed by the reproduction of the same experiment with sesamin, the natural inhibitor of CYP3A4, revealing comparable results [46]. The inhibitory effect of ketoconazole on vitamin E metabolism has further been observed in an *in vivo* model. Here, rats were supplemented with ketoconazole (50 mg/kg body weight) together with α-TOH (10 mg/kg body weight), γ-TOH (10 mg/kg body weight) or mixture of different T3s (29.5 mg/kg body weight). Ketoconazole significantly reduced the catabolism of all applied vitamin E forms resulting in impaired urinary excretion of the respective CEHCs [99]. Beside its inhibition, the pharmacological induction of CYP3A4 represents another way to modulate vitamin E metabolism. Birringer and coworkers demonstrated that 50 μmol/L rifampicin, an inducer of CYP3A4 activity [100], induced the degradation of all-*rac*-α-TOH

in HepG2 cells fivefold [47]. In this study, the cell culture medium has been preconditioned with 100 μmol/L α-TOH for 10 days, as the standard medium was deficient for α-TOH [47]. Further, an indirect approach for the modulation of vitamin E metabolism via the modification of CYP3A4 expression could be realized by triggering PXR, a nuclear receptor that regulates the expression of metabolic enzymes and transporters involved in the metabolism of xenobiotics and endobiotics [101, 102]. Landes and coworkers showed that γ-T3 as well as rifampicin acts as PXR agonists, thus upregulating CYP3A4 mRNA expression in HepG2 liver cells [81]. Given the fact that enhanced mRNA expression of CYP3A4 results in enhanced enzymatic activity, the stimulation of PXR by various pharmacological agonists or antagonists could also modulate the hepatic metabolism of vitamin E. In summary, the direct or indirect regulation of CYP3A4 by various pharmacological means represents an effective way to modify the hepatic vitamin E metabolism.

3.3. Nonmodifiable factors influencing handling of vitamin E

The handling of vitamin E is also influenced by nonmodifiable factors. These are aging, gender, and individual genetics. Published data in this area are sparse but interesting.

3.3.1. Aging

The aging process is characterized by nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [103]. In particular, the mitochondrial dysfunction leads to higher formation of reactive oxygen species (ROS) and enhanced oxidative damage [104]. Both processes can be diminished by the antioxidant function of vitamin E [105]. Consequently, two questions arise: (i) can vitamin E modulate the aging processes or prevent age-related diseases? This has been subject of several reviews [106–109]. (ii) And how is the concentration, distribution, and function of vitamin E modulated by the aging process? In humans, age-dependent changes of α-TOH plasma concentrations are known. In healthy aged humans, the α-TOH plasma concentrations are higher than in younger individuals [110–113]. However, this might be due to the age-related increase of plasma cholesterol concentrations, as the age-related increase in α-TOH plasma concentrations disappear after adjustment for cholesterol plasma concentrations [112] or serum lipids [113]. Traber et al. suggested that α-TOH plasma concentrations are more dependent on control mechanisms for plasma lipids rather than on α-TOH absorption [113]. Hospitalized elderly patients [114] as well as older persons with cognitive impairments (dementia or Alzheimer's disease [115, 116]) have low α-TOH plasma concentrations [117]. However, an unfavorable nutrient status of the hospitalized patients was discussed as the cause of the lower α-TOH plasma concentrations.

Several studies analyzed the age-dependent changes of α-TOH tissue concentrations and handling in mice [37, 117–119] and rats [120]. In brain [37, 117, 118] and kidney [37, 117], epididymal adipose tissue [117] and aortic vessel wall [120], a consistent increase in α-TOH was found with age. In old rats, however, an age-dependent increase in intestinal absorption was found [121]. This was considered as a "*self-protective age-dependent adaption*" [120], which

is thought to counteract increased oxidative stress during aging. In the liver and heart, however, data are conflicting: while some found increased concentrations [37, 119, 120], Takahashi et al. found decreased values [117]. Two studies also analyzed the age-dependent regulation of genes, known to be involved in vitamin E handling, which are α-TTP, ABCA1, and Cyp4f14 (murine orthologue of CYP4F2) [117] as well as NPC1, NPC2, and LPL [37]. Takahashi et al. found increasing (mice with the age of 3–12 month) and then decreasing (12—24 months) α-TTP protein levels in the liver, while mRNA expression was stable over age [117]. Overall, Cyp4f14 mRNA expression decreased during aging (60% decrease in mRNA expression at the age of 24 months compared to the age of 3 weeks), while ABCA1 mRNA expression slightly increased (20% in the same age range as measured for Cyp4f14) [117]. The authors concluded that the age-related changes of hepatic α-TOH levels cannot be explained by the metabolism of α-TOH via Cyp4f14. König et al. analyzed protein expression in kidney tissue or its lysosomal membranes and found a significant decrease of NPC1 and NPC2, but a prominent increase in LPL (361% compared with the tissue from younger mice) [37]. The increased expression of LPL may explain the accumulation of α-TOH in aged mice. Furthermore, NPC1 and NPC2 may be responsible for the transport of α-TOH from the endosomes to the cytosol [69] and their reduced expression may explain the accumulation of α-TOH in lysosomal membranes [37]. In summary, there are age-dependent changes in α-TOH tissue and plasma concentrations and also in the expression of genes responsible for vitamin E handling; however, the underlying regulatory processes are not unraveled completely yet.

3.3.2. Gender

The sex-dependent differences in vitamin E handling were described recently by Schmölz et al. [6] and will be summarized here briefly for humans only. While intake of vitamin E in total is higher in men than in women [122], the intake per kcal is higher for women than for men [123]. The absorption of α-TOH seems not to be influenced by sex, but is mainly regulated by downstream regulatory processes (likely by hepatic sorting or metabolism) [113]. The data on serum concentrations of vitamin E are inconsistent: while some researchers reported elevated α-TOH serum concentrations for women compared to men [124, 125], others found contradictory results [123]. Sex-dependent regulation of vitamin E metabolism is specific for the different forms of vitamin E. Women degrade γ-TOH to a higher degree than men, while the metabolism of α-TOH seems to be independent [87]. Two mechanisms may be relevant for sex-dependent regulation of vitamin E metabolism: the hormonal status of individuals and the activation of the CYP enzymes involved in vitamin E metabolism [6]. Further studies could illuminate gender-specific differences in more detail. In the light of the discovery of vitamin E as a factor that limits female fertility, this is of special interest.

3.3.3. Genetics

The influence of genetics on vitamin E handling was summarized in detail in a recent review (for more details, please see [6]). Therefore, only a short overview will be provided here. Interindividual differences in the handling of vitamin E can be caused by individual genetic constitutions. Polymorphisms in genes, which are responsible for vitamin E handling such as

CYP4F2 [126], NPC1L1 [127], and CD36 [128] are likely to contribute to variations in vitamin E status. The best-studied gene in this context is α-TTP, as its genetic variability may cause AVED. Two genetic variants are known, which are located in or nearby the proposed tocopherol-binding domain and cause reduced α-TOH serum concentrations [129]. Furthermore, mutations in the promoter region of α-TTP (with increased or decreased activity) were also reported [130]. In summary, vitamin E handling is influenced by several mechanisms, one of which is the variability of genes involved in these processes. This might held responsible for interindividual differences in vitamin E serum concentrations.

3.4. Pathophysiological factors influencing handling of vitamin E

3.4.1. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease encompasses a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is a clinical symptom characterized by a pattern of steatosis, inflammation, and hepatocyte ballooning, which can result in the development of cirrhosis and liver cancer [131]. Although the molecular mechanisms of NASH development remain poorly understood, studies provide evidence for a critical role of oxidative stress together with an impaired antioxidative response [132, 133]. In line with this, Erhardt and coworkers observed significantly lower plasma levels of α-TOH and other antioxidants in NASH patients compared to healthy controls [134]. Given the fact that an induction of CYP3A4 or CYP4F2 results in decreased vitamin E concentrations in the human body, it has been expected that NASH leads to an enhanced activity or expression of these enzymes. Thus, Woolsey and coworkers investigated the enzymatic activity as well as the mRNA expression of CYP3A4 in NASH patients [135]. The authors used liver biopsies for mRNA analyses and determined the concentration of 4β-hydroxycholesterol in plasma as an endogenous biomarker for CYP3A4 activity. Interestingly, NASH patients showed a 37% reduced enzymatic activity of CYP3A4 as well as a 69% lower CYP3A4 mRNA expression compared to healthy controls [135]. Unfortunately, there is no further data on the activity or the expression of CYP4F2 in NASH patients. However, Athinarayanan and coworkers investigated the influence of two different CYP4F2 genotypes (V433 M and W12G) on vitamin E plasma concentrations in NASH patients [136–138]. The V433 M genotype was associated to higher baseline levels of vitamin E, indicating lower enzymatic activity compared to the W12G genotype [136–138]. Thus, the authors hypothesized that the W12G genotype in NASH patients could explain the lower vitamin E plasma concentrations. However, this hypothesis has been disproved by the finding that the vitamin E plasma concentrations of NASH patients did not differ between the two CYP4F2 genotypes [136–138]. Based on the available data, CYP4F2 and CYP3A4 seem to have no influence on vitamin E plasma concentrations during the NASH development. Next to the CYPs, α-TTP could also be involved in a potential mechanism explaining the observation of Erhardt and coworkers mentioned above. In line with this, Ban and coworkers used a rat model to investigate whether an exposure to hyperoxia (>95% O_2 for 48 h), an established stimulus for ROS production [139], could alter the expression of hepatic α-TTP [140]. Indeed, hyperoxia decreased the expression of α-TTP mRNA in rat liver, while α-TTP protein expression remained unchanged [140]. As oxidative stress and ROS

formation are crucial factors for NASH development, lowering α-TTP expression by ROS could explain the lower vitamin E levels in NASH patients. In summary, the concentration of vitamin E and other antioxidants is reduced in NASH patients by yet not fully understood molecular mechanisms, potentially involving α-TTP. Nevertheless, recent human intervention trials provide evidence that vitamin E treatment could improve primary NASH outcomes (i.e., steatosis, inflammation, hepatocellular ballooning, and fibrosis) [137, 138].

3.4.2. Cancer

The current data on vitamin E as a potential agent for cancer therapy are inconsistent. While *in vitro* and early epidemiological studies provided evidence for cell growth-inhibiting, antiproliferative and pro-apoptotic effects of vitamin E in cancer treatment [141–145], more recent investigations reported contradictory results [146–148]. These findings were further sustained by the "Selenium and Vitamin E Cancer Prevention Trial (SELECT)," a randomized intervention study to determine the long-term effect of a supplementation of vitamin E (400 IU/d all-*rac*-α-tocopheryl-acetate) and selenium (200 μg/d L-selenomethionine) on the risk of prostate cancer in healthy men. Interestingly, the authors observed an increased incidence for prostate cancer in subjects supplemented with vitamin E [149]. Beside the investigations on beneficial effects of vitamin E in cancer therapy, almost nothing is known about the influence of cancer on human vitamin E homeostasis. An early study by Knekt, who investigated the association of vitamin E serum concentrations and the risk for different types of female cancer, showed an inverse relation between α-TOH serum concentrations and cancer risk [150]. Thus, women with the lowest α-TOH levels were at enhanced risk for cancer compared to those with higher α-TOH levels. Indeed, this association was restricted to cancer outcomes in tissues and organs, which were not exposed to estrogens [150]. Thus, Knekt hypothesized that low vitamin E levels could represent a potential risk factor for several, but not all types of cancer [150]. Nevertheless, the molecular mechanisms underlying this impairment of vitamin E serum concentrations in cancer patients remain unclear. The enhanced metabolic conversion of vitamin E might represent a mechanistic explanation. In line with this, investigations of tissues from cancer patients showed elevated expression of CYP3A4 [151] and CYP4F2 [152], the two major enzymes of vitamin E catabolism. Unfortunately, vitamin E serum concentrations have not been determined in these studies. Further, *in vitro* studies provided evidence that cancer also affects transporters for vitamin E, such as the tocopherol-associated protein (TAP) [153]. Tissue samples from prostate cancer patients showed significantly lower TAP mRNA expression compared to healthy controls, indicating that cancer may affect the intracellular transport of vitamin E. In addition, the overexpression of TAP in prostate cancer cells leads to a significant reduction of cell growth, while a TAP knockdown by small interfering RNA increased their growth [153]. Interestingly, these effects appeared without additional vitamin E treatment, indicating that TAP not only mediates vitamin E transport but also functions as a vitamin E-independent tumor suppressor gene [153]. In summary, the promising cancer preventive effects of vitamin E shown *in vitro* have not been confirmed in recent *in vivo* trials. Nevertheless, cancer could probably be associated with reduced vitamin E concentrations in the human body, because of an enhanced vitamin E catabolism and/or the alteration of its intracellular transport. However, further investigations are required to validate these results.

3.4.3. Disorders of lipoprotein metabolism

After its intestinal absorption, the transport of vitamin E, including its transfer to and its export from the liver as well as the subsequent distribution of vitamin E in the human body, strictly depends on different lipoproteins [7]. Thus, disorders of the lipoprotein metabolism can lead to disturbances of vitamin E homeostasis. Abetalipoproteinemia or Bassen-Kornzweig syndrome is a rare form of neurodegenerative ataxia with a strong impact on the hepatic handling of vitamin E. Abetalipoproteinemia is caused by mutations in the gene encoding for the microsomal triglyceride transfer protein (MTP), which is required for the assembly and secretion of the apolipoprotein B (apoB) forms in the liver and the intestine [154]. The apoB forms are the primary apolipoproteins associated to chylomicrons or VLDL, IDL, and LDL, respectively, and are thus essential for the distribution of vitamin E in the human body [7, 155]. As a result of the disturbed intestinal absorption and hepatic excretion of all lipid soluble molecules, patients with abetalipoproteinemia show vitamin E deficiency as well as low serum concentrations of cholesterol and triglycerides [156]. Next, the hepatic handling of vitamin E can be affected by familial hypobetalipoproteinemia. This lipoprotein disorder is caused by mutations in the *APOB* gene, leading to disturbances of translation of the apoB proteins and/or impaired secretion of VLDL [157]. Thus, familial hypobetalipoproteinemia displays the same clinical features as abetalipoproteinemia. In summary, lipoprotein disorders exert clear impact on the hepatic and systemic handling of vitamin E.

3.4.4. Other relevant pathophysiological factors

AVED is a neurological disorder, which has for the first time been described in a 12-year-old boy with cerebellar ataxia and low serum vitamin E concentrations. Interestingly, the boy showed no lipid malabsorption or a lack of lipoproteins, like it has been observed in abetalipoproteinemia [158]. Subsequent studies identified a mutation in the *TTPA* gene, the gene encoding for α-TTP, as the disease causing factor [159]. Thus, AVED patients have impaired expression of α-TTP, leading to impaired incorporation of vitamin E (α-TOH) into VLDL as well as a higher metabolic conversion and excretion of vitamin E [154]. In addition, AVED patients show very low plasma vitamin E concentrations together with normal absorption rates for vitamin E in the absence of intestinal malabsorption and abetalipoproteinemia [2, 154]. In summary, AVED represents a clinical condition that includes altered hepatic handling of vitamin E without affecting lipoprotein homeostasis.

4. Conclusion

In the last decades of vitamin E research, the liver appeared as the central organ for the uptake, distribution, metabolism, and storage of vitamin E. Thus, it is also a starting point for various strategies for the modulation of the vitamin E homeostasis. Based on current knowledge, we identified physiological, nonphysiological as well as pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis (a brief schematic overview is provided in **Figure 1**). Nevertheless, further studies

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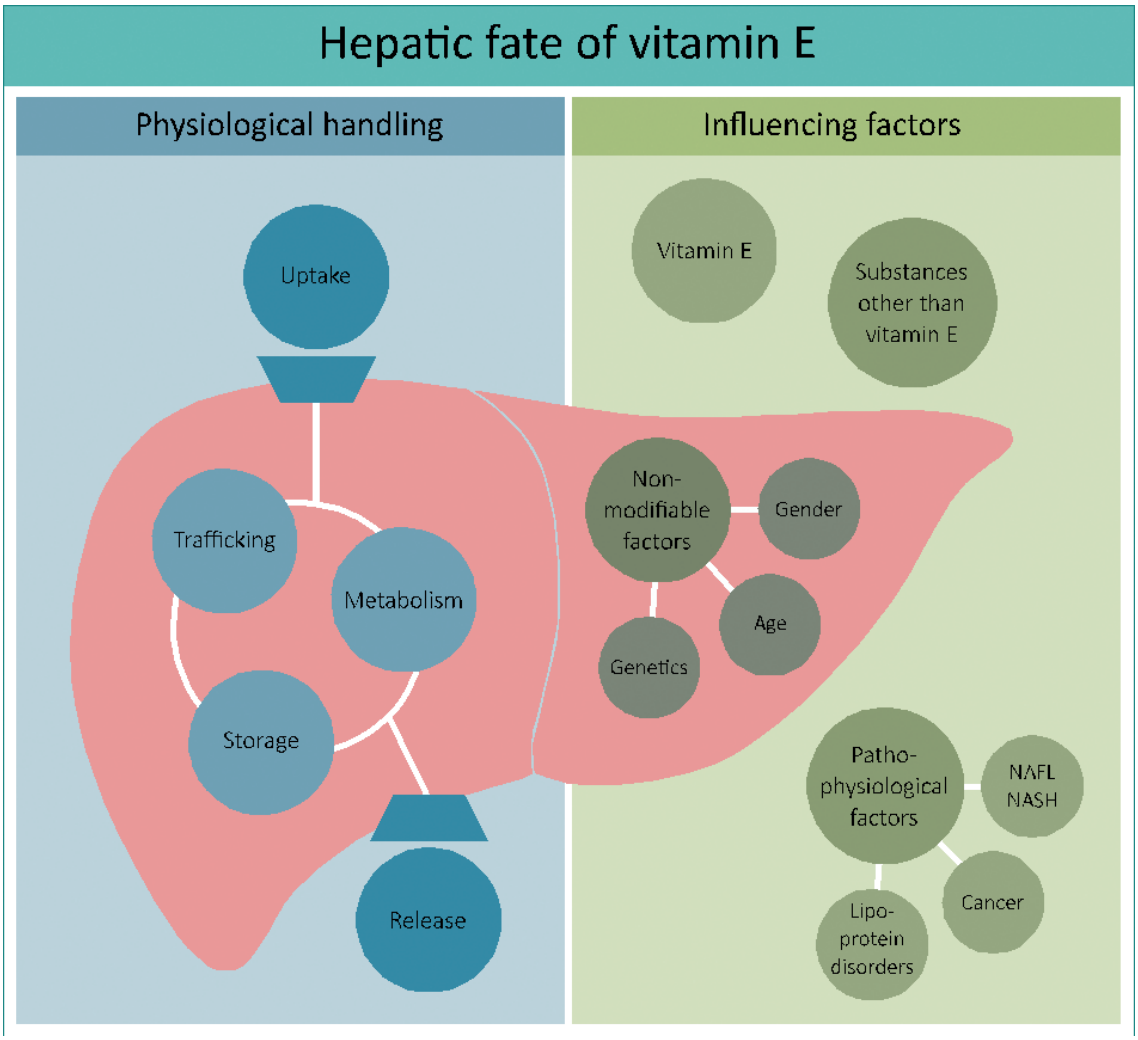

Figure 1. The crucial role of the liver in vitamin E homeostasis.

are needed to unravel the molecular mechanisms underlying the described disturbances of hepatic vitamin E handling by various factors.

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Long-chain metabolites of vitamin E: Interference with lipotoxicity via lipid droplet associated protein PLIN2

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ABSTRACT

The long-chain metabolites of vitamin E (LCM) emerge as a new class of regulatory metabolites and have been considered as the active compounds formed during vitamin E metabolism. The bioactivity of the LCM is comparable to the already established role of other fat-soluble vitamins. The biological modes of action of the LCM are far from being unraveled, but first insights pointed to distinct effects and suggested a specific receptor, which in turn lead to the aforementioned hypothesis. Here, a new facet on the interaction of LCM with foam cell formation of THP-1 macrophages is presented. We found reduced levels of mRNA and protein expression of lipid droplet associated protein PLIN2 by α-tocopherol (α-TOH), whereas the LCM and the saturated fatty acid, stearic acid, increased expression levels of PLIN2. In a lipotoxic setup (0–800 μM stearic acid and 0–100 μM α-TOH or 0–5 μM α-13′-COOH) differences in cellular viability were found. A reduced viability was observed for cells under co-treatment of α-TOH and stearic acid, whereas an increased viability for stearic acid incubation in combination with α-13′-COOH was observed. The striking similarity of PLIN2 expression levels and worsened or mitigated lipotoxicity, respectively, revealed a protective effect of PLIN2 on basal stearic acid-induced lipotoxic conditions in PLIN2 knockdown experiments. Based on our results, we conclude that α-13′-COOH protects cells from lipotoxicity, at least partially via PLIN2 regulation.

Herewith another facet of LCM functionality was presented and their reputation as regulatory metabolites was further established.

1. Introduction

Recently, a new perspective on vitamin E and its metabolism was postulated [1]. The metabolites, which are formed during hepatic degradation of tocopherols and tocotrienols, were thought to be just products of vitamin E excess. However, currently a change of this paradigm suggests that in particular the long-chain metabolites (LCM), the first metabolites occurring in vitamin E metabolism, may be the activated and thereby functional molecules in the family of vitamin E derivatives. This concept is appealing, as it has already been accepted for other fat soluble vitamins, such as vitamin A or D [2–4].

Vitamin E is a collective term summarizing eight highly similar structures, all of which consist of a chromanol ring-system and an aliphatic side-chain. However, the different forms of vitamin E differ in the saturation of their side-chain, leading to the saturated tocopherols (TOH) and the unsaturated tocotrienols (T3). The methylation pattern of the chromanol ring-system determines the α-, β-, γ- or δ-forms. The hepatic metabolism of vitamin E is principally independent of these features, whereas its efficiency highly depends on the type of methylation (α-TOH is the form with the lowest catabolic rate [5]). In more detail, an oxidative modification of the side-chain via cytochrome P450-dependent enzymes (CYP4F2/CYP3A4) leads to the formation of the LCM, which are in the case of α-TOH, α-13′-hydroxychromanol (α-13′-OH) and α-13′-carboxychromanol (α-13′-COOH). A more detailed overview is provided in a recent review [5].

The biological action of the LCM is far from being unraveled.

Abbreviations: α-13′-OH, α-13′-hydroxychromanol; α-13′-COOH, α-13′-carboxychromanol; LCM, long-chain metabolites of vitamin E; TOH, tocopherol; T3, tocotrienols

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However, several comprehensive studies have been published in recent years, mainly covering the topics of interaction of the LCM with inflammation [6–10], cancer [11,12], handling of pharmaceuticals [13], and macrophage foam cell formation [14], which is a hallmark in the progression of atherosclerosis. In brief, macrophages within the arterial wall are loaded with lipids, mainly originating from oxidized lipoprotein particles (e.g., low density lipoproteins (LDL); for more details, the reader is referred to [15]). These lipids can be stored in so-called cytosolic lipid droplets. Through the light microscope, a cell filled with lipid droplets appears to be foamy, which was eponymous for foam cells. The lipid droplets are organelles composed of a phospholipid monolayer, a lipid core containing triglycerides and sterol esters, as well as proteins, which are integrated in the phospholipid monolayer [16]. One of these proteins is PLIN2 (formerly adipophilin or adipose differentiation related protein (ADRP)), which was first identified by Jiang et al. in 1992 [17,18].

When the lipid loading capacity of cells is exceeded, a mechanism called lipotoxicity is induced [19]. The impaired cellular signaling, as well as mitochondrial and ER dysfunction may lead to cell death [20]. In the case of atherogenesis, this causes the formation of the necrotic lipid core of atherosclerotic plaques. In the initial phase of lipotoxicity, the storage of lipids in lipid droplets is protective as free fatty acids are esterified to triglycerides and are thus removed from active signaling [19,21].

To get a deeper insight into the biological actions of the LCM, we focused on the regulatory effects of α-13′-COOH on the expression of PLIN2, its interference with stearic acid-induced lipotoxicity and the possible connections between both mechanisms.

2. Materials and methods

2.1. Chemicals

If not indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Fisher Scientific (Schwerte, Germany), or Merck Millipore (Darmstadt, Germany).

2.2. Cell culture

THP-1 monocytes (ATCC, Manassas, VA), cultivated in RPMI 1640 supplemented with 10% (v/v) FBS and 0.1 mg/ml penicillin/streptomycin/L-glutamine [22] were differentiated into macrophages using 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 50 μM β-mercaptoethanol [23]. After 96 h, macrophages were incubated with serum-free supplemented medium and the test compounds as indicated in the figures and were harvested for further processing as described below.

2.3. Incubation

Stearic acid (C18:0; Alfa Aesar, Haverhill, MA) was dissolved in pure ethanol and complexed to fatty acid-free bovine serum albumin (Sigma-Aldrich) at a molar ratio of 4:1 in Krebs-Ringer bicarbonate buffer. α-TOH and LCM were dissolved in DMSO. For incubation, the compounds were mixed with supplemented RPMI 1640 medium without serum in the concentrations indicated in the figures.

2.4. Cytotoxicity

THP-1 macrophages were incubated with the respective test compounds in 24-well (standard) or 48-well (transfection) plates; at the end of the incubation period, the cells were washed twice with serum-free supplemented medium. The treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/ml in PBS, 50 μl or 25 μl per well, respectively) in 500 μl or 250 μl serum-free supplemented medium was performed for 4 h. Then the medium was exchanged by 1 ml or 0.5 ml isopropanol and was thoroughly mixed for 10 min, before a centrifugation step (5 min, 300 × *g*, room temperature) was applied. The solutions were aliquoted (in quadruplicates or triplicates of 100 μl each) to a 96-well plate. Absorption was measured at 570 nm. Viability was calculated by setting the untreated control to 100%. The EC_{50} values for stearic acid under the influence of a defined compound concentration were calculated using a sigmoidal fit for every biological replicate. Only those experiments were included, which revealed a clear EC_{50} value (three out of five biological replicates). EC_{50} values were obtained as whole numbers with no decimal places.

2.5. Concentrations for cell culture studies

The concentrations of the compounds were determined by absorption measurement in pure ethanol. The wavelengths and attenuation coefficients used are 292 nm and $\varepsilon = 3060$ for α-13′-OH and α-13′-COOH.

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Hilden, Germany) as described [24]. cDNA synthesis was performed using Revert Aid First strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and 500 ng/μl oligo-dT primers as described [25].

2.7. Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Schwerte, Germany) as described [25,26]. Primers (PLIN2, RPL37A, Supplementary Table S1) were purchased from Invitrogen (Karlsruhe, Germany). PCR results were analyzed using the LightCycler software version 1.5.0.39.

2.8. Transfection

Transfection of THP-1 macrophages was performed as described by Maeß et al. [27] with slight modifications. Cells were differentiated for 24 h using 100 ng/ml PMA. Transfection was performed using $3 * 10^6$ cells, 1% human serum (Sigma-Aldrich), 2 μg siRNA (PLIN2 Stealth siRNA ADFP HSS174700, 5288746 or Stealth RNAi Negative Control Low GC, 12935200; Thermo Fisher Scientific) and mouse T cell nucleofector medium (Lonza, Basel, Switzerland). Transfected cells were seeded in 48-well plates using 200 μl of cultivation medium to prefill wells and 100 μl of cell suspension was added to each well. The incubation with test compounds started 72 h after transfection.

2.9. Immunoblotting

Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) and samples were processed for Western blotting as described earlier [6]. The proteins were separated by SDS-PAGE and transferred to PVDF membrane (VWR, Darmstadt, Germany). Primary antibodies against PLIN2 (mouse anti-ADRP AP125, 1:50) and α-tubulin (mouse anti-α-tubulin clone B-5-1-2, 1:5000) were purchased from PROGEN (Heidelberg, Germany) and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse labeled with horseradish peroxidase, 1:5000) from DAKO (Hamburg, Germany) were used. SignalBoost™ Immunoreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for enhancing chemiluminescence signals for PLIN2.

2.10. Flow cytometry to measure neutral lipids via nile red

After incubation, cells were detached by Accutase I treatment (Sigma-Aldrich). Following washing steps with PBS, cells were stained with nile red solution of 1 µg/ml concentration, incubated for 10 min and washed again. A flow cytometric analysis was performed for neutral lipids in a range of 570 to 590 nm.

2.11. Immunofluorescence

Cells were differentiated on glass cover slips in 24-well plates and incubated as described before; cells were fixed with paraformaldehyde and stained using BODIPY 493/503 (20 µg/ml). Cover slips were mounted on slides and a set of ten pictures per cover slide was taken using a fluorescence microscope. Images were transformed to greyscale, inverted and a threshold was set. The size and number of lipid droplets were quantified by Image J using the analyze particles function.

2.12. Extraction of cellular lipids and fatty acid analysis

Cells were differentiated and incubated as described before; cells were scraped in PBS. Total cellular lipids were extracted as described by Dittrich et al. for blood lipids [28]. Due to the small amount of cellular lipids, the thin layer chromatography was skipped, and the gas chromatographic analysis was performed following the FAME preparation.

2.13. Isolation of garcinoic acid and semi-synthesis of α-LCM

Garcinia kola seeds were a gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid from the African bitter nut *Garcinia kola* and syntheses of the LCM were performed as described [12,29]. Purity of all LCM used was higher than 95%, as confirmed by HPLC-MS.

2.14. Statistics

Data are presented either as means ± standard deviation or as means ± standard error of the mean (SEM) of independent experiments as indicated. In order to test for statistical significance, paired Student's *t*-tests were performed using Microsoft Excel 2010. For the analyses of immunofluorescence data, a nested t-test was performed using R.

3. Results

The LCM of vitamin E emerged as regulatory metabolites with distinct and specific effects [30]. The main goal of the present study was to enlighten another aspect of their biological activity and to strengthen the new perspective on LCM as 'activated' or 'executive' metabolites, similar to the metabolites of vitamin A or D.

It has been reported previously that the LCM modulate foam cell formation in THP-1 macrophages, among others by inducing the expression of CD36 [14]. Using these experiments as a starting point, the present study focused on the regulation of another lipid metabolism-related protein, namely adipophilin (adipose differentiation related protein, ADRP or PLIN2) and the impact of the LCM on saturated fatty acid-induced lipotoxicity, as an important event in lipid-driven diseases [20].

PLIN2 is a lipid droplet-associated protein. Based on studies showing effects of vitamin E on lipid-related proteins and lipid metabolism, we investigated the effect of α-TOH and its metabolites on PLIN2. We found that PLIN2 expression was significantly reduced by α-TOH, after 24 h, mRNA and protein levels decreased by 47% to 26%, respectively ($p < 0.01$; Fig. 1). The LCM however, induced PLIN2 expression on both levels in a range of 1.6- to 3.8-fold ($p < 0.05$). The expression of PLIN2 is known to be induced by fatty acids, such as

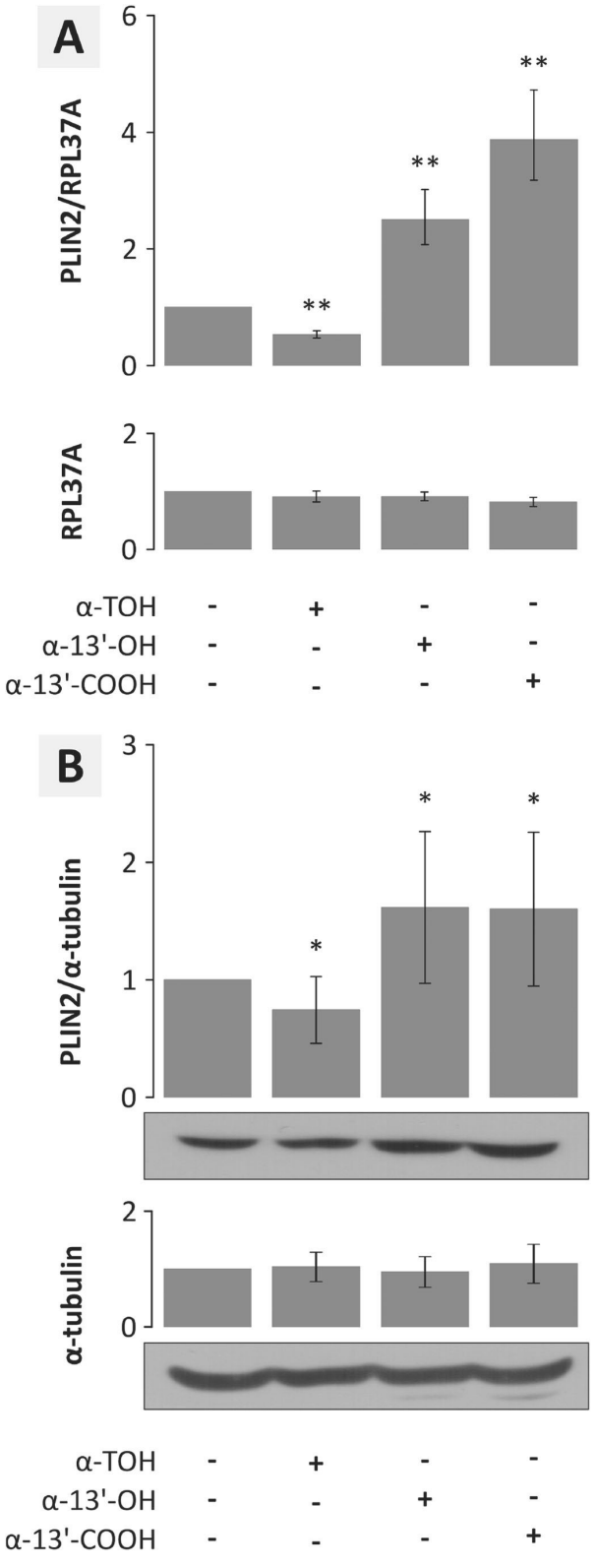

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Fig. 1. PLIN2 expression is induced by α-LCM but reduced by α-TOH.
Human THP-1 macrophages were incubated with 100 μM α-TOH, 10 μM α-13′-OH or 5 μM α-13′-COOH for 24 h and processed for mRNA (A) or protein (B) expression analysis. mRNA expression of PLIN2 was reduced under α-TOH treatment by 47% (SEM min 7%, SEM max 6%) and by 26% ± 28% on protein level. In contrast, the LCM α-13′-OH induced the PLIN2 expression of mRNA level by 250% (SEM min 43%, SEM max 51%) and 162% ± 65% for protein level. More pronounced effects were obvious with α-13′-COOH: an increase of mRNA expression by 388% (SEM min 69%, SEM max 85%) and of protein expression by 160% ± 65% was found. (A) PLIN2 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions. Error bars display calculated maximum and minimum expression levels of mean expression levels of three independent biological experiments each measured twice. (B) PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four independent biological experiments each measured twice are shown. *, $p < 0.05$; **, $p < 0.01$ (vs. control).

stearic acid. Hence, we were interested in the regulation of PLIN2 under stearic acid and LCM treatment. Therefore, THP-1 macrophages were pre-treated with α-TOH or the respective LCM for 24 h, followed by a co-incubation with stearic acid for an additional 24 h before protein expression analysis via Western blot (Fig. 2). For both pre-treatments (α-TOH and α-13′-COOH), the co-incubation with stearic acid and LCM resulted in expression values similar to that of stearic acid alone. Similar results were achieved by neutral lipid staining of cells using nile red. The cellular levels of neutral lipids were not influenced by α-TOH but were increased 1.5- to 1.8-fold by α-13′-COOH and stearic acid, respectively. The co-incubation with α-TOH and α-13′-COOH did not affect the stearic acid-induced accumulation of neutral lipids. Furthermore, we analyzed the relative fatty acid composition in THP-1 macrophages, which were incubated with 100 μM α-TOH or 5 μM α-13′-COOH as single compounds or in combination with 150 μM stearic acid for 48 h in total. As expected, significant increase of C18:0 (stearic acid) in the stearic acid treated samples was found (~3.7-fold; $p < 0.001$; data not shown). A very small, but significant difference ($p < 0.01$) in the relative amount of C16:1 (palmitoleic acid) and C18:1 (oleic acid) was observed, when the co-incubated samples were compared (α-TOH vs. stearic acid (3.5% and 21.2%, respectively) or α-13′-COOH vs. stearic acid (3.7% or 21.8%, respectively; data not shown). We also analyzed the size and the number of lipid droplets using BODIPY staining and fluorescence microscopy (Fig. 2E and F). We found an increase in the number of lipid droplets when stearic acid was applied (~3-fold, $p < 0.001$). The size of lipid droplets increased in the presence of α-13′-COOH (~4-fold, at least $p < 0.01$).

Stearic acid is known to induce lipotoxicity, when the concentrations applied exceed the cell's capacity of saturated fatty acid handling. We demonstrated this by incubating the cells with stearic acid in concentrations of up to 800 μM. Thereby, the treatment with a concentration range for α-TOH (0 to 100 μM) or α-13′-COOH (0 μM to 5 μM) made the impact of LCM on lipotoxicity obvious (Fig. 3). While α-TOH worsened the stearic acid-induced lipotoxicity significantly (decrease of EC_{50} value by 139 μM for 50 μM α-TOH), α-13′-COOH partially protected the cells significantly (increase of EC_{50} value by 214 μM for 1 μM α-13′-COOH).

Keeping in mind that the LCM can induce PLIN2, we wondered whether the reduced lipotoxicity of stearic acid by the LCM is mediated via PLIN2. Therefore, knockdown studies of PLIN2 were performed and the impact of its knockdown on stearic acid-induced lipotoxicity was tested. The knockdown was followed by an incubation regime similar to the approach used for Fig. 3 (incubation with 0 μM to 800 μM stearic acid). It was very clear that the cells treated with siRNA against PLIN2 were more prone to the lipotoxic effect of stearic acid than the controls (16% decreased viability by PLIN2 knockdown for 400 μM stearic acid; $p < 0.01$; Fig. 4).

To study the contribution of the PLIN2 knockdown on the protective effect of the LCM on stearic acid-induced lipotoxicity, the transfected cells were pre-incubated with the α-13′-COOH for 24 h and different concentrations of stearic acid were applied for a further 24 h (Fig. 5). The effective stearic acid concentration was calculated for several viabilities (40% to 85%) for both transfections (control siRNA and PLIN2 siRNA) and these were plotted against the respective LCM concentration. The slope for the defined viabilities across the LCM concentration was calculated and these were averaged within the siRNA treatments. A significant difference between these slopes was found (siCTRL: grey line, siPLIN2: dotted line; $p < 0.001$). This means that although a protective effect of α-13′-COOH is still observed under PLIN2 knockdown, it is by far less pronounced than in cells transfected with control siRNA. Therefore, it can be assumed that α-13′-COOH protects from stearic acid-induced lipotoxicity at least partially via the regulation of PLIN2 protein levels.

4. Discussion and conclusions

Recent studies on the metabolism of vitamin E revealed the physiological presence of the LCM α-13′-OH and α-13′-COOH in human blood [7,14]. We therefore hypothesize that the LCM are available at the site of action, e.g. in the case of atherogenesis at intimal macrophages. A recent study provided convincing evidence for the specific and distinct signaling mediated by LCM even at concentrations lower than their precursors [30]. Based on these results, a specific, not yet identified receptor for LCM was proposed. To elucidate the biological and molecular mechanisms of the LCM in more detail, a study on the regulatory effect of the LCM on the scavenger receptor CD36 [14] was used as a starting point for the experiments described here. We aimed to get a deeper insight into the regulation of foam cell formation by the LCM and focused on the regulation of PLIN2 at basal conditions and under load with the saturated stearic acid. Therefore, we used the LCM and α-TOH in concentrations which have been already used in other studies [7,14,31,32].

The expression of the lipid droplet-associated protein PLIN2 was assessed in human THP-1 macrophages after α-TOH and α-LCM treatment under basal conditions, i.e. incubation without serum. To the best of our knowledge, we are the first to describe the inhibition of PLIN2 expression by α-TOH and the induction of PLIN2 mRNA and protein level by the α-LCM. The neutral lipid accumulation precisely followed the regulation of PLIN2, as measured after incubation of 48 h by nile red staining and subsequent flow cytometric analysis. Due to delayed accumulation of neutral lipids (no significant increase in lipid levels were observed after 24 h (data not shown), but prominent accumulation was found after 48 h; Fig. 2), we conclude that the LCM induce PLIN2 expression, which in turn leads to lipid accumulation. This is plausible since PLIN2 is known to elevate cellular lipid levels by inhibiting β-oxidation [33] and lipolysis [34]. Thus, the lipid storage capacity of the cells is increased by the LCM via induced PLIN2 expression, followed by increases in neutral lipid accumulation. In a recent study, Bartolini et al. treated HepG2 cells with α-TOH and found an increase in lipid accumulation (Oil Red O staining) [35]. Unfortunately, no experiments using the LCM have been reported in this context.

In contrast to the aforementioned experiments, we focused on experiments under fatty acid stimuli. While stearic acid incubation led to the expected increase in PLIN2 protein expression [36] on protein level and in neutral lipid accumulation [37], the co-incubation with stearic acid and α-13′-COOH showed no additive effect (Fig. 2), which was also reflected by the gas chromatographic analysis of the cells (data not shown). This can be explained by mechanisms each specific for LCM and stearic acid. The readout (PLIN2 expression and lipid accumulation) may lead to the same results, but the underlying mechanisms are likely to be distinct. For stearic acid, a direct interaction with the PLIN2 protein via specific binding pockets has been described [38], while

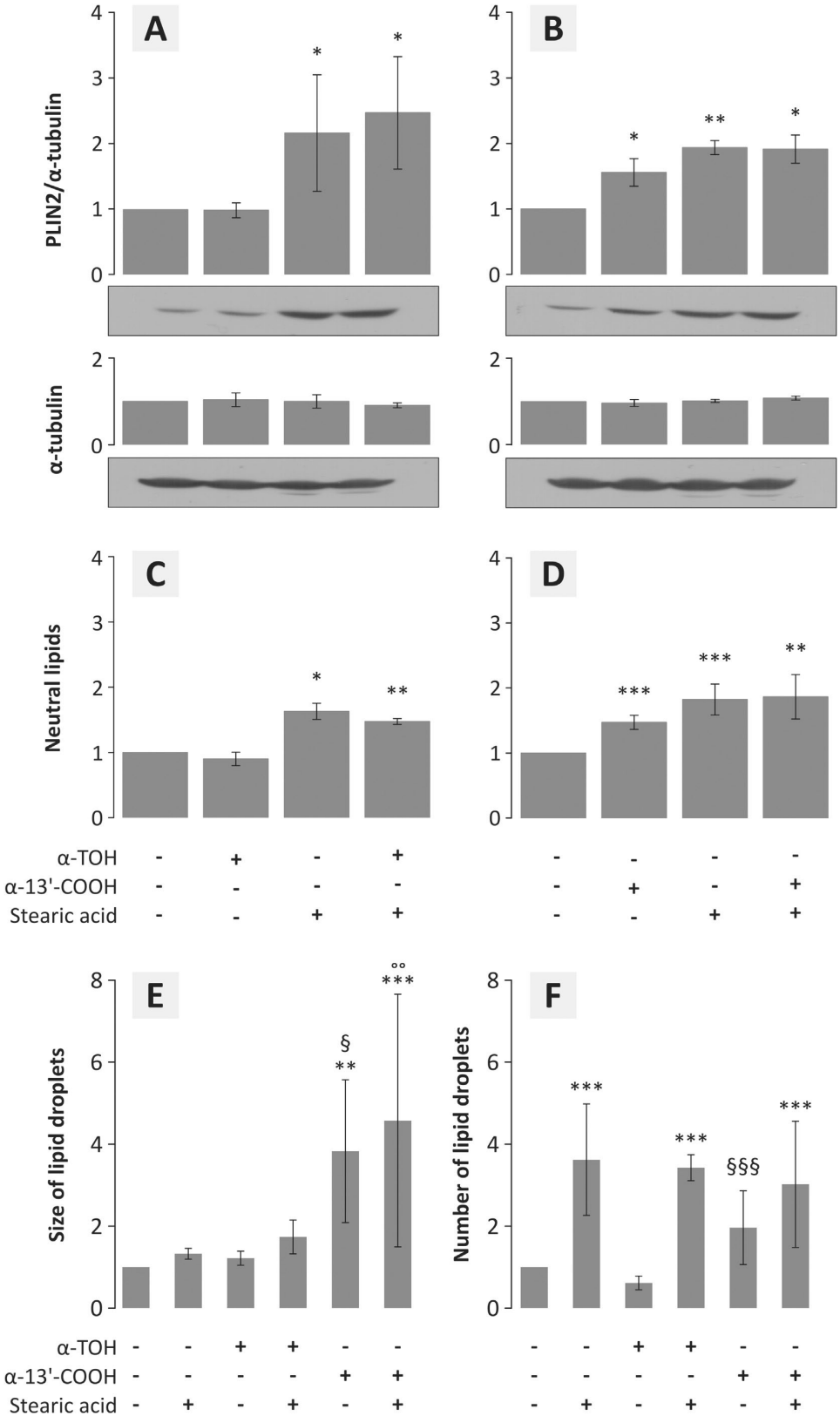

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Fig. 2. PLIN2 expression and neutral lipid accumulation is induced by stearic acid and α-13′-COOH.
Human THP-1 macrophages were incubated with 100 μM α-TOH (A, C, E, F) or 5 μM α-13′-COOH (B, E, F) or 2.5 μM α-13′-COOH (D) for 24 h and with 150 μM stearic acid for an additional 24 h in the presence or absence of the compounds. Afterwards, cells were processed for protein expression analysis via Western blot (A + B), neutral lipid staining (C + D) or immunofluorescence (E + F).
(A) While α-TOH did not regulate PLIN2 protein expression, stearic acid-induced PLIN2 by 216% ± 66% and the combinatory incubation resulted in an induction of 222% ± 67%. (B) The LCM α-13′-COOH increased the PLIN2 protein expression by 156% ± 21%, while stearic acid upregulated PLIN2 expression by 194% ± 11%. In combination, both compounds increased PLIN2 protein expression by 191% ± 22%. PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four (A) or three (B) independent biological experiments each measured once are shown.
(C) While α-TOH did not regulate neutral lipid accumulation, stearic acid induced neutral lipids by 163% ± 13% and the combinatory incubation resulted in an induction of 147% ± 5%. (D) α-13′-COOH induced neutral lipid accumulation by 147% ± 11%, while stearic acid-induced neutral lipid accumulation by 182% ± 24%. In combination, both compounds induced neutral lipid accumulation by 186% ± 34%. Neutral lipid accumulation was calculated relative to the control set to 1. Mean expression levels of three (C) or five (D) independent biological experiments each measured once are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs. control).
(E) The size of lipid droplets was increased by α-13′-COOH by 383% ± 174% or by α-13′-COOH and stearic acid by 457% ± 308%. (F) The number of lipid droplets was increased by stearic acid (362% ± 136% for stearic acid, 342% ± 32% for stearic acid and α-TOH, 302% ± 154% for stearic acid and α-13′-COOH). Size and number of lipid droplets were calculated relative to the control which was set to 1. Three biological replicates were prepared and a set of ten pictures per sample was taken. The data were evaluated using a nested *t*-test. p-Values are only shown for biologically relevant comparisons: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs. control); § vs. α-TOH; ° vs. stearic acid.

analogous studies are pending for the LCM. However, it is not yet established whether this influences neutral lipid accumulation or PLIN2 expression. Each mechanism may also be regulated or influenced by the respective other compound, which in turn may lead to the non-additive effect seen under the co-treatment setup. Another important aspect might be the heterogeneity of lipid droplets [39,40], which could be influenced by the LCM. Lipid droplets vary not only in size or contact sites to other organelles, e.g. ER [41], but also in lipid [40] and protein [42] composition. Interestingly, PLIN2 is localized on lipid droplets of all sizes (starting with pre-lipid droplets) [41,42]. We also report here that the LCM induce the mean size of intracellular lipid droplets. This may contribute to the protective effect of the LCM in the lipotoxic setup described here.

We focused on the interaction of α-TOH and LCM with stearic acid-induced lipotoxicity. We speculated that the LCM may induce a conversion of saturated fatty acids to monounsaturated fatty acids (e.g. C16:0 to 16:1 or C18:0 to C18:1). This has been considered to reduce lipotoxicity of saturated fatty acids [43]. Since the gain of viability by the conversion of C18:0 to C18:1 is higher than for C16:0 to C16:1 [44], we decided to use stearic acid (C18:0) for lipotoxicity experiments in order to reduce the compensatory effect of the conversion. For this purpose, cells were treated with a compound-concentration-matrix of stearic acid (0 to 800 μM) and α-TOH (0 to 100 μM) or α-13′-COOH (0 to 5 μM). Stearic acid-induced lipotoxicity, measured by the MTT viability test, has been investigated [43]. Here, we describe for the first time that stearic acid-induced lipotoxicity was worsened by α-TOH, whereas α-13′-COOH reduced the effect of stearic acid. So far, neither the effects of vitamin E nor its metabolites on lipotoxicity have been investigated in detail, thus the underlying mechanisms are an object of speculation. Besides the regulatory induction of PLIN2, the inhibition of apoptosis via unknown mechanisms may also contribute to the inhibition of lipotoxicity. Results from an analogous study designed by Rabkin et al. [43] were striking. Rabkin also induced lipotoxicity via stearic acid, but oleic acid (C18:1) was used for rescuing cardiomyocytes from death. Surprisingly, the intracellular lipid pattern measured under co-incubation of stearic acid and oleic acid was similar to our results for α-13′-COOH in neutral lipid accumulation, which is a non-additive effect. Keeping in mind the low similarity in their structure, the similar findings for the LCM and oleic acid are unexpected.

We would like to point the reader's attention to the impact of fatty acid distribution of PLIN2 binding on the surface and size of lipid droplets. The binding affinity of PLIN2 for oleic acid is twice that of stearic acid [45], and introducing saturated acyl chains to the phospholipids of the monolayer forming the surface of lipid droplets impairs PLIN2 binding due to the condensation of the phospholipid monolayer covering lipid droplets [46]. However, larger intracellular lipid droplets have a higher proportion of saturated fatty acids in their monolayer than smaller ones [47]. Unfortunately, fatty acid profile of cells treated with 100 μM α-TOH, 5 μM α-13′-COOH or 150 μM stearic acid or the combination of vitamin E derivate and stearic acid did not complete the puzzle. The most obvious finding was the relative increase of stearic acid, when stearic acid was added to the cells. Under treatment with stearic acid, α-13′-COOH also minimally increased the relative amount of the monounsaturated form of C16:0 (C16:1, +0.2%) and C18:0 (C18:1, +0.6%) compared to the α-TOH treated samples. It is known that the monounsaturated fatty acids are less toxic than the saturated forms [44]. This might explain the improved viability of cells treated with α-13′-COOH to some extent. However, keeping the effect sizes in mind, one must admit that it is most likely not a single mechanism that prevents the cells from lipotoxicity.

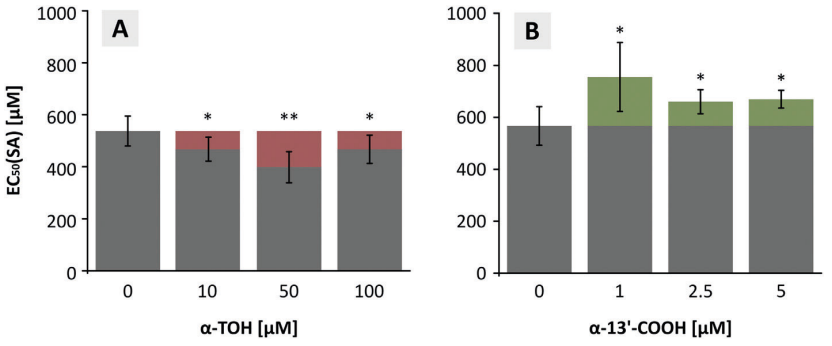

Fig. 3. Stearic acid-induced lipotoxicity is partially reduced by α-13′-COOH but not by α-TOH.
Human THP-1 macrophages were incubated with 0 to 100 μM α-TOH (A) or 0 to 2.5 μM α-13′-COOH (B) for 24 h, followed by a co-incubation with 0 to 800 μM stearic acid for further 24 h. This incubation matrix was assessed by MTT cytotoxicity tests. (A) Treatment of THP-1 macrophages with increasing concentrations of stearic acid resulted in reduced cell viability. The same holds true for cells which were co-treated with α-TOH; here, α-TOH decreased stearic acid-induced lipotoxicity significantly (decrease of EC_{50} value by 70–139 μM) $p < 0.05$ to $p < 0.01$). (B) Again, the concentration-dependent reduction of viability under stearic acid treatment was seen. When cells were co-incubated with α-13′-COOH the viability of the cells was significantly increased (increase of EC_{50} value by 91–214 μM $p < 0.05$). *, $p < 0.05$; **, $p < 0.01$; (vs. control).

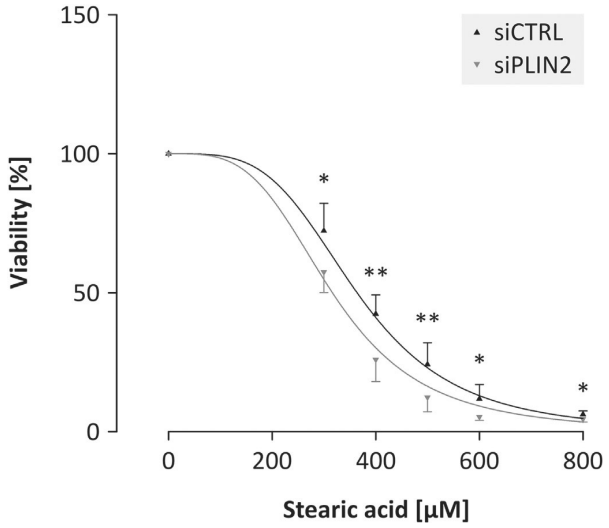

Fig. 4. PLIN2 partially protects from stearic acid-induced lipotoxicity.
Human THP-1 macrophages were transfected with control siRNA (siCTRL) or PLIN2 siRNA (siPLIN2) and incubated with increasing concentrations of stearic acid (0 to 800 µM) for 24 h. Afterwards, the cells were treated with MTT solution and the readout was performed as described in the Materials and Methods section. With increasing concentrations of stearic acid, the viability of control cells decreased (300 µM stearic acid: 72.26% ± 9.9% viability, 500 µM stearic acid: 24.21% ± 5.1% viability). Cells treated with PLIN2 siRNA were more prone to stearic acid-induced lipotoxicity (300 µM stearic acid: 57.47% ± 7.4% viability, $p < 0.05$ (control vs. PLIN2 siRNA treatment), 500 µM stearic acid: 12.39% ± 5.3% viability, $p < 0.01$ (control vs. PLIN2 siRNA treatment)). Error bars display standard deviations of mean viability levels of four independent biological experiments each measured once. *, $p < 0.05$; **, $p < 0.01$ (vs. siCRTL).

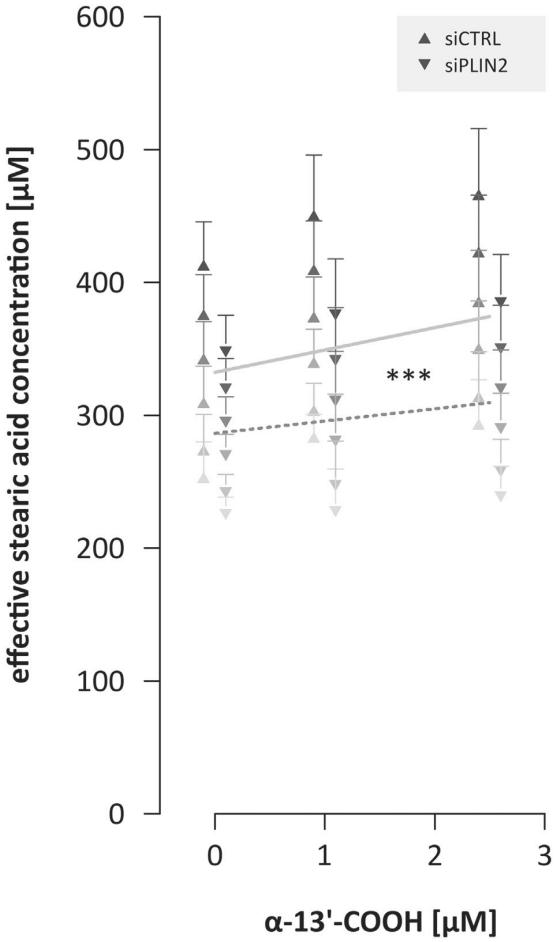

Fig. 5. α-13′-COOH partially protects from stearic acid-induced lipotoxicity via PLIN2.
Human THP-1 macrophages were transfected with control siRNA (siCRTL) and PLIN2 siRNA (siPLIN2) and incubated with 0 to 2.5 µM α-13′-COOH and 0 to 800 µM stearic acid. A plot showing the dependency of the viability from stearic acid concentration was calculated for each LCM concentration. A logarithmic fit was performed, and the effective concentrations of stearic acid for 40% to 85% viability were calculated. For each viability, the slope for the concentration dependency of LCMs on the effective concentration of stearic acid was calculated. The mean for each effective concentration of stearic acid was obtained from four biological replicates. Finally, the mean was calculated across all experiments, which were treated with the same siRNA. The comparison of both slopes, siCRTL (grey line) vs. siPLIN2 (dotted line) dependent on LCM concentration, and the effective concentration of stearic acid revealed a significant difference (***, $p < 0.001$).

The effect of α-TOH or LCM on stearic acid-induced lipotoxicity is accompanied with the regulation of PLIN2 expression. Therefore, we asked whether PLIN2 might be the crux of the matter and thus set up knockdown experiments for PLIN2. At basal conditions (stearic acid concentration gradient only, no incubation with α-13′-COOH), a worsening of stearic acid-induced lipotoxicity was found, which is likely due to a loss in lipid storage capacity forced by the knockdown of PLIN2. Knockdown of PLIN2 has been described as influencing cellular lipid levels and size of lipid droplets as well as their number [33,48]. It has also been suggested that PLIN2 plays a role in the expansion of lipid droplet size [49]. In summary, this could lead to the disruption of stearic acid-induced lipotoxicity by the knockdown of PLIN2. However, it has been described that other homologs of PLIN2, e.g. TIP47 [50] take on its task if PLIN2 is knocked down. This might be the reason why the decrease in stearic acid-induced lipotoxicity by the knockdown of PLIN2 is relatively mild. Taken together, PLIN2 is protective against stearic acid-induced lipotoxicity to some extent.

A cross-comparison of the respective cell viabilities between Figs. 3 and 4 highlights the increased sensitivity of transfected cells towards stearic acid treatment. It is known that electroporation (the transfection method used here) is able to transfer lipids between membrane leaflets [51], which may induce further perturbations in lipid metabolism or handling. Overall, this might contribute to the higher sensitivity of transfected cells to mediators of lipotoxicity.

Next, the stearic acid lipotoxicity tests were conducted in the presence of the knockdown of PLIN2 and LCM incubation. While the LCM-dependent blocking of lipotoxicity was observed under both conditions (control and PLIN2 knockdown), the effective stearic acid concentrations needed to achieve a certain viability, were significantly reduced by the knockdown of PLIN2 across all α-13′-COOH concentrations tested. This leads to two conclusions: (i) PLIN2 expression protects macrophages from stearic acid-induced lipotoxicity, and (ii) the LCM partially protect macrophages from lipotoxicity via the induction of PLIN2. It might be striking that the reduction in lipotoxicity by α-13′-COOH seems to not be coupled with an additive induction of neutral lipid accumulation. On the other hand, it has been shown that the cellular lipid content is not a determinant of lipotoxicity-induced cell death [43].

The underlying signaling pathways, which are responsible for the distinct LCM effects are currently the focus of our research. Our latest study indicated that a molecular receptor for the LCM might exist [30]. However, our most recent unpublished findings revealed insights into more complex molecular interactions, which require a comprehensive

evaluation that is ongoing.

Overall, we have made several new observations: First, PLIN2 protects macrophages from stearic acid-induced lipotoxicity. Second, α-TOH triggers stearic acid-induced lipotoxicity in macrophages. Third, we found evidence for a complex link between the α-LCM, PLIN2 and lipotoxicity. We provide first evidence for the potentially protective effects of the α-LCM by inducing lipid storage capacity and thereby decreasing the proneness to lipotoxicity. Our data also show that PLIN2 contributes at least partially to this phenomenon. Our results will contribute to the growing knowledge on the modes of action of the LCM, which we consider as a new class of regulatory metabolites.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.05.002.

Conflict of interest statement

The authors declare no competing interests.

Transparency document

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Method Article

Simple and rapid real-time monitoring of LPL activity *in vitro*

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A B S T R A C T

Since elevated plasma triglycerides are an independent risk factor for cardiovascular diseases, lipoprotein lipase (LPL) is an interesting target for drug development. However, investigation of LPL remains challenging, as most of the commercially available assays are limited to the determination of LPL activity. Thus, we focused on the evaluation of a simple *in vitro* real-time fluorescence assay for the measurement of LPL activity that can be combined with additional cell or molecular biological assays in the same cell sample. Our procedure allows for a more comprehensive characterization of potential regulatory compounds targeting the LPL system.

The presented assay procedure provides several advantages over currently available commercial *in vitro* LPL activity assays:

1. 12-well cell culture plate design for the simultaneous investigation of up to three different compounds of interest (including all assay controls).
2. 24 h real-time acquisition of LPL activity for the identification of the optimal time point for further measurements.
3. Measurement of LPL activity can be supplemented by additional cell or molecular biological assays in the same cell sample.

A R T I C L E I N F O

Method name: Cell culture based real-time fluorescence assay for the measurement of LPL activity
Keywords: Lipoprotein lipase (LPL), LPL activity assay, Fluorescence, Real-time assay
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Abbreviations: ANGPTL, angiopoietin-like; FBS, fetal bovine serum; FFA, free fatty acid; FI, fluorescence intensity; LPL, lipoprotein lipase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PPAR, proliferator-activated receptor; PSG, L-glutamine-penicillin-streptomycin; RFU, relative fluorescence units; VLDL, very low-density lipoprotein.

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Specification Table

Subject area:	*Biochemistry, Genetics and Molecular Biology*
More specific subject area:	*Investigation of lipoprotein lipase activity*
Method name:	*Cell culture based real-time fluorescence assay for the measurement of LPL activity*
Name and reference of original method:	*Name: Abcam Lipoprotein Lipase Assay Kit (Fluorometric) (ab204721)* *Source:* [1]

Method details

Background

Lipoprotein lipase (LPL) mediates the release of free fatty acids (FFAs) from triglyceride-rich lipoproteins, like chylomicrons and very low-density lipoproteins (VLDL). Therefore, LPL represents a key enzyme for the regulation of cellular lipid homeostasis by providing FFAs for cellular energy supply and intracellular energy storage as well as a control mechanism for plasma triglyceride levels [2]. The activity of LPL is primarily regulated by post-translational modifications. The family of angiopoietin-like (ANGPTL) proteins, whose expression is controlled by peroxisome proliferator-activated receptors (PPARs), appeared as potent physiological inhibitors of LPL activity [3–5]. LPL dysfunction or dysregulation can result in elevated plasma triglycerides [6,7]. There is growing evidence that elevated plasma triglycerides are an independent risk factor for cardiovascular diseases [8,9], making the LPL system an interesting target for drug development [10,11]. Hence, determination of LPL activity is a useful tool for the identification of potential lead compounds from natural or synthetic origins. Commercially available kits for the measurement of LPL activity are based on radiolabeled (^{3}H or ^{14}C), fluorogenic or chromogenic substrates [12–14]. These substrates are degraded by LPL and their reaction products can be detected at defined times [15]. Unfortunately, most commercial kits are optimized for post heparin plasma samples and are therefore not suitable for initial characterization of potential drug compounds in *in vitro* systems. Next, the few commercial LPL assays that are optimized for *in vitro* application require cell harvesting and homogenization. Thus, cells cannot be used for further cell or molecular biological investigations, which would allow a more comprehensive characterization of the respective test compound [1,16]. For example, the measurement of LPL activity combined with subsequent RT-qPCR or Western blot analyses can serve as a useful tool for the identification of transcriptional regulators of LPL activity.

For this reason, we decided to develop a simple cell culture based real-time fluorescence assay for the measurement of LPL activity that can be combined with cell and molecular biological analyses of the same cell sample. In our method, LPL activity is measured using a fluorescently labeled and quenched LPL substrate in combination with isolated VLDL for stimulation of LPL activity.

Required reagents and equipment

(1) *VLDL isolation*
- 50 ml fasted blood sample
- EDTA monovettes
- Centrifuge applicable for at least 1870 × g
- Ultracentrifuge thick wall tubes
- Ultracentrifuge
- Ultracentrifuge rotor applicable for at least 200,000 × g

(2) *Cell culture*
- 12-well cell culture plates
- Adherent cells
- Fetal bovine serum (FBS)
- L-glutamine-penicillin-streptomycin (PSG)
- Cell type specific cell culture medium
- Cell type specific phenol red free cell culture medium (for THP-1 macrophages: RPMI-1640, R7509, Sigma Aldrich)

- VLDL isolated form blood samples of healthy volunteers (alternatively: order commercially available VLDL)
- Fluorescently labeled, quenched LPL substrate (ab214552, Abcam, Cambridge UK)
- Orlistat (O4139, Sigma Aldrich; negative control)

(3) *Fluorescence-based real-time measurement of LPL activity*

- Microplate reader coupled to an atmospheric control unit applicable for fluorescence measurements (Ex/Em = 485/520 nm)
- Microplate reader temperature: 37 °C
- CO2 concentration: 5% (v/v)

Procedure

The description of the following experimental procedure will be illustrated by the human THP-1 macrophage cell model. However, this method may be suitable for various adherent cell lines that express active LPL.

(1) *Isolation of VLDL (alternatively: order commercially available VLDL)*

Note: VLDL is an essential part of the assay procedure. It is used as positive control, because stimulation of LPL activity by VLDL has been already established as positive control for plasma measurements of LPL activity [12].

- Use 50 ml blood obtained from fasted donors with plasma triglyceride concentrations of > 0.90 mmol/l

Note: Plasma triglyceride concentrations of > 0.90 mmol/l was previously described as a suitable range for VLDL-based LPL assays [12].

- Collect the blood in 9 ml EDTA-monovettes (02.1066.001, Sarstedt)
- Centrifuge blood samples at 1870 × g for 10 min at 15 °C for plasma separation
- Transfer plasma into 4 ml thick-wall polycarbonate tubes (355,645, Beckmann Coulter) for ultracentrifugation
- Ultracentrifugation is performed for 4 h at 15 °C and 269 200 × g (50,000 rpm, used rotor: Type 50.4 Ti, Beckman Coulter)
- Collect separated VLDL in 2 ml tubes

Note: The separated VLDL phase is very narrow and can easily be mixed with the plasma fraction below. Be careful not to shake the thick-wall tubes when taking them out of the ultracentrifuge. Use a 1 ml pipette for collecting the VLDL phase by placing the pipette tip on the tube wall and moving it carefully around the tube.

- Determine the protein concentration of the VLDL samples (*e.g.,* Lowry or Bradford assay)
- Isolated VLDL can be stored under nitrogen atmosphere at 4 °C

Note: It is necessary to utilize the isolated VLDL within one week for the respective experimental procedures to ensure high VLDL quality and to avoid lipid oxidation.

(2) *Cell culture*

- The assay procedure described below is based on the use of 12-well cell culture plates (92,012, TPP Techno Plastic Products, Trasadingen, Switzerland)
- Use 1 × 106 THP-1 monocytes per well for macrophage differentiation (add 100 ng/ml phorbol-12-myristate-13-acetate (P1585, Sigma Aldrich) and 50 μmol/l β-mercaptoethanol (4227.3, Carl Roth) together with RPMI-1640 (R8758, Sigma Aldrich) cell culture medium supplemented with 10% (v/v) FBS Superior (S0615, Sigma Aldrich) and 0.1% (v/v) PSG solution (G1146, Sigma Aldrich))

Note: The number of wells used for the assay depends on the experimental design. However, there are four fixed controls included in each assay run to ensure reliability of the procedure: (i) an untreated control, (ii) the orlistat negative control without VLDL, (iii) the VLDL positive control, and (iv) the orlistat negative control in combination with VLDL. Keep in mind to include these four controls in your experimental design. The current 12-well assay design including all assay controls allows the simultaneous analysis of up to three different test compounds.

- THP-1 monocytes are differentiated for 96 h in 2 ml cell culture medium per well
- Remove cell culture supernatant from the fully matured THP-1 macrophages
- Wash cells twice with phosphate-buffered saline (PBS)
- Pre-incubate cells according to the respective experimental procedure (here for 24 h) in 1 ml phenol red-free RPMI-1640 medium (R7509, Sigma Aldrich) under serum-free conditions
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: It is necessary to replace standard RPMI-1640 cell culture medium by phenol red-free RPMI-1640 for incubation to avoid fluorescence interferences. For the basic assay procedure without any test compounds, cells are only pre-incubated with 50 µM orlistat in the respective wells for 24 h.

- Add VLDL in a concentration equivalent to 50 µg/ml protein together with 0.5 µl quenched, fluorescently labeled LPL substrate to the pre-incubated cells
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: The LPL substrate used for the experimental procedure is a standardized, commercially available product from Abcam (Cambridge, UK ab214552). It is a component of the commercial LPL assay kit offered by the company and therefore validated for reliable functionality. Nevertheless, we recommend to pooling LPL substrates of different batches to reduce variability between measurements. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured fluorescence intensity (FI) values are proportional to the amount of hydrolyzed substrate and thus LPL activity. The use of commercially available substrates for determination of LPL activity has also been described in other methodical approaches [12,13].

(3) *Fluorescence based real-time measurement of LPL activity*

Note: In our experimental setup, the FLUOstar Omega microplate reader coupled to an atmospheric control unit (BMG Labtech, Ortenberg, Germany) was used for FI determination. As an initial step, a suitable measurement procedure should be prepared for the respective experimental design of every assay procedure. In our approach, we determined FI values of the used wells hourly over 24 h at Ex/Em = 485/520 nm (recommended wavelength for the LPL substrate). As an additional preparation step, a temperature of 37 °C and a CO_2 concentration of 5% (v/v) should be set at least one hour before each measurement.

- Place the cell culture plate in the plate reader and start the prepared measurement procedure.
- The determined FI values can be used for the assessment of LPL activity for any test compound.
- Adherent cells can be used for subsequent investigations.

Note: The described assay conditions do not require harvesting of the cells. Thus, LPL activity measurement can be supplemented by additional cell or molecular biological analyses for a more comprehensive characterization of the test compounds. We have currently performed Nile red staining and cell viability assays after the determination of LPL activity. However, the combination with other molecular biological methods, such as Western blot, RT-qPCR or any other *in vitro* application is possible in principle.

Method validation

The aim of this study was to establish a simple fluorescence-based *in vitro* assay to allow the initial characterization of potential regulatory compounds targeting the LPL system. We decided to develop this procedure because commercially available LPL assays were not suitable for this demand.

Preliminary experiments revealed that the standard RPMI-1640 cell culture medium had to be replaced by phenol red-free RPMI-1640 for cell incubation to avoid fluorescence interferences. To enable determination of LPL activity, we used a quenched, fluorescently labeled LPL substrate (similar approach as described in [12,13]) in our incubation procedure. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured FI values are proportional to the amount of hydrolyzed substrate and thus LPL activity. Further, we decided to use VLDL as positive control, because stimulation of LPL activity by VLDL has already been shown in plasma measurements [12]. In addition, orlistat, a well-established and clinically used LPL inhibitor [17–19] was added as a negative control.

For the initial establishment of the assay procedure, human THP-1 macrophages were treated as described in the section "Supplemental Material/and or additional information". After 24 h real-time measurement, we noticed that incubation with VLDL (in a concentration equivalent to 50 µg/ml protein) enhanced the measured FI values, indicating increased LPL activity, in a time dependent manner. Fluorescence intensity values of the untreated control and the VLDL-treated sample (positive control) evolved in a significantly different range ($p < 0.01$) (Fig. 1(A)). Maximum FI values have been determined after 24 h as an increase to 268.75 ± 24 relative fluorescence units (RFU) in the VLDL-incubated sample compared to 187 ± 14.5 RFU in the untreated control. As expected, orlistat (negative control) significantly blocked LPL activity compared to VLDL-treatment and the untreated control. Fluorescence intensity values of the VLDL-incubated sample and the combination of VLDL and orlistat evolved over time in a significantly different range ($p < 0.001$). After 24 h, FI value for the cells incubated with a combination of VLDL and orlistat was determined at 150 ± 1.3 RFU and as significantly lower ($p < 0.01$) compared to the VLDL-incubated cells (268.75 ± 24 RFU) (Fig. 1(A)). These results indicate that our methodical approach and the used assay controls are suitable for the *in vitro* measurement of LPL activity.

As a further validation of the established assay procedure, we decided to use GW0742 as a reference test compound. GW0742 is a well-studied PPAR-δ agonist [20,21], that enhances the expression of ANGPTL4 mRNA [22], a potent physiological and endogenously produced inhibitor of LPL. Consequently, stimulation of THP-1 macrophages with the PPAR-δ agonist GW0742 should enhance ANGPTL4 expression, in turn causing a reduction of cellular LPL activity [5]. To confirm the reported effects of GW0742 under our conditions, human THP-1 macrophages were treated with 100 nM GW0742 and harvested at different time points for RT-qPCR analysis. As expected, GW0742 enhanced ANGPTL4 mRNA expression already after 1 h by approximately 20-fold ($p < 0.001$) and after 24 h by approximately 200-fold ($p < 0.001$) (Fig. 1(B)). To examine the effects of GW0742 on cellular LPL activity, we expanded our initial working procedure by an additional 24 h pre-incubation with 100 nM GW0742. In line with our initial experiment, we were able to generate similar FI values for untreated control cells, VLDL-incubated cells as well as cells cultured with orlistat alone or in combination with VLDL. GW0742 treatment slightly, but not significantly increased basal LPL activity (206 ± 20 RFU *vs.* 230 ± 16 RFU) compared to the untreated control. However, combination of GW0742 and VLDL did significantly reduce the induction of LPL activity by VLDL ($p < 0.05$). The strongest reduction was achieved after 24 h, where GW0742 reduced FI values of VLDL incubated cells from 291.3 ± 29 RFU to the control level of 196.6 ± 20 RFU (Fig. 1(C)). In summary, the observed effect of GW0742 on cellular LPL activity is consistent to the literature.

For the combination of the LPL assay procedure outlined here with additional cell or molecular biological methods, we studied cell viability at the end of the LPL activity measurement. For this, methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed after completing the 24 h real-time measurement. None of the applied compounds did significantly reduce cell viability compared to the untreated control. Treatment with orlistat led to a slightly but not significantly reduced cell viability of 85% of the control, representing the lowest viability in our experimental setup (Fig. 1(D)). According to ISO 10993-5:2009, a reduction of cell viability by 15% is not regarded as a cytotoxic effect [23]. For final validation of the assay procedure, we combined our LPL assay

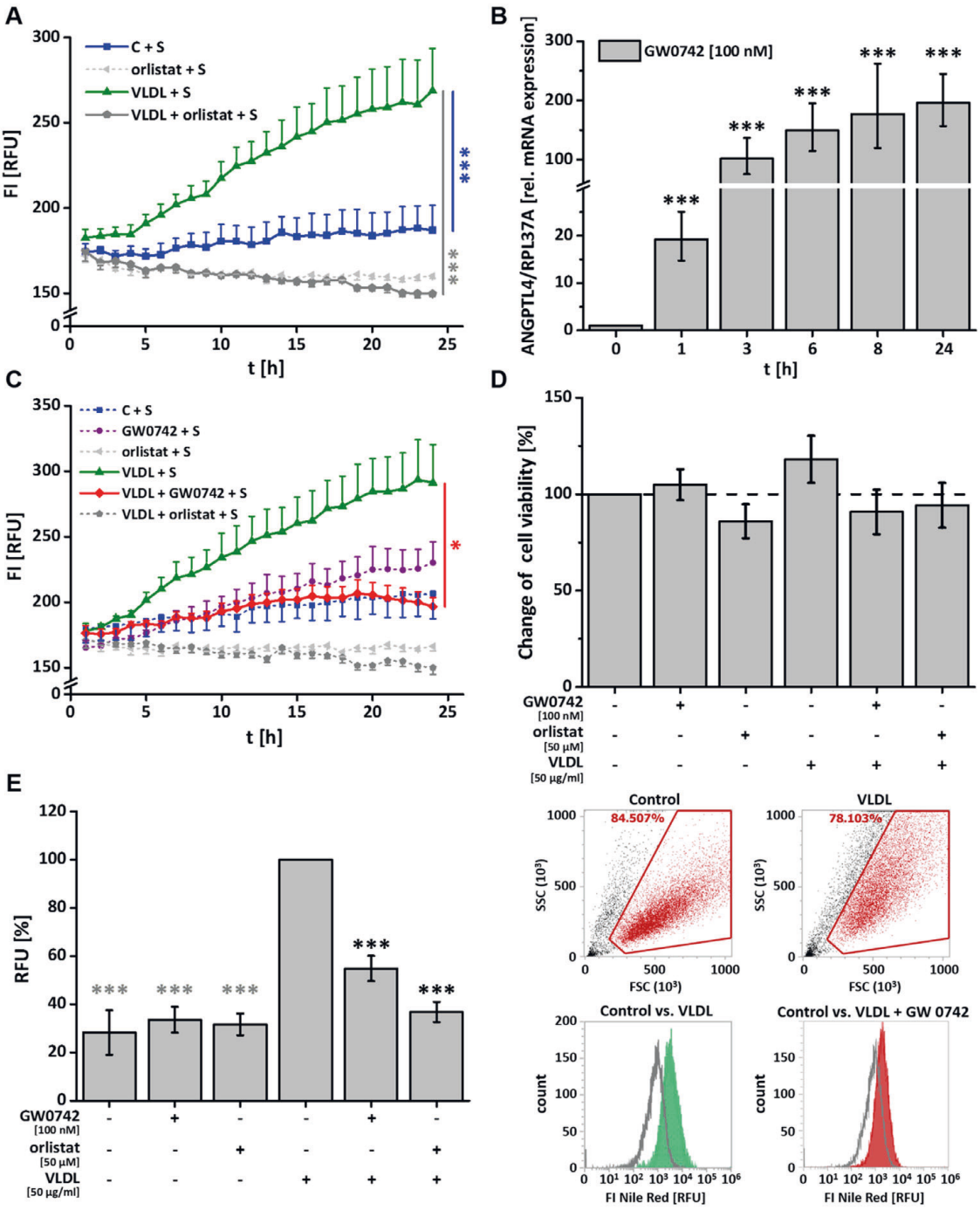

Fig. 1. *Establishment and validation of an in vitro real-time fluorescence assay for the measurement of LPL activity.* (A) Initial establishment of the LPL assay procedure. Human THP-1 macrophages were pre-incubated with 50 µM orlistat (negative control). After 24 h, VLDL (positive control, protein concentration of 50 µg/ml) and the fluorescently labelled LPL subtrate were added to the corresponding wells. Fluorescence intensity (FI) of each well was determined hourly over 24 h at Ex/Em = 485/520 nm ($n = 4$; *** $p < 0.001$ *vs.* VLDL incubation). All further experiments were performed for assay validation, using GW0742 as reference test compound. (B) RT-qPCR of human THP-1 macrophages to investigate ANGPTL4 (LPL inhibitor) mRNA expression after GW0742 treatment ($n = 4$, *** $p < 0.001$, *vs.* untreated control). ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). (C) LPL activity assay with GW0742 as test compound ($n = 3$, * $p < 0.05$ *vs.* VLDL incubation). (D) MTT assay for the assessment of cell viability after measurement of LPL activity ($n = 3$). (E) Measurement of the accumulation of neutral lipids accumulation by flow cytometry using Nile red staining after completion of the real-time LPL activity assay ($n = 3$, *** $p < 0.001$, *vs.* VLDL treatment).

with a subsequent measurement of neutral lipid accumulation by flow cytometry using Nile red staining. This experimental setting was chosen from reports on VLDL-induced cellular accumulation of neutral lipids via LPL [24,25]. Further, ANGPTL4 has been shown to reduce the uptake of triglyceride-derived fatty acids from VLDL by human THP-1 macrophages [26]. Consequently, VLDL treatment should enhance neutral lipid accumulation, while GW0742 treatment should prevent this. As expected, incubation of THP-1 macrophages with VLDL induced accumulation of neutral lipids by almost threefold compared to the untreated control. Co-incubation with GW0742 significantly reduced VLDL-induced accumulation of neutral lipids to 54% ($p < 0.001$). Co-incubation with orlistat resulted also in a significant reduction of VLDL-induced accumulation of neutral lipids to 36% ($p < 0.001$). As expected, the relative amount of neutral lipids in cells treated with GW0742 or orlistat but without VLDL did not differ from the control (Fig. 1(E)). The obtained results confirm that our LPL assay can be easily combined with other cell and molecular biological methods to produce more comprehensive information on the interaction of a compound of interest with the cellular LPL system.

Conclusion

We here provide a simple and rapid fluorescence-based *in vitro* assay for the assessment of the interactions of test compounds with the LPL system. The assay procedure provides several advantages over currently available *in vitro* LPL assays: (i) 12-well cell culture plate design for the simultaneous investigation of up to three different test compounds (including all assay controls); (ii) 24 h real-time acquisition of LPL activity data for the identification of the optimal time point for further measurements; and (iii) LPL activity measurement can be complemented by additional cell and molecular biological analyses using the same cell samples. Nevertheless, we are aware that the current assay design has limitations and needs further improvements:

(i) In our experiments, VLDL was isolated from only a single normolipidemic male donor with plasma triglyceride concentrations of 0.90 mmol/l. However, the approach could be improved by using a mixture of VLDL obtained from multiple donors as described in [12]. Here, samples from ten normolipidemic donors (plasma triglycerides < 1.75 mmol/l) were pooled to create a more representative mean VLDL substrate for their assay procedure. Further, the use of a VLDL pool from different donors may also reduce the variation of the lipid and apolipoprotein composition between individuals.

(ii) For further optimization of VLDL composition, the triglyceride content of the VLDL should be determined before. Di Filippo and coworkers used seven VLDL pools with varying triglyceride concentrations ranging from 0.45 to 3.45 mmol/l for the determination of LPL activity in post-heparin plasma [12]. The authors report that LPL activity reached a steady level in the range between 1.5 and 2.2 mmol/l, while lower concentrations (< 0.90 mmol/l) decreased LPL activity and high concentrations (> 2.7 mmol/l) slightly increased LPL activity in post-heparin plasma. To avoid distortion of the measured LPL activity by suboptimal triglyceride concentrations of the applied VLDL, the authors determined 1.8 mmol/l as the optimal triglyceride concentration for their assay. Hence, optimization of the triglyceride concentrations in the VLDL could improve our assay.

(iii) The assessment of LPL activity in the current assay design is based on the comparison of FI values for the different compounds and controls at various times. This might be sufficient for a first impression of the effect of compounds on LPL activity and is therefore suitable for the intention of our assay procedure. Nevertheless, exact quantification of LPL activity based on the calculation of the released amount of substrate over time, as it has already been described for plasma measurements [1,12,13], should be an aim for future improvements. Unfortunately, no detailed information about their fluorescently labeled LPL substrate (concentration, composition, exact chemical name/structure etc.) is available from the supplier (Abcam, Cambridge UK). It was therefore not possible to adapt the procedure for the use of standard calibration curves as described in the assay manual to our conditions [1].

(iv) For the here presented LPL activity assay, a mean coefficient of variation (CV) for inter-assay variability was 10.6% for low (untreated control) and 12% for high values (VLDL treatment).

Although both CVs are below 15%, which is in general acceptable for biological assays [27], inter-assay variability is an issue, in particular for high FI values. In general, the higher the measured FI values the higher the variation between each measurement. We were able to partially reduce this problem using a pool of different batches of the LPL substrate. Given the fact that the current assay procedure is only designed for initial compound screening and not as a diagnostic tool, the variation between each measurement seems acceptable. Nevertheless, reproducibility and the accuracy of the assay procedure should further be improved.

(v) All experiments and optimizations for the current assay procedure were performed with human THP-1 macrophages. However, to fully understand the global impact of a given drug on lipolysis the use of further LPL-expressing cells, like adipocytes, may be necessary.

Despite of its limitations, our assay design can serve as a reliable tool for *in vitro* measurements of the effects of test compounds modulating the activity of the LPL system.

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Declaration of Competing Interest

The authors declare to have no competing interests.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.100865.

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The vitamin E long-chain metabolite α-13′-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system

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ABSTRACT

The α-tocopherol-derived long-chain metabolite (α-LCM) α-13′-carboxychromanol (α-13′-COOH) is formed via enzymatic degradation of α-tocopherol (α-TOH) in the liver. In the last decade, α-13′-COOH has emerged as a new regulatory metabolite revealing more potent or even different effects compared with its vitamin precursor α-TOH. The detection of α-13′-COOH in human serum has further strengthened the concept of its physiological relevance as a potential regulatory molecule. Here, we present a new facet on the interaction of α-13′-COOH with macrophage foam cell formation. We found that α-13′-COOH (5 μM) increases angiopoietin-like 4 (ANGPTL4) mRNA expression in human THP-1 macrophages in a time- and dose-dependent manner, while α-TOH (100 μM) showed no effects. Interestingly, the mRNA level of lipoprotein lipase (LPL) was not influenced by α-13′-COOH, but α-TOH treatment led to a reduction of LPL mRNA expression. Both compounds also revealed different effects on protein level: while α-13′-COOH reduced the secreted amount of LPL protein via induction of ANGPTL4 cleavage, i.e. activation, the secreted amount of LPL in the α-TOH-treated samples was diminished due to the inhibition of mRNA expression. In line with this, both compounds reduced the catalytic activity of LPL. However, α-13′-COOH but not α-TOH attenuated VLDL-induced lipid accumulation by 35%. In conclusion, only α-13′-COOH revealed possible antiatherogenic effects due to the reduction of VLDL-induced foam cell formation in THP-1 macrophages. Our results provide further evidence for the role of α-13′-COOH as a functional metabolite of its vitamin E precursor.

1. Introduction

Almost 100 years after its initial discovery and classification as a vitamin in 1922, the biological functions and the benefit of vitamin E for human health remain a matter of debate [1]. On the occasion of this 100th anniversary in 2022, a new perspective on vitamin E and its metabolism has recently been postulated by a group of renowned scientists in the field [2]. It is suggested that especially the long-chain metabolites (LCMs) – the first metabolites formed in vitamin E metabolism – are not only side-products of vitamin E excess formed for excretion, but may represent activated and therefore functional molecules of their vitamin precursors [2]. This concept seems plausible, as it has already been accepted for other fat soluble vitamins like vitamins A and D [3–5].

The group of vitamin E is formed by eight compounds with high structural similarity, comprising a chromanol ring system linked to an aliphatic chain. Based on the saturation of the side chain, compounds are divided in tocopherols (TOHs) (saturated side chain) and tocotrienols (T3s) (unsaturated side chain). Furthermore, classification as α-, β-, γ- and δ-forms of TOHs and T3s is determined by the methylation pattern of the chromanol ring system. Tocopherols and T3s are metabolized in the liver, with α-TOH possessing the lowest catabolic rate of all vitamin

Abbreviations: 13′-COOH, 13′-carboxychromanol; 13′-OH, 13′-hydroxychromanol; ANGPTL, angiopoietin-like; CD36, cluster of differentiation 36; CYP, cytochrome P450; FBS, fetal bovine serum; FFA, free-fatty acid; FI, fluorescence intensity; GIP, glucose-dependent insulinotropic polypeptide; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HSPG, heparan sulfate proteoglycan; LCM, tocopherol-derived long-chain metabolite; LPL, lipoprotein lipase; LRP, low-density lipoprotein receptor-related protein; PBMC, peripheral blood mononuclear cells; PCSK, pro-protein convertase subtilisin/kexin; PPAR, peroxisome proliferator-activated receptor; PLIN2, perilipin-2; RFU, relative fluorescence units; RPMI, Roswell Park Memorial Institute; RT-qPCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; T3, tocotrienol; TOH, tocopherol; VLDL, very low-density lipoproteins.

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E forms [6]. Although the exact mechanisms of metabolic degradation remain poorly understood, it is generally accepted that oxidative modification of the side chain via cytochrome P450 (CYP)-dependent enzymes (CYP4F2 and CYP3A4) leads to the formation of the LCMs 13′-hydroxychromanol (13′-OH) and 13′-carboxychromanol (13′-COOH) (extensively reviewed in [5,6]).

Interestingly, only α-TOH seems to have vitamin property due to its ability to preserve fertility in rats as well as preventing *ataxia with vitamin E deficiency* (AVED) in humans, making it the most important form of vitamin E [7]. The health-promoting effects of α-TOH were traditionally attributed to its strong antioxidant potential, although more recent investigations have also revealed non-antioxidant functions, like the modulation of gene expression and enzyme activities (reviewed in [2]). However, in the last decade, α-13′-COOH has emerged as a new regulatory metabolite, revealing more potent or even different effects compared with its vitamin precursor (reviewed in [5]). The detection of α-13′-COOH in human serum has further strengthened the concept of its physiological relevance as a potential regulatory molecule [8]. Although the biological function of α-13′-COOH is far from being unraveled, several studies have shown an interaction with inflammation [9], cancer [10] and the handling of pharmaceuticals [11]. In addition, two recent investigations in human THP-1 macrophages have identified the lipid metabolism-related proteins cluster of differentiation 36 (CD36) and perilipin-2 (PLIN2) as further regulatory targets of α-13′-COOH [8,12]. While Wallert et al. revealed that α-13′-COOH is involved in the modulation of foam cell formation at least in parts via an upregulation of CD36 expression [8], Schmölz et al. showed that α-13′-COOH is able to protect cells from stearic acid-induced lipotoxicity by upregulation of PLIN2 expression [12]. Interestingly, both studies also provided evidence that α-13′-COOH could be involved in the regulation of cellular neutral lipid accumulation by a hitherto-unknown mechanism independent of CD36 and PLIN2.

Cellular neutral lipid accumulation is predominantly driven by free-fatty acids (FFAs), which are obtained from chylomicrons and very low-density lipoproteins (VLDL) via enzymatic processing of their core triglycerides by lipoprotein lipase (LPL) on the cellular surface [13]. In vivo, LPL is produced in parenchymal cells and transported towards the vascular surface by the glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1). Here, catalytically-active LPL dimers are stabilized due the interaction with GPIHBP1, heparan sulfate proteoglycans (HSPGs) and substrate lipoproteins (extensively reviewed in [14]). However, in vitro studies have revealed that besides the normal production mechanism of LPL in vivo, macrophages are also able to produce and secrete functional LPL [15,16]. Due to its key role in cellular energy supply as well as the modulation of plasma triglyceride levels, LPL activity is under tight regulatory control. Several members of the family of angiopoietin-like (ANGPTL) proteins – with ANGPTL4 being the most prominent form in human macrophages – have emerged as potent physiological inhibitors of LPL activity [17,18]. Among others, the expression of ANGPTL4 is induced by FFAs via peroxisome proliferator-activated receptors (PPARs) as part of a feedback mechanism to prevent lipid overload within the cells [19,20].

There is growing evidence that elevated levels of triglyceride-rich lipoproteins represent causal risk factors for the development of cardiovascular diseases, making the LPL system and its associated regulator proteins interesting targets for investigations in that field [21,22]. Especially in macrophages, elevated accumulation of lipids due to the enhanced degradation of triglyceride-rich lipoproteins and the subsequent uptake of FFAs can promote foam cell formation and therefore the development of atherosclerosis [22,23]. In line with this, macrophage-derived foam cells have been identified as the primary source of LPL in human atherosclerotic plaques [15]. The present study will therefore focus on the potential regulation of LPL and ANGPTL4 by α-TOH and its LCM α-13′-COOH, as well as the impact of both compounds on VLDL-induced neutral lipid accumulation in human THP-1 macrophages as an important part of the development of lipid-driven diseases.

2. Materials and methods

2.1. Ethics statement

The collection of blood samples was conducted according to the principles expressed in the Declaration of Helsinki. The study protocol was approved by the ethical committee of the Friedrich Schiller University Jena (registration no. 2019-1548).

2.2. Chemicals

Unless indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Darmstadt, Germany), Thermo Fisher Scientific (Darmstadt, Germany), or Merck Millipore (Darmstadt, Germany).

2.3. Isolation of garcinoic acid and semi-synthesis of α-13′-COOH

The LCM α-13′-COOH was obtained via semi-synthesis from the natural compound garcinoic acid, also known as δ-tocotrienolic acid (δ-T3-13′-COOH). Garcinoic acid was isolated from the seeds of *Garcinia kola E. Heckel*, which were a kind gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid and subsequent synthesis of α-13′-COOH were performed as described in [10,24]. A purity of α-13′-COOH higher than 95% was confirmed by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

2.4. Determination of compound concentrations for cell culture studies

The concentrations of α-TOH and α-13′-COOH were determined weekly by absorption measurement in pure ethanol with a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The wavelengths and attenuation coefficients used were 292 nm and $\varepsilon = 3060$ for both compounds.

2.5. Cell culture

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultivated in Roswell Park Memorial Institute (RPMI)-1640 cell culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) Superior and 0.1% (v/v) penicillin-streptomycin-glutamine (PSG) solution. Cells were cultured at 37 °C in a humidified 5% CO_2/95% air atmosphere. Subsequent differentiation into macrophages was initiated by adding 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 50 μM β-mercaptoethanol for 96 h. After 96 h, fully matured macrophages were incubated with serum-free supplemented RPMI-1640 and the test compounds as indicated in the figures and were harvested for further processing as described below.

2.6. Incubation

α-Tocopherol, α-13′-COOH, orlistat and GSK3787 (Cayman Chemical, Hamburg, Germany) were dissolved in DMSO. For incubation of cells, compounds were mixed with serum-free supplemented RPMI-1640 medium in the concentrations indicated in the figures.

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Qiagen, Venlo, Netherlands) including on-column DNase I (Qiagen) digestion as described in [25]. cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) and 500 ng/μl oligo-dT primers as described in [26].

2.8. Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) analyses were run on a LightCycler 480 II instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix as described in [26,27]. Primers for LPL, ANGPTL4 and RPL37A (reference gene) (Supplementary Table S1) were purchased from Thermo Fisher Scientific. PCR results were analyzed using the LightCycler software version 1.5.0.39 (Roche Diagnostics).

2.9. Immunoblotting

2.9.1. Sample preparation

2.9.1.1. Cell lysate. Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) containing 1% protease inhibitor and mixed 3:1 with SDS sample buffer (6.26% 1 M Tris-HCl, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue).

2.9.1.2. Cell culture supernatant. Protein precipitation was performed according to the protocol of Wessel and Flügge, with slight modifications [28]. A total volume of 700 µl cell culture supernatant was collected for each sample and mixed with 700 µl methanol as well as 175 µl chloroform by vortexing. Subsequent centrifugation at 13.000 ×*g* for 5 min using an Eppendorf 5417 R centrifuge (Eppendorf, Hamburg, Germany) resulted in the separation of three phases: (i) an upper aqueous/methanol phase, (ii) a protein layer, and (iii) a lower chloroform phase. The upper phase was carefully removed, and the remaining liquid was mixed with another 700 µl methanol by vortexing. After an additional centrifugation step at 13.000 ×*g* for 5 min, excess solvents were removed, and protein pellets were dried for 15 min at 55 °C. The samples were reconstituted in 80 µl 1× SDS sample buffer by resuspending and vortexing. After a short centrifugation, the samples were incubated at 70 °C for 5 min using an Eppendorf Themomixer comfort and subsequently placed on ice.

2.9.2. Western blotting

The proteins were separated by SDS-PAGE, using a 12% acrylamide gel and transferred to PVDF membrane (VWR, Darmstadt, Germany) using a transfer buffer containing 0.25 M Tris, 1.92 M glycine, 0.1% SDS and 20% methanol (pH 8.3). Primary antibodies against ANGPTL4 (rabbit anti-ANGPTL4; Abcam ab206420, Cambridge, UK; 1:500), LPL (rabbit anti-LPL; Abcam ab172953; 1:1000) and α-tubulin (mouse anti-α-tubulin clone B-5-1-2; BD Biosciences, Heidelberg, Germany; 1:5000) as well as secondary antibodies (swine anti-rabbit and rabbit anti-mouse labeled with horseradish peroxidase; DAKO, Hamburg, Germany; 1:5000) were used for investigating the proteins of interest. SignalBoost Immunoreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for enhancing chemiluminescence signals of ANGPTL4 and LPL antibodies, while the α-tubulin antibody was incubated in a hybridization buffer containing 0.5% milk powder and PBS (0.137 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4, pH 7.4).

2.9.3. Detection

For detection, Pierce ECL Western Blotting Substrate and CL-XPosure Films (Thermo Fisher Scientific) were applied. The exposure time for ANGPTL4 and LPL was 30 min, and for α-tubulin 1 min.

2.10. Isolation of very low-density lipoproteins

Very low-density lipoproteins were isolated from the blood of a fasted 28-year-old male and 31-year-old female with plasma triglyceride levels > 0.90 mmol/l, which was previously described as a suitable range for LPL assays [29,30]. For this, a total of 50 ml blood was collected in 9 ml EDTA-monovettes (Sarstedt, Nümbrecht, Deutschland). Blood samples were centrifuged at 1870 ×*g* for 10 min at 15 °C for plasma separation. Subsequently, plasma was transferred into 4 ml thickwall polycarbonate tubes (Beckmann Coulter, Brea, CA, USA) for ultracentrifugation. Ultracentrifugation was performed for 4 h at 15 °C and 269,200 ×g using an Optima LE 80 K ultracentrifuge (Beckmann Coulter) together with a Type 50.4 Ti rotor (Beckmann Coulter). Separated VLDL particles from both donors were pooled in 5 ml tubes (Eppendorf) and subsequently stored under nitrogen atmosphere at 4 °C. The protein concentration of the isolated VLDL was determined using Bio Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's operating instructions. Isolated VLDL was used within one week for the respective experimental procedures.

2.11. Measurement of lipoprotein lipase activity

Measurement of lipoprotein lipase activity was performed according to the procedures described in [30]. Human THP-1 monocytes were differentiated in a twelve-well cell culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) as described above, using 1×10^6 cells in 2 ml RPMI-1640 (plus 10% FBS and 0.1% PSG) per well. Subsequently, cell culture medium was removed and the fully matured macrophages were washed twice with PBS. Next, cells were pre-incubated according to the respective experimental design indicated in the figures in phenol red-free RPMI-1640 medium under serum-free conditions. Very low-density lipoproteins in a concentration equivalent to 50 µg/ml protein together with 0.5 µl quenched LPL substrate (Abcam ab214552) were added to the pre-incubated cells. The twelve-well plate was placed in the FLUOstar Omega microplate reader coupled to an atmospheric control unit (BMG Labtech), providing an atmosphere with 5% CO_2 at 37 °C. Fluorescence intensity (FI) of each well was determined hourly over 24 h at Ex/Em = 485/520 nm.

2.12. Flow cytometry to measure neutral lipids using Nile red

Measurement of cellular neutral lipid content was performed after completion of the LPL assay procedure. Cells were washed twice with PBS and detached by accutase treatment (30 min at 37 °C). Detached cells were collected in 2 ml tubes (Eppendorf), centrifuged (5 min, 400 ×*g*) and washed with PBS (this step was repeated twice). Next, cells were stained with 1 µg/ml Nile red solution, incubated for 10 min and washed again with PBS. Two additional wells with 1×10^6 cells (untreated and VLDL-treated) were included throughout the whole assay procedure to serve as unstained controls for Nile red staining. Flow cytometric analysis of neutral lipids was performed by measuring the emission at 570 to 590 nm using the Attune NxT Flow Cytometer (Thermo Fisher Scientific). Measurement results were analyzed using the Attune NxT software version 2.2.0.8543 (Thermo Fisher Scientific). A detailed description of the gating strategy used for generating flow cytometric data is provided in the Supplement.

2.13. Statistics

Data are presented as either means ± standard deviation (Figs. 3, 4, 5, 6, 7 B and C) or means ± standard error of the mean (Figs. 1, 2, and 7 A) of independent experiments as indicated. To test for statistical significance of the data shown in Figs. 1, 2, 3, 4, 6, 7 A and C, repeated measurement one-way Anova with Dunnett's post hoc test was performed using OriginPro software version 9.1G (OriginLab, Northampton, USA). To test for statistical significance of the data shown in Figs. 5 and 7 B, repeated measurement two-way Anova with Tukey's post hoc test was performed using SPSS software version 19.0 (IBM Deutschland GmbH, Ehningen, Germany). All experiments were performed in at least three independent biological replicates.

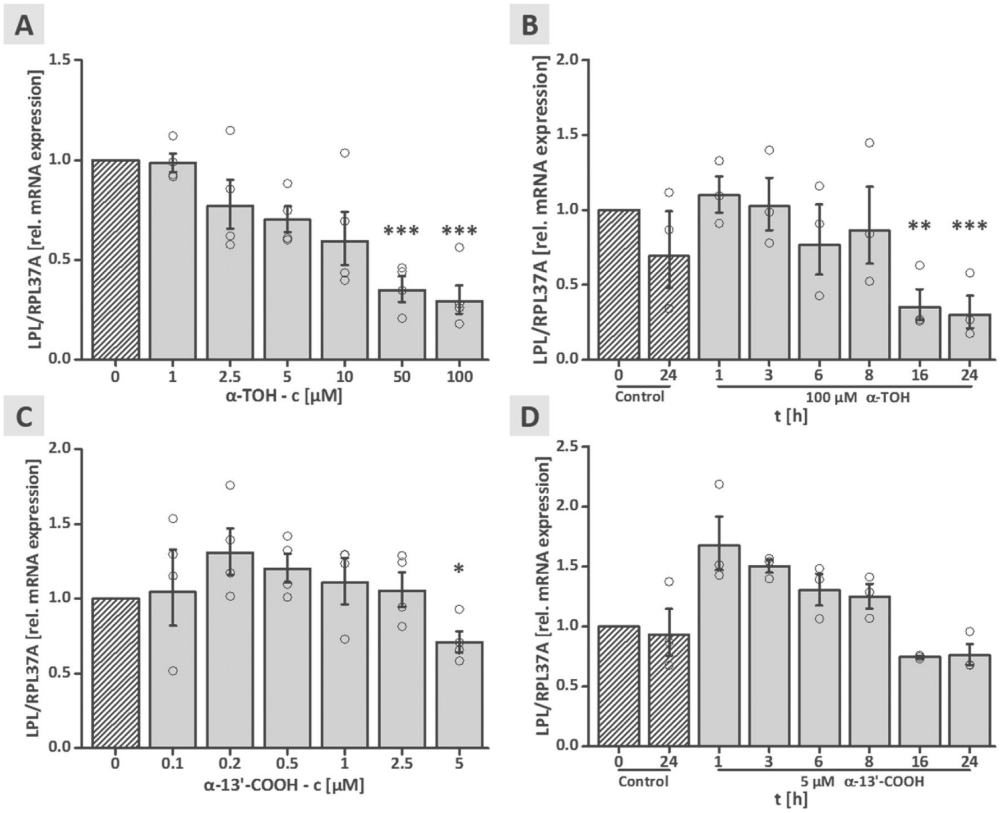

Fig. 1. LPL mRNA expression is reduced by α-TOH but not by α-13′-COOH. The influence of α-TOH and α-13′-COOH on LPL mRNA expression was investigated in a dose- and time-dependent manner under serum-free conditions. LPL mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Treatment with α-TOH in a concentration of 50 μM ($p < 0.001$) and 100 μM ($p < 0.001$) significantly reduced LPL mRNA expression, respectively. Lower α-TOH concentrations (2.5–10 μM) reduced LPL mRNA expression to a lower, non-significant extent (A). Time-dependent investigation of LPL mRNA expression confirmed the observed inhibitory effect of α-TOH at a concentration of 100 μM. Treatment with α-TOH significantly reduced LPL mRNA expression at the 16 h ($p < 0.01$) and 24 h ($p < 0.001$) time-points, respectively (B). In contrast to α-TOH, neither ascending concentrations of α-13′-COOH – aside from a small decrease with 5 μM – nor time-dependent investigation of the highest concentration used revealed significant effects of α-13′-COOH on LPL mRNA expression. Only marginal but non-significant induction of LPL mRNA expression was observed after a short time incubation with α-13′-COOH (1 and 3 h) at a concentration of 5 μM (C and D). Mean expression levels of four (A and C) or three (B and D) independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM). To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (A and C vs. control; B and D vs. 0 h control).

3. Results

The LCM α-13′-COOH emerged as a regulatory metabolite with specific effects that are more potent and even different to the effects of its vitamin precursor α-TOH [8–12,31]. Therefore, one of the main goals of the present study was to strengthen the new perspective on vitamin E metabolites as 'activated' or 'functional' metabolites by revealing another facet of their biological activity. It has recently been reported that α-13′-COOH is involved in regulating different aspects of the cellular lipid homeostasis – namely foam cell formation and lipotoxicity – via modulation of CD36 and PLIN2 expression [8,12]. In addition, there has also been evidence of the involvement of α-13′-COOH in regulating cellular neutral lipid accumulation by a hitherto-unknown mechanism independent of CD36 and PLIN2 (the reader is referred to section '1 Introduction'). Based on these results and the fact that lipid metabolism-related proteins represent regulatory targets of α-13′-COOH, the present study focused on the regulation of LPL and ANGPTL4 by α-TOH and its LCM α-13′-COOH. Furthermore, the impact of both compounds on VLDL-induced neutral lipid accumulation in human macrophages was investigated to extend the knowledge on foam cell formation from the previous works of Wallert et al. and Schmölz et al. [8,12].

3.1. LPL mRNA expression is reduced by α-TOH but not by α-13′-COOH

Lipoprotein lipase represents a key enzyme for the release of FFAs from triglyceride-rich lipoproteins and is therefore indirectly related to intracellular lipid storage [13]. Based on previous studies in THP-1 macrophages showing an impact of α-TOH and α-13′-COOH on neutral lipid accumulation, we investigated the effect of these compounds on LPL mRNA expression in a dose- and time-dependent manner by RT-qPCR (under serum-free conditions). First, human THP-1 macrophages were treated with ascending concentrations of either α-TOH (1–100 μM) or α-13′-COOH (0.1–5 μM) for 24 h. DMSO was used as the vehicle control (Fig. 1 A and C). In addition, THP-1 macrophages were harvested at different time points (1–24 h), using 100 μM α-TOH or 5 μM α-13′-COOH for incubation, respectively. DMSO vehicle control was prepared for the 0 h and 24 h time points (Fig. 1 B and D). Lipoprotein lipase mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown).

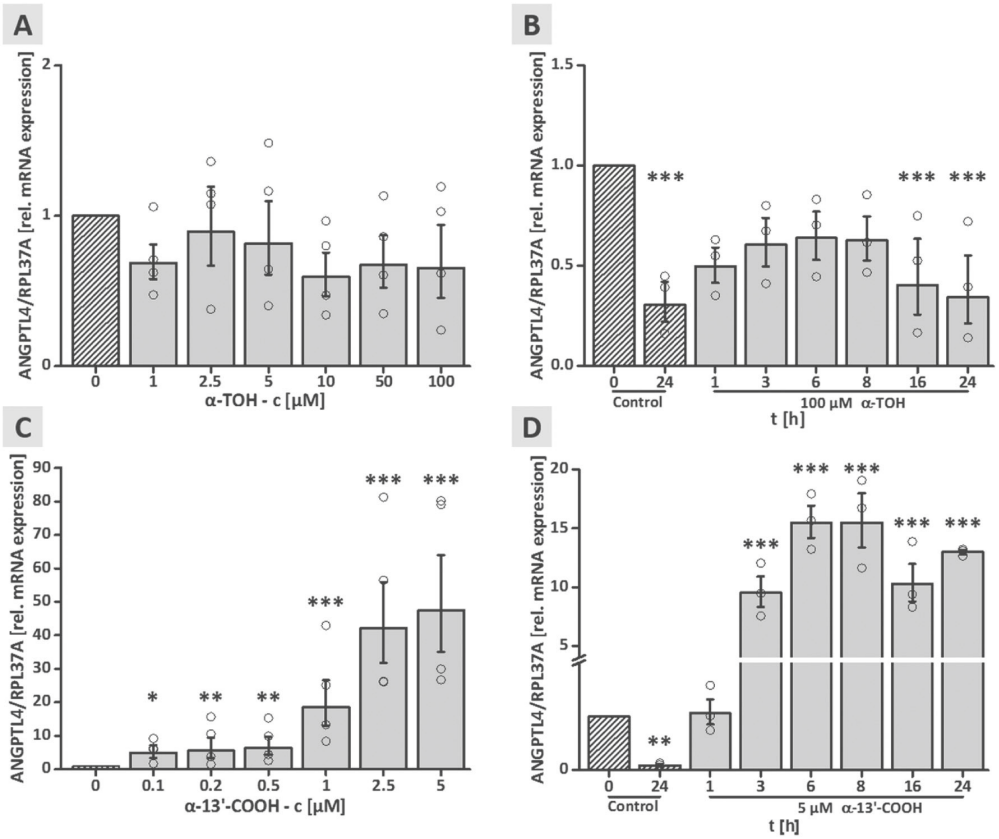

Fig. 2. ANGPTL4 mRNA expression is induced by α-13′-COOH but not by α-TOH. The influence of α-TOH and α-13′-COOH on ANGPTL4 mRNA expression was investigated in a dose- and time-dependent manner under serum-free conditions. ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Neither ascending concentrations nor time-dependent investigation of the highest concentration used revealed significant effects of α-TOH on ANGPTL4 mRNA expression (A and B). In tendency, α-TOH slightly reduced ANGPTL4 mRNA expression at all investigated concentrations, where the lowest (1 μM) and highest concentrations (100 μM) revealed nearly similar effects (A). Non-significant reduction of ANGPTL4 mRNA expression was also observed for time-dependent investigation after treatment with 100 μM α-TOH. Interestingly, ANGPTL4 mRNA expression was affected by serum-free conditions. Serum depletion for 24 h (24 h control) resulted in a significant reduction of ANGPTL4 mRNA expression ($p < 0.001$) compared to the 0 h control (B). In contrast to α-TOH, ascending concentrations of α-13′-COOH significantly enhanced ANGPTL4 mRNA expression in THP-1 macrophages ($p < 0.05$ to 0.001) (C). The effect of 5 μM α-13′-COOH was further investigated over different times. As already observed, 24 h serum depletion resulted in a significant reduction of ANGPTL4 mRNA expression compared with the 0 h control ($p < 0.01$). Nevertheless, treatment with 5 μM α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points – aside from 1 h ($p < 0.001$) (D). Mean expression levels of four (A and C) or three (B and D) independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM). To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (A and C vs. control; B and D vs. 0 h control).

Treatment with high concentrations of α-TOH (50 and 100 μM) significantly reduced LPL mRNA expression to 0.3-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.001$) and 0.3-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.001$), respectively. Lower concentrations of α-TOH (2.5–10 μM) had no significant effect on LPL mRNA expression (Fig. 1 A). Time-dependent investigation of LPL mRNA expression confirmed the observed inhibitory effect of α-TOH at a concentration of 100 μM. Treatment with α-TOH significantly reduced LPL mRNA expression at the 16 h and 24 h time points to 0.4-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.01$) and 0.4-fold (SEM min 0.01-fold, SEM max 0.1-fold, $p < 0.001$), respectively (Fig. 1 B). In contrast to α-TOH, neither ascending concentrations of α-13′-COOH – aside from a small decrease with 5 μM to 0.7-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.05$) – nor time-dependent investigation of the highest concentration used revealed significant effects on LPL mRNA expression. Only a marginal but non-significant induction of LPL mRNA expression was observed after a short incubation period with α-13′-COOH (1 and 3 h) at a concentration of 5 μM (Fig. 1 C and D).

3.2. ANGPTL4 mRNA expression is induced by α-13′-COOH but not by α-TOH

Based on the initial results on LPL mRNA expression, we further explored the impact of both compounds on the expression of ANGPTL4, a potent physiological inhibitor of LPL activity. An upregulation of ANGPTL4 mRNA expression by α-TOH or α-13′-COOH could also represent an effective way of regulating cellular lipid storage [32,33]. Therefore, the influence of α-TOH and α-13′-COOH on ANGPTL4 mRNA expression was investigated in dose- and time-dependent manner by RT-qPCR (under serum-free conditions). First, human THP-1 macrophages were treated with ascending concentrations of either α-TOH (1–100 μM) or α-13′-COOH (0.1–5 μM) for 24 h. DMSO was used as vehicle control (Fig. 2 A and C). Next, THP-1 macrophages were harvested at different time points (1–24 h), using 100 μM α-TOH or 5 μM α-13′-COOH for

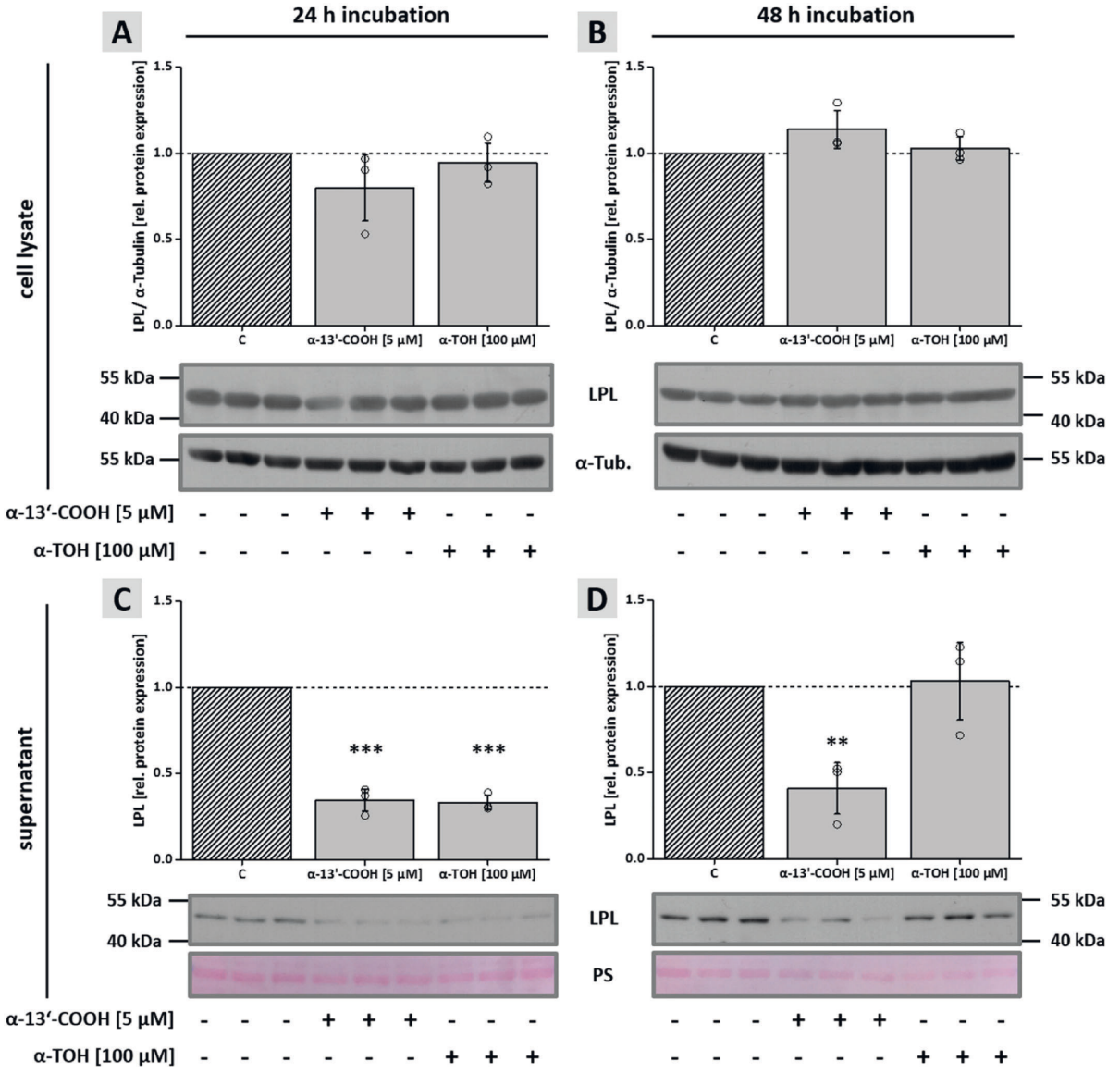

Fig. 3. The amount of secreted LPL protein is reduced by α-13′-COOH and α-TOH. Interestingly, expression of LPL protein (≈53 kDa) in the cell lysate was affected neither by α-TOH nor α-13′-COOH treatment after 24 h and 48 h incubation (A and B). However, Western blots of the corresponding cell culture supernatants revealed that both compounds affected the amount of secreted LPL protein (C and D). In line with the results from mRNA analysis, α-TOH treatment significantly reduced the secreted amount of LPL protein after 24 h compared with the vehicle control ($p < 0.001$) (C). After 48 h, the amount of secreted LPL was restored to the control level (D). Interestingly, α-13′-COOH also significantly reduced the secreted LPL amount after 24 h to the same extent as α-TOH compared with the vehicle control ($p < 0.001$) (C). In contrast to α-TOH, significant reduction of the secreted LPL amount was still present after 48 h in the α-13′-COOH-treated samples ($p < 0.01$) (D). Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control; lanes 4 to 6: 5 µM α-13′-COOH; lanes 7 to 9: 100 µM α-TOH). Mean relative protein expression levels of three independent biological experiments are shown (transparent points). Data is presented as means ± standard deviation. To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (vs. DMSO control). Abbreviations: α-Tub, α-tubulin; C, DMSO control; kDa, kilodalton; PS, Ponceau S staining.

incubation, respectively. DMSO vehicle control was prepared for the 0 h and 24 h time points (Fig. 2 B and D). ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Neither ascending concentrations nor time-dependent investigation of the highest concentration used revealed significant effects of α-TOH on ANGPTL4 mRNA expression. In tendency, α-TOH slightly reduced ANGPTL4 mRNA expression at all investigated concentrations, with the lowest (1 µM) and the highest concentration (100 µM) revealing nearly similar effects. Non-significant reduction of ANGPTL4 mRNA expression was also observed for time-dependent investigation after treatment with 100 µM α-TOH, aside from the late time points (16 h and 24 h). Interestingly, ANGPTL4 mRNA expression was affected by serum depletion. Serum-free conditions for 24 h (24 h control) resulted in a reduction of

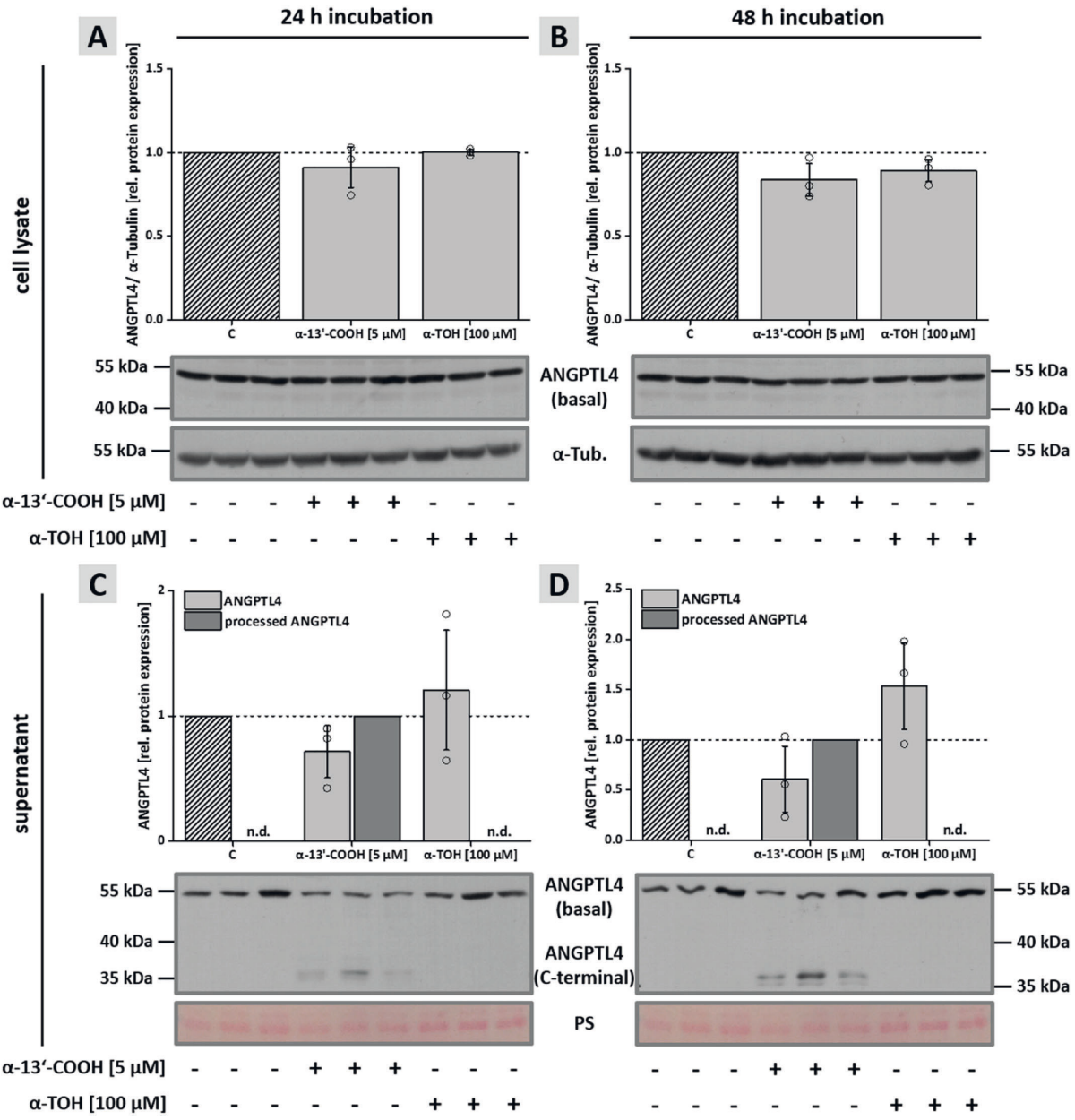

Fig. 4. Processing of ANGPTL4 protein is induced by α-13′-COOH but not by α-TOH. In line with the results from mRNA analysis, α-TOH treatment did not significantly affect ANGPTL4 (≈50 kDa) expression in cell lysates as well as secretion of ANGPTL4 protein to cell culture supernatant compared with the vehicle control (A to D). Surprisingly, α-13′-COOH also had no effect on ANGPTL4 protein expression in cell lysates after 24 h and 48 h incubation (A and B). Furthermore, Western blots of the corresponding cell culture supernatants revealed that α-13′-COOH slightly, but not significantly reduced the secretion of basal ANGPTL4 protein after 24 h and 48 h incubation (C and D). However, an additional band representing the C-terminal fragment of ANGPTL4 (≈37 kDa) could be exclusively detected in supernatants of α-13′-COOH treated samples. The amount of secreted C-terminal fragments further increased after 48 h incubation (C and D). Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control; lanes 4 to 6: 5 µM α-13′-COOH; lanes 7 to 9: 100 µM α-TOH). Mean relative protein expression levels of three independent biological experiments are shown (transparent points). Data is presented as means ± standard deviation. To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used (vs. DMSO control A and D, C and D only for basal ANGPTL4). For quantifying the amount of C-terminal ANGPTL4 cleavage product, α-13′-COOH treatment and not DMSO control was used as reference (C and D, processed ANGPTL4). For this, the relative amount of C-terminal ANGPTL4 in the α-13′-COOH treated samples were set to one as a fixed value, while the absence of C-terminal ANGPTL4 in DMSO control and α-TOH treated samples were marked as not detectable (n.d.). Because of the missing values for C-terminal ANGPTL4 in DMSO control and α-TOH treated samples, no calculation of statistical significance was possible (C and D, processed ANGPTL4). Abbreviations: α-Tub, α-tubulin; C, DMSO control; kDa, kilodalton; n.d., not detectable; PS, Ponceau S staining.

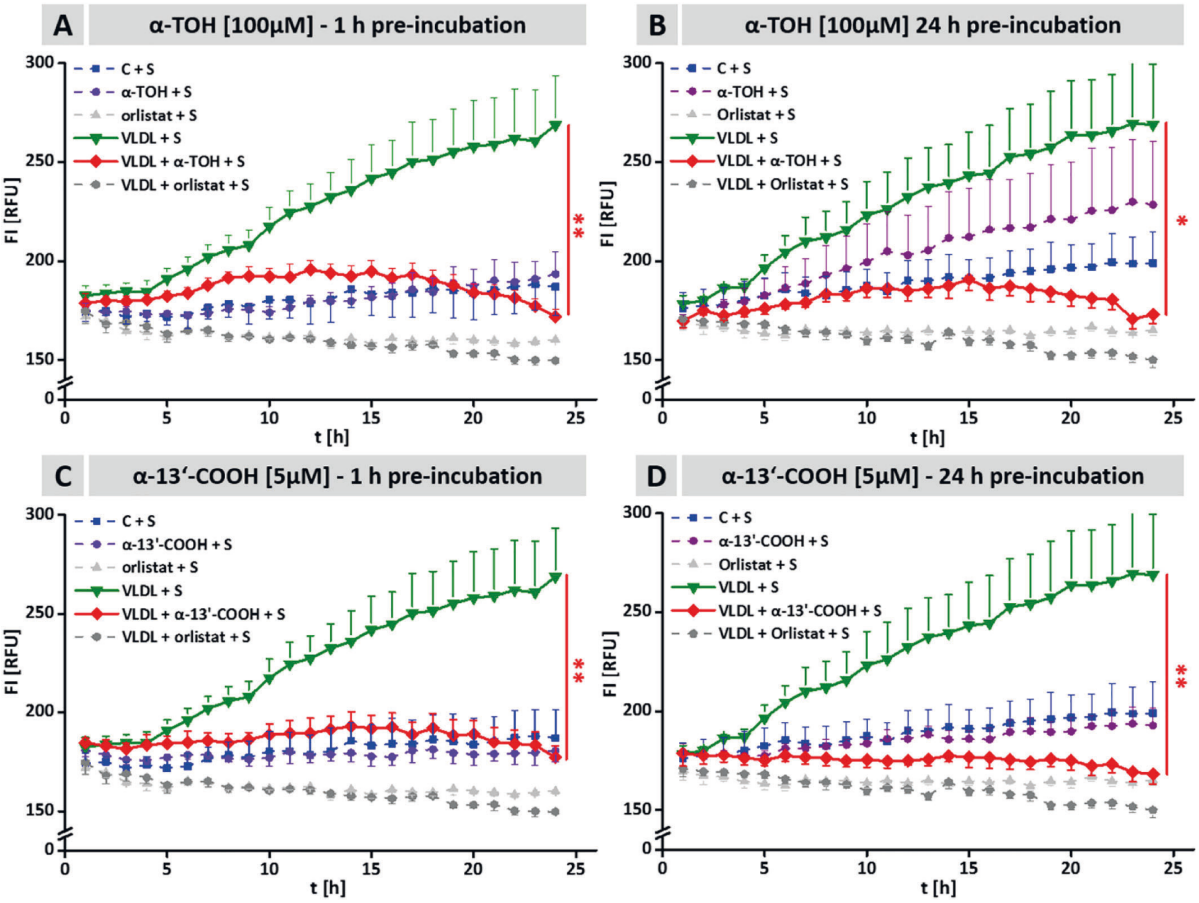

Fig. 5. Lipoprotein lipase activity is reduced by α-13′-COOH and α-TOH. Both α-TOH (100 µM) and α-13′-COOH (5 µM) appeared as potent inhibitors of VLDL-induced LPL activity. Already after 1 h pre-incubation with the two compounds, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH or α-13′-COOH evolved in a significantly different range ($p < 0.01$) (A and C). Elongation of the pre-incubation time with α-TOH or α-13′-COOH to 24 h revealed similar results. Again, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH ($p < 0.05$) or α-13′-COOH ($p < 0.01$) evolved in a significantly different range (B and D). As expected, orlistat (negative control) significantly blocked LPL activity compared with VLDL treatment and the vehicle control (significance not shown). Mean FI values of four independent biological experiments are shown. Data is presented as means ± standard deviation. In order to test for statistical significance, a repeated measurement two-way Anova with Tukey post hoc test was used. *, $p < 0.05$; **, $p < 0.01$ (vs. VLDL). Abbreviations: C, untreated control; FI, fluorescence intensity; RFU, relative fluorescence units; S, fluorescence-labeled LPL substrate.

ANGPTL4 mRNA expression to 0.4-fold (SEM min 0.1-fold, SEM max 0.2-fold, $p < 0.001$) compared to 0 h control, which almost corresponds to the values of 16 h and 24 h α-TOH treatment. Therefore, the observed time-dependent reduction of ANGPTL4 mRNA expression is probably not attributed to α-TOH but rather to serum depletion (Fig. 2 A and B). In contrast to α-TOH, ascending concentrations of α-13′-COOH significantly increased ANGPTL4 mRNA expression in THP-1 macrophages. Already 0.1 µM α-13′-COOH enhanced ANGPTL4 mRNA expression to 4.9-fold (SEM min 1.5-fold, SEM max 2.2-fold, $p < 0.05$), while treatment with 5 µM increased ANGPTL4 mRNA expression to 47.5-fold (SEM min 12.2-fold, SEM max 16.6-fold, $p < 0.001$) (Fig. 2 C). The effect of 5 µM α-13′-COOH was further investigated at different time points. As already observed, 24 h serum depletion resulted in a significant reduction of ANGPTL4 mRNA expression to 0.1-fold (SEM min 0.2-fold, SEM max 0.2-fold, $p < 0.001$) compared with the 0 h control. Nevertheless, treatment with 5 µM α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points – aside from 1 h – between 9.5-fold and 15.5-fold ($p < 0.001$). At the 24 h time point, α-13′-COOH treatment increased ANGPTL4 mRNA expression to 13.0-fold (SEM min 0.2-fold, SEM max 0.2-fold, $p < 0.001$). Time-dependent experiments showed a substantially lower increase of ANGPTL4 mRNA expression than dose-dependent experiments, indicating that the high induction of ANGPTL4 in dose-dependent experiments is partially the result of the 24 h serum depletion (Fig. 2 D).

3.3. The amount of secreted LPL protein is reduced by α-TOH and α-13′-COOH

In order to examine whether the observed changes in LPL mRNA expression are also present at the protein level, we performed Western blot analyses of cell lysates and cell culture supernatants of human THP-1 macrophages. Cell culture supernatants were analyzed because LPL protein is secreted in its monomeric form and subsequently anchored on the cellular surface by the interaction with GPIHBP1 and HSPGs. Here, LPL is converted to its catalytically-active dimeric form [14]. Hence, the reduced secretion of LPL monomers by α-TOH or α-13′-COOH treatment could also result in a reduction of LPL activity. In order to investigate LPL protein, human THP-1 macrophages were treated with 5 µM α-13′-

COOH or 100 μM α-TOH for 24 h or 48 h under serum-free conditions. DMSO was used as vehicle control. Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control, lanes 4 to 6: 5 μM α-13′-COOH, and lanes 7 to 9: 100 μM α-TOH). α-Tubulin and Ponceau S staining were used as loading controls for cell lysates and cell culture supernatants, respectively. Expression of LPL was detected using a recombinant antibody (Abcam ab172953) against the C-terminal residue of the protein. Interestingly, the expression of LPL protein (≈53 kDa) in the cell lysate was affected neither by α-TOH nor α-13′-COOH treatment after 24 h and 48 h incubation (Fig. 3 A and B). However, Western blots of the corresponding cell culture supernatants revealed that both compounds affected the amounts of secreted LPL protein. In line with the results from mRNA analyses, α-TOH treatment significantly reduced the secreted amount of LPL protein after 24 h to 0.33-fold ± 0.04-fold ($p < 0.001$) compared with the vehicle control (Fig. 3 C). After 48 h, the secreted amount of LPL protein was restored to the control level (Fig. 3 D). Surprisingly, α-13′-COOH also significantly reduced the secreted LPL amount after 24 h to the same extent as α-TOH (to 0.35-fold ± 0.06-fold, $p < 0.001$) compared with the vehicle control (Fig. 3 C). In contrast to α-TOH, the significant reduction of secreted LPL protein was still present after 48 h in the α-13′-COOH-treated samples (to 0.41-fold ± 0.15-fold, $p < 0.01$) (Fig. 3 D).

3.4. Processing of ANGPTL4 protein is induced by α-13′-COOH but not by α-TOH

Based on the observed induction of ANGPTL4 mRNA expression, we were interested in exploring whether the unexpected reduction of LPL protein secretion by α-13′-COOH could be explained by an ANGPTL4-dependent mechanism. The ANGPTL4 protein (≈50 kDa) comprises a C-terminal (≈37 kDa) and an N-terminal domain (≈15 kDa) with distinct functions. The N-terminal domain is responsible for the inhibition of LPL activity due to the conversion of catalytically-active LPL to the inactive form [34], while the C-terminal domain mediates anti-angiogenic functions [35]. Both domains are connected by a linker, which can be cleaved by pro-protein convertases subtilisin/kexin (PCSKs) [36]. In order to investigate whether ANGPTL4 expression and cleavage is affected by α-TOH or α-13′-COOH, we performed Western blot analyses of THP-1 cell lysates as well as cell culture supernatants. Cell culture supernatants were studied because ANGPTL4 is also a secreted protein that is expected to follow the similar route to the cell surface as LPL [37]. To ensure specific detection of the ANGPTL4 protein, we had to use a recombinant antibody (Abcam, ab206420) against the C-terminal region of the protein, although the N-terminal fragment would be of greater interest for the inhibition of LPL activity. Unfortunately, no specific recombinant antibody against the N-terminal fragment was commercially available. However, previous studies have shown that if the C-terminal fragment (≈37 kDa) can be detected, the N-terminal fragment (≈15 kDa) is also present [38]. For the investigation of ANGPTL4 protein, human THP-1 macrophages were treated with 5 μM α-13′-COOH or 100 μM α-TOH for 24 h or 48 h under serum-free conditions. DMSO was used as vehicle control. Each of the presented Western blot images comprises three independent biological replicates (lanes 1–3 DMSO control, lanes 4–6 5 μM α-13′-COOH, and lanes 7–9 100 μM α-TOH). α-Tubulin and Ponceau S staining were used as loading controls for cell lysate and cell culture supernatant, respectively.

In line with the results from mRNA analysis, α-TOH treatment did not significantly affect ANGPTL4 (≈50 kDa) expression in cell lysates as well as secretion of ANGPTL4 protein to cell culture supernatant compared with the vehicle control (Fig. 4 A to D). Surprisingly, α-13′-COOH also had no effect on ANGPTL4 protein expression in cell lysates after 24 h and 48 h incubation (Fig. 4 A and B). Furthermore, Western blots of the corresponding cell culture supernatants revealed that α-13′-COOH slightly, but not significantly reduced the secretion of basal ANGPTL4 protein after 24 h (to 0.71-fold ± 0.21-fold) and 48 h (to 0.61-fold ± 0.33-fold) incubation (Fig. 4 C and D). However, the most interesting observation was the appearance of an additional band, representing the C-terminal fragment of ANGPTL4 (≈37 kDa), exclusively in the supernatants of α-13′-COOH treated samples. The amount of C-terminal ANGPTL4 further increased with 48 h incubation time (Fig. 4 C and D). In summary, while α-13′-COOH-triggered induction of ANGPTL4 mRNA expression did not affect the expression of full-length ANGPTL4, the amount of the secreted processed C-terminal form was increased.

3.5. Lipoprotein lipase activity is reduced by α-TOH and α-13′-COOH

Based on the results from mRNA and protein investigations, α-TOH and α-13′-COOH seem to affect the LPL system through different mechanisms. The catalytic activity of LPL is under tight regulatory control at the transcriptional and post-translational level, among others by ANGPTL4 [32,39]. The function of LPL is predominantly considered to be anti-atherogenic [40]. However, LPL has also been associated with pro-atherogenic effects [41]. Especially in macrophages, enhanced degradation of triglyceride-rich lipoproteins and subsequent uptake of FFAs represents a risk factor for foam cell formation [17,22,23]. We were therefore interested in ascertaining whether α-TOH and α-13′-COOH affect the catalytic activity of LPL in human THP-1 macrophages. In our macrophage model, VLDL isolated from human donors was used as a physiological ligand for the stimulation of LPL activity. Furthermore, orlistat – a well-established and clinically-used LPL inhibitor – was included as a negative control [42]. In the experimental procedure, human THP-1 macrophages were pre-incubated with α-TOH (100 μM), α-13′-COOH (5 μM) or orlistat (50 μM, negative control) for 1 h (Fig. 5 A and C) or 24 h (Fig. 5 B and D) under serum-free conditions to investigate the effect of short- and long-term exposure to the two compounds. DMSO was used as vehicle control. Subsequently, VLDL in a concentration equivalent to 50 μg/ml protein together with 0.5 μl quenched, fluorescence-labeled LPL substrate was added to the pre-incubated cells. Analysis of LPL activity was carried out by hourly determination of fluorescence intensity as an equivalent for the processed substrate over 24 h.

Both α-TOH (100 μM) and α-13′-COOH (5 μM) appeared as potent inhibitors of VLDL-induced LPL activity. Already after 1 h pre-incubation with the two substances, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH or α-13′-COOH evolved in a significantly different range ($p < 0.01$) (Fig. 5 A and C). For α-TOH, the highest reduction of FI values was determined after 24 h from 268.8 ± 24.7 relative fluorescence units (RFU) in the VLDL-treated sample compared with 172.0 ± 3.7 RFU in the combination of α-TOH and VLDL (Fig. 5 A). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 268.8 ± 24.7 RFU in the VLDL-treated sample compared with 177.2 ± 5.8 RFU in the combination of α-13′-COOH and VLDL (Fig. 5 C). Elongation of the pre-incubation time with α-TOH or α-13′-COOH to 24 h revealed similar results. Again, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH ($p < 0.05$) or α-13′-COOH ($p < 0.01$) evolved in a significantly different range (Fig. 5 B and D). For α-TOH, the highest reduction of FI values was determined after 24 h from 269.0 ± 30.3 RFU in the VLDL-treated sample compared with 173.0 ± 4.7 RFU in the combination of α-TOH and VLDL (Fig. 5 B). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 269.0 ± 30.3 RFU in the VLDL-treated sample compared with 168.3 ± 5.2 RFU in the combination of α-13′-COOH and VLDL (Fig. 5 D). As expected, orlistat (negative control) significantly blocked LPL activity compared with VLDL treatment and the vehicle control (significance not shown). These results show that α-TOH and α-13′-COOH both inhibit the catalytic activity of LPL in human THP-1 macrophages.

3.6. VLDL-induced neutral lipid accumulation is attenuated by α-13′-COOH but not by α-TOH

It is well established that VLDL promotes the accumulation of neutral lipids in macrophages [43–45]. Furthermore, enhanced VLDL levels have been associated with pro-atherogenic effects like foam cell formation [22,46]. Based on the observed inhibition of LPL activity by α-TOH and α-13′-COOH, we were interested in ascertaining whether both compounds could attenuate neutral lipid accumulation in the presence of VLDL oversupply. Because the determination of neutral lipid accumulation was accomplished in the same sample, human THP-1 macrophages were treated as described in the experimental procedure for measuring LPL activity. Analyses were carried out by flow cytometry after Nile red staining. As expected, VLDL treatment significantly increased neutral lipid accumulation in human THP-1 macrophages compared with the vehicle control ($p < 0.001$). After 1 h pre-incubation with α-TOH (100 μM) or α-13′-COOH (5 μM), both compounds were unable to reduce VLDL-induced neutral lipid accumulation. Interestingly, co-incubation with α-TOH even increased the accumulation of neutral lipids to 114% ± 10% compared with VLDL alone (100%, $p < 0.01$) and with the combination of VLDL and α-13′-COOH (101% ± 5%, $p < 0.05$) (Fig. 6 A). After 24 h pre-incubation, VLDL-induced neutral

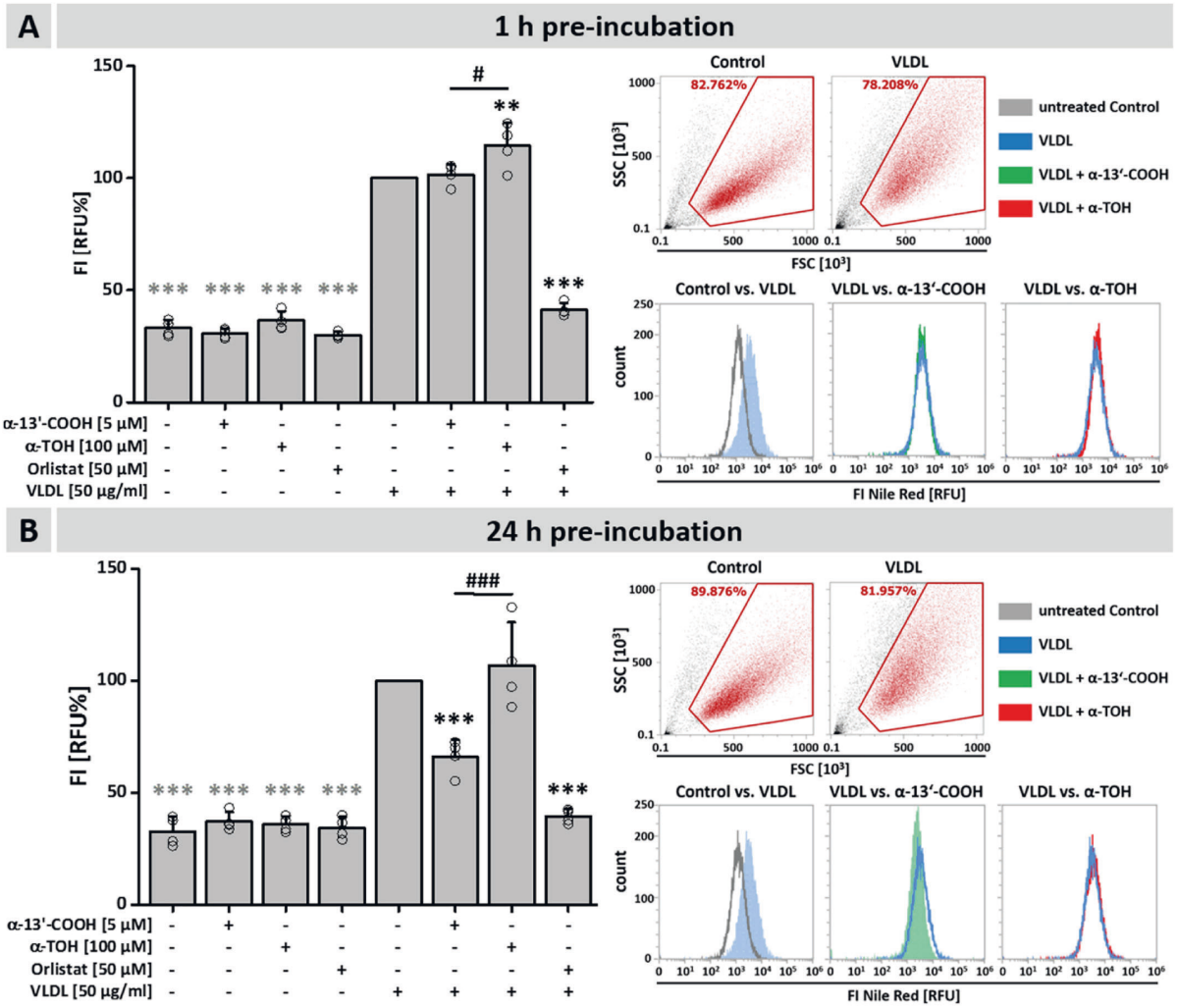

Fig. 6. VLDL-induced neutral lipid accumulation is attenuated by α-13′-COOH but not by α-TOH. As expected, VLDL treatment significantly increased neutral lipid accumulation in human THP-1 macrophages compared with the vehicle control ($p < 0.001$) (A and B). After 1 h pre-incubation, neither co-incubation with α-TOH (100 μM) nor α-13′-COOH (5 μM) reduced VLDL-induced neutral lipid accumulation. Interestingly, co-incubation with α-TOH even increased the accumulation of neutral lipids compared with VLDL alone ($p < 0.01$) and with the combination of VLDL and α-13′-COOH ($p < 0.05$) (A). After 24 h pre-incubation, VLDL-induced neutral lipid accumulation was significantly reduced by co-incubation with α-13′-COOH ($p < 0.001$). Interestingly, co-incubation with α-TOH again slightly increased neutral lipid accumulation compared with VLDL alone. There was also a significant difference between the effects of α-TOH and α-13′-COOH ($p < 0.001$) (B). As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages after 1 h and 24 h pre-incubation time ($p < 0.001$) (A and B). Mean FI values [%] of four independent biological experiments are shown (transparent points). Data is presented as means ± standard deviation. In order to test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (vs. control), $^{\#}$, $p < 0.05$, $^{\#\#\#}$, $p < 0.001$ (vs. α-TOH). The cell population of interest was identified using a gating strategy based on forward (FSC) and sideward scatter (SSC) data. Abbreviations: RFU, relative fluorescence units.

lipid accumulation was significantly reduced by co-incubation with α-13′-COOH to 66.0% ± 7.6% ($p < 0.001$). Surprisingly, co-incubation with α-TOH did not reduce neutral lipid accumulation, contrasting the expectation based on the LPL activity data. Again, co-incubation with α-TOH slightly increased neutral lipid accumulation to 106% ± 19% compared with VLDL alone. There was also a significant difference between the effect sizes of α-13′-COOH compared with α-TOH ($p < 0.001$) (Fig. 6 B). As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages after 1 h (41% ± 3%) and 24 h (40% ± 3%) pre-incubation time (p < 0.001) (Fig. 6 A and B). Taken together, VLDL-induced neutral lipid accumulation in THP-1 macrophages was attenuated by 24 h pre-incubation with α-13′-COOH, while α-TOH showed no effect.

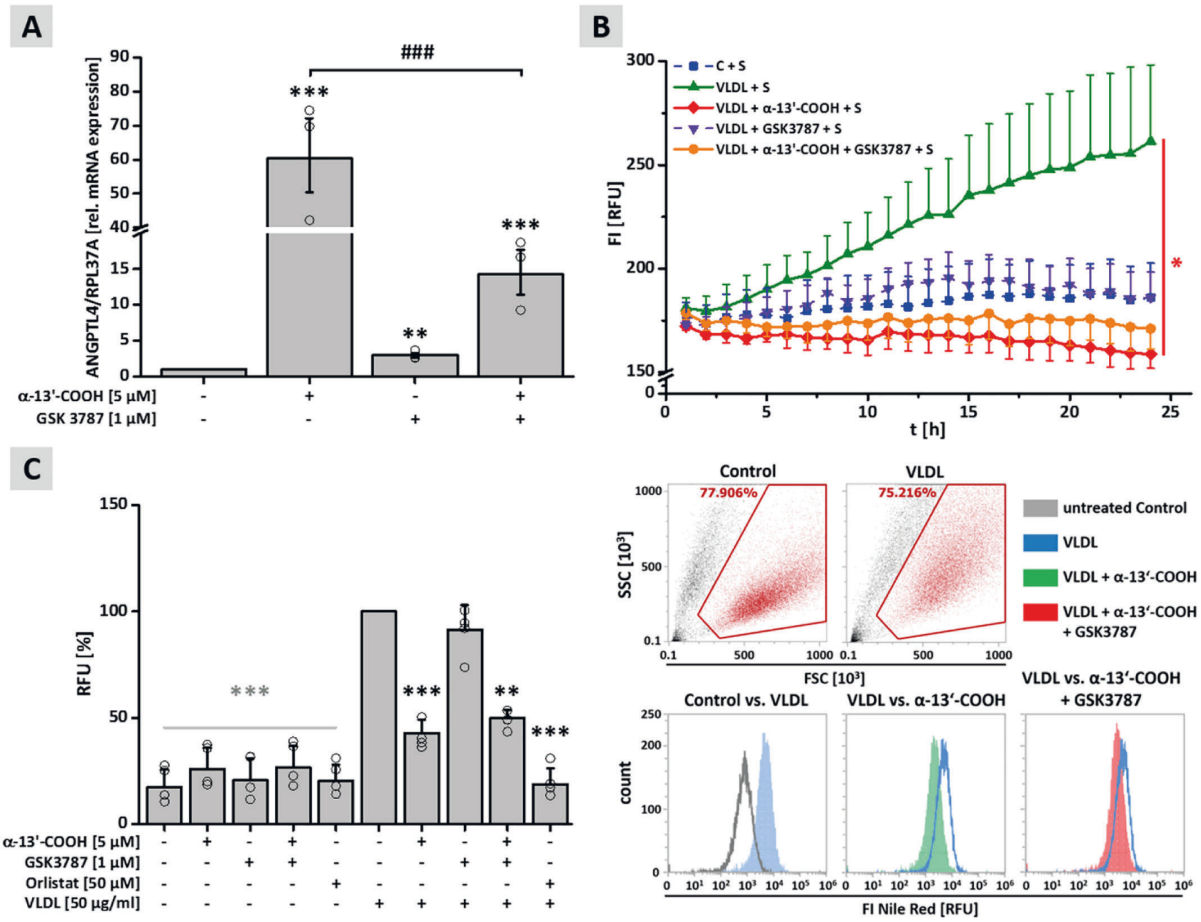

Fig. 7. PPARδ is involved in the transcriptional regulation of ANGPTL4 but does not affect the α-13′-COOH-mediated inhibition of LPL activity and neutral lipid accumulation. ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown) (A). As expected from our previous results, α-13′-COOH significantly increased ANGPTL4 mRNA expression (p < 0.001) compared with the vehicle control. However, the combination of α-13′-COOH and the PPARδ antagonist GSK3787 attenuated this effect, resulting in a significantly lower induction of ANGPTL4 mRNA expression (p < 0.001) compared with the vehicle control. Interestingly, the PPARδ antagonist GSK3787 alone also increased ANGPTL4 mRNA expression (p < 0.01) compared with the vehicle control (A).

Analysis of LPL activity was carried out by hourly determination of fluorescence intensity over 24 h (B), with subsequent analysis of neutral lipid accumulation via flow cytometry (C). As expected from our previous results, FI values of the VLDL-treated sample (positive control) and the combination with α-13′-COOH evolved in a significantly different range ($p < 0.05$). However, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on VLDL-induced LPL activity. For better clarity, VLDL-free treatments as well as orlistat negative controls are not shown. However, orlistat significantly blocked LPL activity compared with VLDL treatment and the vehicle control ($p < 0.001$) (B). As already observed, co-incubation with α-13′-COOH significantly reduced VLDL-induced neutral lipid accumulation (p < 0.001). However, in line with the results from the determination of LPL activity, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on VLDL-induced triglyceride accumulation. There was still a significant reduction ($p < 0.01$) in the combination of α-13′-COOH, GSK3787 and VLDL compared with the VLDL-treated samples. As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages (C). The cell population of interest was identified using a gating strategy based on forward (FSC) and sideward scatter (SSC) data.

Mean expression levels of three independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM) (A). Mean FI values of four independent biological experiments are shown (B). Mean FI values [%] of four independent biological experiments are shown (transparent points) (C). Data is presented as means ± standard deviation (B and C). In order to test for statistical significance, either a repeated measurement one-way Anova with Dunnett's post hoc test was performed using OriginPro 9.1G software (A and C) or a repeated measurement two-way Anova with Tukey post hoc test was performed using SPSS 19.0 software (B). *, $p < 0.05$; **, p < 0.01; ***, $p < 0.001$ (vs. control (A) or vs. VLDL (B and C)), $^{###}$, p < 0.001 (vs. α-13′-COOH (A)). Abbreviations: C, untreated control; FI, Fluorescence intensity; RFU, relative fluorescence units; S, fluorescence-labeled LPL substrate.

3.7. PPARδ is involved in the transcriptional regulation of ANGPTL4 but does not affect α-13′-COOH-mediated inhibition of LPL activity and neutral lipid accumulation

Due to the promising effects of α-13′-COOH in preventing excessive lipid accumulation in human THP-1 macrophages, we were further interested in ascertaining the signaling mechanism underlying these effects. We first investigated the transcriptional regulation of ANGPTL4, based on the strong induction of ANGPTL4 mRNA expression in response to α-13′-COOH treatment. It is well-established that transcriptional regulation of ANGPTL4 is predominantly mediated by PPARs [17,47–49], with PPARδ representing the predominant PPAR form in human THP-1 macrophages [50]. We were therefore interested in determining whether the stimulation of PPARδ by α-13′-COOH could be responsible for the observed induction of ANGPTL4 mRNA expression as well as the inhibition of LPL activity and triglyceride accumulation. For mRNA investigations, human THP-1 macrophages were pre-incubated with GSK3787 (1 μM) – a specific and potent antagonist for PPARδ [51] – for 1 h under serum-free conditions. Subsequently, α-13′-COOH (5 μM) was added to the pre-incubated cells for another 24 h. DMSO was used as vehicle control (Fig. 7 A). As expected from our previous results, α-13′-COOH significantly increased ANGPTL4 mRNA expression to 60.0-fold (SEM min 9.9-fold, SEM max 11.9-fold) ($p < 0.001$) compared with the vehicle control. However, the combination of α-13′-COOH and GSK3787 attenuated this effect, resulting in a lower induction of ANGPTL4 mRNA expression to only 14.2-fold (SEM min 2.8-fold, SEM max 3.5-fold) ($p < 0.001$) compared with the vehicle control. The difference of the effect sizes between α-13′-COOH alone and in combination with GSK3787 was also significant ($p < 0.001$). Interestingly, the PPARδ antagonist GSK3787 alone also increased ANGPTL4 mRNA expression to 2.9-fold (SEM min 0.3-fold, SEM max 0.34-fold) ($p < 0.01$) compared with the vehicle control. Hence, PPARδ seems to be at least partly involved in the α-13′-COOH-dependent induction of ANGPTL4 mRNA expression (Fig. 7 A).

Based on this promising result, we were further interested in whether co-incubation with GSK3787 could also reduce the α-13′-COOH mediated effects on LPL activity and neutral lipid accumulation. Therefore, human THP-1 macrophages were treated as described for the measurement of LPL activity with slight modifications. The α-TOH treatment was replaced by GSK3787. Furthermore, two additional samples with co-incubation of α-13′-COOH and GSK3787 were included in the experimental setup. These samples were pre-incubated with GSK3787 for 1 h and subsequently treated with α-13′-COOH for an additional 24 h. Analysis of LPL activity was carried out by hourly determination of FI over 24 h (Fig. 7 B), with subsequent analysis of neutral lipid accumulation via flow cytometry (Fig. 7 C). As expected from our previous results, FI values of the VLDL-treated sample (positive control) and the combination with α-13′-COOH evolved in a significantly different range ($p < 0.05$). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 261.5 ± 36.8 RFU in the VLDL-treated sample compared with 158.8 ± 6.9 RFU in the combination of α-13′-COOH and VLDL (Fig. 7 B). However, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on LPL activity. There was still a reduction of the FI values from 261.5 ± 36.8 RFU in the VLDL-treated sample compared with 171.0 ± 9.1 RFU in the combination of α-13′-COOH, GSK3787 and VLDL. However, orlistat significantly blocked LPL activity compared with VLDL treatment and the vehicle control ($p < 0.001$). For better clarity, VLDL-free treatments as well as orlistat negative controls are not shown (Fig. 7 B). As already observed, co-incubation with α-13′-COOH significantly reduced VLDL-induced neutral lipid accumulation to 42.8% ± 6.2% ($p < 0.001$). In line with the results from the determination of LPL activity, co-incubation of α-13′-COOH and GSK3787 was unable to attenuate the inhibitory effect of α-13′-COOH on VLDL-induced neutral lipid accumulation. There was still a significant reduction to 49.7% ± 4.1% ($p < 0.01$) in the combination of α-13′-COOH, GSK3787 and VLDL compared with the VLDL-treated samples. As expected, orlistat significantly blocked neutral lipid accumulation in human THP-1 macrophages (18.6% ± 7.5%) (Fig. 7 C). Taken together, the induction of ANGPTL4 mRNA expression by α-13′-COOH is diminished by pre-incubation with the PPARδ antagonist GSK3787. However, GSK3787 failed to attenuate the inhibitory effects of α-13′-COOH on LPL activity and neutral lipid accumulation.

4. Discussion

In a recently-postulated hypothesis on the significance of vitamin E metabolism in humans, LCMs were suggested as activated and therefore functional molecules of their vitamin precursors that must be taken into consideration for a correct interpretation of physiological effects of vitamin E in humans [2]. The concept of a central metabolic activation in the liver and the subsequent distribution of functional metabolites in the body is supported by the presence of α-13′-COOH in human serum [8]. Therefore, α-13′-COOH could theoretically be provided to various sites of action, including intimal macrophages, an important factor in the development of atherosclerosis. Two previous studies have already revealed an involvement of α-13′-COOH in the regulation of foam cell formation at least in part via the modulation of CD36 and PLIN2 expression [8,12]. However, there is also evidence of further mechanisms independent of CD36 and PLIN2. Using the above-mentioned investigations as a starting point, our aim was to elucidate the regulatory potential of α-13′-COOH and its vitamin precursor α-TOH on VLDL-mediated foam cell formation, focusing on the regulation of LPL and ANGPTL4.

As the regulatory potential of α-TOH and α-13′-COOH on lipid metabolism-related genes has already been shown in previous studies, we initially investigated the effect of both compounds on LPL and ANGPTL4 mRNA expression. Interestingly, the two compounds revealed different effects on the investigated genes. While α-TOH reduced LPL mRNA expression, it had no effect on the expression of ANGPTL4 (Figs. 1 and 2). By contrast, α-13′-COOH treatment strongly enhanced ANGPTL4 mRNA expression and did not affect LPL (Figs. 1 and 2). To the best of our knowledge, we are the first to describe an inhibition of LPL mRNA expression by α-TOH as well as the induction of ANGPTL4 mRNA expression by its LCM α-13′-COOH. Interestingly, ANGPTL4 mRNA expression was also affected by serum-free conditions. Especially in the α-TOH treated samples, the observed time-dependent reduction of ANGPTL4 mRNA expression is probably not attributed to compound treatment but rather to serum depletion. However, treatment with α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points, although the effect of serum depletion was present. Since α-TOH and α-13′-COOH revealed their strongest effects on the mRNA level at a non-toxic concentration of 100 μM or 5 μM, respectively, we decided to use these concentrations for further experiments. In addition, identical concentrations of both compounds have also been used in other studies [8,10–12,31].

Observations at the protein level revealed a more complex picture. Since LPL and ANGPTL4 are both secreted proteins, occurrence in cell lysates and cell culture supernatants was investigated. Surprisingly, the protein expression of LPL was not affected by α-TOH in cell lysates, although it would have been expected from the previous mRNA results. However, Western blots of the corresponding supernatant showed that α-TOH reduced the secretion of LPL monomers after 24 h pre-incubation (Fig. 3). Previous studies – especially in adipocytes – have shown that the modulation of LPL mRNA expression also results in changes of LPL protein synthesis or activity [52,53]. We therefore conclude that the reduced amount of LPL monomers in cell culture supernatant after 24 h α-TOH treatment probably resulted from diminished LPL mRNA expression. We further conclude that α-TOH mediates its inhibitory effect on LPL protein expression via direct transcriptional regulation, as the compound did not affect ANGPTL4 mRNA and protein expression (Fig. 4). In line with our mRNA results, α-13′-COOH treatment did not

affect LPL expression in cell lysates. However, the amount of LPL monomers in the cell culture supernatant diminished after 24 h and 48 h (Fig. 3). Intracellular expression of full-length ANGPTL4 (≈50 kDa) was also not affected by α-13′-COOH treatment, although it would have been expected from mRNA results. Interestingly, Western blots of the corresponding cell culture supernatants revealed an enhanced amount of C-terminal ANGPTL4 (≈37 kDa) (Fig. 4). It should be noted that we had to use a recombinant antibody against the C-terminal region of the protein to ensure specific detection. Unfortunately, we were unable to find a commercial antibody for the specific and reliable detection of the N-terminal ANGPTL4 fragment, although this fragment would have been of greater interest for LPL inhibition. However, it was shown that if the C-terminal fragment is detectable, the N-terminal fragment (≈15 kDa) is also present [38]. We conclude that the induction of ANGPTL4 mRNA expression by α-13′-COOH results in a subsequent increase of protein expression, although basal levels of full-length ANGPTL4 protein remained unchanged. The increase of ANGPTL4 protein amount is probably masked by enhanced processing and the subsequent secretion of cleavage products (C-terminal and the N-terminal fragments), indicated by the detection of C-terminal ANGPTL4 fragments in the supernatant of α-13′-COOH-treated samples. Makoveichuk et al. reported similar results in a study in human THP-1 macrophages. Here, treatment with the PPARδ agonist GW501516 enhanced ANGPTL4 mRNA expression, resulting in an increased secretion of the C-terminal ANGPTL4 fragments to the cell culture supernatant. Intracellular expression of full-length ANGPTL4 was not affected [17]. Furthermore, we hypothesize that the enhanced cleavage of ANGPTL4 after α-13′-COOH treatment is responsible for the reduced amount of LPL monomers in cell culture supernatant. The availability of a sufficient amount of LPL monomers on the cell surface is essential for the formation of dimeric LPL structures and therefore the functionality of the enzyme [14]. It is generally accepted that ANGPTL4 inhibits LPL due to the conversion of catalytically-active LPL dimers to inactive monomers [17,32,34,38,54], although the exact mechanism is a matter of debate. Among others, the most prominent explanatory approach is that ANGPTL4 is initially activated by cleavage of its full-length form. Afterwards, the N-terminal fragment of ANGPTL4 catalyzes a conformational switch from LPL dimers to inactive monomers on the cellular surface [34,54]. However, Dijk et al. provided evidence for a different mechanism, showing that ANGPTL4 promotes the degradation of LPL monomers. Therefore, enzymatic activity of LPL would be reduced due to the lesser availability of monomeric structures for the formation of catalytically active dimers. In their experiments, CHO-pgsA-745 cells were co-transfected with LPL and ANGPTL4. Processing of ANGPTL4 – indicated by the detection of C-terminal ANGPTL4 fragments in supernatant – reduced the extracellular amount of LPL monomers due to enhanced LPL degradation inside the cell [37]. In a later investigation, cleavage of ANGPTL4 was identified as the essential part of this mechanism, as ANGPTL4 silencing remarkably diminished the degradation of LPL in primary adipocytes [55]. In our own experiments, stimulation of ANGPTL4 cleavage by α-13′-COOH also reduced the amount of LPL monomers in cell culture supernatant. However, the intracellular amount of monomeric LPL remained unchanged. This was also observed by Dijk et al. after co-plating of CHO-pgsA-745 cells transfected with either ANGPTL4 or LPL. In this experimental setting, enhanced ANGPTL4 cleavage only reduced the amount of LPL monomers in the culture medium, while intracellular LPL was not affected [37]. The authors concluded that ANGPTL4 cleavage might also promote extracellular degradation of LPL monomers, which could also represent an explanation for our results. This concept is supported by another investigation by Dijk et al., showing that ANGPTL4 promotes LPL degradation through a PCSK-dependent mechanism. Due to the occurrence of PCSK5 and PCSK6 on the cellular surface [56,57], degradation of LPL by PCSKs outside the cell also seems plausible [55]. Nevertheless, α-13′-COOH treatment could also promote the intracellular degradation of LPL, resulting in a diminished secretion of LPL monomers to the cell culture supernatant, although no change of the intracellular LPL level was detectable. However, there is no proof for this hypothesis. Based on the observations of Dijk et al. and our own results, we conclude that α-13′-COOH promotes the degradation of LPL monomers via induction of ANGPTL4 cleavage, although the exact location and mechanism remains unclear. Therefore, α-13′-COOH mediates its inhibitory effect on LPL protein expression via a post-translational ANGPTL4-dependent mechanism.

It was further shown that cleavage of ANGPTL4 also improves its inhibitory potential on LPL activity [37,54]. Co-transfection of CHO-pgsA-745 cells expressing full-length ANGPTL4 together with LPL strongly reduced LPL activity in cell lysates and cell culture medium compared with cells without ANGPTL4 expression. Western blots of the cell culture medium revealed that the highest reduction of LPL activity was accomplished in the samples in which ANGPTL4 cleavage products (C-terminal fragments) were also detectable. In parallel, the amount of LPL monomers was reduced [37]. This is perfectly in line with our own results. Here, α-13′-COOH treatment strongly reduced VLDL-induced LPL activity after short-term (1 h) and long-term (24 h) pre-incubation (Fig. 5). We therefore conclude that α-13′-COOH reduces the catalytic activity of LPL due to the diminished availability of LPL monomers in the culture medium, resulting from an induction of ANGPTL4 cleavage and subsequent LPL degradation. Short-term (1 h) and long-term (24 h) pre-treatment with α-TOH reduced LPL activity to nearly the same extent as α-13′-COOH (Fig. 5). However, the inhibitory effect of α-TOH was accomplished by a different mechanism and at much higher concentrations. As already described, α-TOH treatment reduced LPL mRNA expression, resulting in the subsequent reduction of LPL monomers in cell culture supernatant, at least after 24 h incubation. Previous studies have revealed that the transcriptional regulation of LPL expression is also linked to the regulation of its catalytic activity [52,53]. In an adipocyte model, treatment with the gastrointestinal hormone glucose-dependent insulinotropic polypeptide (GIP) together with insulin enhanced LPL mRNA and protein expression, resulting in a subsequent induction of LPL activity. These effects were diminished after treatment with the transcription inhibitor actinomycin D, showing that the transcriptional regulation of LPL expression was responsible for the observed effects on LPL activity [53]. In line with this concept, a further investigation in adipocytes isolated from dexamethasone-treated Sprague-Dawley rats showed a reduction of LPL mRNA expression followed by a subsequent reduction of LPL activity [52]. We therefore conclude that α-TOH reduces the catalytic activity of LPL due to its inhibitory effect on LPL mRNA expression.

Changes in LPL activity are closely connected to changes in intracellular lipid accumulation, since LPL represents a key enzyme for the cellular supply with free-fatty acids from triglyceride-rich lipoproteins. Elevations of plasma VLDL in patients with diabetes mellitus type 2 or metabolic syndrome are associated with an increased risk of atherosclerosis [58]. It has also been shown that VLDL enhances lipid accumulation in vitro, making it a good test stimulus for our THP-1 macrophage model [59]. Based on the results of our previous experiments, we hypothesized that α-TOH and α-13′-COOH can prevent excessive intracellular lipid accumulation in human THP-1 macrophages in the presence of VLDL oversupply. Our hypothesis is strengthened by the observations of Wallert et al. on oxLDL-induced neutral lipid accumulation in THP-1 macrophages. Here, oxLDL oversupply increased cellular neutral lipid accumulation, while co-incubation with 5 μM α-13′-COOH dampened the effect [8]. Unfortunately, no data on the influence of α-TOH on neutral lipid accumulation in the presence of a comparable lipoprotein stimulus was available. However, Schmölz et al. showed that α-TOH as well as α-13′-COOH treatment alone enhanced neutral lipid accumulation in THP-1 macrophages [12]. As expected from our previous results, α-13′-COOH treatment diminished VLDL-induced lipid accumulation after 24 h pre-incubation, although there was no effect after 1 h pre-incubation (Fig. 6). We therefore conclude that α-13′-COOH reduced VLDL-induced lipid accumulation by an

ANGPTL4-dependent inhibition of LPL activity. Furthermore, a sufficiently long pre-incubation time with α-13′-COOH seems important for the development of its regulatory potential on lipid accumulation. However, α-TOH revealed contrary results to what would have been expected from our previous investigations. Although the catalytic activity of LPL was reduced, no reduction of VLDL-induced lipid accumulation was observed after 1 h and 24 h pre-incubation with α-TOH (Fig. 6). However, previous studies have shown that LPL is able to enhance cellular lipoprotein uptake via pathways independent of its catalytic activity, requiring LPL protein as a molecular bridge between lipoproteins and cellular receptors or proteoglycans [60–64]. As an example, Beisiegel et al. showed that HepG2 cells and fibroblasts absorbed triglyceride-rich lipoproteins – including chylomicron remnants and VLDL – via a low-density lipoprotein receptor-related protein (LRP)-dependent mechanism. In this study, LPL enhanced the binding of both lipoproteins to LRP. This bridging effect appeared to be independent of the catalytic activity of LPL but to be dependent on the protein itself [60]. In order to investigate the LPL bridging effect in vivo, Merkel et al. created a catalytically inactive form of human LPL by introducing a mutation in its catalytic domain. Selective expression of this inactive LPL in the muscle of *LPL*$^{-/-}$ mice resulted in a reduction of plasma VLDL and triglyceride levels by 33% compared with control mice. In line with this, triglyceride concentration in the muscle of mice expressing catalytically inactive LPL was also increased [61]. Based on the observations of Beisiegel et al. and Merkel et al., we conclude that the presence or absence of LPL protein on the cellular surface seems to be another important factor to modulate neutral lipid accumulation inside the cell, independent of its catalytic activity. Although α-TOH and α-13′-COOH inhibited LPL activity, only α-13′-COOH also diminished intracellular lipid accumulation, at least after long-term pre-incubation. A plausible explanation for this difference may be found in the comparison of LPL protein in the cell culture supernatant: while the amount of LPL monomers was still reduced after 48 h α-13′-COOH treatment, the secreted amount of LPL was restored to the control level in the α-TOH-treated samples. Based on the aforementioned studies, LPL-VLDL bridging potentially may increase intracellular lipid accumulation in the α-TOH-treated samples, although the catalytic activity of LPL was inhibited. However, it cannot be excluded that α-TOH enhances intracellular lipid accumulation through an LPL-independent mechanism.

Due to the promising effects of α-13′-COOH in preventing excessive lipid accumulation in human THP-1 macrophages, we were further interested in ascertaining the signaling mechanism underlying these effects. It is well established that transcriptional regulation of ANGPTL4 is predominantly mediated by PPARs [17,47–49]. The peroxisome proliferator-activated receptor δ represents the predominant PPAR form in human THP-1 macrophages, because its expression is increased during monocyte differentiation compared with the other PPAR forms [50]. Stimulation of THP-1 macrophages with PPARδ agonists also increased ANGPTL4 mRNA expression [17]. Interestingly, several studies have demonstrated that VLDL-induced lipid accumulation in macrophages is attenuated by a pre-treatment with PPARδ agonists [59,65]. However, the observations on this topic are contradictory. We hypothesize that α-13′-COOH can affect the LPL system as well as intracellular lipid accumulation through a PPARδ-dependent mechanism. In order to test this hypothesis, the effect of co-incubation of α-13′-COOH with GSK3787 – a selective antagonist of PPARδ [51] – was investigated on mRNA expression and at the functional level, i.e. measurement of LPL activity and neutral lipid accumulation. Although co-incubation with GSK3787 attenuated the induction of ANGPTL4 mRNA expression, GSK3787 failed to reduce the inhibitory effects of α-13′-COOH on LPL activity and neutral lipid accumulation. We therefore concluded that the induction of ANGPTL4 mRNA expression by PPARδ is at least one – but not the crucial – mechanism for α-13′-COOH-dependent regulation of LPL-mediated lipid homeostasis. However, it cannot be excluded that the increase of ANGPTL4 mRNA expression in the combination of α-13′-COOH and GSK3787 is still sufficient to induce the inhibitory effects of α-13′-COOH at the functional level. Based on the sparse knowledge about the interaction of α-13′-COOH with intracellular signaling pathways, we can only speculate about an alternative route to the PPARδ-mediated mechanism. In a previous investigation, Schmölz et al. provided convincing evidence that the specific effect of α-13′-COOH depends on its complete molecular structure, including the chromanol ring system, aliphatic side chain and the carboxylic group. If one of the three components was missing, no regulatory effect of α-13′-COOH could be observed [31]. Therefore, Schmölz et al. proposed the hypothesis of the existence of a specific, hitherto-unidentified cellular receptor for α-13′-COOH and other LCMs of vitamin E [12,31]. If this concept holds true, α-13′-COOH would be able to activate indirect signal transduction pathways, among others by G protein-coupled receptors or the activation of ion channels. Especially the activation of ion channels would be of interest, since calcium represents an important co-factor for the enzymatic activity of PCSKs [66,67]. In addition, it has been shown that ANGPTL4 promotes the degradation of LPL through a PCSK-dependent mechanism, independent of its transcriptional regulation [55]. Overall, the direct regulation via PPARδ seems unlikely, but based on the combination of the mentioned observations, α-13′-COOH could probably affect LPL-mediated lipid homeostasis via other signaling pathways, such as calcium-dependent mechanisms. However, further studies are needed to prove this hypothesis.

Despite having shown convincing effects of α-13′-COOH on the ANGPTL4-dependent regulation of cellular lipid homeostasis, there is still a limitation of the overall story. Although there are a variety of hints that ANGPTL4 is responsible for the α-13′-COOH-mediated effects on cellular lipid homeostasis, final validation via small interfering RNA (siRNA) knockdown of *ANGPTL4* is missing. Unfortunately, we were not able to establish a reliable *ANGPTL4* knockdown model in human THP-1 macrophages that can be combined with our LPL activity assay as well as the measurement of neutral lipid accumulation. The knockdown of *ANGPTL4* was impeded by the effect of serum depletion on ANGPTL4 mRNA expression. Hence, there was no detectable difference between basal expressions of *ANGPTL4* in the vehicle control compared with the *ANGPTL4* siRNA-transfected sample under serum-free conditions. In our opinion, ANGPTL4 mRNA expression level was already too low due to serum depletion. Furthermore, initial experiments (data not shown) revealed that *ANGPTL4* siRNA knockdown was unable to compensate the induction of ANGPTL4 mRNA expression by α-13′-COOH. Further, we like to point out that the human THP-1-like macrophage model used for the experiments presented here cannot cover the complex biochemical, physiological or metabolic processes affecting LCM functionality in living organisms. We are also aware that as an acute myeloid leukemia cell line, THP-1-like macrophages may have several differences compared to primary human macrophages, such as peripheral blood mononuclear cells (PBMCs). Nevertheless, since the main aim of the present study was to generate first data on fundamental regulatory processes mediated by the α-TOH derived LCM α-13′-COOH, the use of human THP-1-like macrophages as a reliable and easy to handle in vitro model was justified. Hence, the results generated in this study allow, at least in parts, conclusions to the in vivo situation.

5. Conclusion

Here we provide new insights into the regulatory role of α-13′-COOH and its vitamin precursor α-TOH in another facet of lipid metabolism. Interestingly, the two compounds affected the LPL system through different regulatory mechanisms: While α-TOH affected LPL expression via transcriptional regulation, α-13′-COOH triggered post-translational regulation of LPL via ANGPTL4. Furthermore, both molecules effectively reduced the catalytic activity of LPL. However, only α-13′-COOH was able to protect human THP-1 macrophages against excessive lipid accumulation in the presence of VLDL oversupply (Fig. 8).

As already observed by others [8,12,31], much higher doses of α-TOH (100 μM) were required to preserve regulatory effects compared

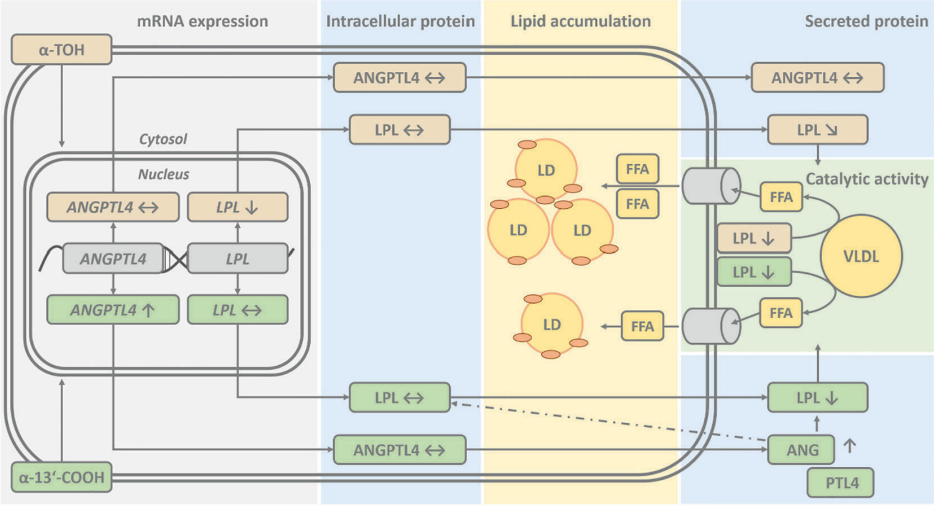

Fig. 8. Schematic overview on the proposed regulatory activities of α-13′-COOH and its precursor α-TOH on LPL-dependent foam cell formation of macrophages. We found that α-13′-COOH (5 μM) increases the expression of angiopoietin-like 4 (ANGPTL4) mRNA in human THP-1 macrophages in a time- and dose-dependent manner, while α-TOH (100 μM) shows no effect. In contrast, the mRNA of lipoprotein lipase (LPL) is not influenced by α-13′-COOH, but α-TOH reduces expression of LPL mRNA. Both compounds also reveal different effects on protein levels: while α-13′-COOH reduces the amount of secreted LPL protein via cleavage-mediated activation of ANGPTL4 protein, the amount of secreted LPL in the α-TOH-treated samples was diminished via direct transcriptional regulation. Further, both compounds reduce the catalytic activity of LPL. However, only α-13′-COOH (5 μM) but not α-TOH (100 μM) attenuate VLDL-induced lipid accumulation in the presence of VLDL. Abbreviations: FFA, free fatty acid; LD, lipid droplet; VLDL, very-low density lipoprotein.

with α-13′-COOH (5 μM). Although their exact mode of action remains unknown, our results support the concept that the LCMs of vitamin E – including α-13′-COOH – represent the activated and therefore functional forms of their vitamin precursors. Since only α-13′-COOH and not α-TOH prevented excessive lipid accumulation in macrophages, we also provide evidence for an atheroprotective potential of this regulatory metabolite due to the reduction of VLDL-induced foam cell formation. Nevertheless, further studies are required to elucidate the underlying regulatory pathways and the physiological relevance of α-13′-COOH.

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CRediT authorship contribution statement

Stefan Kluge: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Martin Schubert:** Writing – review & editing. **Lisa Börmel:** Investigation. **Stefan Lorkowski:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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Research Paper

The vitamin E derivative garcinoic acid from *Garcinia kola* nut seeds attenuates the inflammatory response

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ABSTRACT

The plant *Garcinia kola* is used in African ethno-medicine to treat various oxidation- and inflammation-related diseases but its bioactive compounds are not well characterized. Garcinoic acid (GA) is one of the few phytochemicals that have been isolated from *Garcinia kola*.

We investigated the anti-inflammatory potential of the methanol extract of *Garcinia* kola seeds (NE) and purified GA, as a major phytochemical in these seeds, in lipopolysaccharide (LPS)-activated mouse RAW264.7 macrophages and its anti-atherosclerotic potential in high fat diet fed ApoE$^{-/-}$ mice.

This study outlines an optimized procedure for the extraction and purification of GA from *Garcinia kola* seeds with an increased yield and a purity of > 99%. We found that LPS-induced upregulation of iNos and Cox2 expression, and the formation of the respective signaling molecules nitric oxide and prostanoids, were significantly diminished by both the NE and GA. In addition, GA treatment in mice decreased intra-plaque inflammation by attenuating nitrotyrosinylation. Further, modulation of lymphocyte sub-populations in blood and spleen have been detected, showing immune regulative properties of GA.

Our study provides molecular insights into the anti-inflammatory activities of *Garcinia* kola and reveals GA as promising natural lead for the development of multi-target drugs to treat inflammation-driven diseases.

1. Introduction

Natural products obtained from plants are widely used in folk medicine. The number of novel natural products described every year is large and systematic efforts are needed to elucidate their effectiveness and functions as bioactive principles or lead structures for drug development. A good example for the use of extracts in phytomedicine is the African plant *Garcinia kola* [1], which was first described for its antimicrobial properties by Hussain et al., in 1982 [2]. Until today several additional effects, such as radical scavenging [3], anti-oxidative [4] and anti-inflammatory properties [5], have been reported. Since this plant contains several bioactive compounds, namely garcinoic acid (GA) [6], it represents an interesting source to study putative pharmacological actions [7].

Our compound of interest, GA, also known as *trans*-13′-carboxy-δ-tocotrienol, contains an oxidative modification at its side chain and is a principle hepatic metabolite of dietary δ-tocotrienol (T3) [6]. In addition to tocopherols (TOH), T3s represent a less abundant form of vitamin E. TOHs and T3s, which differ in the saturation of the side chain, are further divided into α-, β-, γ- and δ-forms showing specific methylation patterns of the chromanol ring. However, in the last decades T3s have been described to potently interfere with inflammation and

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oxidative stress *in vitro* [8,9] and in animal models [10,11]. In addition, T3s affect macrophage recruitment [12] – a key event in atherosclerosis. In line with this data, anti-atherosclerotic effects of T3s have been shown in ApoE$^{-/-}$ mice by Shibata and colleagues [13].

Recent studies demonstrated that carboxylation of the side chain significantly increases the anti-inflammatory capacity of TOHs [14–16]. Similar effects have been demonstrated for GA, an oxidized δ-T3, which inhibits mPGES-1 *in vitro* [17]. Therefore, we investigated the anti-inflammatory effects of GA in comparison to the methanol extract of *Garcinia kola* seeds (NE) in LPS-activated RAW264.7 macrophages to elucidate the contribution of the latter phytochemical. Further, we studied the effectiveness of GA in decreasing inflammation-related formation of atherosclerotic plaques using an atherosclerotic mouse model to estimate the potential of GA as a promising new therapeutic lead molecule against inflammation-driven diseases.

2. Materials and Methods

2.1. Chemicals

If not indicated otherwise, chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), or Merck Millipore (Darmstadt, Germany).

2.2. Extraction of Garcinia kola seeds and isolation of GA

2.2.1. Standard preparation of NE

The standard preparation of the NE was performed according to published procedures [4,18,19] (Suppl. Fig. S1).

2.2.2. Optimized preparation of NE

NE from *Garcinia kola* seeds was obtained using Bligh and Dyer extraction [20]. Thus, 100 g crushed seeds and methanol/chloroform (400 ml/800 ml) were shaken for 4 h. After filtering, 400 ml of a 2% (w/v) NaCl solution was added and the mixture was shaken vigorously for 5 min. The chloroform phase was dried using Na_2SO_4 and the solvent was evaporated (Suppl. Fig. S1).

2.2.3. Isolation and purification of GA

Purification of GA was performed as reported with slight modifications [4,18,19]. In brief, seed extract was dissolved in methanol/chloroform (95%/5%, v/v) and applied to a silica gel column to isolate a crude product. Presence of GA in collected eluates was tested using thin-layer chromatography with dichloromethane/methanol (95%/5%, v/v) as solvent. Subsequently, re-chromatography of GA-containing aliquots was performed on a silica gel using a hexane/acetone (65%/35%, v/v) mixture. GA was characterized by high-performance liquid chromatography coupled with mass spectrometry (Fig. 1 and flow chart in Suppl. Fig. S1).

2.2.4. Liquid chromatography coupled with tandem MS (LC-MS/MS) analysis

The LC-MS/MS system consisted of a Dionex UltiMate 3000 UHPLC system coupled to a Bruker AmaZon SL Ion trap mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) source (Bruker, Karlsruhe, Germany). The chromatography utilized a Kinetex F5 Core-Shell column (2.1 × 100 mm, 2.6 µm) from Phenomenex (Aschaffenburg, Germany) connected to a SecurityGuard ULTRA cartridge (Phenomenex). The solvent system consisted of methanol/formic acid (1000:1 v/v, A) and H_2O/formic acid (1000:1 v/v, B). The separation was performed with a multi-step gradient scheme as follows: 0 min, 70% B; 3 min, 70% B; 5 min, 80% B; 10 min 80% B; 12 min, 90% B; 18 min, 90% B; 20 min, 100% B (flow rate 0.2 ml/min). Data were analyzed using basic peak monitoring and negative polarity APCI (dry gas temperature: 250 °C, flow: 4.2 l/min; nebulizer pressure: 34.8 psi; vaporizer temperature: 380 °C; capillary voltage: 4000 V; end plate offset: 500 V) with Bruker Compass Data Analysis software version 4.2.

2.3. RAW264.7 macrophage culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA) were cultivated as described previously [16]. For experiments, cells were incubated as indicated in the figure legends and harvested for further processing as described below. For detailed information, see the Suppl. Materials and Methods section.

2.4. Animal experiments and treatment

All animal procedures were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia (Ethic number E/1658/2016/B) and was performed in accordance with the Australian code for care. To investigate the effect of GA on the progression of atherosclerosis, eight weeks old male apolipoprotein E knockout (ApoE$^{-/-}$) mice (C57Bl/6 background, 25–28 g), ad libitum fed a HFD (22% fat and 0.15% cholesterol, SF00-219, Specialty Feeds, Western Australia, Suppl. Table S1) for further eight weeks, have been used. Mice were randomly assigned to receive 1 mg/kg body weight of GA (n = 9) or vehicle (PBS + 0.8% DMSO, n = 9) via intraperitoneal (IP) injection weekly. At the age of 16 weeks, mice have been anesthetized using ketamine (50 mg/kg, Parnell Laboratories, NSW, Australia) and xylazine (10 mg/kg, Troy Laboratories, NSW, Australia). Blood and tissue samples were collected and processed as described below. Grouping of animals and quantifications were blinded from the responsible researchers throughout the study.

2.5. RNA isolation, cDNA synthesis and quantitative real-time PCR (RT-qPCR)

Total RNA isolation (Qiagen, Hilden, Germany), cDNA synthesis (Fermentas, St. Leon-Rot, Germany) and RT-qPCR analysis (LightCycler 480 II instrument, Roche Diagnostics, Mannheim, Germany) have been performed as described previously [16]. Primers (Suppl. Table S2) were purchased from Invitrogen (Karlsruhe, Germany).

2.6. Immunoblotting

Cell harvesting, sample preparation and antibody usage are according to Wallert et al. [16] For the detection of α-tubulin (55 kDa), Cox2 (72 kDa) and iNOS (130 kDa), PageRuler™ Prestained Protein Ladder (10–180 kDa) from Thermo Fisher Scientific (Schwerte, Germany) was used.

2.7. Quantification of nitric oxide (NO) formation using Griess assay

Griess assay was used to measure nitrite in the supernatant of RAW264.7 macrophages (Enzo Life Science) and in murine plasma samples (Promega). Macrophages were incubated with either solvent (DMSO), 1.25 µg/ml NE or 2.5 µM GA in serum free high glucose DMEM for 4 h followed by a combined incubation with 100 ng/ml LPS for 20 h. For Griess assay, collected cell supernatants were prepared as outlined in Wallert et al. [16]. Murine plasma samples were prepared according to manufacturer's protocol. Absorbance was measured at 540 nm using a Fluostar omega plate reader (BMG Labtech, Offenbach, Germany and Adelaide, Australia).

2.8. Quantification of prostanoids using reversed phase UPLC-MS/MS and ELISA

Thromboxane (Tx)B_2 release from RAW264.7 macrophages was measured using an ELISA Kit from Enzo life Sciences (Lörrach, Germany) according to the manufacturer's protocol. In addition,

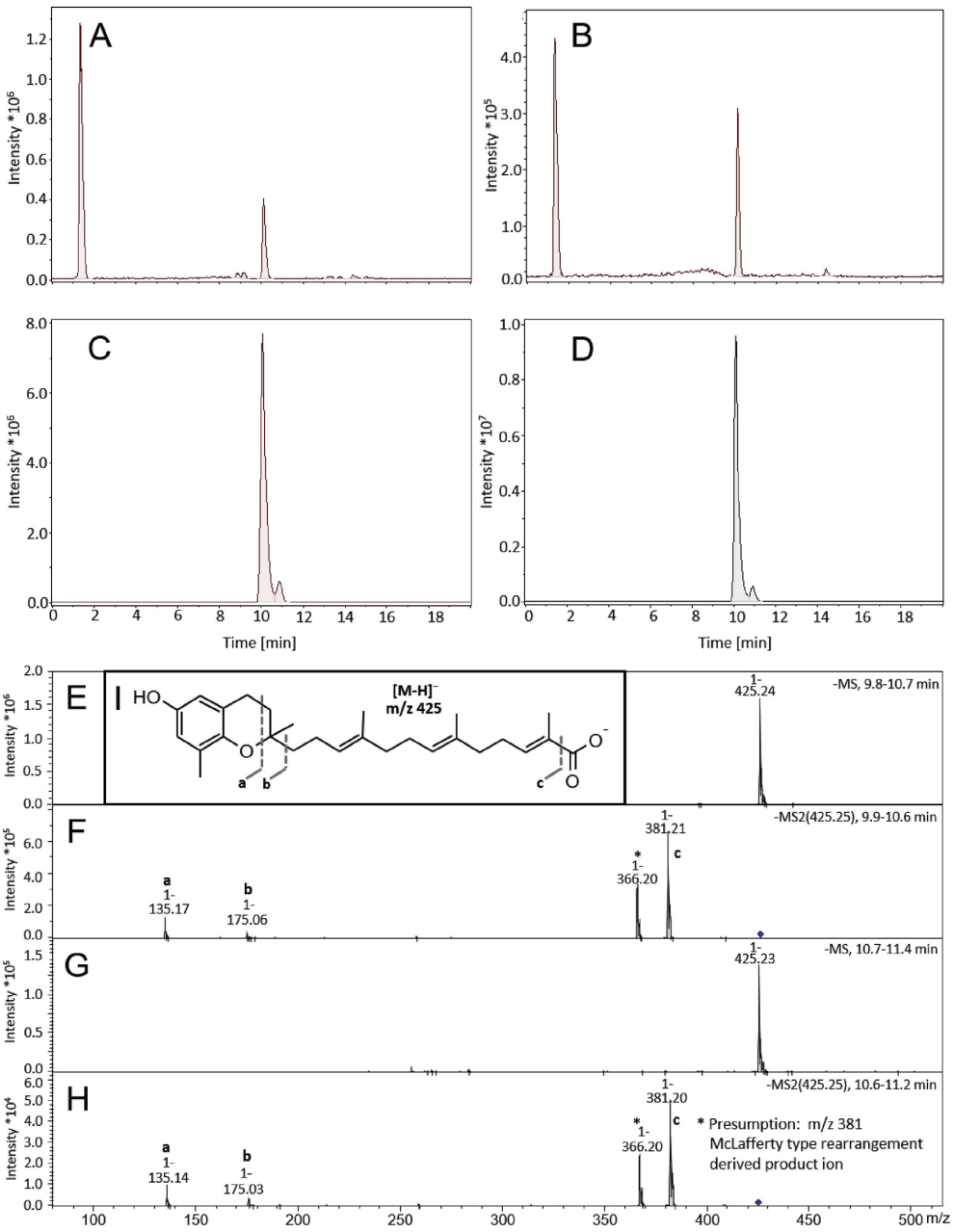

Fig. 1. Bligh and Dyer extraction increased the yield of garcinoic acid (GA) isolated from *Garcinia kola* seeds at high purity. Representative LC-MS chromatograms of the *Garcinia kola* seeds extracts obtained by the standard procedure **(A)** and by Bligh and Dyer extraction **(B)**. Panel **(C)** and **(D)** show LC-MS chromatograms of the purified GA obtained from crude methanol extract from *Garcinia kola* seeds according to the procedures used for **(A)** and **(B)**, respectively. Mass spectra of the purified GA were obtained from the LC-MS chromatogram **(D)** for two peaks with retention times of 9.8–10.6 min (main peak, **E**) and 10.7–11.4 min (minor peak, **G**). MS/MS fragmentation spectra of **(E)** and **(G)** are shown in panels **(F)** and **(H)**. The fragmentation is indicated on structure **(I)**, respectively.

prostaglandins and TxB_2 were extracted from RAW264.7 cell supernatants and murine plasma, separated on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters, Milford, MA) using an Acquity™ UPLC system (Waters), and detected using a QTRAP 5500 MS (Sciex, Darmstadt, Germany) equipped with an electrospray ionisation source as previously described [16].

2.9. Histology and immunohistochemistry

For histology and immunohistology transversal cryosections of O.C.T. embedded aortic sinus (6 µm) were prepared (Zeiss MICROM HM 550). For detailed information see Suppl. Materials and Methods section.

2.9.1. Histological staining

For H&E staining, fixed sections (70% EtOH, 10% formalin, 5% glacial acetic acid, 15% dH_2O, 5 min) were washed twice in 70% EtOH (30 s) and dH_2O (5 min) and stained with Mayer's Hematoxylin (2 min), followed by Puff's Eosin solution (20 s), dehydrated and cleaned using 100% EtOH and Xylene, respectively. Finally, samples were mounted with Depex to ensure their longevity. To stain collagen content, samples were fixed in 10% formalin for 20 min and placed in 0.1% PSR (0.5 g Sirius Red in 500 ml Picric Acid Solution) solution for 1 h. After differentiation with 0.01 M HCl, samples were washed, dehydrated, cleaned and mounted as described above. Lipid content was determined using isopropanol/dH_2O diluted (3:2 v/v) and filtered ORO. Samples were fixed (10% formalin, 4 min) and washed in 60% isopropanol (25 s) before staining with ORO (1 h), differentiated in 60% isopropanol, washed, counterstained with Mayer's hematoxylin (45 s) and mounted in Aquatex (Merck Millipore, Bayswater, VIC, Australia).

2.9.2. Immunohistochemical staining

Quantification of plaque stability, intra-plaque inflammation and cell infiltration was determined using antibodies against nitrotyrosine, Interleukin (IL)1β, vascular cell adhesion protein (VCAM)-1, monocyte chemoattractant protein (MCP)-1 and the macrophage marker CD68. Fixed sections were washed with PBS, blocked with 3% hydrogen peroxide, washed with PBS supplemented with 0.05% Tween20 and blocked with 10% horse or rabbit serum according to host of antibody species. After Avidin and Biotin blocking (Vector Laboratories, Burlingame, CA, USA), except for IL1β and MCP-1 staining, primary antibodies, diluted in blocking solution, were applied. Afterwards, washed samples were incubated with the respective secondary antibody diluted in blocking solution for 30 min, followed by conversion of the chromophoric horseradish peroxidase substrate diaminobenzidine (Vector Laboratories). Finally, samples were counterstained, dehydrated with 95% EtOH followed by 100% EtOH (twice), cleaned using Xylene (twice) and mounted with Depex. Respective IgG and omit antibody controls have been performed (Suppl. Fig. S2). Positive stained area of four sections per sample were analyzed using OPTIMAS version 6.2 VideoPro-32 system.

2.10. Flow cytometer analysis to investigate immunologic cell pattern

Antibodies were purchased from BD Bioscience if not otherwise indicated. Blood was collected in 0.5 M anti-coagulant ethylenediaminetetraacetic acid (EDTA) using cardiac puncture. Spleen was removed and stored on ice (PBS, 2 mM EDTA, 0.1% bovine serum albumin, BSA). Within 1 h of collection, cells from blood and spleen have been isolated, red blood cells were lysed (BD FACS lysing solution), and remaining cells were filtered and plated for further staining. Total monocyte/macrophage population was detected using CD11b-FITC and CD115-PE-Cy7 antibodies (Biolegend, San Diego, CA, USA). In addition, Ly6C-PB staining was performed to separate pro- and anti-inflammatory monocytes sub-populations. B cell lymphocytes have been gated using CD19-PE (BD Bioscience) staining. T cell lymphocytes were categorized in CD4 (PB) and CD8 (PerCP) positive cells as well as natural killer T (NKT) cells via NK1.1-PE-Cy7 and T-cell receptor β (TCR-β) staining. NK1.1-PE-Cy7 positive and TCR-β negative cells were gated as natural killer (NK) cells (Suppl. Fig. S3). Cell populations were analyzed using flow cytometry (FACSCanto II, BD Biosciences, USA) and analyzed with BD FACS DIVA software version 8.0.1.

2.11. Lipid measurement

Blood samples were taken as described above and centrifuged (300 × *g*, 10 min, room temperature) to separate plasma within 1 h of collection. Total serum cholesterol, LDL, HDL and triglycerides were measured using COBAS Integra 400 Plus blood chemistry analyzer (Roche Diagnostics, Australia).

2.12. Statistics

Data are presented either as means ± standard deviation (SD) or as means ± standard error of the mean (SEM) of independent experiments. In order to test for statistical significance, paired Student's t-tests was performed using Microsoft Excel 2010. ANOVA followed by Tukey post-hoc tests with logarithmized values and one-way ANOVA with multiple comparisons were used as outlined in the respective figure legends.

3. Results

In the here outlined study we pursued two aims. Firstly, the optimization of the extraction procedure of GA starting from *Garcinia kola* seeds to ensure the most effective use of these rare seeds. Secondly, the characterization of GA, as one of the main phytochemicals in *Garcinia kola* seeds, by focusing on anti-inflammatory effects and its potential as a drug for the treatment of inflammation-driven diseases.

3.1. Bligh and Dyer extraction increases the yield of GA

The extraction method of GA from *Garcinia kola* seeds firstly published by Terashima et al. [4] and slightly modified by Birringer et al. yields up to 0.38% GA[18]. The isolation of the GA follows in principle two steps: (i) the methanol extraction of the seed, and (ii) the purification of GA. Using our optimized approach, we were able to increase the quantity of NE 2.3-fold and the purified GA 6.6-fold (Suppl. Fig. S1) and were able to highly purify GA by silica gel chromatography (> 99%). Chromatographic separation revealed a major peak with *m/z* 425.4 $[M\text{-}H^+]^-$ at a retention time (RT) of 10.2 min representing GA and a minor peak at a RT of 10.8 min with identical mass. As the MS/MS spectra of the two peaks showed identical fragmentation patterns, we assume that the minor compound is a stereoisomer (either the *cis/trans* or the diastereomeric form) of GA (Fig. 1).

3.2. Purified GA blocks the LPS-induced expression of inflammatory mediators in murine macrophages

Previous studies demonstrated anti-inflammatory potential of a *Garcinia kola* seeds extract in rats [21]. One of the major compounds in this extract is GA, a T3 metabolite [18]. It has been shown that T3s are highly potent in blocking the inflammatory response of macrophages [8]. Therefore, we investigated the effects of both the NE and purified GA on LPS-induced inflammatory response in murine RAW264.7 macrophages. First, we analyzed the effect of GA on the expression of classic LPS-responsive genes such as Il6, Il1β, Tnfα, Cox2 and iNos, which encode pro-inflammatory mediators (Suppl. Table S2). Neither the NE nor GA affected basal expression levels of these marker genes (Fig. 2A–F, white bars). As expected, LPS significantly induced the expression of the genes of interest. The LPS response was efficiently blocked by both the NE and even stronger by GA as indicated in the figures (Fig. 2A–F, grey bars). Expression of Tnfα was significantly blocked to similar extent by different concentrations of GA (Fig. 2A, right column), whereas the NE tended to decrease Tnfα expression (Fig. 2A, left column). GA blocked the LPS-induced expression of LPS-responsive genes Il6, Il1β, Cox2 and iNos dose-dependently in concentrations of 1, 2, and 5 μM. At a concentration of 5 μM, GA significantly decreased Il6, Il1β, Cox2 and iNos RNA expression to 30% ($p < 0.05$), 39% ($p < 0.001$), 30% ($p < 0.05$) and 3% ($p < 0.05$), respectively (Fig. 2B–E, right column).

3.3. NE and GA differently affect the LPS-induced upregulation of iNos and Cox2 protein expression and secretion of respective signaling molecules

Further, we investigated the effect of NE and GA on post-translational expression of iNos and Cox2. In non-stimulated RAW264.7

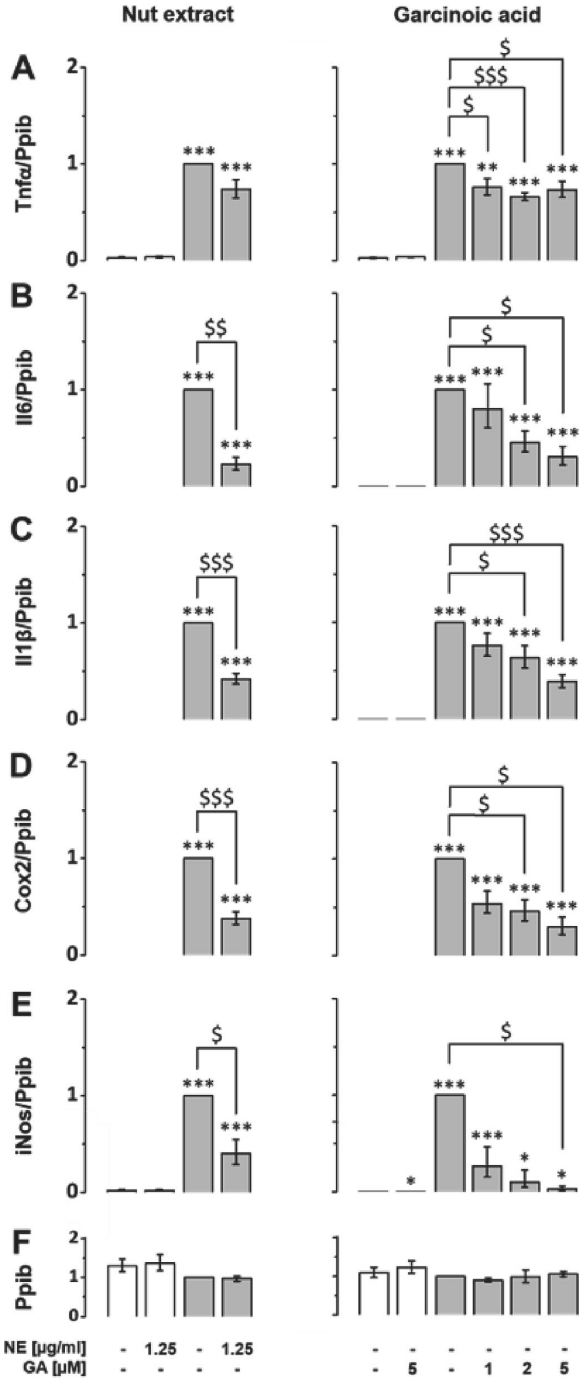

Fig. 2. Lipopolysaccharide-induced upregulation of Il6, Il1β, Cox2, iNos and Tnfα mRNA expression is blocked by the NE and isolated GA. RAW264.7 were pre-incubated with either NE (left column) GA (right column) or solvent (DMSO) for 24 h (white bars). For LPS-induced experiments, macrophages were co-incubated with 100 ng/ml LPS and either solvent, NE or GA at the doses indicated for another 24 h (grey bars). Samples co-cultured with solvent and LPS were defined as one. Expression levels of the inflammatory response genes **(A)** Tnfα, **(B)** Il6, **(C)** Il1β, **(D)** Cox2 and **(E)** iNos were measured using RT-qPCR and normalized to the mRNA expression of the reference gene **(F)** peptidylprolyl isomerase B (Ppib). Error bars display calculated minimum and maximum of SEM (SEM ± min, max) expression levels of four independent biological experiments, each measured in one or two technical replicates. *, $p < 0.05$; **$p < 0.01$; ***, $p < 0.001$ (*vs.* solvent control); $^{\$}$, $p < 0.05$; $^{\$\$}$, $p < 0.01$; $^{\$\$\$}$, $p < 0.001$ (*vs.* LPS treatment). Student's t-test was performed for statistical analysis.

macrophages, protein levels of iNos and Cox2 were neither detectable nor modulated by GA or NE. Treatment with NE and GA decreased LPS-induced protein expression of Cox2 to 62% ± 23% (left) and 67% ± 13% (right, $p < 0.0001$; Fig. 3A), respectively. The iNos protein expression was significantly diminished by NE (68% ± 10%, left) and GA (17% ± 11%, right, Fig. 3B, $p < 0.0001$) to a similar extent.

Since Cox2 and iNos expression regulate production and release of respective signaling molecules, the effect of NE and GA on the release of NO, TxB_2 and different prostanoids have been measured (Fig. 3C–E). While basal NO (Fig. 3C), TxB_2 (Fig. 3D) and prostaglandin (Fig. 3E, white bars) levels remained unchanged in the presence of NE and GA, treatment with LPS significantly elevates these signaling molecules in the supernatant of RAW264.7 macrophages ($p < 0.0001$, Fig. 3C–E). The LPS-induced production of NO was significantly decreased from 32.2 ± 3.7 µM (LPS control) to 26.0 ± 5.8 µM ($p < 0.05$) by NE and even more effectively by GA to 6.2 ± 5.7 µM ($p < 0.01$; Fig. 3C, grey bars). The release of TxB_2 was inhibited in LPS-activated macrophages by NE and GA to 37.3% ± 25.2% ($p < 0.01$) and 9.1% ± 7.8% ($p < 0.001$) remaining activity, respectively (Fig. 3D, grey bars). In addition, the effect of NE and GA on the release of different prostaglandins was measured by ultraperformance liquid chromatography-coupled tandem mass spectrometry (UPLC-MS/MS). Co-incubation of LPS and GA decreased the release of PGE_2 and PGD_2 (Fig. 3E) almost to baseline levels, 0.09 ± 0.03 RU ($p < 0.05$) and 0.05 ± 0.02 RU ($p < 0.01$), respectively, whereas NE decrease the prostaglandin levels to 0.53 ± 0.10 RU (PGE_2) and 0.49 ± 0.11 RU (PGD_2). To exclude that the potent inhibition of Cox-derived eicosanoids in LPS-activated macrophages depends on a direct inhibition of Cox isoenzymes, we determined the effect of GA on the activity of isolated bovine Cox1 and human recombinant COX2 in a cell-free assay. GA did not affect Cox1 and only weakly inhibited Cox2 (89 ± 13% residual activity) at a concentration of 10 µM (data not shown).

3.4. Local anti-inflammatory effects of GA in atherosclerotic lesions of aortic sinus

Based on the significant anti-inflammatory effects of GA shown here *in vitro*, we investigated its impact on the development of atherosclerotic plaques in male $ApoE^{-/-}$ mice fed with high fat diet (HFD). Under our experimental conditions body and organ weight (Suppl. Fig. S4A) as well as the plasma lipid profile (Suppl. Fig. S4B) of mice remained unchanged. Focusing on plaque morphology and stability, no significant differences were detected for morphological parameters including total lesion size, necrotic core area and lipid content analyzed using Hematoxylin and Eosin (H&E) and Oil Red O (ORO) staining, respectively. Collagen content (PSR), vascular cell adhesion protein (VCAM)-1 and cluster of differentiation (CD) 68, a marker for macrophage infiltration, remained unchanged (Fig. 4A). Analysis of intraplaque inflammatory profile revealed no change in monocyte chemoattractant protein (MCP)-1 and interleukin (IL)1β levels. However, treatment with GA significantly decreased nitrotyrosine level - a marker for inflammatory stress - in atherosclerotic plaques to 50% compared to the control group (Fig. 4B and Suppl. Fig. S2, $p < 0.05$).

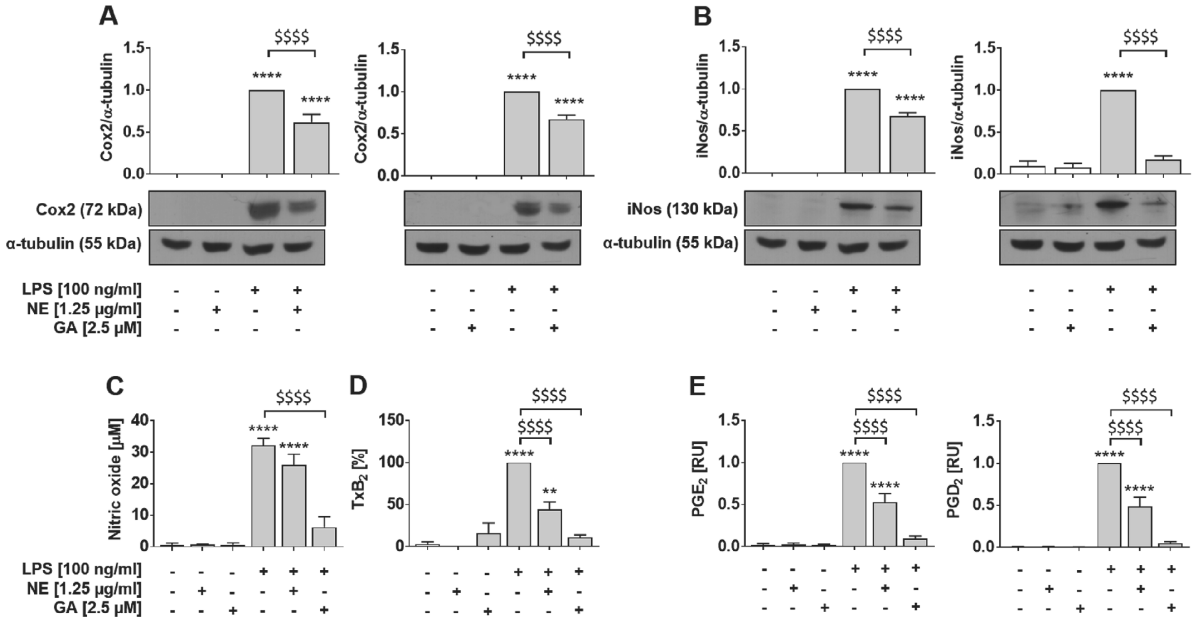

Fig. 3. Lipopolysaccharide-induced expression of iNos and Cox2 protein and secretion of respective signaling molecules are more potently blocked by GA compared to NE. RAW264.7 macrophages were incubated with either solvent (DMSO, white bars), NE or GA, or co-incubated with 100 ng/ml LPS (grey bars). The NE and GA decreased the protein expression of **(A)** Cox2 and **(B)** iNos after 24 h pre-incubation with NE or GA followed by 14 h and 24 h co-incubation with LPS, respectively. Samples incubated with LPS were defined as reference and were set as one. Protein levels were normalized to α-tubulin for quantification and representative Western blots are shown (for un-chopped versions see Suppl. Fig. S6). **(C)** Basal NO production, determined using Griess assay, were affected neither by the NE nor GA, whereas LPS-induced the formation of NO was significantly decreased by NE and even more effectively by purified GA. Treatment of RAW264.7 macrophages to measure released **(D)** TxB_2 and **(E)** PGs into culture supernatants followed the description in Fig. 2 except for use of 2.5 μM GA. Neither the NE nor GA altered the basal release of prostanoids. Treatment with LPS significantly induced TxB_2 and PG levels in the supernatant of macrophages and was set to 100% or one, respectively. Both, NE and GA decreased the release of TxB_2, PGE_2 and PGD_2 by LPS-activated macrophages Means of three independent biological experiments measured in two technical replicates **(A,B)**, three **(C)**, six **(D)** or four to five **(E)** independent biological experiments are shown; error bars display SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ ****, $p < 0.0001$ (*vs*. control); $, $p < 0.05$, $$, $p < 0.01$, $$$, $p < 0.001$, $$$$, $p < 0.0001$ (*vs*. LPS treatment). Student's t-test and ANOVA followed by Tukey post-hoc tests with logarithmized values was performed for statistical analysis.

3.5. GA does not affect systemic levels of iNos and Cox signaling molecules

As shown in Fig. 3, GA significantly blocked Cox2 and iNos pathways in LPS-activated macrophages. To determine if these effects occur in our atherosclerotic mouse model, plasma levels of NO (Fig. 5A) and prostanoids (Fig. 5B–D) have been measured. Baseline prostanoid concentrations in plasma increased under atherosclerotic conditions 8-fold (TxB_2, $p > 0.01$), 1.4-fold (6 keto $PGF_{1\alpha}$, n.s.) and 8-fold (PGE_2, $p > 0.001$, Suppl. Fig. S5). However, treatment with GA did neither alter NO levels (Fig. 5A) nor changed prostanoid levels in mice (Fig. 5B–D).

3.6. GA differentially affects systemic and localized inflammation

Changes in the distribution of immune cells such as lymphocytes and monocytes and the modulation of their sub-populations play a pivotal role in atherosclerosis. Systemic (blood) and local (spleen) cell population have been changed after GA treatment in at least partly different extent. In blood, the total population of monocytes/macrophages as well as the pro-inflammatory $Ly6c^{high}$ and the anti-inflammatory $Ly6c^{low}$ monocyte sub-population remain unchanged (Fig. 6A). There is no change in B cell and CD8 T cell population. However, CD4 positive T cells are significantly downregulated in the GA-treated group by 14.8% ($p < 0.05$) and the CD4/CD8 ratio decreases from 2.1 ± 0.1 (control) to 1.9 ± 0.05 (GA treatment, $p = 0.077$). Further, natural killer (NK) and natural killer T (NKT) cells were significantly up-regulated 3.2% ± 0.4% vs. 5.6% ± 0.8%; 0.2% ± 0.02% vs. 0.6% ± 0.2%, respectively ($p < 0.05$, control vs. GA-treated group). In contrast, local (spleen) population of analyzed cell types remained unchanged, except for NK cells which were significantly upregulated (2.0% ± 0.1% vs. 2.8% ± 0.1%; $p < 0.001$, control vs. GA treatment).

4. Discussion and conclusions

Garcinia kola is known in Africa as a 'traditional' medicinal plant [7]. One of the significant phytochemicals found in *Garcinia kola* seeds is GA [7,18,19]. In order to obtain high yields of GA from *Garcinia kola* seeds, we developed an optimized extraction procedure for GA to ensure the most effective use of the seeds. Using our improved approach, we significantly increased the yield (6.6 fold) and purity (> 99%) of extracted GA (Fig. 1 and Suppl. Fig. S1). Extracted GA is purer than the commercially available product as well as the GA received using former protocols, consequently indicating that the *Garcinia kola* seeds were utilized optimally by our extraction method. Having reasonable amounts of pure GA accessible opens new application possibilities, such as (i) *in vitro* and *in vivo* studies as well as (ii) using GA as a starting product for the synthesis of the long-chain metabolites of vitamin E (LCMs) as described by Mazzini et al. [6] Synthesis of the LCMs is essential to study their physiological role, as the LCMs are not commercially available.

The bioactivity of the *Garcinia kola* plant has been studied *in vitro* as well as in animal and human studies, as recently reviewed [22]. Different studies demonstrated the radical scavenging, anti-oxidative and

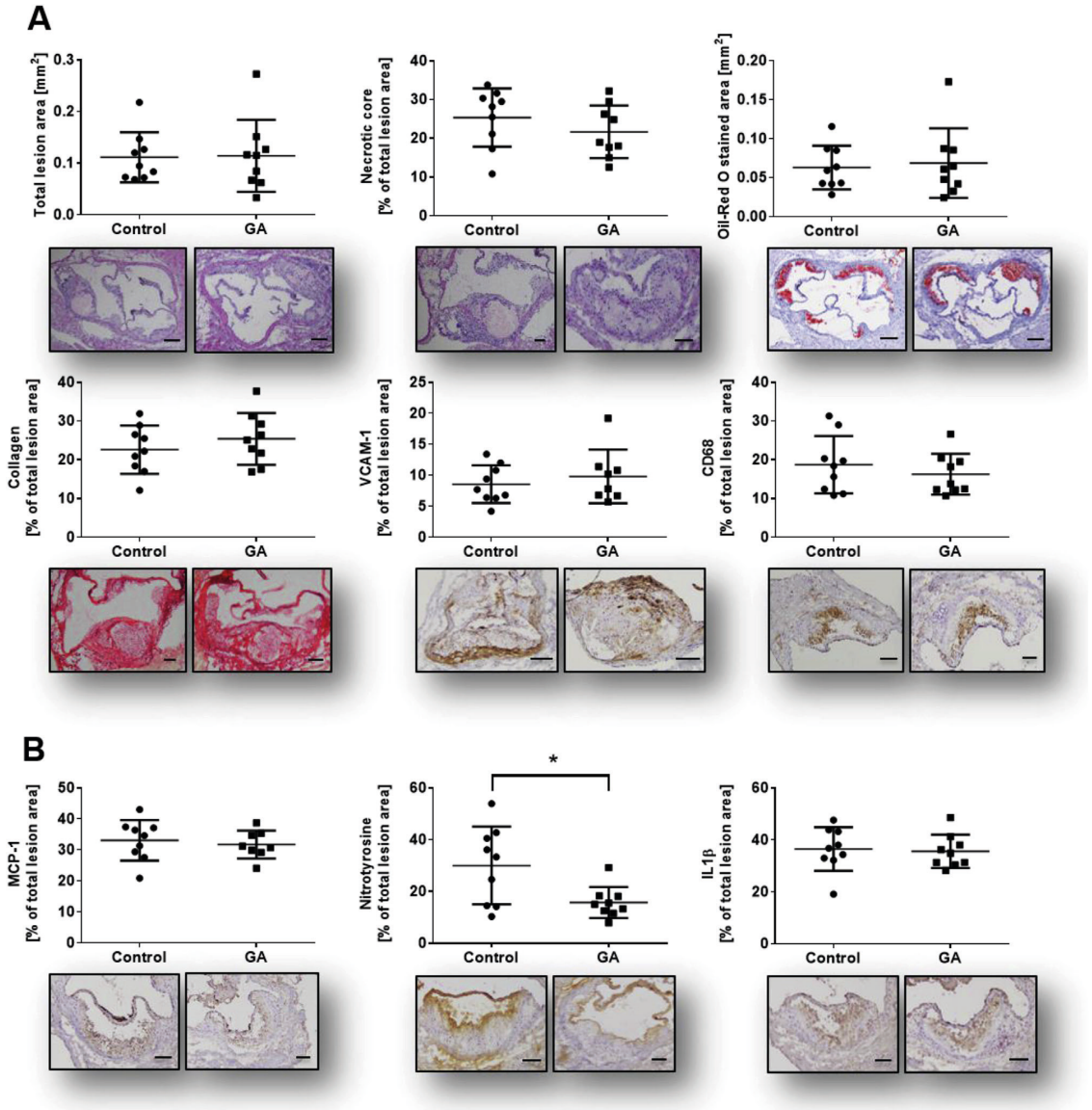

Fig. 4. Plaque morphology, stability and inflammatory profile of lesions. Frozen OCT embedded aortic sinus sections (6 μm) have been stained as follows: **(A)** Characterization of plaque morphology, stability and inflammation status has been analyzed using histological (Hematoxylin and Eosin; H&E, Oil Red O; ORO, Picro Sirus Red; PSR) and immunohistochemical staining (VCAM-1, CD68, MCP-1, nitrotyrosine, IL1β). No significant changes could be detected in all morphological parameters including total lesion size, necrotic core area (H&E), lipid content (ORO) and collagen content (PSR). GA application decreased inflammatory status as shown by a significant downregulation of nitrotyrosine level in the treatment group vs. control group. In contrast, adhesion marker (VCAM-1), macrophage content (CD68) and further inflammatory markers such as MCP-1 and IL1β remain unchanged. Single dots represent the mean of three to four sections per mouse. Error bars display calculated standard deviation. *, $p < 0.05$, scale bar 100 μm and 200 μm (total lesion size and ORO), magnification 100×. One-way ANOVA with multiple comparisons were calculated.

anti-inflammatory potential of the *Garcinia kola* plant, and the seeds in particular [3,7]. It has been shown that a methanol extract of the seeds reduces the LPS-induced NO production in the human macrophage cell line U937[3] and in rats [21], which is possibly mediated by one of its main phytochemicals, such as GA. Data on anti-inflammatory effects of isolated GA are rare. Until recently its anti-inflammatory potential was merely postulated based on its anti-oxidative effects [4]. To demonstrate the importance of GA for the reported anti-inflammatory effects of *Garcinia kola* seeds, we directly compared the effects of NE and GA *in vitro*. Our data revealed that both GA and NE interfere with anti-inflammatory signaling in LPS-stimulated RAW264.7 macrophages, with GA being slightly more efficient compared to NE (Figs. 2 and 3). Birringer et al. have shown that less than 1% of the *Garcinia kola* seeds is constituted by GA [18]. Since GA is soluble in organic solvents the percentage of GA in our NE is higher. Taking this into account, a significant contribution of GA to the anti-inflammatory effects of the NE is evident.

GA combines structural similarities of both, δ-T3 and the LCM of

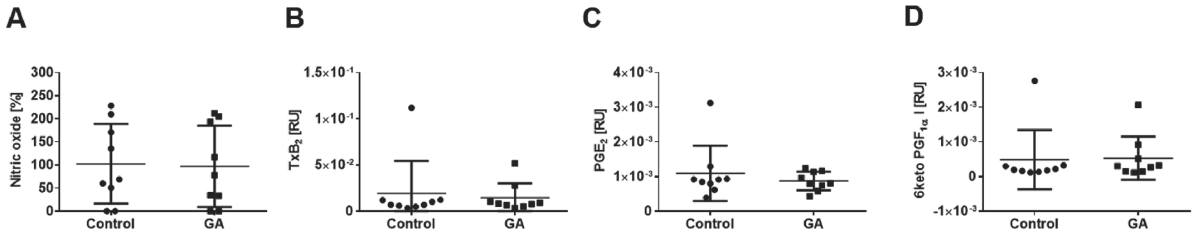

Fig. 5. GA does not affect systemic levels of iNos and Cox signaling molecules. Plasma samples from ApoE$^{-/-}$ mice fed with HFD and in parallel injected with GA or vehicle for eight weeks have been measured to determine **(A)** NO levels **(B)** thromboxane levels as well as **(C + D)** prostaglandin (PGE_2 and 6-keto $PGF_{1\alpha}$) levels. Mean of basal NO levels of vehicle control group was set as 100%. Treatment with GA did neither alter NO levels in mice (102.7% ± 28.8% vs. 97.1% ± 29.3%; control vs. treatment) nor the prostanoid levels (TxB_2: 1.939E-02 ± 1.162 E−02 vs. 1.443E-02 ± 0.523E-02, PGE_2: 1.092E-03 ± 0.2662 E−03 vs. 0.873E-03 ± 0.0908E-03, 6 keto $PGF_{1\alpha}$: 0.488E-03 ± 0.29 E−03 vs. 0.531E-03 ± 0.2088E-03; control vs. GA). Error bars display calculated SD. Student's t-test and ANOVA followed by Tukey post-hoc tests with logarithmized values was performed for statistical analysis.

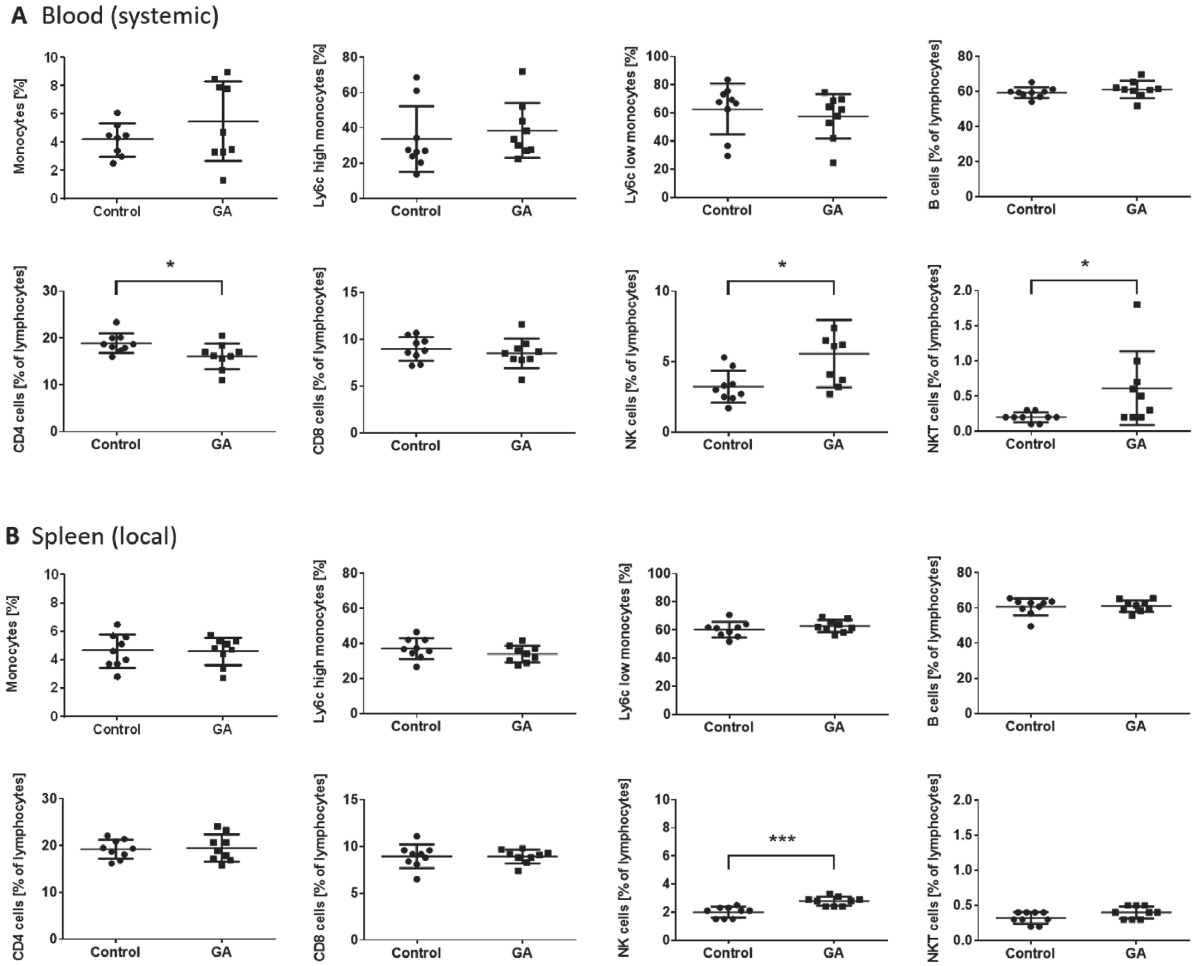

Fig. 6. GA modulates systemic and localized inflammation to different extent. Interactions of GA treatment with immune cell distribution were analyzed using flow cytometry. Systemic (blood) and local (spleen) cell population have been quantified using monocyte/macrophage, B cell and T cell specific fluorescent staining. **(A)** In blood total monocyte/macrophage population and sub-populations remained unchanged. There is no change in B cell and CD8 T cell population. CD4 positive T cells are significantly downregulated in GA-treated mice, 16.1% ± 0.9% compared to control group 18.9% ± 0.7%, whereas nature killer (NK) and natural killer T (NKT) cells were significantly upregulated 3.2% ± 0.4% vs. 5.6% ± 0.8%; 0.2% ± 0.02% vs. 0.6% ± 0.2%, respectively (control vs. GA-treated group). **(B)** In contrast local (spleen) population of tested cell types remained unchanged, except for an increase in NK cells (2.0% ± 0.1% vs. 2.8% ± 0.1%). Error bars display calculated SD. *, $p < 0.05$, ***, $p < 0.001$ (control vs. GA). Student's t-test was used.

vitamin E α-13′-COOH, with respect to the methylation pattern of the chromanol ring as well as the saturation and the terminal oxidation of the side chain, respectively. Within the group of vitamin E, δ-T3 is probably the most effective in inhibiting pro-inflammatory pathways [23]. For example, expression of Cox2, production of cytokines and release of PGE_2 and NO was inhibited in LPS-activated RAW264.7 macrophages by δ-T3 more effectively than by α-TOH, α-T3 and γ-T3 [9]. In line with these findings, Qureshi et al. have shown that Tnfα serum levels and expression of Il6, Il1β, iNos and Tnfα were effectively decreased by δ-T3 in LPS-stimulated peritoneal macrophages obtained from BALB/c mice [24]. Furthermore, GA (δ-TE-13'-COOH) has been described as the most potent 5-lipoxygenase (5-LO) inhibitor within the group of vitamin E metabolites [25].

α-13'-COOH is claimed to be a bioactive molecule since significant effects on lipid metabolism and homeostasis as well as inflammation have been reported [14,16,26,27]. In the study of Jiang and colleagues, the LCMs δ- and γ-13′-COOH were characterized as potent anti-inflammatory agents due to their ability to inhibit the COX2 pathway in IL1β-stimulated human lung adenocarcinoma A549 cells [14]. In addition, our group has recently shown that α-13′-COOH blocks the LPS-induced expression of inflammatory marker genes, proteins and related signaling molecules in LPS-stimulated RAW264.7 macrophages [16]. Similar anti-inflammatory properties have been recently discussed for the structurally related GA [28]. Here, we demonstrate that GA reduced the LPS-induced mRNA expression of iNos and other crucial inflammatory pathways (Fig. 2), followed by the inhibition of NO and other signaling molecules (Fig. 3C–E).

Based on the promising anti-inflammatory effects of GA *in vitro* and previous studies demonstrating anti-atherosclerotic effects of T3s[13], we tested the potential of GA as a treatment against the inflammation-driven disease atherosclerosis. For the *in vivo* application, a maximum dose of 1 mg/kg of GA was used; the dose of GA was chosen with respect to its low solubility in aqueous solutions and the need of using DMSO as solvent. We used the highest concentration of 0.8% DMSO that is allowed for long-term application in mice by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia. Due to the limited solubility of GA in aqueous solutions containing only 0.8% DMSO we had to limited the dose of GA to 1 mg/kg. Calculated to the total amount of blood (2 ml) in mice, with 1 mg/kg GA injected intraperitoneally, initial plasma concentrations of GA up to an equivalent of 35 μM could be achieved. We decided to use the highest possible dose of 1 mg/kg (35 μM) GA. However, we cannot exclude that the distribution and clearance of GA changes the actual concentration achievable *in vivo* and that higher concentrations are needed to achieve suitable concentrations *in vivo* resulting in anti-atherogenic properties of GA. We found a significant reduction of intra-plaque nitrotyrosine levels (Fig. 4B) – a marker for NO production as expected from our *in vitro* studies. Since elevated nitrotyrosine levels have been reported in human atherosclerotic lesions, its contribution to cardiovascular disease as a linking mechanism between inflammation and development of atherosclerosis has been discussed [29]. Other inflammatory marker, such as IL1β and MCP-1, remained unchanged in the plaque, although GA has been shown to block these pathways *in vitro* (Fig. 2C). The total plaque size was not affected by GA treatment, despite the apparent interference with local NO production. Overall, it can be assumed that the decrease of intra-plaque inflammation by GA was not sufficient to significantly impact the plaque size. As recently shown by Pein et al., GA potently inhibits 5-LO [25]. An association of the expression of 5-LO and the progression of atherosclerotic plaque formation [30], and more precisely plaque instability [31], in patients has been shown. However, there are discrepancies of 5-LO expression in atherosclerotic plaques of humans and mice. Whereas the expression of 5-LO is upregulated in carotid plaques in humans, there is no difference in the expression levels in wild-type mice (C57Bl/6) compared to atherosclerotic mice strains ($ApoE^{-/-}$ and $ApoE^{-/-}/LDLR^{-/-}$) fed a HFD [32]. Indeed, the $5\text{-}LO^{-/-}/ApoE^{-/-}$ knockout model or pharmacological inhibition of 5-LO in mice failed to show an impact on the progression of atherosclerosis [33]. Therefore, evidence for a contribution of 5-LO to the formation of atherosclerotic plaques is still on demand.

As shown in Fig. 3C–E, GA efficiently blocks the production of NO and prostanoids in LPS-activated macrophages *in vitro*. However, neither NO nor prostanoid levels are changed by GA in murine plasma samples. The atherosclerotic mouse model used in our study is characterized by a low-level chronic inflammation. Therefore, baseline NO and prostanoid plasma levels are at least 100-fold lower compared to levels in LPS-activated macrophages. The lack of excessive pre-activation could be a reason why we did not see inhibitory effects of GA on systemic pro-inflammatory signaling molecules in our study. Whether GA would be more effective in acute inflammation (e.g. sepsis), which is closer to our *in vitro* model, needs further investigation. We can also not exclude that a different treatment regime or animal model of atherosclerosis would lead to different results.

Monocytes play a pivotal role in atherogenesis. We found the total monocyte population and the $Ly6C^{low}$ and $Ly6C^{high}$ sup-populations in blood and spleen to be unchanged. Effects of α-TOH [34] and γ-T3[12] on monocyte and macrophage recruitment have been shown, respectively, but nothing was known for GA. In addition, we observed that lymphocyte populations, especially T cells, NKT and NK cells, are modulated under GA treatment. This demonstrates the importance of oxidative modifications of the side chain for vitamin E metabolites on regulatory effects. In blood, GA treatment decreased CD4 positive cells and the CD4/CD8 cell ratio without regulation the CD8 positive cell population. This finding goes in line with the earlier reported effects of vitamin E in Brown Norway rats [35] and chickens [36]. Further, NK and NKT cells, which are important for the defence against tumour cells, are significantly increased. Since these cells are known to regulate immune responses, their upregulation shows the involvement of GA on the immune system. In support, effects of α-TOH on increased NK cell activity and their tumorolytic activity in mice have been shown [37].

In summary, our improved procedure for the extraction of GA from *Garcinia kola* seeds enabled us to perform *in vitro* and *in vivo* investigations using pure GA. For the first time, we clearly demonstrate that both the NE and GA efficiently affect acute inflammation by inhibiting LPS-induced pro-inflammatory pathways *in vitro*. However, in atherosclerosis as a model of low level chronic inflammation this effect is not sufficient to make a significant difference in plaque size and plaque stability. Therefore, further studies are required to unravel the effects of GA in inflammation-driven diseases using different animal models to shed new light on the molecular modes of action of GA and to verify the *in vivo* importance of our findings.

Authors contribution

MW, JB, SK, LS, YCC, MZ, AS, AM, MS, MT, HP and AK performed the experiments. MW and SL designed the study. MW, MB, KP and SL supervised the project. MW wrote the manuscript. MW, JB, SK, LS, YCC, MZ, AS, AM, MS, AK, OW, MB, KP and SL carefully read and evaluated the manuscript and discussed the results.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101166.

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REVIEW
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Diversity of Chromanol and Chromenol Structures and Functions: An Emerging Class of Anti-Inflammatory and Anti-Carcinogenic Agents

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Natural chromanols and chromenols comprise a family of molecules with enormous structural diversity and biological activities of pharmacological interest. A recently published systematic review described more than 230 structures that are derived from a chromanol ortpd chromenol core. For many of these compounds structure-activity relationships have been described with mostly anti-inflammatory as well as anti-carcinogenic activities. To extend the knowledge on the biological activity and the therapeutic potential of these promising class of natural compounds, we here present a report on selected chromanols and chromenols based on the availability of data on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis. The chromanol and chromenol derivatives seem to bind or to interfere with several molecular targets and pathways, including 5-lipoxygenase, nuclear receptors, and the nuclear-factor "kappa-light-chain-enhancer" of activated B-cells (NFκB) pathway. Interestingly, available data suggest that the chromanols and chromenols are promiscuitively acting molecules that inhibit enzyme activities, bind to cellular receptors, and modulate mitochondrial function as well as gene expression. It is also noteworthy that the molecular modes of actions by which the chromanols and chromenols exert their effects strongly depend on the concentrations of the compounds. Thereby, low- and high-affinity molecular targets can be classified. This review summarizes the available knowledge on the biological activity of selected chromanols and chromenols which may represent interesting lead structures for the development of therapeutic anti-inflammatory and chemopreventive approaches.

Keywords: chromanols, chromenols, inflammation, cancer, molecular targets

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INTRODUCTION

Chromanols and chromenols are collective terms for about 230 structures derived from photosynthetic organisms like plants, algae, cyanobacteria, fungi, corals, sponges, and tunicates (Birringer et al., 2018). Both compound classes are formed by a cyclization of substituted 1,4-benzoquinones. While 6-hydroxy-chromanols are derived from a 2-methyl-3,4-dihydro-2*H*-chromen-6-ol structure, 6-hydroxy-chromenols are derived from 2-methyl-2*H*-chromen-6-ol (**Figure 1**). The respective bicyclic core structure is associated to a side-chain with varying chain length and modifications, resulting in a great diversity of chromanol and chromenol derivates (Birringer et al., 2018). In a systematic review, Birringer and coworkers were the first implying the great potential of these structures by providing a comprehensive overview of the structural diversity and chemical transformation of all 230 chromanols and chromenols known at that time together with their natural source. The aim of the comprehensive review was rather the detailed description of the complexity of this group of compounds than an outline of their biological activity. Based on this systematic review, the intention of our review was to more selectively describe the effects of this class of natural products on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis, and the underlying molecular modes of action for selected chromanols and chromenols. Our review therefore represents a useful and relevant addition to the work of Birringer et al., focusing on the evaluation of selected compounds with known biological activity as possible lead structures for putative therapeutic approaches. Based on the mentioned inclusion criteria, we here focus on tocopherol (TOH) and tocotrienol (T3) structures, sargachromanols, amplexichromanols, and sargachromenols, which show structure-activity relationships with mostly anti-inflammatory as well as anti-carcinogenic activities.

Tocopherols and T3s differ in the saturation of the side-chain and form in its entirety the group of vitamin E. Based on the methylation pattern of the chromanol ring system α-, β-, γ-, δ-forms of TOHs and T3s can be distinguished. Oxidative modifications of the terminal side-chain increase anti-inflammatory activities. Therefore, hepatic metabolites of vitamin E are supposed to have important physiological activities and will also be included in this review. Sargachromanols (SCA), sargachromenols (SCE), and amplexichromanols (AC) have a tocotrienol-derived backbone implying similar biological activities. Our review focuses in more detail on the current knowledge about the biological activity as well as on potential regulatory pathways and molecular targets of chromanols and chromenols.

Abbreviations: α-T-13′-COOH, α-13′-carboxychromanol; α-T-13′-OH, α-13′-hydrochromanol; δ-T3-13′-COOH, garcinoic acid; AC, amplexichromanol; BMDM, bone marrow-derived macrophages; CEHC, carboxyethyl-hydroxychromanol; JNK, c-Jun N-terminal kinase; CoA, coenzyme A; COX, cyclooxygenases; CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; ICM, intermediate-chain metabolite; IL, interleukin; iNOS, inducible nitric oxide synthase; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; LCM, long-chain metabolite; LPS, lipopolysaccharide; LO, lipoxygenases; MAPKs, mitogen-activated kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; NO, nitric oxide; PARP-1, poly-[ADP-ribose]-polymerase 1; PG, prostaglandin; PMA, phorbol-12-myristat-13-acetate; PMNL, polymorphonuclear neutrophils; ROS, reactive oxygen species; SCA, sargachromanols; SCE, sargachromenols; SCM, short-chain metabolites; TX, thromboxane; TOH, tocopherol; TNF-α, tumor necrosis factor α; T3, tocotrienol.

CHROMANOL AND CHROMENOL STRUCTURES

Chromanols

Tocopherols and Tocotrienols

Vitamin E, more precisely *RRR*-α-tocopherol, has been identified in 1922 as a vital factor for fertility in rats (Evans and Bishop, 1922). Vitamin E does naturally occur in various plant-derived foods, such as oils, nuts, germs, seeds as well as vegetables and, in lower amounts, fruits. Thus, vitamin E represents the most widely distributed and abundant chromanol in nature. The term vitamin E comprises different lipophilic molecules that consist of the chromanol ring structure with a covalently bound phytyl-like side-chain. Depending on the saturation of the C-16′ side-chain, these molecules are classified as TOH, T3s (**Figure 2**), and vitamin E related structures named tocomonoenols and marine-derived TOHs. Tocopherols are characterized by a saturated phytyl side-chain whereas tocomonoenols, marine-derived TOHs and T3 are unsaturated at either the terminal isoprene unit or have three double bonds within the side-chain (Fujisawa et al., 2010; Kruk et al., 2011). Further, the methylation pattern of the chromanol ring determines the classification as α-, β-, γ-, and δ-forms of TOHs and T3s. Although several similar molecules form the group of vitamin E, only α-TOH seems to have vitamin property in animals and humans. For instance, in rats α-TOH preserves fertility, whereas in humans the deficiency disease *ataxia with vitamin E deficiency* (AVED) is prevented by α-TOH supplementation (Azzi, 2019).

For a long time, the health-promoting effects of vitamin E were only attributed to its antioxidant properties, but more recent studies revealed additional non-antioxidant functions of vitamin E. It is evident that vitamin E modulates gene expression and enzyme activities and also interferes with signaling cascades (Brigelius-Flohé, 2009; Zingg, 2019). Examples for these regulatory effects are the suppression of inflammatory mediators, reactive oxygen species (ROS) and adhesion molecules, the induction of scavenger receptors as well as the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (reviewed in Glauert, 2007; Rimbach et al., 2010; Wallert et al., 2014b; Zingg, 2019).

All forms of vitamin E undergo metabolic degradation in the liver. Although the detailed mechanisms remain poorly understood, the principles of the degradation of vitamin E to vitamer-specific physiological metabolites with intact chromanol ring (the nomenclature as α-, β-, γ- and δ-metabolites is used as described for the metabolic precursors in order to distinguish the

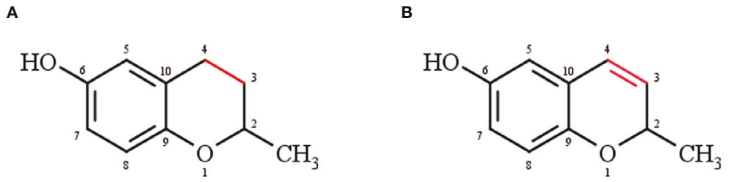
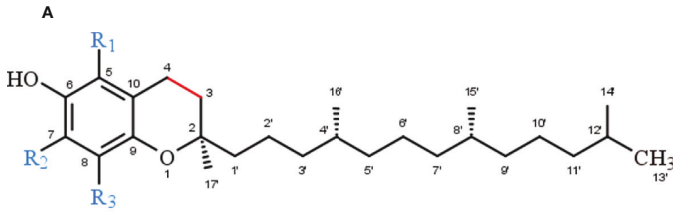
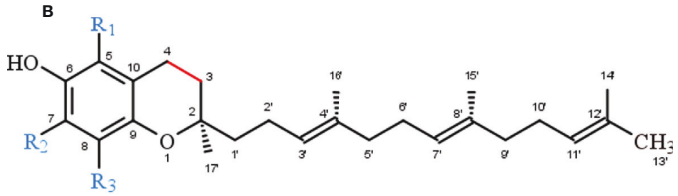


FIGURE 1 | (A) Chromanol (2-methyl-3,4-dihydro-2*H*-chromen-6-ol) and **(B)** chromenol (2-methyl-2*H*-chromen-6-ol) core structure.



-tocopherol	R_1	R_2	R_3
α	$-CH_3$	$-CH_3$	$-CH_3$
β	$-CH_3$	-H	$-CH_3$
γ	-H	$-CH_3$	$-CH_3$
δ	-H	-H	$-CH_3$

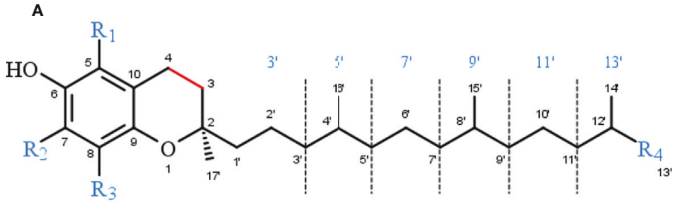


-tocotrienol	R_1	R_2	R_3
α	$-CH_3$	$-CH_3$	$-CH_3$
β	$-CH_3$	-H	$-CH_3$
γ	-H	$-CH_3$	$-CH_3$
δ	-H	-H	$-CH_3$

FIGURE 2 | Core structure of **(A)** tocopherol and **(B)** tocotrienol forms.

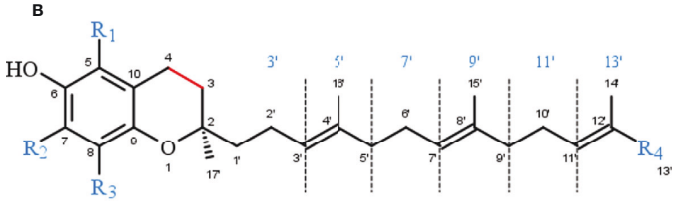
different forms of vitamin E metabolites) is widely accepted (**Figure 3**). Thus, enzymatic modifications are restricted to the side-chain (extensively reviewed in (Kluge et al., 2016; Schmölz et al., 2016)). α-Tocopherol is the main form of vitamin E in the human body due to its higher binding affinity to the α-tocopherol transfer protein (Hosomi et al., 1997). Thus, we will focus on the metabolic conversion of α-TOH in the following. Nevertheless, it should be noted that all forms of vitamin E (TOHs as well as T3s) follow the same metabolic route. However, due to the unsaturated side-chain, the degradation of T3s requires further enzymes such as 2,4 dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase, which are also

A



α/β/γ/δ -metabolite	R_4	sidechain length
-13'-T-OH	-OH	13
-13'-T-COOH	-COOH	13
-11'-T-COOH	-COOH	11
-9'-T-COOH	-COOH	9
-7'-T-COOH	-COOH	7
-5'-T-COOH	-COOH	5
-3'-T-COOH	-COOH	3

B



α/β/γ/δ -metabolite	R_4	sidechain length
-13'-T3-OH	-OH	13
-13'-T3-COOH	-COOH	13
-11'-T3-COOH	-COOH	11
-9'-T3-COOH	-COOH	9
-7'-T3-COOH	-COOH	7
-5'-T3-COOH	-COOH	5
-3'-T3-COOH	-COOH	3

FIGURE 3 | Core structure of **(A)** tocopherol and **(B)** tocotrienol metabolites. The substitutions R1 to R3 are given in **Figure 2 (A)** and **(B)**.

involved in the metabolism of unsaturated fatty acids (Birringer et al., 2002). The initial step of α-TOH modification *via* ω-hydroxylation in the endoplasmic reticulum leads to the formation of the long-chain metabolite (LCM) α-13′-hydroxychromanol (α-T-13′-OH). It is supposed that this hydroxylation is catalyzed by cytochrome P450 (CYP)4F2 and CYP3A4 (Parker et al., 2000; Sontag and Parker, 2002). After its transfer from the endoplasmic reticulum to the peroxisome, α-T-13′-OH is converted to α-13′-carboxychromanol (α-T-13′-COOH) *via* ω-oxidation, likely *via* a two-step mechanism involving alcohol and aldehyde dehydrogenases. α-T-13′-OH and α-T-13′-COOH have been found in human serum (Wallert et al., 2014a; Ciffolilli et al., 2015; Giusepponi et al., 2017), supporting the idea of a more complex physiologic role of vitamin E with physiological relevance of its metabolites for various processes. In healthy humans α-TOH is the most abundant form of vitamin E, occurring in concentrations of about 20–30 μM in serum (Péter et al., 2015). However, supplementation of α-TOH increases α-TOH serum concentration in humans up to 90 μM (Dieber-Rotheneder

et al., 1991). Following supplementation, the hepatic metabolism is enhanced to protect the liver from excessive accumulation of α-TOH. Consequently, metabolites of vitamin E are formed and accumulate in turn in human serum. The LCMs α-T-13′-OH and α-T-13′-COOH were found in concentrations of 1–7 nM and 1–10 nM at baseline, respectively, whereas supplementation of α-TOH increased serum concentrations of the LCMs up to 12–32 nM and 3–55 nM, respectively (Wallert et al., 2014a; Ciffolilli et al., 2015; Giusepponi et al., 2017). Recent studies showed that the active metabolites of vitamin E exert effects on lipid metabolism, apoptosis, proliferation, and inflammatory processes as well as xenobiotic metabolism (Wallert et al., 2014a; Jang et al., 2016; Podszun et al., 2017; Schmölz et al., 2017). Finally, α-T-13′-COOH is excreted *via* bile and feces or is further degraded *via* several rounds of oxidation to the hydrophilic short-chain metabolite α-carboxyethyl-hydroxychromanol (CEHC), which is largely excreted *via* urine (Zhao et al., 2010; Johnson et al., 2012; Jiang, 2014). Another characteristic of the hepatic degradation of vitamin E is that the metabolites are chemically modified. In particular, the LCMs and the short-chain metabolites (SCMs) have been found as sulfated or glucuronidated conjugates in different biological matrices (Galli et al., 2002; Wallert et al., 2014a). Freiser and Jiang (2009) reported that more than 75% of γ-CEHC in the plasma of γ-T3-supplemented rats occurred in conjugated form. Further, also the LCMs, especially 13′-COOH and 11′-COOH metabolites were found as conjugates. Conjugation (sulfation or glucoronidation) seems to occur in the liver in parallel to the β-oxidation of the side-chain of vitamin E (Freiser and Jiang, 2009).

Beside the mentioned LCMs, intermediate-chain metabolites (ICMs) and SCMs that are formed *via* hepatic degradation of the different vitamin E forms, and vitamin E is also the precursor of quinones, representing another class of vitamin E-derived metabolites that exhibit antioxidant activity. Vitamin E quinones, in particular α-TOH-derived quinones, are formed as byproducts of α-TOH oxidation during peroxidation reactions in *in vitro* systems (Liebler et al., 1990; Infante, 1999). In addition, these metabolites can also be synthesized by photosynthetic organisms (Liebler et al., 1990). Although the knowledge on this group of tocopherol-derived metabolites is sparse, α-TOH quinone has been described as an essential enzymatic cofactor for fatty acid desaturase (Liebler et al., 1990).

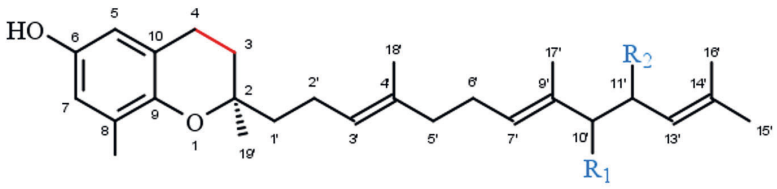
The natural compound δ-T3-13′-COOH, also known as δ-garcinoic acid or δ-tocotrienolic acid, shares structural similarity with the δ-T-LCM δ-T-13′-COOH, the second LCM originating from the hepatic metabolism of δ-TOH. As described previously, hepatic metabolism of tocotrienols follows that of tocopherols. Consequently, δ-T3-13′-COOH is formed during the degradation of δ-T3. Since the concentration of δ-T3 in human plasma is below 1% compared to α-TOH, the physiological relevance of δ-T3-13′-COOH in humans is likely low. So far, the detection of δ-T3-13′-COOH in human blood is still pending. However, local accumulation of δ-T3-13′-COOH in cells or tissues cannot be excluded. δ-T3-13′-COOH can be obtained in relatively high amounts and purity from the seeds of *Garcinia kola E. Heckel* (Bartolini et al., 2019; Wallert et al., 2019), a plant that is used in traditional African ethnomedicine (extensively reviewed in Kluge et al., 2016). This compound can be used as precursor for the semi-synthesis of α- and δ-LCMs (including α-T-13′-OH, α-T-13′-COOH, δ-T-13′-OH, and δ-T-13′-COOH) for experimental use *in vitro* and in mice and is therefore important for vitamin E metabolite research (Maloney and Hecht, 2005; Birringer et al., 2010). Further, δ-T3-13′-COOH also appeared to be a potent anti-inflammatory (Wallert et al., 2019) and anti-proliferative agent (Mazzini et al., 2009) and has been shown to act as an inhibitor of DNA polymerase β (Maloney and Hecht, 2005), indicating that δ-T3-13′-COOH may disturb base excision repair in tumor cells. A recent preprint of Bartolini et al. described δ-T3-13′-COOH as a potent agonist of PXR, which is known to be involved in inflammatory processes (Bartolini et al., 2019).

Sargachromanols

Sargachromanols (SCA) comprise a group of chromanols that occur in the brown algae family *Sargassaceae* (**Figure 4**). Their high structural diversity results from various side-chain modifications, leading to their classification from SCA-A to SCA-S. The entirety of sargachromanols has been isolated from *Sargassum siliquastrum* and has been classified *via* two-dimensional nuclear magnetic resonance experiments (Jang et al., 2005; Im Lee and Seo, 2011). The extensive analysis revealed detailed structural differences between the sargachromanols. For example SCA-C contains a 9′-hydroxyl group with *R*-configuration in the side-chain, while SCA-F has a methoxy group at C-9′ and a hydroxyl group with *R*-configuration at C-10′ (extensively reviewed in Birringer et al., 2018). SCAs have been reported to exhibit various biological activities, including anti-oxidative (Lim et al., 2019) (SCA-G), anti-osteoclastogenic (Yoon et al., 2012b; Yoon et al., 2013) (SCA-G), anti-inflammatory (Yoon et al., 2012a; Lee et al., 2013; Heo et al., 2014) (SCA-G and SCA-D), as well as anti-diabetic (Pak et al., 2015) (SCA-I) ones. To the best of our knowledge, metabolism of sargachromanols in humans or animals has not been investigated.

Amplexichromanols

Amplexichromanols represent a small group of hydroxylated T3 derivatives found in different parts of *Garcinia* plants. For instance, lipophilic extracts from the bark of *Garcinia amplexicaulis* were used to isolate γ-AC and δ-AC (**Figure 5**). The chemical structure of γ-AC and δ-AC are similar to γ-T3 and δ-T3, respectively, but carry two additional hydroxyl groups at C-13′ and C-14′. In an initial *in vitro* experiment, δ-AC reduced vascular endothelial growth factor induced cell proliferation in low nanomolar concentrations, while γ-AC had no effect. This observation probably indicates distinct efficiencies for the different amplexichromanols (Lavaud et al., 2013). However, further experiments revealed strong anti-oxidative potential for both compounds (Lavaud et al., 2015), but nothing is known about the metabolization, systemic distribution, tissue accumulation, or excretion of amplexichromanols so far.

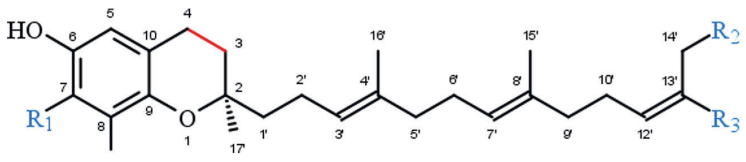


Sargachromanol	R_1	R_2
D	-OH (R)	-OH (S)
E	-OH (R)	-OH (R)
F	-OMe	-OH (S)
G	=O	-OH (S)

FIGURE 4 | Core structure of sargachromanol forms.

Chromenols

Chromenols consist of a 2-methyl-2*H*-chromen-6-ol core that is associated with a side-chain with varying chain length and varying chemical modifications, leading to high structural diversity. The multitude of these compounds can be obtained from photosynthetic organisms like plants, algae, cyanobacteria, fungi, corals, sponges, and tunicates (Birringer et al., 2018). As the current knowledge on the biological functions of chromenol structures is sparse, this review will exemplarily focus on the most studied sargachromenols (**Figure 6**). Similar to their chromanol counterparts, sargachromenols were named after the brown algae species *Sargassum serratifolium*, from which they have been isolated first (Kusumi et al., 1979). Just like sargachromanols, sargachromenols comprise a molecule class of high structural diversity due to different side-chain modifications. In the first systematic review on the field of chromanols and chromenols, Birringer and coworkers described 15 sargachromenols, 13 compounds with marine origin (brown algae) and two with marine and plant origin (Birringer et al., 2018). As an example, δ-SCE, a structure consisting of a δ-chromenol ring system with an unsaturated side-chain containing a carboxy group at C-15′, is widely distributed in algae of the *Sargassaceae* family but can also be obtained from plants like *Iryanthera juruensis*. Another interesting sargachromenol is dehydro-δ-T3, or Sargol, which is supposed to serve as a biosynthetic precursor for most of the sargachromenols and is occurring in brown algae (Birringer et al., 2018). Brown algae from the *Sargassaceae* family have been used in traditional Asian medicine as well as in health promoting diets, revealing a variety of biological functions (Kim et al., 2014). For example, ethanolic extracts from the *Sargassaceae* species *Myagropsis myagroides*, an alga that grows at the coast of East Asia, revealed potent anti-inflammatory activity. After HPLC-based separation, sargachromenols



-amplexichromanol	R_1	R_2	R_3
γ	$-CH_3$	-OH	$-CH_2OH$
δ	-H	-OH	$-CH_2OH$

FIGURE 5 | Core structure of amplexichromanol forms.

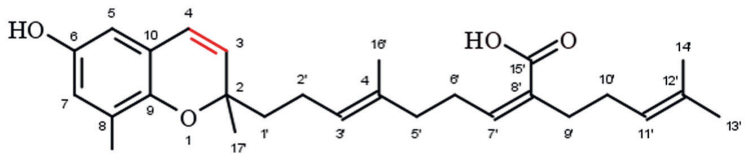


FIGURE 6 | Molecular structure of δ-sargachromenol.

(mostly δ-SCE) have been identified as the most potent anti-inflammatory compounds within these extracts, based on their inhibitory effect on nitric oxide (NO) production in lipopolysaccharide (LPS)-treated immortalized murine microglial BV-2 cells (Kim et al., 2014). Beside their anti-inflammatory activity, anti-carcinogenic (Hur et al., 2008), anti-photoaging (Kim et al., 2012), and anti-cholinesterase activities (Choi et al., 2007) have been described for SCEs. Further, sargachromenols isolated from *Sargassum macrocarpum* mediate nerve-growth-factor-driven neuronal growth in pheochromocytoma of rat adrenal medulla derived PC12D cells (Tsang et al., 2005).

BIOLOGICAL ACTIVITY OF NATURAL CHROMANOLS AND CHROMENOLS

Based on published data, we have chosen signaling pathways that are central for inflammation, apoptosis, cell proliferation, and carcinogenesis (**Figure 7**). Respective effects of tocopherol-

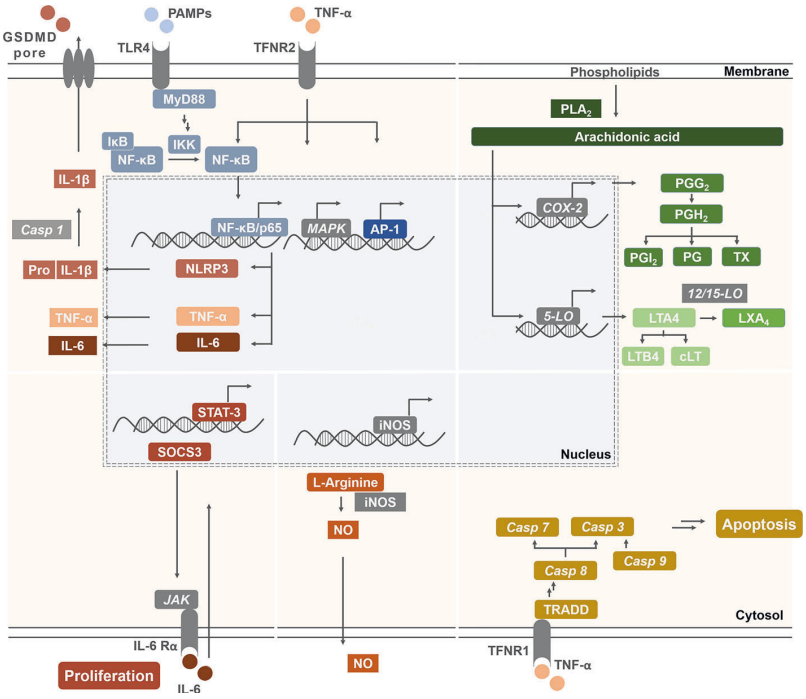


FIGURE 7 | Schematic illustration of signaling targets, pathways, and molecules involved in inflammatory response and cancer progression. Pathways were chosen due to known interactions with the compounds of interest. Inflammatory signaling molecules are interleukins (IL), tumor necrosis factor-α (TNF-α), nitric oxide (NO), prostaglandins (PG), prostacyclin (PGI_2), tromboxanes (TX), leukotriens (LT), and lipoxins (LX). Their expression, synthesis, or release depends, among others, on the activation of NF-κB, NLR family pyrin domain containing 3 (NLRP3) inflammasome, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and lipoxygenases (LO). In addition, membrane receptors, such as cytokine or epidermal growth factor receptors regulate the activation of nuclear receptor signal transducer and activator of transcription (STAT) 3 as well as extrinsic or intrinsic pathways which trigger the activation of caspases (Casp). These mediators are therefore important factors for modulating the balance between cell proliferation and apoptosis, which is essential to prevent carcinogenesis.

derived (T) and tocotrienol-derived (T3) chromanol and chromenol structures on nuclear receptors and target enzymes were screened and are discussed in the following.

Inflammation

Inflammation is essential for wound healing as well as defense and clearance of pathogens (Kunnumakkara et al., 2018). However, excessive and persistent inflammation is a driving force for many chronic diseases. In addition to obvious inflammatory diseases such as rheumatoid arthritis, it is well accepted that cancer, Alzheimer's disease, and metabolic syndrome-related diseases like atherosclerosis, non-alcoholic fat liver disease, and diabetes mellitus type 2 are triggered by chronic low-grade inflammation (Kunnumakkara et al., 2018). As systemic inflammation is a complex process, this review refers only to inflammatory pathways that have been studied for chromanol and/or chromenol structures. Key regulatory factors and mediators of inflammatory processes in this context are receptors that sense proinflammatory stimuli, e.g. the toll-like receptors (TLRs), intracellular signaling molecules, like mitogen-activated protein kinases (MAPKs), and transcription factors, such as NF-κB or nuclear factor erythroid 2-related factor 2 (Nrf2). Further, enzymes that produce pro-inflammatory mediators such as prostaglandins (PGs) and leukotrienes (LTs) play a central role during the coordinated orchestra of the inflammatory process. This includes cyclooxygenases (COX) and lipoxygenases (LO). Other key players of inflammation are cytokines which are secreted by various cells and affect the interaction and communication between the different types of cells involved in inflammation (Aggarwal, 2009; Kunnumakkara et al., 2018). Important pro-inflammatory cytokines are interleukin (IL)-1β, IL-6, and IL-8 as well as tumor necrosis factor-α (TNF-α). Another important signaling molecule in inflammatory processes is nitric oxide (Aggarwal, 2009). In the following, chromanol and chromenol structures regulating the expression of key pro-inflammatory enzymes and the respective formation of signaling molecules are outlined.

Chromanols

A detailed overview on the biological activities of chromanols linked to inflammation is provided in **Table 1**.

Tocopherols and Tocotrienols

Data available for TOHs and T3s correlate with their abundance in humans. Therefore, α- and γ-TOH as well as their respective T3 forms were mostly investigated so far. α-Tocopherol is regarded as the only form within the group of vitamin E that has been shown to mediate actual vitamin E function (Azzi, 2019). Further, α-TOH is considered as the most abundant vitamin E form in human nutrition, followed by γ-TOH. Relevance of T3s as anti-inflammatory compounds has just recently come to fore of research and will be presented in the following sections.

Tocopherols. For many years, TOHs were solely known for their anti-oxidative capacity. However, Azzi and colleagues discovered additional gene regulatory effects of α-TOH that are independent from its capacity as an antioxidant. α-TOH revealed distinct effects on nitric oxide- and eicosanoid-mediated inflammation. For instance, α-TOH (10 μM) decreased the expression level of inducible nitric oxide synthase (iNOS) in LPS-stimulated murine RAW264.7 macrophages (Jiang et al., 2000). However, others could not confirm the observed alteration of iNOS expression using 5 μM (Wallert et al., 2015), 20 μM (Schmölz et al., 2017), or even 100 μM (Ciffolilli et al., 2015) α-TOH. In line with this, iNOS-mediated formation of nitric oxide remained unchanged in RAW264.7 macrophages by coincubation with α-TOH (Jiang et al., 2000; Ciffolilli et al., 2015; Wallert et al., 2015). In contrast, the formation of PGE_2 was blocked by 23 to 100 μM α-TOH in LPS-stimulated RAW264.7 macrophages (Jiang et al., 2000; Yam et al., 2009; Ciffolilli et al., 2015; Wallert et al., 2015), but not in IL-1β-stimulated A549 epithelial cells (Jiang et al., 2008). Unexpectedly, upstream-regulated COX-2 expression and activity remained unchanged in RAW264.7 macrophages at concentrations of 23 to 100 μM α-TOH. Furthermore, cytokine-mediated inflammation was not regulated by α-TOH (Yam et al., 2009), except for an inhibition of IL-1β gene expression in RAW264.7 macrophages using 100 μM (Ciffolilli et al., 2015; Wallert et al., 2015). Beside external stimuli, induction of inflammation, mainly *via* the TLR4-NF-κB signaling pathway, senescence of cells, and aging are also known triggers of inflammation (Lasry and Ben-Neriah, 2015). Indeed, 24-months-old mice are characterized by an increased inflammatory state compared to younger mice (six months). Application of 500 ppm α-TOH acetate lowered aging-induced increases of nitric oxide and PGE_2 plasma levels as well as COX-2 activity compared to 24-months-old mice fed 30 ppm (Beharka et al., 2002). In line with this, supplementation with 800 mg α-TOH/kg/d in elder humans for 30 days lead to significantly lower levels of PGE_2 in plasma and peripheral blood mononuclear cells compared to vehicle-treated controls (Meydani et al., 1990).

The second most abundant form of vitamin E, γ-TOH, is more prominent for its anti-inflammatory capacity compared to α-TOH. Release of nitric oxide by LPS-stimulated RAW264.7 cells was significantly inhibited using 10 μM γ-TOH (Jiang et al., 2000). Release of eicosanoids inflammation, more precisely PGE_2, in LPS-stimulated RAW264.7 cells and in IL-1β-stimulated A549 cells was inhibited by 10 μM (IC_{50} 7.5 μM) (Jiang et al., 2000) and 25–40 μM (IC_{50} 4–7 μM) (Jiang et al., 2000; Jiang et al., 2008), respectively. However, COX-2 expression (Jiang et al., 2000; Jiang et al., 2008) and activity (Jiang et al., 2000; Jang et al., 2016) remained unchanged in LPS-stimulated RAW264.7 macrophages, whereas COX-2 activity was inhibited by 50 μM γ-TOH in IL-1β-stimulated A549 epithelial cells (Jiang et al., 2008). Azoxymethane-induced IL-6 production was dampened in BALB/c mice by a γ-TOH-enriched diet (Jiang et al., 2013).

δ-tocopherol (20 μM) significantly decreased LPS-induced expression of iNOS (by 60% at mRNA and by 48% at protein level) and formation of nitric oxide (by 36%) in RAW264.7 macrophages (Schmölz et al., 2017). Jiang et al. reported an inhibition of COX-2 activity, but not COX-2 expression in IL-1β-stimulated A549 cells (Jiang et al., 2008), whereas Jang et al.

TABLE 1 | Overview on the biological activities of chromanols linked to inflammation.

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
α-TOH								
LPS iNOS PE RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS NO PrD RAW264.7 20 μM no inhibition (Wallert et al., 2015)	LPS COX-2 PE, A RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS COX-2 GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS PGE_2 PrD RAW264.7 100 μM inhibition (Wallert et al., 2015)	IL-1β PGE_2 PrD A549 cells 50 μM no inhibition (Jiang et al., 2008)	LPS pro IL-1β GE RAW264.7 100 μM inhibition (Wallert et al., 2015)	LPS IL-6 GE RAW264.7 100 μM no inhibition (Wallert et al., 2015)	LPS TNF-α GE RAW264.7 100 μM no inhibition (Wallert et al., 2015)
LPS iNOS PE RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS NO PrD RAW264.7 10 μM no inhibition (Jiang et al., 2000)	LPS COX E, A RAW264.7 50 μM no inhibition (Jiang et al., 2000)	LPS COX-2 A m_PM 500 ppm inhibition (Beharka et al., 2002)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Jiang et al., 2000)	Age PGE_2 PrD human 800 mg/d inhibition (Meydani et al., 1990)	LPS pro IL-1β GE RAW264.7 100 μM inhibition (Ciffolilli et al., 2015)	LPS IL-6 PrD RAW264.7 23 μM no inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 23 μM induction (Yam et al., 2009)
LPS iNOS E RAW264.7 20 μM no inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 20 μM induction (Schmölz et al., 2017)	LPS COX-2 E RAW264.7 23 μM no inhibition (Yam et al., 2009)		LPS PGE_2 PrD RAW264.7 23 μM induction (Yam et al., 2009)	LPS PGE_2 PrD RAW264.7 100 μM inhibition (Ciffolilli et al., 2015)		LPS IL-6 GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS TNF-α GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)
LPS iNOS GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS NO PrD RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)			LPS PGE_2 PrD m_PM 500 ppm inhibition (Beharka et al., 2002)				
	LPS NO PrD m_PM 500 ppm inhibition (Beharka et al., 2002)							
β-TOH								
				IL-1β PGE_2 PrD A549 cells 50 μM no inhibition (Jiang et al., 2008)				
γ-TOH								
LPS iNOS PE RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS NO PrD RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS COX E + A RAW.264.7 50 μM no inhibition (Jiang et al., 2000)	IL-1β COX-2 A A549 cells 50 μM inhibition (Jiang et al., 2008)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 cells 25 μM inhibition (Jiang et al., 2008)		AOM IL-6 PrD BALB/c mice 0.1% of diet inhibition (Jiang et al., 2013)	
		IL-1β COX-2 PE A549 cells 40 μM no inhibition (Jiang et al., 2008)	LPS COX A A549 10 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 cells 40 μM inhibition (Jiang et al., 2000)				

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
δ-TOH								
LPS iNOS E RAW264.7 20 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 20 μM inhibition (Schmölz et al., 2017)	IL-1β COX-2 PE A549 cells 40 μM no inhibition (Jiang et al., 2008)	IL-1β COX-2 A A549 cells 50 μM inhibition (Jiang et al., 2008)	IL-1β PGE_2 PrD A549 cells 25 μM inhibition (Jiang et al., 2008)				
α-T3								
	LPS NO PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)	IL-1β COX-2 PE A549 cells 10 μM no inhibition (Jiang et al., 2008) LPS COX-2 GE RAW264.7 23.5 μM inhibition (Yam et al., 2009)	LPS COX-2 PE RAW264.7 23.5 μM no inhibition (Yam et al., 2009)				LPS IL-6 PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)
γ-T3								
	LPS NO PrD RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS COX-2 GE RAW264.7 24 μM inhibition (Yam et al., 2009) LPS COX-2 PE RAW264.7 24 μM no inhibition (Yam et al., 2009)	LPS COX-2 GE m_BMDM 1 μM inhibition (Kim et al., 2018)	LPS PGE_2 PrD RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS PGE_2 PrD m_BMDM 1 μM inhibition (Kim et al., 2018)	LPS pro IL-1β GE/PrD m_BMDM 1 μM inhibition (Kim et al., 2018) LPS pro IL-1β PrD m_BMDM 1 μM inhibition (Kim et al., 2016) LPS IL-1β PrD m_BMDM 1 μM inhibition (Kim et al., 2016)	LPS IL-6 PrD RAW264.7 24 μM inhibition (Yam et al., 2009) diabetes IL-6 PrD *db/db* mice 0.1% of diet inhibition (Kim et al., 2016)	LPS TNF-α PrD RAW264.7 24 μM no inhibition (Yam et al., 2009) diabetes TNF-α PrD *db/db* mice 0.1% of diet inhibition (Kim et al., 2016)
δ-T3								
	LPS NO PrD RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS COX-2 GE RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS COX-2 PE RAW264.7 25 μM no inhibition (Yam et al., 2009)	LPS PGE_2 PrD RAW264.7 25 μM inhibition (Yam et al., 2009)			LPS IL-6 PrD RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 25 μM induction (Yam et al., 2009)
α-T-13′-OH								
LPS iNOS E RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS NO PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS COX-2 E RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)		LPS PGE_2 PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)		LPS IL-1β PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS IL-6 PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS TNF-α PrD RAW264.7 10 μM no inhibition (Ciffolilli et al., 2015)

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
LPS iNOS E RAW264.7 10 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 10 μM inhibition (Schmölz et al., 2017)							
α-T-13′-COOH								
LPS iNOS PE RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS NO PrD RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS COX-2 PE RAW264.7 5 μM inhibition (Wallert et al., 2015)	IL-1β COX-2 A platelet 10 μM inhibition (Pein et al., 2018)	LPS PGE_2 PrD RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS PGE_2 PrD h_monocytes 10 μM no inhibition (Pein et al., 2018)	LPS pro IL-1β GE RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS IL-6 GE RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS TNF-α GE RAW264.7 5 μM no inhibition (Wallert et al., 2015)
LPS iNOS E RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS COX-2 A RAW264.7 5 μM no inhibition (Wallert et al., 2015)	– COX-2 A enzyme 10 μM no inhibition (Pein et al., 2018)					
δ-T-13′-OH								
LPS iNOS E RAW264.7 10 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 10 μM inhibition (Schmölz et al., 2017)							
δ-T-13′-COOH								
LPS iNOS E RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 5 μM inhibition (Schmölz et al., 2017)	IL-1β COX-2 A A549 4 μM inhibition (Jiang et al., 2008)	– COX-2 A enzyme 5 μM inhibition (Jang et al., 2016)					
		– COX-2 A enzyme 4 μM inhibition (Jiang et al., 2008)						
δ-T-9′-COOH								
		– COX-2 A enzyme 20 μM no inhibition (Jiang et al., 2008)	IL-1β COX-2 A A549 6 μM inhibition (Jiang et al., 2008)					
α-T-5′-COOH								
		– COX-2 A enzyme 140 μM inhibition (Jiang et al., 2008)						

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
α-T-3′-COOH								
TNF-α iNOS PE EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	TNF-α NO PrD RAEC cells 100 μM inhibition (Grammas et al., 2004) TNF-α/LPS NO PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)			LPS PGE_2 PrD RAEC cells 100 μM inhibition (Grammas et al., 2004)	LPS PGE_2 PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)			
γ-T-3′-COOH								
TNF-α iNOS E EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	LPS NO PrD RAW264.7 10 μM no inhibition (Jiang et al., 2000) LPS NO PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	IL-1β COX-2 A A549 50 μM inhibition (Jiang et al., 2000) IL-1β COX-2 PE A549 50 μM no inhibition (Jiang et al., 2000)	– COX-2 A enzyme 450 μM inhibition (Jiang et al., 2008)	LPS PGE_2 PrD RAW264.7 10 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 40 μM inhibition (Jiang et al., 2000)			
δ-T3-13′-COOH								
LPS iNOS E RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS NO PrD RAW264.7 5 μM inhibition (Wallert et al., 2019)	– COX-2 A enzyme 9.8 μM inhibition (Jang et al., 2016) LPS COX-2 E RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS COX-2 A h_monocytes 10 μM no inhibition (Pein et al., 2018)	LPS PGE_2 PrD RAW264.7 5 μM inhibition (Wallert et al., 2019) HFD PGE_2 PrD m_APOE$^{-/-}$ 1 mg/kg no inhibition (Wallert et al., 2019)	LPS PGE_2 PrD h_monocytes 300 nM inhibition (Pein et al., 2018)	LPS pro IL-1β GE RAW264.7 5 μM inhibition (Wallert et al., 2019) HFD IL-1β PrD m_APOE$^{-/-}$ 1 mg/kg no inhibition (Wallert et al., 2019)	LPS IL-6 GE RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS TNF-α GE RAW264.7 5 μM inhibition (Wallert et al., 2019)
SCA D								
LPS iNOS PE RAW264.7 15 μM inhibition (Heo et al., 2014)	LPS NO PrD RAW264.7 15 μM inhibition (Heo et al., 2014)	LPS COX-2 PE RAW264.7 15 μM inhibition (Heo et al., 2014)		LPS PGE_2 PrD RAW264.7 15 μM inhibition (Heo et al., 2014)		LPS IL-1β PrD RAW264.7 60 μM inhibition (Heo et al., 2014)	LPS IL-6 PrD RAW264.7 30 μM inhibition (Heo et al., 2014)	LPS TNF-α PrD RAW264.7 60 μM inhibition (Heo et al., 2014)
SCA E								
LPS iNOS PE RAW264.7 29 μM inhibition (Lee et al., 2013)	LPS NO PrD RAW264.7 29 μM inhibition (Lee et al., 2013)	LPS COX-2 PE RAW264.7 29 μM inhibition (Lee et al., 2013)		LPS PGE_2 PrD RAW264.7 29 μM inhibition (Lee et al., 2013)		LPS IL-1β PrD RAW264.7 12 μM inhibition (Lee et al., 2013)		LPS TNF-α PrD RAW264.7 29 μM inhibition (Lee et al., 2013)

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated		Cytokine-mediated		
iNOS	**NO**	**COX-2**	**PGE_2**	**IL-1β**	**IL-6**	**TNF-α**
SCA G						
LPS iNOS PE RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS NO PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS COX-2 PE RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS PGE_2 PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS IL-1β PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS IL-6 PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS TNF-α PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)
δ-AC						
				LPS IL-1β PrD monocytes 1 μM inhibition (Richomme et al., 2017)		LPS TNF-α PrD monocytes 10 μM inhibition (Richomme et al., 2017)

The effects of the respective compounds on inflammation have been divided into activities mediated by nitric oxide (iNOS, NO), eicosanoids (COX-2, PGE_2), and cytokines (IL-1β, IL-6, TNF-α). The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type, tissue, mouse, or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. When no stimulus was used or was required for the studies, the respective row is marked with a dash. The following abbreviations are used: A, activity; A549, human adenocarcinoma alveolar basal epithelial cells; BALB/c mice, albino laboratory-bred strain of the house mouse; $Apoe^{-/-}$ mice, apolipoprotein E deficient mice; BMDM, bone marrow derived macrophages; COX-2, cyclooxygenase 2; EOC-20, epithelial ovarian cancer cells; E, expression; GE, gene expression; HFD, high-fat diet; h, human; iNOS, inducible nitric oxide synthase; IL, interleukin; db/db mice, leptin receptor activity deficient mice; LPS, lipopolysaccharides; m, murine; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; NO, nitric oxide; ppm, parts per million; PM, peritoneal macrophages; PrD, production; PGE_2, prostaglandin E_2; PE, protein expression; RAEC, rat aortic endothelial cells; TNF-α, tumor necrosis factor α.
All results obtained from in vivo studies are marked in gray.

did not observe altered COX-2 activity after δ-TOH treatment using a human recombinant enzyme-based assay (Jang et al., 2016). However, formation of PGE_2 was significantly blocked (IC_{50} 1–3 μM) (Jiang et al., 2008). The least abundant form of tocopherols, β-TOH has been rarely studied regarding its anti-inflammatory capacity. Studies available so far did not reveal any anti-inflammatory effects of β-TOH (Jiang et al., 2008).

Tocotrienols. Recent publications reported a more pronounced anti-inflammatory capacity of T3s compared to TOHs, with γ-T3 and α-T3 showing the strongest effects. α-, δ-, and γ-T3 significantly decreased LPS-mediated formation of nitric oxide (by 11%, 31%, 19%, respectively) and PGE_2 (by 30%, 55%, 20%, respectively) in RAW264.7 macrophages treated with 23.5 μM of the respective compound (Yam et al., 2009) as well as bone marrow-derived macrophages (BMDMs) using 1 μM of γ-T3 (Kim et al., 2018). Expression of COX-2 mRNA was inhibited by α-, δ-, and γ-T3, whereas protein expression remained unchanged (Jiang et al., 2008; Yam et al., 2009; Kim et al., 2018). In addition, cytokine-driven inflammation is also dampened by α-, δ-, and γ-T3, which reduced the release of IL-6 and TNF-α in LPS-stimulated RAW264.7 cells. However, γ-T3 reduced expression of IL-6 and TNF-α mRNA as well as the secretion of IL-6, but not of TNF-α in this cell model (Yam et al., 2009). Furthermore, first reports suggest inhibitory effects of γ-T3 on the NLR family pyrin domain containing 3 (NLRP3) inflammasome. In brief, 1 μM γ-T3 suppressed mRNA expression of pro-IL-1β and -18 as well as respective formation of active IL-1β and -18. This has been observed in LPS/nigericin- as well as LPS/palmitate-stimulated BMDMs and *db/db* mice fed with a diet containing 0.1% γ-T3 for eight weeks (Kim et al., 2016; Kim et al., 2018).

Metabolites of Tocopherols and Tocotrienols

We here present a report on selected structures formed during hepatic catabolism of vitamin E, for which data on the biological activity was available. Metabolites formed during physiological hepatic metabolism of vitamin E are highly potent anti-inflammatory compounds with different efficiencies, depending on their methylation pattern (Azzi, 2019) and the number of isoprene units forming the side-chain (Schmölz et al., 2017). Metabolism of non-α-TOH forms of vitamin E is more pronounced, resulting from the lower affinities of these molecules to the α-tocopherol transfer protein. However, α-metabolites revealed significant anti-inflammatory properties. The most widely studied metabolites are the LCMs α-T-13′-OH and -COOH and the short-chain metabolites α- and γ-3′-T-COOH, likely due to their presence in plasma, feces, and urine, respectively, which may account for their physiological relevance (Jiang et al., 2007).

Long- and Intermediate-Chain Tocopherol-Derived Metabolites. Birringer and coworkers showed the relevance of the terminal oxidative modification of the side-chain for biological activity (Birringer et al., 2018). During the hepatic metabolism of TOHs, T-13′-OH are the first metabolites that are formed; these LCMs show distinct effects that are different from those of their respective metabolic precursor (for details, see **Chapter 2.1.1. *Tocopherols and Tocotrienols***). Both, α- and δ-T-13′-OH significantly decreased mRNA (29–72% and 87%, respectively) and

protein (40–53% and 53%, respectively) expression of iNOS and the production of nitric oxide (56–69% and 49%, respectively) in LPS-stimulated murine RAW264.7 macrophages at a concentration of 10 μM, thus showing comparable effect sizes independent from the methylation pattern of the chromanol ring system (Ciffolilli et al., 2015; Schmölz et al., 2017). Furthermore, α-T-13′-OH significantly decreased expression of COX-2 mRNA and protein (64% and 49%, respectively), IL-1β (64%) and IL-6 (68%) mRNA, and the production of PGE_2 (55%) (Ciffolilli et al., 2015).

Notably, the length of the side-chain is important for the mediation of anti-inflammatory effects. Accordingly, both α-T-13′-COOH (5 μM) and δ-T-13′-COOH (5 μM) significantly decreased expressions of iNOS and COX-2 mRNAs as well as proteins in murine LPS-stimulated RAW264.7 macrophages (Wallert et al., 2015; Schmölz et al., 2017). Further, δ-T-13′-COOH inhibited the activity of purified recombinant COX-2 enzyme (5 μM [Jiang et al., 2008; Jang et al., 2016]) as well as in human lung adenocarcinoma A549 cells (4 μM, [Jiang et al., 2008]). Interestingly, the activity of recombinant COX-2 enzymes remained unchanged by α-T-13′-COOH (5–10 μM) (Wallert et al., 2015; Pein et al., 2018). LPS-induced production of the respective signaling molecules, nitric oxide and PGE_2, was completely blocked in murine macrophages (5 μM), but not in LPS-activated human primary monocytes (10 μM) (Pein et al., 2018). In addition, 5-LO-induced formation of pro-inflammatory leukotrienes was dampened by α-T-13′-COOH in LPS-stimulated monocytes (LTB_4), activated human neutrophils, activated human blood, zymosan-induced mouse peritonitis (LTC4), as measured in plasma and exudate, and ovalbumin-induced bronchial hyperreactivity in mice (Pein et al., 2018). Effective concentrations of α-T-13′-COOH, that inhibit 5-LO product formation *in vitro*, were in a range that was detected for the metabolite in human and mice serum without supplementation (<0.3 μM). Furthermore, expression of pro-IL-1β was down-regulated by 5 μM α-T-13′-COOH, whereas IL-6 and TNF-α remained unchanged (Wallert et al., 2015).

Degradation of the LCMs of different vitamin E forms results in formation of respective ICMs that are further processed to SCMs. These metabolic end-products do not accumulate in plasma or tissues and their physiological relevance is therefore considered as less important. Hence, data on these metabolites are scarce. To date, anti-inflammatory effects, *i.e.* the inhibition of COX-2 activity (IC_{50} 6 μM), by δ-9′-T-COOH have been reported in human lung adenocarcinoma A549 cells (Jiang et al., 2008).

Long- and Intermediate-Chain Tocotrienol-Derived Metabolites. Within the group of T3-derived metabolites, the LCM δ-T3-13′-COOH (*i.e.* garcinoic acid) is the most potent anti-inflammatory compound of the ones studied so far. Expression of iNOS (by 97%), COX-2 (by 70%), pro-IL-1β (by 61%), IL-6 (by 70%), and TNF-α (by 25%) mRNA was decreased by 5 μM δ-T3-13′-COOH in LPS-stimulated murine RAW264.7 macrophages. Consequently, protein expression of iNOS (by 83%), COX-2 (by 33%), and the respective formation of NO (by 81%), PGE_2 (by 90%) and thromboxane (TX)B_2 (by 91%) were dampened in LPS-stimulated murine RAW264.7 macrophages (Wallert et al., 2019). Formation of PGE_2 in LPS-stimulated monocytes was inhibited already by 300 nM δ-T3-13′-COOH (Pein et al., 2018). In line with this, δ-T3-13′-COOH also inhibited activity of microsomal PGE_2 synthase (by nearly 70%) at a concentration of 10 μM in a cell-free assay using microsomes of IL-1β-stimulated human lung adenocarcinoma A549 cells as an enzyme source (Alsabil et al., 2016; Pein et al., 2018). However, in *Apoe*$^{-/-}$ mice fed a high fat diet with 1 mg/kg δ-T3-13′-COOH for 8 weeks neither nitric oxide, PGE_2, TXB_2 nor IL-1β concentrations in plasma were altered compared to the control group (Wallert et al., 2019). However, contrary data exist also for the alteration of prostaglandins following inhibition of COX-2 activity: IC_{50} 9.8 μM (Jang et al., 2016) and IC_{50} >10 μM (Pein et al., 2018).

Short-Chain Tocopherol-Derived Metabolites. 5′-T-COOH (CMBHC) and 3′-T-COOH (CEHC) are the SCMs. Physiologically formed γ-3′-T-COOH was mainly detected in urine. Supplementation of α-TOH enhances the hepatic metabolism of α-TOH, which in turn increases degradation of α-TOH and excretion of α-5′-T-COOH and α-3′-T-COOH *via* urine. Both, α-5′-T-COOH (IC_{50} 140 μM) and γ-3′-T-COOH (IC_{50} 450 μM) showed marginal inhibitory effects on human recombinant COX-2 activity (Jiang et al., 2008). However, in IL-1β-stimulated A549 cells, γ-3′-T-COOH (50 μM) exhibited stronger inhibition of COX-2 activity. Formation of PGE_2 was also inhibited in IL-1β-stimulated A549 (50 μM), LPS-stimulated RAW264.7 (10 μM), as well as TNF-α-stimulated RAEC (IC_{50} 59 μM) and EOC-20 cells (IC_{50} 66 μM) (Jiang et al., 2000; Grammas et al., 2004). The TNF-α-induced release of nitric oxide was blocked in EOC-20 (IC_{50} 58 μM) and RAEC cells (IC_{50} 56 μM) by α-3′-T-COOH, whereas 100 μM γ-3′-T-COOH inhibited production of nitric oxide in EOC-20 cells by 10% only (Grammas et al., 2004). In contrast, both α-3′-T-COOH and γ-3′-T-COOH decreased production of nitric oxide in LPS-stimulated EOC-20 cells (Grammas et al., 2004). Notably, lower concentrations did not alter production of nitric oxide (Jiang et al., 2000; Grammas et al., 2004).

Sargachromanols

The sargachromanol forms D, E, and G isolated from *Sargassum siliquastrum* also exert anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages in a concentration-dependent manner. Sargachromanol forms D, E, and G inhibited expression of iNOS protein to 30–50% with concentrations of 15, 12.5, and 20 μM, respectively. In contrast, inhibitory effects on the formation of the respective signaling molecule nitric varies compound-dependent between 10 and 90% (Lee et al., 2013), with SCA E being the most effective (Yoon et al., 2012a; Lee et al., 2013; Heo et al., 2014). Within the inflammatory eicosanoid pathway, expression of COX-2 was inhibited by 15% by SCA D and G and up to 90% by SCA E. The IC_{50} for the formation of COX-2-derived PGE_2 was 15 μM (SCA D [Heo et al., 2014]), 12.5 μM (SCA E [Lee et al., 2013]), and 20 μM (SCA G [Yoon et al., 2012a]), respectively. The LPS-induced production of TNF-α, IL-6 and IL-1β was effectively blocked by SCA D (IC_{50} >60, >20–25, and 40 μM, respectively

[Heo et al., 2014]), E (IC_{50} >25 μM, not investigated and >15 μM, respectively [Lee et al., 2013]), and G (IC_{50} 40, 20, and 20 μM, respectively [Yoon et al., 2012a]). The total inflammatory capacity, as determined by the expression of iNOS and COX-2, the production of their respective signaling molecules, nitric oxide and PGE_2, as well as the production of cytokines leads to the following estimation of compound effectiveness: SCA E > D > G.

Amplexichromanols

Amplexichromanols can be distinguished as α-, β-, γ-, δ-forms. δ-Amplexichromanols have been shown to inhibit the secretion of TNF-α (IC_{50} <10 μM) and IL-1β (IC_{50} 10 μM) in LPS-stimulated monocytes (Richomme et al., 2017). To the best of our knowledge, there are no reports on anti-inflammatory effects of the other forms of AC.

Chromenols

Compared to the complex group of structures comprising the chromanol family, chromenol structures are less ubiquitous. Sargachromenol is described here as a representative of the chromenols with anti-inflammatory effects. An ethanolic extract of *Myagropsis myagroides* inhibited nitric oxide-, eicosanoid-, and cytokine-mediated pathways and the inflammatory response (**Table 2**), with sargachromenol being the lead compound in the extract (Kim et al., 2014). Further studies using isolated sargachromenol from different sources confirmed the results obtained by Kim et al. For instance, sargachromanol isolated from the marine brown alga *Sargassum serratifolium* inhibited peroxinitrite anion-mediated albumin nitration with an IC_{50} of 5 μM (Ali et al., 2017). Furthermore, the COX-2 pathway was inhibited using 50 μM and 100 ppm sargachromenol isolated from *Sargassum micracanthum* (Yang et al., 2013) and *Iryanthera juruensis* seeds (Silva et al., 2007), respectively. Here, the effect sizes of 70 and 84% found by Yang *et al*. and Silva *et al.*, respectively, are comparable with respect to the inhibition of the expression of COX-2 protein. For the respective signaling molecule PGE_2 an IC_{50} value of 30 μM was defined (Yang et al., 2013). In addition, inhibitory effects were observed for the expression of iNOS protein (95%) and the formation of nitric oxide (IC_{50} 82 μM) (Yang et al., 2013).

Carcinogenesis

For the evaluation of anti-carcinogenic effects of chromanol and chromenol structures, key apoptotic pathways, such as cleavage of poly-[ADP-ribose]-polymerase 1 (PARP-1), caspases 3, 7, 8, and 9 as well as anti-proliferative and cytotoxic properties on cancer cell lines and further markers of carcinogenesis marker in mice were evaluated (**Figure 7**). In addition, large-scaled human trials investigating preventive and therapeutic effects of some tested compounds will be discussed in the following chapter.

Chromanols

A detailed overview on the biological activities of chromanols linked to carcinogenesis is provided in **Table 3**.

Tocopherols and Tocotrienols

Like the mediation of anti-inflammatory effects, anti-carcinogenic actions were profoundly investigated for α-TOH

TABLE 2 | Overview on the biological activities of chromenols linked to inflammation.

Nitric oxide		Eicosanoid-mediated		Cytokine-mediated		
iNOS	**NO**	**COX-2**	**PGE2**	**IL-1β**	**IL-6**	**TNF-α**
Sargachromenol						
LPS iNOS PE BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS NO PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS COX-2 E BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS PGE_2 PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS IL-1β PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS IL-6 PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS TNF-α PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)
LPS iNOS PE RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS NO PrD RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS COX-2 PE RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Yang et al., 2013)			
	peroxynitrite NO PrD BSA nitrition 2.5 μM inhibition (Ali et al., 2017)	– COX-2 A enzyme 100 ppm inhibition (Silva et al., 2007)				

The effects of the respective compounds on inflammation have been divided into activities mediated by nitric oxide (iNOs, NO), eicosanoids (COX-2, PGE_2), and cytokines (IL-1β, IL-6, TNF-α). The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. In the publications where no stimulus was used or was required for the studies, the respective row is marked with a dash. The following abbreviations are used: A, activity; BSA, bovine serum albumin; BV-2, brain microglial cells transformed by recombinant retrovirus (v-raf/v-mic); COX-2, Cyclooxygenase 2; E, expression; iNOS, induce able nitric oxide synthase; IL-1β, interleukin 1β; IL-6, interleukin 6; LPS, lipopolysaccharides; m, murine; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; NO, nitric oxide; PrD, production; PGE_2, prostaglandin E_2; PE, protein expression; TNF-α, tumor necrosis factor α.

TABLE 3 | Overview on the biological activities of chromanols linked to carcinogenesis.

Apoptosis/Necrosis mediated					Proliferation		Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**				
α-TOH								
PARP-1 CL MDA-MB-231 MCF-7 cells 23 μM no induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 μM no induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 μM no induction (Husain et al., 2011)	Casp7 CL SW 480 cells HCT-116 100 μM no induction (Campbell et al., 2006)	MDA-MB-435 > 2000 μM MCF-7 cells 290 μM no inhibiton (Guthrie et al., 1997)	MDA-MB-435 230 μM no inhibition (Nesaretnam et al., 1995)		h_cc cells 200 μM no reduction (Campbell et al., 2006)
PARP-1 CL SW 480 cells HCT-116 100 μM no induction (Campbell et al., 2006)	Casp8 CL SW 480 cells HCT-116 100 μM no induction (Campbell et al., 2006)		Casp3 CL SW 480 cells HCT-116 100 μM no induction (Campbell et al., 2006)		m_NB2A cells inhibition h_ SaOs-2 cells no inhibition 50 μM (Azzi et al., 1993)	Du-145 cells LNCaP cells CaCo-2 cells 25 μM inhibition SaOs-2 cells no inhibition (Gysin et al., 2002)		MCF-7, MCF-7-C3 50 μM no reduction (Birringer et al., 2003)
					PC-3 HTB-82 50 μM inhibition (Galli et al., 2004) MDA-MB-231 MCF-7 cells 46.5 μM no inhibition (Loganathan et al., 2013)	MCF-7 cells 23 μM no inhibition (Nesaretnam et al., 1998) HT-29 100 μM inhibition (Campbell et al., 2006)		
β-TOH								
					Du-145 cells LNCaP cells SaOs-2 cells 25 μM inhibition (Gysin et al., 2002)	m_NB2A cells h_ SaOs-2 cells 50 μM no inhibition (Azzi et al., 1993)		MCF-7, MCF-7-C3 50 μM no reduction (Birringer et al., 2003)
γ-TOH								
PARP-1 CL SW 480 cells HCT-116 100 μM induction (Campbell et al., 2006)	Casp8 CL SW 480 cells HCT-116 100 μM induction (Campbell et al., 2006)		Casp3 CL SW 480 cells HCT-116 100 μM induction (Campbell et al., 2006)	Casp7 CL SW 480 cells HCT-116 100 μM induction (Campbell et al., 2006)	PC-3 cells HTB-82 cells 1 μM inhibition (Galli et al., 2004)	h_cc cells 100 μM inhibition (Campbell et al., 2006)	HCT-116, HT-29 50 μM no inhibition (Jang et al., 2016)	h_cc cells 100 μM reduction (Campbell et al., 2006)
					Du-145 cells LNCaP cells CaCo-2 cells SaOs-2 cells 25 μM inhibition (Gysin et al., 2002)	tumor count m_BALB/c 0.1% diet reduction (Jiang et al., 2013)	PC-3, LNCaP 50 μM inhibition (Jiang et al., 2012)	MCF-7, MCF-7-C3 50 μM no reduction (Birringer et al., 2003)
δ-TOH								
							MCF-7, MCF-7-C3 50 μM no reduction (Birringer et al., 2003)	HCT-116 inhibition HT-29 no reduction 50 μM (Jang et al., 2016)
α-T3								

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation		Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**				
PARP-1 CL, MDA-MB-231, MCF-7 cells 23.5 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		MDA-MB-435 211.9 µM MCF-7 cells 14.1 µM inhibition (Guthrie et al., 1997)	m_B16(F10) 110 µM inhibition (He et al., 1997)	SCID mice 200 mg/kg no reduction (Husain et al., 2011)	MiaPaCa-2, 50 µM no reduction (Husain et al., 2011)
PARP-1 CL MiaPaCa-2 50 µM no induction (Husain et al., 2011)					MDA-MB-231 22.5 µM MCF-7 cells 26.1 µM inhibition (Loganathan et al., 2013)	MCF-7 23.5 µM no inhibition (Nesaretnam et al., 1998)		MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)
β-T3								MiaPaCa-2, 50 µM reduction (Husain et al., 2011)
γ-T3								
PARP-1 CL, MDA-MB-231, MCF-7 cells 24.2 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)	Casp9 CL PC-3, LNCaP 20 µM induction (Jiang et al., 2012)	Casp3 A MCF-7, MCF-7-C3 50 µM induction (Birringer et al., 2003)	Casp7 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	SKBR3, BT474 5 µM inhibition (Alawin et al., 2016)	rh_RLh-84 50 µM inhibition (Sakai et al., 2004)	MiaPaCa-2, 50 µM reduction (Husain et al., 2011)	PC-3, LNCaP 20 µM reduction (Jiang et al., 2012)
PARP-1 CL MiaPaCa-2 50 µM induction (Husain et al., 2011)	Casp8 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	Casp9 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		m_B16(F10) 20 µM inhibition (He et al., 1997)	PC-3 32 µM inhibition (Yap et al., 2008)		
PARP-1 CL PC-3, LNCaP 20 µM induction (Jiang et al., 2012)	Casp8 CL rh_RLh-84 25 µM induction (Sakai et al., 2004)		Casp3 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)		MDA-MB-231 11.4 µM MCF-7 cells 15.4 µM inhibition (Loganathan et al., 2013)	MCF-7 14.6 µM inhibition (Nesaretnam et al., 1998)		
PARP-1 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)			Casp3 CL rh_RLh-84 25 µM induction (Sakai et al., 2004)		MDA-MB-435 73.2 µM MCF-7 cells 4.9 µM inhibition (Guthrie et al., 1997)			
δ-T3								
PARP-1 CL, MDA-MB-231, MCF-7 cells 25.2 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		MDA-MB-435 226.8 µM MCF-7 cells 5 µM inhibition (Guthrie et al., 1997)	PC-3 41 µM LNCap 75 µM inhibition (Yap et al., 2008)		MiaPaCa-2, 50 µM reduction (Husain et al., 2011)
PARP-1 CL MiaPaCa-2 50 µM induction					m_B16(F10) 10 µM inhibition (He et al., 1997)	MCF-7 25.2 µM inhibition (Nesaretnam et al., 1998)		

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation	Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**			
(Husain et al., 2011)							
					MDA-MB-231 MCF-7 cells 17 μM inhibition (Loganathan et al., 2013)		
α-T-13'-OH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 μM no induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 μM no induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 μM no induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 μM no induction (Birringer et al., 2010)		m_C6 cells 10 μM reduction (Mazzini et al., 2009)	THP-1 MΦ 100 μM no reduction (Wallert et al., 2014a)
α-T-13'-COOH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 μM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 μM induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		THP-1 MΦ 7.4 μM reduction (Wallert et al., 2014a)	HepG2 cells 13.5 μM reduction (Birringer et al., 2010)
δ-T-13'-OH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 μM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 μM no induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		THP-1 100 μM no reduction (Schmölz et al., 2017)	HepG2 cells 50 μM no reduction (Birringer et al., 2010)
δ-T-13'-COOH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 μM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 μM induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 μM induction (Birringer et al., 2010)		HCT-116 HT-29 8.9/8.6 μM reduction (Jang et al., 2016)	HepG2 cells 6.5 μM reduction (Birringer et al., 2010)
PARP-1 CL HCT-116 20 μM induction (Jang et al., 2016)		Casp9 CL HCT-116 20 μM induction (Jang et al., 2016)				m_C6 cells 10 μM reduction (Mazzini et al., 2009)	HCT-116 HT-29 8.9/8.6 μM reduction (Jang et al., 2016)
						THP-1 11.1 μM reduction (Schmölz et al., 2017)	
α-T-3'-COOH (tocopherol derived)							
					PC-3 cells HTB-82 cells 1 μM inhibition (Galli et al., 2004)		m_C6 cells 10 μM reduction (Mazzini et al., 2009)
γ-T-3'-COOH (tocopherol derived)							
					PC-3 cells HTB-82 cells 1 μM inhibition (Galli et al., 2004)		m_C6 cells 10 μM reduction (Mazzini et al., 2009)
δ-T3-13′-COOH							

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation	Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**			
PARP-1 CL HCT-116 20 μM induction (Jang et al., 2016)		Casp9 CL HCT-116 20 μM induction (Jang et al., 2016)				m_C6 cells 10 μM reduction (Mazzini et al., 2009)	HCT-116 HT-29 16/17 μM reduction (Jang et al., 2016)
SCA E							
PARP-1 CL h_HL-60 25 μM induction (Heo et al., 2011)		Casp9 CL h_HL-60 25 μM induction (Heo et al., 2011)	Casp3 CL h_HL-60 25 μM induction (Heo et al., 2011)		h_HL-60 50 μM inhibition (Heo et al., 2011)		
α-AC							
							HepaRG 10 μM no reduction (Richomme et al., 2017)

The effects of the respective compounds on carcinogenesis have been divided into apoptosis-mediated (PARP-1, caspases 3, 7, 8, and 9) activities as well as activities affecting proliferation and viability. The content of each cell of the table in the apoptosis section is constructed as follows (read from top to bottom): (i) investigated parameter; (ii) cell type model used for investigation; (iii) used concentration of the respective compound; (iv) observed effect on the studied parameter; (v) reference. The content of each table cell in the proliferation as well as viability section is constructed as follows (read from top to bottom): (i) cell type model used for investigation; (ii) used concentration of the respective compound; (iii) observed effect on the studied parameter; (iv) reference. The following abbreviations are used: A, activity; A549, adenocarcinomic human alveolar basal epithelial cells; BALB/c mice, albino laboratory-bred strain of the house mouse; LNCap, androgen-sensitive human prostate adenocarcinoma cells; Casp, caspase; MCF-7-C3, caspase 3 reconstituted MCF-7 cells; CL, cleavage; cc, colon cancer; MDA-MB-23, epithelial human breast cancer cell line; NB2A, fast-growing mouse neuroblastoma cell line; h, human; MCF-7, human breast cancer cell line established by Michigan Cancer Foundation-7; SKBR3, human breast cancer cell line isolated by the Memorial Sloan–Kettering Cancer Centere; BT-474, human breast carcinoma ductal cell line; SaOs-2, human cell line derived from primary osteosarcoma; SW-480, human cell line established from a lymph node metastasis; HCT-116, human colon cancer cell line; HT-29, human colorectal adenocarcinoma cell line; CaCo-2, human epithelial colorectal adenocarcinoma cells; THP-1, human immortalized monocyte-like cell line; HL-60, human leukemia cell line; HepG2, human liver cancer cell line; MiaPaCa-2, human pancreatic cancer cell line; Du-145, human prostate cancer cell line; PC-3, human prostate cancer cell line; B16(10), mouse skin melanoma cells; m, murine; m_C6, murine glial cancer cell line; PARP-1, poly (ADP-Ribose)-polymerase 1; HTB-82, rhabdomyosarcoma cell line; rh_RLh-84, rat hepatoma cell line; HepaRG, terminally differentiated hepatic cells derived from a human hepatic progenitor cell line.
All results obtained from in vivo studies are marked in gray.

and γ-TOH, due to their biological relevance. Screening of multiple human breast cancer cell lines (Nesaretnam et al., 1995; Guthrie et al., 1997; Nesaretnam et al., 1998; Birringer et al., 2003; Loganathan et al., 2013) and the human osteosarcoma cell line Saos-2 (Azzi et al., 1993; Gysin et al., 2002) revealed no anti-proliferative effects or alteration of cell viability using α-TOH (4.6–230 μM), whereas Campbell et al. found controversial results for different human colon cancer cell lines using different assays (Campbell et al., 2006). However, the colon cancer cell lines HT-29 (Campbell et al., 2006) and CaCo-2 (Gysin et al., 2002) treated with 100 μM (48 h), 200 μM (5 h), and 25 μM (24 h) α-TOH showed significantly induced cell death and dampened proliferation. In addition, proliferation of different human prostate cancer cells was significantly inhibited by α-TOH. In brief, 25–50 μM α-TOH inhibited proliferation of PC-3 cells (41%, 24 h) (Galli et al., 2004), Du-145 cells (50%, 24 h), and LNCaP cells (48%, 48 h) (Gysin et al., 2002). Furthermore, α-TOH (50 μM) significantly inhibited the growth of murine neuroblastoma NB2A cells by 50% (Azzi et al., 1993), and rhabdomyosarcoma HTB-82 cells by 32% (Galli et al., 2004). However, effects on proliferation and viability seemed to be independent from the cleavage and activity of the apoptosis marker PARP-1 and caspases 3, 7, and 8 in breast cancer (23 μM [Loganathan et al., 2013]), pancreas cancer (50 μM [Husain et al., 2011]), and colon cancer cells (100 μM [Campbell et al., 2006]). β-TOH-treatment of cancer cells revealed similar effects compared to α-TOH. While growth of human prostate cancer cells was significantly inhibited by >40% (Gysin et al., 2002), growth of human osteosarcoma cells was marginally inhibited. In neuroblastoma (Azzi et al., 1993) and breast cancer cells (Birringer et al., 2003) β-TOH did not alter cell viability.

γ-Tocopherol is by far the most potent anti-carcinogenic TOH regarding prostate-cancer. Indeed, viability or rather proliferation of prostate-cancer cell lines PC-3 (1 μM [Galli et al., 2004], 50 μM [Jiang et al., 2012]), CaCo-2, Du-145, LNCaP (25 μM [Gysin et al., 2002]), SW480, HCT-116, HCT-15, and HCT-29 (100 μM [Campbell et al., 2006]) was blocked by γ-TOH. More precisely, 100 μM γ-TOH induced apoptosis in SW480 and HCT-116 cells following the cleavage of PARP-1 as well as caspases 3, 7, and 8 (Campbell et al., 2006). Described effects are most likely tumor-specific, finding no or weak alteration of tumor growth on breast cancer cell lines (Birringer et al., 2003) and colon carcinoma cells (Jang et al., 2016). However, in male BALB/c mice γ-TOH (0.1% of diet) suppressed DSS- and AOM-induced tumor multiplicity of macroscopic adenomas and large adenomatous polyps (>2mm^2) by 60, 85, and 36% (Jiang et al., 2013). Of the tested tumor cell lines, only viability of HCT-116 was inhibited by 50

μM δ-TOH, whereas HT-29 cells, and the breast cancer cell lines MCF-7 and MCF-7-C3 were not affected (Birringer et al., 2003; Jang et al., 2016). Based on the presented data, anti-carcinogenic capacity for different forms of TOHs can be assessed as γ-TOH >> β-TOH > α/δ-TOH.

Despite of the promising results outlined above, it should be noticed that several human trials failed to confirm preventive effects of vitamin E, in particular α-TOH, against cancer. The Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention Study examined whether a daily supplementation of 50 mg α-TOH and/or 20 mg β-carotene could prevent lung cancer in male smokers (Virtamo et al., 2014). However, after five to eight years of supplementation of either α-TOH or β-carotene or the combination of both failed to prevent lung cancer (Virtamo et al., 2014). In addition, other human intervention trails revealed disappointing results, with the Selenium and Vitamin E Cancer Prevention Trial (SELECT) representing a very interesting one. The aim of the SELECT study was to investigate the preventive potential of α-TOH and/or selenium on prostate cancer. In the SELECT trial, healthy men received a daily dose of either 400 IU all-*rac*-α-tocopheryl acetate or 200 μg selenium or a combination of both for an average of 5.5 years (Lippman et al., 2009). Supplementation with both compounds failed to prevent prostate cancer development. Surprisingly, daily supplementation with all-*rac*-α-tocopheryl acetate was slightly, but not significantly, associated with an increased overall risk for prostate cancer (Lippman et al., 2009). Next, in the 7 to 12 years follow-up the subjects who had received a daily dose of 400 IU all-*rac*-α-tocopheryl acetate showed a significantly enhanced risk for prostate cancer (Klein et al., 2011). This result indicates that a dietary supplementation with high doses of this vitamin E derivate could result in an increased risk for cancer.

The T3-rich fraction of palm oil is comprised of all T3 forms (α- [25%], γ- [29%], δ-T3 [14%] relative to the total vitamin E amount) and inhibits the proliferation of the estrogen receptor-negative human breast cancer cell line MDA-MB-435 with an IC_{50} of 180 μg/ml (Nesaretnam et al., 1995). Based on that finding, single forms of T3s were tested regarding their effects on proliferation and viability of carcinoma cell lines. The α-, γ-, and δ-forms of T3s were found to mediate cancer type specific effectiveness, with breast cancer cell lines being most affected by the treatment with TOHs. Viability and proliferation of MDA-MB-231 (IC_{50} 22.5 μM), MCF-7 (IC_{50} 14.1–26.1 μM), and MDA-MB-435 cells (IC_{50}: 211.9 μM) were concentration-dependently affected by α-T3 treatment independent on whether they were responsive to estrogen and estradiol (Guthrie et al., 1997; Nesaretnam et al., 1998; Loganathan et al., 2013). However, whereas cleavage of PARP-1 (Loganathan et al., 2013) has been observed, general involvement of apoptosis has not been described yet (Birringer et al., 2003). Although cleavage of PARP-1 as well as caspases 3 and 8 has been observed in pancreatic MiaPaCa-2 carcinoma cells, 50 μM α-T3 had no effect on cell viability (Husain et al., 2011). In contrast, β-T3 (50 μM) reduced the viability of MiaPaCa-2 cells (Husain et al., 2011). In mice, 200 mg/kg α-T3 did not affect tumor growth of AsPC-1 human pancreatic cancer xenografts (Husain et al., 2011), whereas 110 μM α-T3 suppressed proliferation of murine B16(F10) melanoma cells (He et al., 1997).

Within the group of TOHs and T3s, γ-T3 is the most potent anti-carcinogenic form that affects cell growth of breast, prostate, pancreas, and hepatic cancer cells, likely due to a preferred incorporation of γ-T3 in these cells (Sakai et al., 2004). There is strong evidence for the anti-proliferative effects of γ-T3 on breast cancer cell lines MDA-MB-231 (IC_{50} 11.4 μM), MCF-7 (IC_{50} 15.4 μM) (Loganathan et al., 2013), SKBR3 (IC_{50} 4 μM), BT474 (IC_{50} 4 μM) (Alawin et al., 2016), estrogen receptor-negative MDA-MB (IC_{50} 73.2 μM), and estrogen receptor-positive MCF-7 cells (IC_{50} 4.9 μM) (Guthrie et al., 1997). Others even found complete inhibition of MCF-7 cell growth by γ-T3 at a concentration of 14.6 μM (Nesaretnam et al., 1998). Inhibitory effects on proliferation were at least in part mediated *via* the activation of apoptosis, such as activation of caspase 3 in MCF-7 (25%), and MCF-7-C3 cells (35%) with 50 μM γ-T3 (Birringer et al., 2003). Furthermore, the proliferation of MiaPaCa-2 pancreas cancer cells (Husain et al., 2011), PC-3 prostate cancer cells (IC_{50}: 32 μM, 24 h), and dRLh-84 hepatic cancer cells (IC_{50}: 80–100 μM, 24 h) was suppressed by γ-T3, most likely *via* cleavage of PARP-1, and caspases 3, 7, 8, and 9 (Sakai et al., 2004; Yap et al., 2008) and induction of autophagy (Jiang et al., 2012). In murine B16(F10) melanoma cells (He et al., 1997) and the myelogenous leukemia cell line KBM-5 (Ahn et al., 2007) γ-T3 significantly suppressed proliferation (IC_{50} 20 μM, 24 h). Comparable to γ-T3, δ-T3 inhibits the proliferation of the breast cancer cell lines MDA-MB-435 (IC_{50} 226.8 μM), MDA-MB-231 (IC_{50} 17.4 μM), and MCF-7 cells (IC_{50} 5–25.2 μM) (Guthrie et al., 1997; Nesaretnam et al., 1998; Loganathan et al., 2013), as well as prostate cancer cell lines PC-3 (IC_{50} 41 μM), and LNCaP (IC_{50} 75 μM) (Yap et al., 2008), melanoma B16(F10) cells (IC_{50} 10 μM) (He et al., 1997), and MiaPaCa-2 pancreas cancer cells (IC_{50} 50 μM) (Husain et al., 2011) by the induction of apoptosis, as indicated by the cleavage of apoptosis-mediating PARP-1 as well as caspases 3 and 8 (Husain et al., 2011).

Metabolites of Tocopherols and Tocotrienols

In contrast to the TOH and T3 forms, the respective metabolites have been rarely investigated regarding their anti-carcinogenic properties. The LCMs of TOHs, namely α-T-13′-COOH (20 μM) and δ-T-13′-COOH (20 μM) induced apoptosis *via* the mitochondrial pathway, which was shown by cleavage of PARP-1 and caspases 3, 7, and 9, resulting in decreased viability of HepG2 cells (IC_{50} 13.5 μM and 6.5 μM, respectively, Birringer et al., 2010). In human leukemia-derived THP-1 macrophages, viability was decreased by α-T-13′-COOH (IC_{50} 7.4 μM, Wallert et al., 2014a) and δ-T-13′-COOH (IC_{50} 11.1 μM, Schmölz et al., 2017). In addition, δ-T-13′-COOH increased apoptosis-induced cytotoxicity in HCT-116 (IC_{50} 8.9 μM), HT-29 (IC_{50} 8.6 μM) (Jang et al., 2016), and C6 cells (IC_{50} <10 μM, Mazzini et al., 2009). The T3-derived δ-garcinoic acid decreased the viability of HCT-116, HT-29 (Jang et al., 2016), glioma C6 (Mazzini et al., 2009), and human THP-1 macrophage-like cells (IC_{50} <20 μM, unpublished data) to a similar extent. In BALB/c mice fed with 0.022%, δ-garcinoic acid in the diet, AOM- and DSS-induced

colon tumor growth was decreased (Jang et al., 2016). In contrast to the carboxychromanol structures, the hydroxychromanols were less efficient in the cleavage of apoptosis markers and consequently did not affect the viability of HepG2 cells (Birringer et al., 2010) and THP-1 macrophages (Wallert et al., 2014a; Schmölz et al., 2017) at concentrations up to 50 μM and 100 μM, respectively, whereas an anti-proliferative effect on glioma C6 cancer cells was determined using 10 μM α-T-13′-OH (Mazzini et al., 2009). Short-chain metabolites were found to affect growth of prostate cancer cells PC-3 and rhabdomyosarcoma HTB-82 cells at a concentration of 1 μM (Galli et al., 2004).

Sargachromanols

The group of sargachromanols may serve as anti-carcinogenic agents that suppress cell proliferation as reported for SCA E in HL-60 leukemia cells accompanied by cleavage of PARP-1 as well as caspases 3 and 9 (Heo et al., 2011). However, confirmatory data are pending.

Amplexichromanols

To date, α-AC has been studied only in HepaRG cells, without effects on viability up to concentrations of 10 μM (Richomme et al., 2017). Therefore, studies on anti-carcinogenic effects of amplexichromanols are still on demand.

Chromenols

Within the group of chromenols, δ-sargachromenol is the best-studied one. Previous studies revealed an induction of the cleavage of PARP-1 and caspases along with the induction of apoptosis and reduced cell viability in human skin keratinocyte (HaCaT) cells (Hur et al., 2008). Data obtained from cancer cell lines is still lacking.

INTERFERENCE WITH MOLECULAR TARGETS AND KEY PROTEINS CONNECTING INFLAMMATION AND CARCINOGENESIS

Many signaling molecules involved in inflammatory processes play in parallel also key roles in carcinogenesis. We here exemplarily focus on the interaction of selected chromanols and chromenols with the molecular crosstalk of NF-κB (Jurjus et al., 2016), lipoxygenases (Rådmark et al., 2015; Roos et al., 2016; Merchant et al., 2018), MAPK (Gkouveris and Nikitakis, 2017; Jiménez-Martínez et al., 2019), and the inflammasome (Moossavi et al., 2018; Swanson et al., 2019) due to their accepted involvement in both, inflammation and cancer (**Figure 7**). However, due to the sparse knowledge about their connection to chromanols and chromenols, further topics, like the interaction of tumor and immune cells, adhesion proteins, structure and regulation of tumor microenvironments, mechanisms for programed cell death as well as other prominent signaling pathways (PI3K/Akt/mTOR; PKC; STAT; Wnt/β-catenin), were not considered in this review.

Chromanols

A detailed overview on the interference of chromanols with molecular targets and key enzymes connecting inflammation and carcinogenesis is provided in **Table 4**.

Tocopherols and Tocotrienols

As outlined above, inflammation and carcinogenesis are only marginally affected by α-TOH. This is probably the consequence of a lack of interference of α-TOH with NF-κB. Neither in phorbol-12-myristat-13-acetate (PMA)-stimulated BALBc/3T3 fibroblasts (Azzi et al., 1993), and human pancreatic cancer MiaPaCa-2 cells (Husain et al., 2011), nor TNF-α-stimulated murine myelogenous leukemia KBM-5 cells (Ahn et al., 2007), α-TOH (50 μM), β-TOH (50 μM), or γ-TOH (25 μM) affected NF-κB binding affinity or its activation. In murine RAW264.7 macrophages, 100 μM α-TOH even induced translocation of p65 into the nucleus (Wallert et al., 2015). However, pharmacological doses of α-TOH (500 μM) inhibited NF-κB transcriptional activity as well as the phosphorylation and subsequent degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB)-α, the inhibitor of NF-κB, resulting in decreased NF-κB activation in multifactorially stimulated dendritic cells (Tan et al., 2005). γ-Tocotrienol and δ-T3 significantly decreased NF-κB/p65 binding affinity in MiaPaCa-2 cells and diminished p65 subunit translocation in AsPc-1 cells and tumor tissue. In addition, β-T3 and δ-T3 inhibited the translocation in MiaPaCa-2 cells (Husain et al., 2011). The NF-κB inhibitor IκB-α remained unchanged in the aforementioned study. Within the group of T3s, γ-T3 has been described to affect NF-κB activation and p65 subunit translocation in various cell lines and isolated tissue. For example, γ-T3 (20–40 μM) inhibited the phosphorylation of IκB-α and the nuclear translocation of the p65 subunit following various stimuli, including pro-inflammatory cytokines, tumor promoters, carcinogens, and growth factors in different cell lines (Ahn et al., 2007; Yap et al., 2008; Wang et al., 2015). Further, γ-T3 treatment also increased IκB-α protein expression in epididymal adipose tissues isolated from γ-T3-fed *db/db* mice (Kim et al., 2016) as well as in LPS/palmitate-activated BMDM using 1 μM γ-T3 (Kim et al., 2018). In mice, 400 mg γ-T3/kg, applied orally, sensitized pancreatic tumors to gemcitabine treatment, a drug applied in clinical treatment of pancreatic cancer, by suppressing NF-κB-mediated inflammatory pathways linked to tumorigenesis (Kunnumakkara et al., 2010). The expression of A20 (acronym: TNFAIP3), another inhibitor of NF-κB, was induced by 20 μM γ-T3 in RAW264.7, A549, PC3, and MCF-7 cells (Wang et al., 2015) as well as in peritoneal macrophages obtained from diabetic *db/db* mice fed with a γ-T3-containing diet (0.1%) (Kim et al., 2016).

5-, 12-, and 15-LO pathways mediate the formation of lipid mediators (including leukotrienes, lipoxins, resolvins, protectins, and maresins), which orchestrate inflammation by triggering immune cell recruitment and allergic responses, and/or actively terminating inflammation, i.e. triggering resolution of inflammation. Leukotrienes and the so-called specialized pro-resolving lipid mediators (produced by the tumor-

TABLE 4 | Overview on the interference of chromanols with molecular targets and key enzymes connecting inflammation and carcinogenesis.

NF-κB				NLRP3	MAPKs	Lipoxygenases	
α-TOH							
PMA NF-κB A BALB c/3T3 fibroblasts 50 μM no inhibition (Azzi et al., 1993)	LPS NF-κB PE (Nucleus) RAW264.7 100 μM induction (Wallert et al., 2015)	IL-1β, IL-6, TNF-α, LPS, PGE_2, INF-γ h_DC NF-κB A 500 μM inhibition (Tan et al., 2005)	IL-1β, IL-6, TNF-α, LPS, PGE_2, INF-γ h_DC IκB-α Phos 500 μM inhibition (Tan et al., 2005)		–	–	AA 5-LO PF PMNL 808 μM inhibition (Pein et al., 2018)
					5-LO A enzyme 5 μM inhibition (Reddanna et al., 1985)	5-LO A enzyme >1 μM inhibition (Pein et al., 2018)	
–					AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	
p65 DNA BA MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)							
β-TOH							
PMA NF-κB A Balb c/3T3 fibroblasts 50 μM no inhibition (Azzi et al., 1993)					–	AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
					5-LO A enzyme 750 nM inhibition (Pein et al., 2018)		
					AA 5-LO PF PMNL 57 μM inhibition (Pein et al., 2018)		
γ-TOH							
TNF-α NF-κB Actv KBM-5 25 μM no inhibition (Ahn et al., 2007)					–	AA 5-LO PF PMNL 502 μM inhibition (Pein et al., 2018)	– 5-LO A enzyme 910 nM inhibition (Pein et al., 2018)
					5-LO A enzyme 2–3 μM inhibition (Reddanna et al., 1985)		
					–	AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
					5-LO A enzyme > 50 μM no inhibition (Jang et al., 2016)		
					– 5-LO A enzyme 200 μM inhibition (Jiang et al., 2011)		
δ-TOH							
					–	–	
					5-LO A enzyme 310 nM inhibition (Pein et al., 2018)	5-LO A enzyme > 50 μM no inhibition (Jang et al., 2016)	

(Continued)

TABLE 4 | Continued

NF-κB				NLRP3	MAPKs		Lipoxygenases	
						AA 5-LO PF PMNL 85 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM inhibition (Pein et al., 2018)
α-T3								
– p65 DNA BA MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl AsPC-1 50 μM no inhibition (Husain et al., 2011)	– p65 Trl m_TT 50 μM no inhibition (Husain et al., 2011)			– 5-LO A enzyme 330 nM inhibition (Pein et al., 2018) AA 5-LO PF PMNL 277 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
β-T3								
– p65 DNA BA (Cytosol) MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 DNA BA (Nucleus) MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 Trl m_TT 50 μM no inhibition (Husain et al., 2011)			– 5-LO A enzyme 190 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
– p65 Trl AsPC-1 50 μM no inhibition (Husain et al., 2011)						AA 5-LO PF PMNL 95 μM inhibition (Pein et al., 2018)		
γ-T3								
– p65 Trl PC3 cells 40 μM inhibition (Yap et al., 2008)	TNF-α NF-κB Actv RAW264.7 20 μM inhibition (Wang et al., 2015)	TNF-α NF-κB Actv H1299, A293, MCF-7, U226, SCC4 25 μM inhibition (Ahn et al., 2007)	diabetes IκB-α PE *db/db* mice 0.1% of diet induction (Kim et al., 2016)	LPS/pal, Ng NLRP3 E m_BMDM 1 μM inhibition (Kim et al., 2016)	diabetes p38 Phos db/db mice 0.1% of diet inhibition (Kim et al., 2016)	- 5-LO A enzyme 200 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
– NF-κB Actv mice 400 mg/kg/d inhibition (Kunnumakkara et al., 2010)	– p65 DNA BA MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 Trl AsPC-1 50 μM inhibition (Husain et al., 2011)	TNF-α IκB-α PE RAW264.7, m_BMDM 20 μM induction (Wang et al., 2015)	diabetes NLRP3 E db/db mice derived PM/AT 0.1% of diet inhibition (Kim et al., 2016)	diabetes ERK Phos db/db mice 0.1% of diet inhibition (Kim et al., 2016)	AA 5-LO PF PMNL 132 μM inhibition (Pein et al., 2018)	LPS/pal 5-LO GE BMDM 1 μM no inhibition (Kim et al., 2018)	
– p65 Trl MiaPaCa-2 50 μM no inhibition	– p65 Trl m_TT 50 μM inhibition	TNF-α IκB-α Phos A549, PC3, MCF-7 20 μM inhibition (Wang et al., 2015)	LPS/pal IκB-α PE m_BMDM 1 μM induction (Kim et al., 2018)		TNF-α JNK Phos RAW264.7, m_BMDM 20 μM inhibition			

(Continued)

TABLE 4 | Continued

NF-κB				NLRP3	MAPKs	Lipoxygenases		
(Husain et al., 2011)	(Husain et al., 2011)				(Wang et al., 2015) LPS ERK Phos m_BMDM 0.5 μM inhibition (Kim et al., 2016)			
– IκB-α Phos AsPC-1 50 μM inhibition (Husain et al., 2011)	– IκB-α Phos MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– IκB-α Phos m_TT 50 μM inhibition (Husain et al., 2011)						
δ-T3								
– p65 DNA BA MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 Trl AsPC-1 50 μM inhibition (Husain et al., 2011)	– p65 Trl MiaPaCa-1 50 μM inhibition (Husain et al., 2011)	– IκB-α Phos MiaPaCa-2, 50 μM inhibition (Husain et al., 2011)			– 5-LO A enzyme 170 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
– p65 Trl m_TT 50 μM inhibition (Husain et al., 2011)	– IκB-α Phos MiaPaCa-2, 50 μM inhibition (Husain et al., 2011)	– IκB-α Phos m_TT 50 μM inhibition (Husain et al., 2011)				AA 5-LO PF PMNL 60 μM inhibition (Pein et al., 2018)		
α-T-13′-OH								
						– 5-LO A enzyme 350 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM induction (Pein et al., 2018)
						AA 5-LO PF PMNL 190 nM inhibition (Pein et al., 2018)		
α-T-13′-COOH								
LPS p65 Trl RAW264.7 2.5 μM no inhibition (Wallert et al., 2015)						– 5-LO A enzyme 270 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM inhibition (Pein et al., 2018)
						AA 5-LO PF PMNL 80 nM inhibition (Pein et al., 2018)		
δ-T-13′-OH								
						– 5-LO A enzyme 120 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM induction (Pein et al., 2018)

(Continued)

TABLE 4 | Continued

NF-κB	NLRP3	MAPKs	Lipoxygenases	
		AA 5-LO PF PMNL 540 nM inhibition (Pein et al., 2018)		
δ-T-13′-COOH				
		– 5-LO A enzyme >1 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM induction (Pein et al., 2018)
		AA 5-LO PF PMNL 2 μM inhibition (Pein et al., 2018)	– 5-LO A enzyme 2 μM inhibition (Jang et al., 2016)	Ca^{2+} 5-LO PF HL-60 50 μM inhibition (Jiang et al., 2011)
		– 5-LO A enzyme 0.5–1 μM inhibition (Jiang et al., 2011)		
α-T-5′-COOH				
		– 5-LO A enzyme 750 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
α-T-3′-COOH				
		– 5-LO A enzyme >3 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
δ-T3-13′-COOH				
		– 5-LO A enzyme 35 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL > 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL > 3 μM no inhibition (Pein et al., 2018)
		AA 5-LO PF PMNL 260 nM inhibition (Pein et al., 2018)	– 5-LO A enzyme 57 nM inhibition (Richomme et al., 2017)	AA 5-LO PF PMNL 345 nM inhibition (Richomme et al., 2017)
		– 5-LO A		

(Continued)

TABLE 4 | Continued

NF-κB		NLRP3	MAPKs	Lipoxygenases
				enzyme 1 μM inhibition (Jang et al., 2016)
SCA D				
LPS p65 Phos RAW264.7 60 μM inhibition (Heo et al., 2014)	LPS IκB-α Phos RAW264.7 60 μM inhibition (Heo et al., 2014)		LPS JNK Phos RAW264.7 30 μM inhibition (Heo et al., 2014) LPS ERK Phos RAW264.7 30 μM inhibition (Heo et al., 2014)	
SCA E				
			LPS ERK Phos RAW264.7 58 μM inhibition (Lee et al., 2013) LPS p38 Phos RAW264.7 58 μM inhibition (Lee et al., 2013) LPS JNK Phos RAW264.7 58 μM inhibition (Lee et al., 2013)	
SCA G				
IL-1β p65/p50 Phos MG-63 40 μM inhibition (Yoon et al., 2012b)	IL-1β IκB-α Phos MG-63 20 μM inhibition (Yoon et al., 2012b)		IL-1β ERK Phos MG-63 40 μM inhibition (Yoon et al., 2012b) IL-1β p38 Phos MG-63 20 μM inhibition (Yoon et al., 2012b) IL-1β JNK Phos MG-63	

(Continued)

TABLE 4 | Continued

NF-κB	NLRP3	MAPKs	Lipoxygenases
		40 μM inhibition (Yoon et al., 2012b)	

The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type, tissue, mouse, or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. In the publications where no stimulus was used or was required for the studies, the respective row is marked with "-". Actv, activation; A, activity; AT, adipose tissue; BALB/c mice, albino laboratory-bred strain of the house mouse; AA, arachidonic acid; BA, binding affinity; BMDM, bone marrow derived macrophages; JNK, c-Jun N-terminal kinase; DC, dendritic cells; E, expression; ERK, extracellular-signal regulated kinase; 3T3, murine fibroblast cell line; GE, gene expression; h, human; MCF-7, human breast cancer cell line established by Michigan Cancer Foundation-7; SCC4, human head and neck squamous cell carcinoma cell line; HL-60, human leukemia cell line; H1299, human non-small cell lung carcinoma cell line; MiaPaCa-2, human pancreatic cancer cell line; AsPC-1, human pancreas adenocarcinoma cell line; U226, human peripheral blood myeloma, plasmacytoma cell line; MG-63, human osteosarcoma cell line; INF-γ, interferon γ; IL, interleukin; db/db mice, leptin receptor activity deficient mice; LPS, lipopolysaccharide; LO, lipoxygenase; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; MAPK, mitogen-activated protein kinase; m, murine; KBM-5, murine myelogenous leukemia cell line; Ng, nigericine; NLRP3, NLR family pyrin domain containing 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p65, nuclear factor NF-κB p65 subunit; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; pal, palmitate; PM, peritoneal macrophages; PMA, phorbol-12-myristat-13-acetat; Phos, phosphorylation; PMNL, polymorphonuclear neutrophils; PF, product formation; PGE_2, prostaglandin E_2; PE, protein expression; Trl, translocation; TNF-α, tumor necrosis factor α; TT, tumor tissue.
All results obtained from in vivo studies are marked in gray.

microenvironment, in particular by 15-LO-expressing macrophages of the M2 subtype) have further been shown to play pivotal roles in tumor initiation and development as well as angiogenesis and metastasis (Serhan, 2014; Rådmark et al., 2015; Wculek and Malanchi, 2015; Gilligan et al., 2019). All forms of TOHs inhibit the activity of the isolated 5-LO enzyme in the following sequence of their inhibitory capacity: δ-TOH (IC_{50} 0.31 μM) < β-TOH (IC_{50} 0.75 μM) < α-TOH (IC_{50} 1–5 μM) = γ-TOH (IC_{50} 0.9–3 μM) (Reddanna et al., 1985; Pein et al., 2018). In activated polymorphonuclear leukocytes (PMNL), inhibitory concentrations are 10- to100-fold higher with the following order: β-TOH < δ-TOH < γ-TOH < α-TOH (Pein et al., 2018). However, activity of 12- and 15-LO, which catalyze the formation of 12- and 15-HETE, respectively, remained unaltered by 3 μM TOH in LPS-activated PMNL, except for δ-TOH which inhibited 15-LO with an IC_{50} of 3 μM (Pein et al., 2018). α-, β-, γ-, and δ-T3 appeared as efficient inhibitors of isolated 5-LO, all with IC_{50} values below 0.5 μM, whereas the inhibition of 5-LO product formation in activated PMNL required concentrations of 60 μM (δ-T3) to 277 μM (α-T3) (Pein et al., 2018). 12-Lipoxygenase product formation in PMNL was significantly inhibited by all T3 forms, whereas 15-LO-derived products remained unchanged or were even significantly elevated using concentrations of 3 μM (Pein et al., 2018).

MAPK pathways mediate a multitude of cellular processes, including growth, proliferation, differentiation, migration, apoptosis, and inflammation, in response to external stress signals. Therefore, MAPK pathways represent interesting targets for the development of anti-carcinogenic as well as anti-inflammatory therapeutics. Within the MAPK protein family, extracellular signal-regulated kinase (ERK) represents a prominent target for cancer research, because ERK deregulation is linked to approximately one-third of all human cancers (Dhillon et al., 2007). In addition, ERK affects cellular inflammation *via* modulation of cytokine expression (Kim, 2014). However, the stress-activated kinases, c-Jun N-terminal kinase (JNK) and p38, have emerged as interesting therapeutic targets, due to their involvement in the regulation of inflammation, DNA damage response, and apoptosis (Kaminska, 2005). Inhibitory effects of γ-T3 on the MAPK pathway, more precisely the phosphorylation of ERK, p38 and JNK have been observed in epididymal adipose tissues from γ-T3-fed *db/db* mice (0.1% of the diet), in LPS-activated BMDMs using 0.5 μM γ-T3 (Kim et al., 2016), and in TNF-α-activated RAW264.7 cells (Wang et al., 2015). The relevance of NLRP3 inflammasome activation and subsequent formation of pro-inflammatory cytokines, namely IL-1β and IL-8, in inflammation and related diseases has been shown. γ-T3 decreased NLRP3 inflammasome activation by inhibiting the mRNA and protein expression of the NLRP3 inflammasome in BMDM activated with LPS/palmitate, rather than with LPS/nigericin, in peritoneal macrophages and adipose tissue isolated from γ-T3-fed *db/db* mice (Kim et al., 2016). In addition, in BMDMs treated with chloroquine, an inhibitor of lysosomal degradation, the accumulation of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II, and the degradation of p62 were decreased implying that γ-T3 co-regulates autophagosome formation and inflammasome activation (Kim et al., 2016).

Metabolites of Tocopherols and Tocotrienols

Tocopherols and T3s inhibit the activity of isolated recombinant human 5-LO enzyme 10- to 100-fold more efficiently than in activated PMNL. The respective long-chain TOH- and T3-derived metabolites inhibited isolated 5-LO to a similar extent (Jiang et al., 2011; Jang et al., 2016; Pein et al., 2018). Notably, in activated PMNL, α-T-13′-COOH was the most potent inhibitor of 5-LO activity with an IC_{50} value of 80 nM followed by α-T-13′-OH (190 nM), δ-T-13′-OH (540 nM), and δ-T-13′-COOH (2 μM) (Pein et al., 2018). Treatment of activated PMNL with 3 μM LCM effectively blocked 12- and 15-LO product formation, whereas only the 12-LO pathway was blocked by α-5′-T-COOH, α-3′-T-COOH, and γ-3′-T-COOH (Pein et al., 2018). Conversion of arachidonic acid to leukotrienes *via* 5-LO was blocked by δ-T3-13′-COOH (human recombinant enzyme: IC_{50} 35–57 nM) (Richomme et al., 2017; Pein et al., 2018) and 1 μM (Jang et al., 2016); neutrophils (IC 260–345 nM) (Richomme et al., 2017; Pein et al., 2018), whereas product formation

TABLE 5 | Overview on the interference of chromenols with molecular targets and key enzymes connecting inflammation and carcinogenesis.

NF-κB					**MAPKs**	
Sargachromenol						
TNF-α p65 Trl HUVEC 40 μM inhibition (Gwon et al., 2017)	TNF-α p65 PE HUVEC 40 μM inhibition (Gwon et al., 2017)	LPS p65 Trl BV-2 60 μM inhibition (Kim et al., 2014)	TNF-α IκB-α Phos HUVEC 40 μM inhibition (Gwon et al., 2017)	LPS JNK Phos BV-2 60 μM inhibition (Kim et al., 2014)	LPS p38 Phos BV-2 60 μM no inhibition (Kim et al., 2014)	LPS ERK Phos BV-2 60 μM inhibition (Kim et al., 2014)
TNF-α IκB-α Phos HUVEC 40 μM inhibition (Gwon et al., 2017)	LPS IκB-α Phos BV-2 60 μM inhibition (Kim et al., 2014)					

The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type tissue, mouse, or other models used for the studies; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. The following abbreviations are used. BV-2, brain microglial cells transformed by recombinant retrovirus (v-raf/v-mic); JNK, c-Jun N-terminal kinase; ERK, extracellular-signal regulated kinase; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p65, nuclear factor NF-κB p65 subunit; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; Phos, phosphorylation; PE, protein expression; Trl, translocation; TNF-α, tumor necrosis factor α.

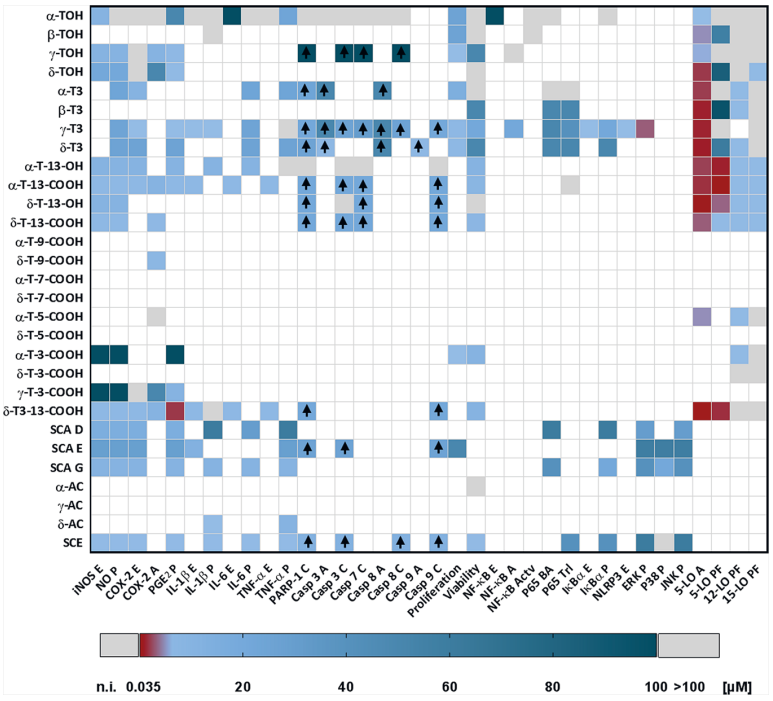


FIGURE 8 | Heatmap illustrating the effectiveness of chromanol and chromenol structures on selected targets. If not indicated otherwise, the plotted effects represent inhibitory effects of the respective compound on distinct parameters; induced parameters are marked with an arrow (↑). The color coding of the presented heat map ranges from high-affinity targets and parameters (effect with <1 μM) presented in red to low-affinity targets and parameters (effects with >1 μM to ≤100 μM) presented in dark blue. If a compound did not affect a specific factor/parameter or showed low effectiveness (>100 μM), the factor/parameter is marked in light gray. Factors and parameters lacking data are marked in white. The heat map is considered as simplified guide for orientation and does not provide a detailed summary of the topic. All concentrations are given micromole (μM). Abbreviations used are: Actv, activation; A, activity; BA, binding affinity; C, cleavage; E, expression; PF, product formation; P, production; T, translocation.

mediated by 12- and 15-LO remained unchanged (Jang et al., 2016). The discrepancy in IC_{50} values in the inhibition of cell-free 5-LO likely depends on the different assay conditions. While Pein et al. analyzed specific 5-LO products by reverse-phase high-performance liquid chromatography with ultraviolet detection, Jang et al. used an indirect colorimetric assay, which determines the formation of hydroperoxides. For SCMs, namely 5′-T-COOH and 3′-T-COOH, no inhibitory effect was observed at the tested concentrations up to 3 μM, except for α-5′-T-COOH (IC_{50} 750 nM) (Pein et al., 2018).

Sargachromanols

Blocking of NF-κB activation with SCAs by inhibiting the phosphorylation of p65 and IκB-α, thereby protecting IκB-α from degradation, has been shown in LPS-activated RAW264.7 macrophages (Heo et al., 2014) and in IL-1β-activated MG-63 osteosarcoma fibroblasts (Yoon et al., 2012b) for SCA D (60 μM) and G (20 μM), respectively. In addition, interference of SCAs D, E, and G with the MAPK pathways, namely phosphorylation of JNK, ERK, and p38, has been observed in LPS-stimulated RAW264.7 macrophages and IL-1β-activated MG-63 osteosarcoma fibroblasts (Yoon et al., 2012b; Lee et al., 2013; Heo et al., 2014).

Chromenols

Like SCAs, δ-SCE has been shown to interfere with the NF-κB and the MAPK pathways. In TNF-α-stimulated endothelial cells (Gwon et al., 2017) and LPS-stimulated microglia cells (Kim et al., 2018), p65 translocation and the phosphorylation of IκB-α were inhibited by 40 μM and 60 μM δ-SCE, respectively. In the same cell models inflammation-induced phosphorylation of JNK and ERK was diminished by δ-SCE, whereas p38 remained unchanged (Kim et al., 2018) (**Table 5**).

LOW AND HIGH-AFFINITY MOLECULAR TARGETS

The heat map in **Figure 8** provides a simplified overview about high- and low-dose bioactivities of the different chromanols and chromenols for a rapid assessment. The selection of compounds and parameters is based on a comprehensive review of the current literature about chromanols and chromenols and focusses on the important biological functions described for these compounds in the context of inflammation and cancer. For reasons of simplification, we did not take into account compound-specific uptake kinetics or cell type- or animal model-specific differences. For more detailed information, the reader is referred to **Tables 1–5** which summarize our current knowledge on the chromanols and chromenols described in the respective sections. For comparison, presented concentrations are IC_{50} values or the lowest reported concentrations affecting the respective parameters.

In the studies considered here, T3s often showed higher effectiveness on the induction or suppression of biological activities linked to inflammation and cancer than TOHs. Furthermore, oxidative modification of the terminal side-chain often substantially increases the anti-inflammatory capacity of respective compounds compared to parental compounds, such as TOHs and T3s. Amplexichromanols, sargachromanols and sargachromenols are also characterized by oxidative modifications of the side-chain, which might rationalize potent interactions with inflammatory targets, which needs further investigation. Notably, regulation of different target genes, proteins, and nuclear receptors can hardly be generalized. For instance, within the group of investigated targets, 5-LO is mostly inhibited by a few compounds, with δ-T3-13′-COOH showing strongest inhibitory effects (IC_{50} 35 nM) and α-TOH showing the least (IC_{50} 1 μM). In contrast, the COX-2-regulated formation of signaling molecules is most efficiently inhibited by γ-T3. In summary, especially 5-LO seems to represent a high affinity (affected at concentrations <1 μM) and therefore specific target for the LCMs of vitamin E. Most of the other observed effects, like mediation of caspase activity, anti-proliferative effects, inhibition of NO formation, are probably the result of a stimulation involving low-affinity targets (affected at concentrations ≥1 μM). However, as implied by the heat map in **Figure 8**, further studies are required for a comprehensive evaluation of the potential of chromanol and chromenol structures to serve as lead structures for the development of future anti-inflammatory therapeutic approaches.

CONCLUSION

For our review, we selected chromanols and chromenols for which data on anti-inflammatory and anti-carcinogenic effects were available in public databases of the scientific literature. The structures of our interests were tocopherols, tocotrienols, and their respective metabolites (which are produced in the liver under physiological and pathophysiological conditions) as well as structurally related compounds including sargachromanols, sargachromenols, and amplexichromanols. Criteria for the evaluation of compounds as possible lead structures for future therapeutic targets were their effects on key inflammatory and apoptotic pathways, proliferation, and interaction with (nuclear) receptor and enzymes that connect inflammation with carcinogenesis. Within this group of selected structures, tocopherols, more precisely α-TOH, are by far the most extensively studied compounds. However, the effects of TOHs are mostly only marginal compared to other compounds described in this review.

It should be noted that the methylation pattern of the chromanol ring system significantly affects inflammation and carcinogenesis. For instance, non-α-TOH and non-α-T3 forms affect eicosanoid- and cytokine-mediated inflammation as well as the cleavage of caspases that mediate apoptosis. Further, T3s are more potent in inhibiting caspase cleavage compared to the respective TOH forms. Tocopherol- and T3-derived metabolites and carboxychromanols more than hydroxychromanols inhibit LO, and in particular 5-LO, effectively and reduce the viability of multiple cancer cell lines. Furthermore, sargachromanols interact

with MAPK and NF-κB pathways, assuming their crosstalk with both, carcinogenesis and inflammation, while sargachromenols mediate anti-carcinogenic effects. Although our knowledge about biological activities of amplexichromanols is sparse, first results indicate their potential for pharmacological applications.

The development of clinically relevant nitric oxide-, eicosanoid-, or cytokine-inhibiting agents or agents that interact with signaling pathways of inflammation is challenging with respect to selectivity and toxicity. Next, although blocking inflammation is meant to be protective, its permanent or long-term inhibition may cause damage to the body (Brasky et al., 2017). Although detrimental effects of naturally occurring chromanols and chromenols cannot be excluded yet, they are less likely for this group of lead compounds in light of the good tolerability of TOHs and T3s at low to moderate doses. Further studies are required to evaluate whether the observed effects of chromanols and chromenols on inflammation and carcinogenesis are indeed beneficial in humans. Until today, no human clinical trials have been published that provide valid information on the biological activity, bioavailability, kinetics, systemic distribution, or local accumulation of these compounds. However, this groups of molecules appears to be promising as lead structures for future anti-inflammatory and/or anti-cancerogenic therapeutic approaches.

LIMITATIONS

Our review is based on a recent systematic review of Birringer et al. (2018), which presented the first comprehensive overview on the diversity of chromanol and chromenol structures and their biological functions. The aim of our review was to more selectively describe the effects on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis and the underlying modes of action for selected chromanols and chromenols. We are aware of the lack of data for a variety of chromenol structures in our overview. We therefore focused on chromanols and chromenols only where adequate data was available that reported anti-inflammatory and anti-carcinogenic properties. For a more detailed description of the structural and chemical properties of all 230 chromanol and chromenol structures, the reader is referred to (Birringer et al., 2018).

AUTHOR CONTRIBUTIONS

MW and SK wrote the manuscript. MW, SK, MS, MB, and SL designed and structured the manuscript, MS, MB, SL, AK, and OW supervised the project and carefully read, evaluated, and discussed the content of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Relation of the works included in this thesis

This thesis comprises eleven works, from which six are original contributions and five are review articles or chapters, respectively. The centrality of the manuscripts with respect to this thesis as well as their rough classification based on the main aspect either being the lipid metabolism or inflammation are depicted in Figure 3.

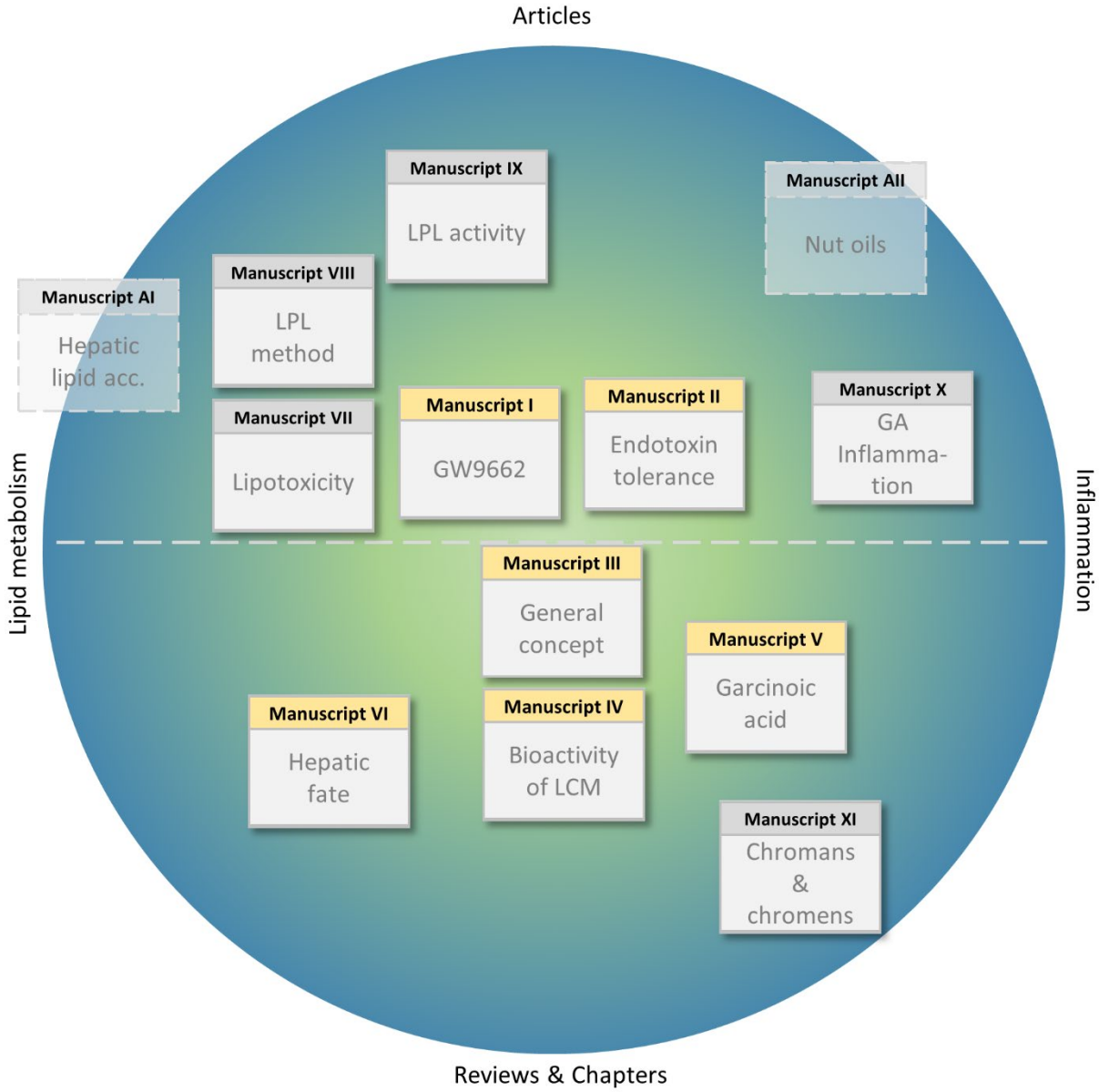

Figure 3: Overview of the centrality and relation of the manuscripts included in this thesis.
Boxes with golden headers indicate manuscripts with contribution as first author. Boxes with silver headers indicate manuscripts with contribution as co-author. Additional co-authored manuscripts are transparent. For the sake of clarity, the manuscripts have been given short titles. The actual titles of the manuscripts can be found in Table 3 below.

The larger framework (refer to the chapter ‘The great framework: non-communicable diseases as consequence of meta-inflammation’) connecting these works and their contributions to this thesis as well as to the scientific field are outlined in the following chapters in detail. Furthermore, the author of this thesis co-authored two additional works (Manuscripts AI and AII; detailed information can be found in the Appendix) that are not directly related to the central topic of research on vitamin E-derived LCM but are of relevance in the broader context of research on lipid metabolism and inflammation, as will be discussed in the following chapters.

Table 3: Full titles and references for the short titles used in Figure 3 of this thesis.

Short title	Title	Reference
GW9662	The PPARγ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662) triggers PLIN2 expression via PPARδ and induces lipogenesis and triglyceride accumulation in human THP-1 macrophages	Schubert, M.; Becher, S.; Wallert, M.; Maeß, M. B.; Abhari, M.; Rennert, K.; Mosig, A. S.; Große, S.; Heller, R.; Grün, M.; Lorkowski, S. (2020) *Molecular Pharmacology* 97 (3), pp. 212–225. (Manuscript I: Schubert et al. 2020)
Endotoxin tolerance	The vitamin E long-chain metabolite α-13'-COOH is a reliable suppressor of CCL2 / MCP-1 and modulates regulatory mechanisms of MAPK and NFκB signaling and the inflammatory response of macrophages	Schubert, M.; Kluge, S.; Birringer M.; Lorkowski, S. (2020) Prepared for publication in *Molecular Nutrition and Food Research.* (Manuscript II: Schubert et al. 2020)
General concept	Long-chain metabolites of vitamin E: metabolic activation as a general concept for lipid-soluble vitamins	Schubert, M.; Kluge, S.; Schmölz, L.; Wallert, M.; Galli, F.; Birringer, M.; Lorkowski, S. (2018) *Antioxidants* 7 (1), p. 10. (Manuscript III: Schubert et al. 2018)
Bioactivity of LCM	Bioactivity of vitamin E long-chain metabolites	Kluge, S.; Schubert, M.; Schmölz, L.; Wallert, M.; Birringer, M.; Lorkowski, S. (2019) *Vitamin E in Human Health. Springer International Publishing,* pp. 61–79. (Manuscript IV: Kluge et al. 2019)
Garcinoic acid	Garcinoic acid: a promising bioactive natural product for better understanding the physiological functions of tocopherol metabolites	Kluge, S.; Schubert, M.; Schmölz, L.; Birringer, M.; Wallert, M.; Lorkowski, S. (2016) *Studies in Natural Products Chemistry. Elsevier,* pp. 435–481 (Manuscript V: Kluge et al. 2016)
Hepatic fate	The hepatic fate of vitamin E	Schmölz, L.; Schubert, M.; Kluge, S.; Birringer, M.; Wallert, M.; Lorkowski, S. (2018) *Vitamin E in Health and Disease. InTech.* pp. 1–30. (Manuscript VI: Schmölz et al. 2018)
Lipotoxicity	Long-chain metabolites of vitamin E: interference with lipotoxicity via lipid droplet associated protein PLIN2	Schmölz, L.; Schubert, M.; Kirschner, J.; Kluge, S.; Galli, F.; Birringer, M.; Wallert, M.; Lorkowski, S. (2018) *Biochimica et biophysica acta. Molecular and cell biology of lipids* 1863 (8), pp. 919–927. (Manuscript VII: Schmölz et al. 2018)
LPL method	Simple and rapid real-time-monitoring of LPL activity *in vitro*	Kluge, S.; Boermel, L.; Schubert, M.; Lorkowski, S. (2020) *MethodsX* 7, p. 100865. (Manuscript VIII: Kluge et al. 2020)
LPL activity	The vitamin E long-chain metabolite α-13'-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system	Kluge, S.; Schubert, M.; Börmel, L.; Lorkowski, S. (2021) *Biochimica et biophysica acta. Molecular and cell biology of lipids* 1866 *(ahead of print)*. (Manuscript IX: Kluge et al. 2021)
GA Inflammation	The vitamin E derivative garcinoic acid from *Garcinia kola* nut seeds attenuates the inflammatory response	Wallert, M.; Bauer, J.; Kluge, S.; Schmölz, L.; Chen, Y.-C.; Ziegler, M.; Searle, A. K.; Maxones, A.; Schubert, M.; Thürmer, M.; Pein, H.; Koeberle, A.; Werz, O.; Birringer, M.; Peter, K.; Lorkowski, S. (2019) *Redox Biology* 24, p. 101166. (Manuscript X: Wallert et al. 2019)
Chromans & chromens	Diversity of chromanol and chromenol structures and functions: an emerging class of anti-inflammatory and anti-carcinogenic agents	Wallert, M.; Kluge, S.; Schubert, M.; Koeberle, A.; Werz, O.; Birringer, M.; Lorkowski, S. (2020) *Frontiers in Pharmacology* 11, p. 362. (Manuscript XI: Wallert et al. 2020)
Additional (not included) studies co-authored by the author of this thesis (AI & AII)		
Hepatic lipid acc.	Differential capability of metabolic substrates to promote hepatocellular lipid accumulation	Hoang, N. A.; Richter, F.; Schubert, M.; Lorkowski, S.; Klotz, L.-O.; Steinbrenner, H. (2019) *European Journal of Nutrition* 58 (8), pp. 3023–3034. (Manuscript AI: Hoang et al. 2019)
Nut oils	In vitro digested nut oils attenuate the lipopolysaccharide-induced inflammatory response in macrophages	Müller, A. K.; Schmölz, L.; Wallert, M.; Schubert, M.; Schlörmann, W.; Glei, M.; Lorkowski, S. (2019) *Nutrients 11* (3). p. 503. (Manuscript AII: Müller et al. 2019)

8 Physiologic and pathophysiologic implications of the works in the context of NCD and meta-inflammation

Focus of the manuscripts included in this thesis was on the aspects of NCD delineated in the introduction (refer to the chapter 'The great framework: non-communicable diseases as consequence of meta-inflammation') and their relevance not only on physiological but also on pathophysiological and pharmacological implications of the LCM for inflammation and lipid metabolism. The broad context of this thesis is research on vitamin E and especially on the LCM and their putative role in the (patho-)physiology of lipid metabolism and immune response and thus the relation to NCD. In the effort to understand the underlying molecular mechanisms and actions, it is crucial not only to use existing experimental tools but also to evaluate them and to develop new approaches. Consequently, respective strategies make up a significant part of this work as outlined below.

8.1 Physiologic functions as research objective

A central topic of this thesis is the elucidation of the biological importance of the vitamin E-derived LCM. The occurrence of the LCM α-13'-COOH in the blood of *healthy* human donors was repeatedly reported, shortly before the beginning of the work on this thesis (Wallert et al. 2014) and during the work (Pein et al. 2018; Giusepponi et al. 2019). The repeated finding in *healthy* subjects indicates a role of this LCM in human physiology and that the occurrence of α-13'-COOH is not due to pathophysiologic alterations of, for example, the hepatic metabolism of TOH. Crucial to the elucidation of physiologic functions is the profound understanding of physiologic metabolic pathways. We therefore comprehensively reviewed the knowledge on the physiological hepatic handling of vitamin E as well as influencing factors (Manuscript VI: Schmölz et al. 2018). The liver represents the central organ in vitamin E handling and formation of LCM and is thus of special interest in this field of research. Furthermore, we summarized knowledge on the physiologic metabolism of the lipid-soluble vitamins (vitamins A, D, K, as well as E) in general, to generate a profound hypothesis on a general mechanism of physiological activation shared by all lipid-soluble vitamins (Manuscript III: Schubert et al. 2018). According to this well-known mechanism for the vitamins A and D and the hypothetical comparable mechanism for vitamin E, the LCM represent the physiologic biologically active form of vitamin E. Following this concept, all recently known and in the future elucidated biological functions of vitamin E-derived LCM may principally have physiologic implications.

However, direct demonstration of physiological functions of the LCM in human is virtually impossible since this would require the complete prevention of formation of the LCM in the living organism. It is unlikely to examine distinct functions of the LCM due to the nature of the key enzymes of LCM formation, the cytochrome P450 oxidases (the reader is referred to the chapter 'Metabolism' for detailed information). Albeit disabling mutations of these enzymes not always result in a phenotype (and thus individuals to serve for research could principally be found), many CYP are redundant with respect to their metabolizing functions and LCM formation may not be affected (Nebert et al. 2013). Further, CYP catalyze the formation of various lipid mediators that have been shown to affect inflammatory mechanisms and lipid metabolism (Bishop-Bailey et al. 2014; Johnson et al. 2015) and thus interfere with the (to date) known functions of LCM. Dissection of specific effects of LCM would be difficult if not impossible. Another conceivable approach would be the identification of subjects with a disabled LCM retention system. The retention of LCM in neutrophils was recently shown (Pein et al. 2018). Tocopherol retention is known to be realized by α-TTP. However, α-TTP gene expression in leukocytes is generally very low (Misaki et al. 2003) suggesting that LCM

retention is based on a distinct mechanism. Interestingly, Sarkisyan and colleagues recently stated that 13'-carboxychromanols form more stable complexes with α-TTP than their precursors. However, α-TOH showed higher affinity to α-TTP in their work (Sarkisyan et al. 2018). If future research should strengthen the evidence for α-13'-COOH retention by α-TTP, individuals suffering from AVED could serve as a 'model system' for research since they lack expression of functional α-TTP (Ouahchi et al. 1995). As outlined above, the mechanism behind LCM retention is not elucidated to date and making use of this approach is thus in distant future.

In consequence of the above delineated obstacles and uncertainties, generation of evidence for physiologic functions is to date merely possible indirectly. In this context, beneficial effects of LCM in cell and animal models of pathologic conditions must be used to draw conclusions about their true physiologic effects. Fundamental findings like the identification of distinct receptors and signaling pathways for compounds of interest of course have implications for physiology as well as pathophysiology.

8.2 Pathophysiologic processes as research objective

As delineated in the above section, models of pathologic conditions as well as tools to elucidate fundamental mechanisms are the indirect opportunities we are (to date) restricted to in order to identify physiological functions. This thesis and all included manuscripts are thus related to pathological conditions and models by one means or another. The overall framework is here created by the joint immunometabolic component of NCD. The implicated diseases have all been connected to vitamin E as outlined in the chapters 'Vitamin E and non-communicable diseases' and 'The great framework: non-communicable diseases as consequence of meta-inflammation' and the central compounds in this work (GA, α-TOH and α-13'-COOH) have repeatedly been connected to related disease patterns in our reviews (Manuscript III: Schubert et al. 2018; Manuscript IV: Kluge et al. 2019; Manuscript V: Kluge et al. 2016; Manuscript VI: Schmölz et al. 2018; Manuscript XI: Wallert et al. 2020). The focus of the experimental work, however, is owing to the history of our group on cardiovascular (and inflammatory) disease. Most of the investigations were carried out in macrophage models since they are the key players in atherosclerosis and thus CVD (Barrett 2020). However, macrophages also play prominent roles in diseases like chronic inflammatory diseases, neurodegenerative diseases, cancer, infection and sepsis (Ardura et al. 2019), and thus respective results have also implications for further immunometabolic disease patterns. The macrophage models generally either focused on macrophage lipid metabolism (Manuscript I: Schubert et al. 2020; Manuscript VII: Schmölz et al. 2018; Manuscript VIII: Kluge et al. 2020; Manuscript IX: Kluge et al. 2021) or on the inflammatory response (Manuscript II: Schubert et al. 2020; Manuscript AII: Müller et al. 2019). These studied mechanisms represent key events in the initiation and progression of atherosclerosis (Barrett 2020) and potential beneficial alteration of these processes by the vitamin E LCM α-13'-COOH (Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021; Manuscript II: Schubert et al. 2020) and the related compound GA (Manuscript V: Kluge et al. 2016; Manuscript X: Wallert et al. 2019) are of high interest. Physiological functions of vitamin E as well as possible pharmacological applications could be extrapolated from respective studies.

As stated earlier, the inflammatory response of macrophages is not only relevant in atherosclerosis, but also in other diseases like asthma (Saradna et al. 2018), sepsis (Qiu et al. 2019) or NAFLD/NASH (non-alcoholic steatohepatitis) (Krenkel and Tacke 2017). The latter is the leading cause of liver cirrhosis and hepatocellular carcinoma. Hence, anti-inflammatory actions of the compounds investigated within this thesis could have implications for the

prevention and treatment of these diseases. Since lipid overload is not only a detrimental condition in the context of atherosclerosis but also in NAFLD (Ipsen et al. 2018), modulation of macrophage lipid metabolism and inflammation by LCM and GA also has implications for this disease (Peters et al. 2018). Macrophages have even been stated as 'role models of pathogenic immunometabolism' in the context of NAFLD (Krenkel and Tacke 2017). NAFLD is characterized by an excessive accumulation of lipids in hepatocytes (Manuscript AI: Hoang et al. 2019) and progression to NASH was initially described by the two-hit hypothesis of a subsequent inflammatory phase (Day and James 1998). However, novel findings support a multiple parallel hits hypothesis with macrophages being key players already in the pathogenesis of NAFLD (Buzzetti et al. 2016; Krenkel and Tacke 2017).

During the work on this thesis, we successfully developed and characterized an *in vitro* model of NAFLD pathogenesis in HepG2 liver cancer cells (Manuscript AI: Hoang et al. 2019) where we included different nutrients and inflammatory stimuli (*vide infra* and refer to the chapter 'Review of the studies and applied methods'). Based on this model and our growing knowledge on macrophage lipid metabolism and inflammation (Manuscript I: Schubert et al. 2020; Manuscript II: Schubert et al. 2020; Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021; Manuscript VIII: Kluge et al. 2020), we have the opportunity to further develop this model to better resemble the current understanding of NAFLD and NASH pathogenesis. Interactions between macrophages and hepatocytes and possible beneficial or pharmacological effects of, amongst others, the LCM or GA could be studied. This supports a currently started project on the regulation of the inflammasome by the LCM and GA. Since activation of the inflammasome is thought to be a key event in the progression of NAFLD (Krenkel and Tacke 2017) and NASH (Luan and Ju 2018), respectively, the project will later focus on these disease patterns. Further experimental bases for these investigations were provided by the elucidation of anti-inflammatory mechanisms of the LCM (Manuscript II: Schubert et al. 2020) as well as of anti-inflammatory actions of GA (Manuscript X: Wallert et al. 2019) in the progress of this thesis. In addition, possible theoretical implications of the LCM and GA in NAFLD and NASH have been discussed in respective reviews (Manuscript V: Kluge et al. 2016; Manuscript VI: Schmölz et al. 2018) which are included in this thesis and a recent review by our group (Wallert et al. 2020).

8.3 Integrating metabolism and inflammation

Non-communicable disease development is characterized by a chronic low-grade inflammation across the life span (Calder et al. 2017; Bennett et al. 2018b). A key factor in chronic low-grade inflammation is dysmetabolism, a state defined by the inability to keep homeostasis. This in turn results in the loss of lipid control, in oxidative stress, inflammation and insulin resistance (van den Brink et al. 2019). The interconnection of these processes is highly conserved throughout evolution (Hotamisligil 2017). Such a conserved mechanism is the relationship between the pleiotropic immune mediator tumor necrosis factor (TNF), the pathogen sensing system of toll-like receptors and the metabolic hormone insulin. TNF and toll-like receptor (TLR) activation is known to block insulin signaling or production and thus cytokines can be regarded as metabolic hormones in the adaption to nutrient status (Hotamisligil 2017). Despite this, we observed in our *in vitro* NAFLD model that TNF is able to augment the fructose-induced lipid accumulation in liver cells, indicating that a low-grade inflammation can support lipid-accumulation in the liver (Manuscript AI: Hoang et al. 2019). The work using this model nicely illustrates the interconnection between monosaccharides, insulin, cytokines and fatty acids and the implications of diet composition and inflammation in NAFLD pathogenesis (Manuscript AI: Hoang et al. 2019). While insulin augmented the lipid

accumulation from glucose in this setup, lipid accumulation from oleic acid or palmitic acid was not promoted. Conversely, excessive fat accumulation and disturbed lipid metabolism (causing inflammation) contribute to the development of insulin resistance (Kojta et al. 2020).

8.3.1 Lipids – Janus-faced characters in the inflammatory process

Lipids occupy a central position in the inflammatory process. Endogenously produced pro-inflammatory bioactive lipids initiate and drive inflammation in order to fend off invading pathogens or initiate regeneration of damaged tissues. This process must be followed by a switch to the production of endogenous anti-inflammatory lipids to terminate inflammation and further drive the regeneration. Insufficient resolution of inflammation results in chronic (low-grade) inflammation, a hallmark in the pathogenesis of NCD (Chiurchiù et al. 2018). Taken together, inflammation is regulated by a complex interplay of different factors, with lipid mediators playing a key role. This complex process can be disturbed by exogenous factors. One mechanism is the immunological reaction to lipopolysaccharides from bacteria sensed and triggered by the well-characterized TLR4 (Rosadini and Kagan 2017). In this favorable process, the lipid component of LPS accounts for most of the stimulatory capacity (Rietschel et al. 1994). However, the activation of TLR4 signaling by other lipids can also be detrimental. Several fatty acids have been reported to activate TLR4 signaling and thus trigger pro-inflammatory mechanisms and the initiation of NCD (Fessler et al. 2009). A substantial finding for the relevance of this mechanism is that mice without functional TLR4 are protected from negative effects of diets rich in saturated fat (Davis et al. 2008). Interestingly, the concept of a direct activation of TLR4 by fatty acids was recently questioned (Lancaster et al. 2018) and the new hypothesis states that TLR4 must be primed by a specific signal and subsequently enhances the inflammatory effects of fatty acids. However, the pro-inflammatory effects of specific endogenous and dietary fatty acids are not questioned.

In contrast, omega-3 poly-unsaturated fatty acids are known to suppress TLR4 mediated pro-inflammatory signaling (Rogero and Calder 2018), underlining that the structure of fatty acids, or lipid-soluble compounds in general, determines their beneficial or detrimental effect. The exact molecular mechanisms behind these effects are still not fully elucidated. Notwithstanding this, lipid compounds have great potential to modulate inflammation in general and the TLR4 pathway in particular. The potential of distinct lipid compounds or extracts on the modulation of the inflammatory response of macrophages to LPS was repeatedly examined during the preparation of this thesis. Interestingly, all examined lipid mixtures and sole lipid-soluble compounds had the potential to suppress key mediators of inflammation in response to LPS *in vitro* (Manuscript II: Schubert et al. 2020; Manuscript X: Wallert et al. 2019; Manuscript AII: Müller et al. 2019). Extracts from different *in vitro* digested nuts were similarly effective, irrespective of their composition, including significant differences in the content of omega-3 PUFA (Manuscript AII: Müller et al. 2019). Comparably, both, the lipid extract from *garcinia kola* nuts as well as the isolated constituent GA suppressed the inflammatory response of macrophages to LPS (Manuscript X: Wallert et al. 2019). Apart from this, the LCM α-13'-COOH shows high potential to suppress inflammatory processes while its precursor α-TOH is considerably less effective in terms of effect size and required concentration (Wallert et al. 2015). Since the carboxyl moiety is the sole difference in the structure of the molecules, anti-inflammatory properties might depend on the recognition of lipid-soluble structures as fatty acids by the cell (refer to the chapter 'Long-chain metabolites of vitamin E'). Taken together, this points either to a 'global mechanism' altering the lipidome and thus responsiveness of the cell, or to a distinct mechanism (*i.e.* receptor or pathway) shared by all of the compounds (except α-TOH) (refer to the chapter 'Future perspectives'). First evidence for the molecular

regulatory mechanisms exerted by the LCM α-13'-COOH leading to the anti-inflammatory effects was obtained within this work (refer also to 'Contribution to the development of the field') (Manuscript II: Schubert et al. 2020).

8.3.2 Lipid droplets – Regulatory cellular substructures in lipid metabolism and inflammation

Lipid droplet formation could contribute to the above-mentioned 'global mechanism'. Growing evidence supports the hypothesis of a complex relationship between lipid droplets and inflammatory signaling (Jarc and Petan 2020). This hypothesis is basically founded on the function of lipid droplets as lipid storage organelles. Two key mechanisms are proposed: Firstly, lipid droplets can serve as a 'sink' for fatty acids, thus preventing the formation of lipid inflammatory mediators. Secondly, fatty acids can be released from their storage form as triglycerides and serve as precursors of lipid inflammatory mediators (Jarc and Petan 2020). We demonstrated that PLIN2 (ADRP) is tightly related to lipid droplet formation and lipid accumulation in our *in vitro* NAFLD model and can thus be regarded as a marker for droplet formation (Manuscript AI: Hoang et al. 2019). This is not restricted to hepatocytes but also applicable to monocytes and macrophages (Buechler et al. 2001; Persson et al. 2007). The lipid accumulation in THP-1 macrophages induced by PPARδ activation is accompanied by an increase in PLIN2 mRNA and protein (Manuscript I: Schubert et al. 2020). Further, we demonstrated that the LCM α-13'-COOH also induces PLIN2 mRNA and protein in THP-1 cells and have nicely shown that neutral lipid accumulation as well as the size of lipid droplets are concomitantly increased (Manuscript VII: Schmölz et al. 2018). In conclusion, α-13'-COOH induces the formation of lipid droplets, hypothetically via PPARδ (refer to the chapter 'Future perspectives'). Hence, this represents two possible mechanistic approaches to explain the anti-inflammatory properties of α-13'-COOH (Manuscript II: Schubert et al. 2020; Wallert et al. 2015).

It is further known that inflammatory stimuli induce the biogenesis of lipid droplets in immune cells (Bozza et al. 2009; Feingold et al. 2012; Guijas et al. 2012). Induction of lipid droplet formation by α-13'-COOH might thus be caused by the activation of the inflammation-related pathways MAPK and NFκB as reported in manuscript II (Manuscript II: Schubert et al. 2020). Lipid droplet formation is further believed to regulate the availability of certain fatty acids and thus the formation of distinct lipid mediators (Jarc and Petan 2020). This might contribute to the repeatedly reported finding that LCM, especially α-13'-COOH, alter the lipid mediator profile of immune cells (Wallert et al. 2015; Jiang et al. 2011; Pein et al. 2018). Conversely, it is conceivable that the action of LCM and related compounds is regulated via lipid droplet formation. Tocopherols have been reported to occur in lipid droplets of human cells (Traber and Kayden 1987) and storage of the lipid-soluble vitamin A as retinyl esters in lipid droplets is well-known (Blaner et al. 2016). Given that the compounds of our interest (α-13'-COOH and GA) carry a carboxyl moiety, esterification in cells cannot be excluded albeit possible reactants are not known to date. The reported accumulation of α-13'-COOH in immune cells (Pein et al. 2018) in a model of peritonitis (a severe inflammatory condition) might thus be associated with the enhanced biogenesis of lipid droplets (*vide supra*).

Further, model systems based on an excess supply of calories, like a high-fat diet, might prevent the proposed beneficial effects of certain fatty acids (Manuscript AII: Müller et al. 2019), LCM (Manuscript II: Schubert et al. 2020; Manuscript IX: Kluge et al. 2021; Manuscript VII: Schmölz et al. 2018) and GA (Manuscript X: Wallert et al. 2019) due to a massively disturbed lipid metabolism and lipid overload in certain tissues and cells. This might account for the results of our study on GA in an atherosclerosis model of high-fat diet fed apolipoprotein E

(ApoE) knockout mice, where the promising *in vitro* effects could in large parts not be resembled *in vivo* (Manuscript X: Wallert et al. 2019). The lipophilic nature of GA hypothetically enables an association to lipid droplets and a phenotype promoting fat storage (high fat diet) might facilitate 'trapping' of GA in lipid storage tissues. This could prevent GA from serving as ligand for hypothetically relevant receptors like the 'fatty-acid sensors' PPARs. The requirement of fatty acid esterification and rehydrolysis to generate PPAR ligands was convincingly demonstrated in adipose triglyceride lipase (ATGL)-deficient (*i.e.* deficient in releasing fatty acids from lipid droplets) mice (Haemmerle et al. 2011). Here, suppressed action of PPARα caused mitochondrial dysfunction, massive cardiac lipid accumulation and lethal cardiomyopathy (Haemmerle et al. 2011). Strikingly, functional impairments have also been reported in ATGL-deficient macrophages (Chandak et al. 2010) and liver (Ong et al. 2014; Ong et al. 2011)[1]. These represent central cell types and tissues in our research on LCM and GA as outlined above. Given the demonstration of a close mechanistic connection between ATGL and PPAR (Mottillo et al. 2012) and that PPAR play a crucial role in the metabolism and function of the respective cells (Manuscript I: Schubert et al. 2020; Montagner et al. 2016; Chawla 2010; Lefere et al. 2020), a role of lipid droplets and PPAR in the effects of the LCM and GA appear obvious.

8.3.3 PPAR – Nuclear receptors at the crossroad of lipid metabolism and inflammation

The PPAR are a subfamily of nuclear receptors represented by the members PPARα, PPARδ (also termed PPARβ) and PPARγ. A variety of compounds have been shown to lead to PPAR activation, however, PPAR are mainly known as sensors for fatty acids and fatty acid-derived metabolites (Lefebvre et al. 2006; Tontonoz and Spiegelman 2008; Neels and Grimaldi 2014). Emphasizing their importance, all PPAR isoforms have been designated as 'master regulators' of metabolism (Kersten 2014; Shao et al. 2016; Fan et al. 2017). In their function as transcription factors, PPAR also regulate various genes in inflammation, glucose metabolism, proliferation and differentiation (Varga et al. 2011; Vrablík and Češka 2015). Due to their diverse regulatory properties, PPAR are implicated in virtually all NCD (Kersten et al. 2000; Cheng et al. 2019) including asthma (Kytikova et al. 2020), NAFLD (Choudhary et al. 2019) and NASH (Boeckmans et al. 2019). Interestingly, the modulatory properties of PPAR in the context of inflammation are so well-accepted that PPAR agonists have even been proposed for the treatment of the cytokine storm in COVID-19 disease (Ciavarella et al. 2020).

Accordingly, there is deep interest in PPAR and their physiologic functions as well as high expectations and hopes set on PPAR as treatment options for various diseases. Conscientious studies on PPAR's modes of action and functions are thus imperative. An established strategy for the elucidation of effects of a compound of interest is the use of specific chemical agonists or antagonists for signaling proteins. While trying to confirm PPARγ involvement in the effects of LCM (among other compounds) on macrophage lipid metabolism, we recognized that the commonly used PPARγ antagonist GW9662 (2-chloro-5-nitro-N-phenylbenzamide) (Leesnitzer et al. 2002; Lea et al. 2004; Nielsen et al. 2006) unexpectedly acted in the fashion of a PPAR agonist (Manuscript I: Schubert et al. 2020). Since its first mention in the year 2000, GW9662 has been referenced in about 1400 publications listed in PubMed, demonstrating the widespread use of this chemical (Figure 4). In combination with our used model system,

[1] Note: The authors of these publications argue against an involvement of lipid mediators released by ATGL in PPARα activation. However, from the author of this thesis' point of view, their experimental setup is not sufficient to draw this conclusion. Individual interpretation of the reported experimental data is recommended.

macrophages, GW9662 has been referenced about 200 times from 2002 until today (Figure 4) (National Center for Biotechnology Information 2020). However, this number may lead to an understatement of the significance since macrophages are key players in virtually all major diseases (Ardura et al. 2019) including NCD (Camps 2014; Castro et al. 2017; Oishi and Manabe 2016) and results from this cell type have thus implications in respective disease models and treatment strategies. Our report on the unexpected opposing effect of GW9662 to the stated action as a PPARγ antagonist in macrophages (Manuscript I: Schubert et al. 2020), as well as the dominant role of PPARδ (*vide infra*), is thus of high relevance for the scientific community. Some existing studies might require reinterpretation in the light of these findings and we provide a basis for better design of experimental studies on PPAR in the future. According to our above outlined interest in NCD, lipid metabolism, inflammation, macrophages and PPAR, this work (Manuscript I: Schubert et al. 2020) is of major importance for our research (Figure 3).

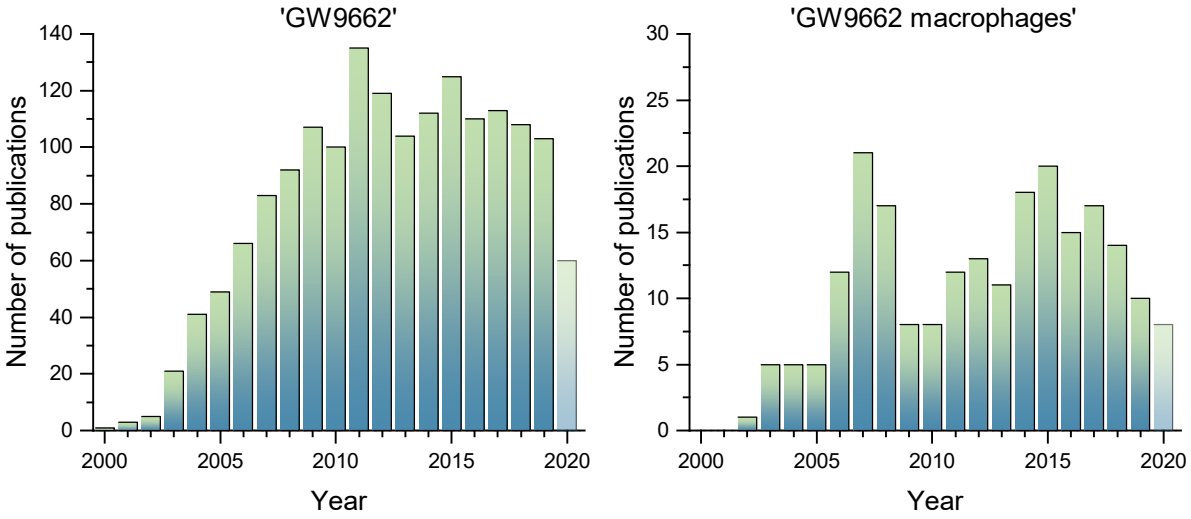

Figure 4: Number of publications found per year from 2000 to 2020 (as of July) for the search terms ‘GW9662’ and ‘GW9662 macrophages’ in the ‘PubMed’ database.

Within the work on GW9662, PPARδ was identified as the dominant PPAR isoform in human and murine macrophages (Manuscript I: Schubert et al. 2020), which is in line with earlier reports on the strong induction of this isoform during macrophage differentiation (Vosper et al. 2001; Adhikary et al. 2015). Since PPAR overlap in their substrate specificity, dominance of a PPAR subtype in a given tissue or cell type determines the effect of a stimulus (Escher and Wahli 2000; Bugge and Mandrup 2010). Accordingly, we identified an off-target agonism of GW9662 on PPARδ (instead of an antagonistic action on PPARγ) to be responsible for the profound alterations in macrophage lipid metabolism by GW9662 (Manuscript I: Schubert et al. 2020). Given that the compounds of our interest, *i.e.* vitamin E-derived LCM (Manuscript II: Schubert et al. 2020; Manuscript IX: Kluge et al. 2021; Manuscript VII: Schmölz et al. 2018) and GA (Manuscript X: Wallert et al. 2019) as well as classical PPAR ligands (Manuscript AII: Müller et al. 2019), have been found to modulate macrophage lipid metabolism as well as the inflammatory response, PPARδ is likely of relevance in the mediation or modulation of these effects. First attempts to confirm a PPARδ contribution to the effects of α-13’-COOH indeed showed promising results (Manuscript IX: Kluge et al. 2021) (refer to the chapter ‘Future perspectives‘ and Figure 6).

8.4 Concluding remarks

Taken together, this thesis and all included manuscripts contribute to the understanding of vitamin E (α-TOH) metabolism and function (Manuscript III: Schubert et al. 2018; Manuscript IV: Kluge et al. 2019; Manuscript V: Kluge et al. 2016; Manuscript VI: Schmölz et al. 2018; Manuscript XI: Wallert et al. 2020), exerted by the putative biologically active metabolite(s) (Manuscript III: Schubert et al. 2018). Particular focus is laid on lipid metabolism (Manuscript I: Schubert et al. 2020; Manuscript VII: Schmölz et al. 2018; Manuscript VIII: Kluge et al. 2020; Manuscript IX: Kluge et al. 2021) and the inflammatory response (Manuscript II: Schubert et al. 2020; Manuscript X: Wallert et al. 2019; Manuscript AII: Müller et al. 2019) of macrophages, representing highly relevant factors in the pathology of NCD. In order to extend the range of available tools for NCD research, an *in vitro* hepatocyte NAFLD model (Manuscript AI: Hoang et al. 2019) as well as a macrophage LPL activity model (Manuscript VIII: Kluge et al. 2020) were established. The usability of these tools (and the existing macrophage inflammation model (Manuscript AII: Müller et al. 2019)) and thus their value for NCD research were verified (Manuscript AI: Hoang et al. 2019; Manuscript VIII: Kluge et al. 2020; Manuscript IX: Kluge et al. 2021). Further, the highly relevant compound for PPAR and thus NCD research (*vide supra*), GW9662 was thoroughly evaluated and limitations of its applicability, especially in macrophages, were communicated to the scientific community. Collectively, this thesis and all included manuscripts provide a solid base for future investigations of not only vitamin E and LCM functions, but also of virtually all promising compounds in the context of NCD, especially with focus on macrophage and liver function. As indicated above, this will not only help to better understand physiologic and pathophysiologic mechanisms but also to evaluate the pharmacological potential of certain compounds. First attempts with an anti-inflammatory compound of the African ethno medicine *Garcinia kola* (Manuscript V: Kluge et al. 2016), *i.e.* GA, showed promising results in the macrophage inflammation model (Manuscript X: Wallert et al. 2019). Based on the overlapping structural features and biological functionalities of GA with the vitamin E-derived LCM (Manuscript V: Kluge et al. 2016; Manuscript XI: Wallert et al. 2020), development and evaluation of a pharmacological lead compound from these with the help of the tools and knowledge from this thesis is a conceivable future goal. However, translation into *in vivo* models is challenging (Manuscript X: Wallert et al. 2019), especially in the context of meta-inflammation (Hotamisligil 2017). This further emphasizes the necessity of thorough examination of promising compounds in appropriate *in vitro* models using reliable experimental tools.

9 Contributions of the manuscripts to achieve the aims of the work

The overall aim of this work was to broaden and evolve the knowledge from the three scientific studies outlined in the chapter ‘Aim of the work’ as starting point in their specific research fields and to further expand the basis for the future research on this new and promising subfield of vitamin E research. The efforts to achieve this aim can be subdivided into three aspects:

9.1 Aspect 1 – Dissemination and delineation of the potential of vitamin E-derived LCM

This aspect aimed to introduce this new and promising research field to a broad audience and delineate its potential. To achieve this, review articles and chapters on the physiology of vitamin E and the formation and possible physiological role of LCM should be published. Further, the pharmacological potential of related compounds and the LCM themselves should

be communicated. Regularly updated views were intended to further generate interest as well as to preserve interest in the field.

The following publications included in this thesis contributed to achieve this aim:

Table 4: Main aspects of the works contributing to aspect 1 of the aim of the thesis.

Main aspect*	Publications (Reference)
Hypothesis-generating: Role of LCM	(Manuscript III: Schubert et al. 2018)
Physiological role of vitamin E	(Manuscript VI: Schmölz et al. 2018)
Pharmacological potential of vitamin E-related compounds	(Manuscript V: Kluge et al. 2016; Manuscript XI: Wallert et al. 2020)
Updates on LCM	(Manuscript III: Schubert et al. 2018; Manuscript IV: Kluge et al. 2019)

* The aspects of these publications overlap. Emphasizing main aspects is intended to improve clarity. LCM: long-chain metabolites (of vitamin E)

9.2 Aspect 2 – Metabolism and regulatory functions

As outlined above, first reports indicated roles of the LCM in macrophage and liver metabolism as well as the inflammatory response. Evolving this knowledge was aim of this work. To achieve this aim, one aspect of this thesis was directed at the better understanding of general mechanisms of lipid metabolism and the inflammatory response. Development and evaluation of respective experimental tools represent a part of this aspect.

The following publications included in this thesis contributed to achieve this aim:

Table 5: Main aspects of the works contributing to aspect 2 of the aim of the thesis.

Aspect	Publications (Reference)
Lipid Metabolism	(Manuscript I: Schubert et al. 2020; Manuscript IX: Kluge et al. 2021; Manuscript AI: Hoang et al. 2019)
Inflammation	(Manuscript II: Schubert et al. 2020; Manuscript X: Wallert et al. 2019; Manuscript AII: Müller et al. 2019)
Experimental tools	(Manuscript VIII: Kluge et al. 2020; Manuscript I: Schubert et al. 2020; Manuscript X: Wallert et al. 2019)

LCM: long-chain metabolites (of vitamin E)

9.3 Aspect 3 – The specific effects of vitamin E-derived LCM

A central aspect of this thesis is the elucidation of biological functions of the vitamin E-derived LCM in order to demonstrate and characterize their potential as physiologic mediators of vitamin E function. The related compound GA can help to achieve this aim (Manuscript V: Kluge et al. 2016) and is thus also of relevance. However, as outlined in the chapters ‘Physiologic and pathophysiologic implications of the works in the context of NCD and meta-inflammation’ and ‘Review of the studies and applied methods’, experimental model systems have pathophysiological character and thus implications of LCM and GA as compounds with pharmaceutical potential are obvious. Given their roles at the crossroads of lipid metabolism and inflammation, LCM (and GA) likely play a role in the pathophysiology and possibly in the treatment of NCD. The elucidation of distinct regulatory mechanisms of these compounds is thus a highly important aim of this work.

The following publications included in this thesis contributed to achieve this aim:

Table 6: Main aspects of the works contributing to aspect 3 of the aim of the thesis.

Aspect	Publications (Reference)
Specific effects of GA on inflammatory processes	(Manuscript X: Wallert et al. 2019)
Specific effects of LCM on inflammatory processes	(Manuscript II: Schubert et al. 2020)
Specific effects of LCM on the lipid metabolism	(Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021)

LCM: long-chain metabolites (of vitamin E)

10 Review of the studies and applied methods

The vitamin E-derived LCM represent a relatively new research field (refer to the chapter 'Aim of the work'), since first reports on biological activities were provided no more than around 10 years ago (Jiang et al. 2008; Birringer et al. 2010; Jiang et al. 2011). In the last decade, the interest increased and further knowledge on the synthesis, analytics and biological functions of this substance class became available (Manuscript V: Kluge et al. 2016; Birringer and Lorkowski 2019; Manuscript IV: Kluge et al. 2019; Manuscript XI: Wallert et al. 2020). However, current efforts to reveal the significance of LCM (aiming finally at physiologic relevance for human and possible pharmacologic applications) are still mainly at the stage of basic research. Already in 1945, Vannevar Bush, Director of the US Office of Scientific Research and Development, stated the following:

'Basic research is performed without thought of practical ends. It results in general knowledge and an understanding of nature and its laws. This general knowledge provides the means of answering a large number of important practical problems, though it may not give a complete specific answer to any one of them. The function of applied research is to provide such complete answers.'

Vannevar Bush (United States. Office of Scientific Research and Development 1945)

Following this definition of basic research, all forms of knowledge gain are valuable since they lead to new hypotheses and may provide the basis to answer future questions as well as for application development. Most of the manuscripts included in this thesis used *in vitro* test systems to gain knowledge on basic principles of cellular metabolism as well as functions of LCM and GA. With an eye to physiologic functions or pharmacologic applications in humans, this type of scientific evidence is less meaningful compared to animal and human trials. Notwithstanding this, when the work on this thesis started in 2015, knowledge on biological functions of the LCM was very sparse and *in vivo* trials could not be advocated from a scientific and ethical point of view. Hence, focus was initially set on gaining more knowledge on distinct functions and mechanisms of action of LCM and GA. In this context, *in vitro* test systems have indisputable advantages. A primary advantage is the reduction of potential confounders being present in complex systems (e.g. 'whole animals') when examining the mechanism of action of a compound. Further, the applied concentration of the compound as well as the cellular environment can be tightly controlled and *in vitro* studies can be performed more rapidly and thus confirmation of the reproducibility of the results is straightforwardly possible. Especially reproducibility of findings is crucial in scientific research even though this principle seems to fade more and more into the background today.

'Non-reproducible single occurrences are of no significance to science.'

Sir Karl R. Popper (Popper 1934)

Taking the guiding principle of the 'Three Rs' (replacement, reduction and refinement) of *in vivo* animal testing into account, robust and reliable *in vitro* results become even more important as solid basis (Russell and Burch 1959). Animal testing should be carefully planned, based on reliable *in vitro* data in order to reduce to a minimum the number of animals needed. However, there are several limitations of *in vitro* systems making *in vivo* testing necessary. This especially applies when trying to examine the physiologic functions of a dietary compound like vitamin E and, in this case, of the metabolites formed. The central question is, if the *in vivo* concentrations even reach the concentration used in *in vitro* studies in respective organs or cells. The fate of a compound after ingestion is complex and processes including absorption,

distribution, metabolism and excretion determine the concentrations in tissues and the whole organism. Most of these processes are well characterized for vitamin E (α-TOH) (Schmölz et al. 2016; Manuscript VI: Schmölz et al. 2018). In contrast, the fate of the LCM in the human body is virtually unknown. Notwithstanding this, the key finding of LCM occurrence in human blood is reproducible (Wallert et al. 2014; Pein et al. 2018; Giusepponi et al. 2019), suggesting a physiologic role of these metabolites. Further, comparable levels were found in mouse blood and a substantial accumulation of α-13'-COOH in immune cells to micromolar levels was recently reported (Pein et al. 2018). These findings indicate that our *in vitro* models of human and murine macrophages (*vide infra*) as well as the used concentrations in these test systems are suitable to examine LCM functions. Basically, test systems and methods should always be chosen according to the specific aim of the study. In the following, the original contributions and manuscripts intended as such included in this thesis are briefly reviewed in this regard.

10.1 Preliminary remarks

Based on their relevance in CVD, but also other NCD (Schultze et al. 2015; Russo and Lumeng 2018; Ponzoni et al. 2018; Li et al. 2018), macrophages were the main model system used in this thesis. Beside the inflammatory response, lipid metabolism of these cells was in the focus. As outlined above, LCM research is still at the stage of early research and thus the aim of virtually all studies included in this thesis is the elucidation of specific responses and mechanisms of the cells to applied compounds. Hence, well characterized and stable (invariable) *in vitro* test systems are needed. In this regard, immortalized cell lines are the method of choice since they have a homogenous genetic background and low phenotype variability (Chanput et al. 2014) as well as the opportunity of uncomplicated propagation over a long time. The reproducibility of results is higher in these cell lines, making them favorable for the initial description of cellular mechanisms. However, the homogeneity of the cells is a drawback in the context of translating results to the *in vivo* situation since individuals (human and wild-type animals) show differences in their genetic background and phenotype. Notwithstanding this, LCM research is in the early stages of development and broadening of basic knowledge is still required. Attempts to translate the initial findings into mouse models are ongoing, however, the results are not always meeting the expectations ((Manuscript X: Wallert et al. 2019) and unpublished data). Due to the sparse knowledge on possible adverse effects of LCM and GA, safety of these substances is not assessable to date and human intervention trials are a long way away. Rather observational approaches in human would probably be feasible earlier, however, they would hardly be meaningful unless the significance of the blood levels of α-13'-COOH (or GA) as biomarker for tissue distribution and function is understood (refer to the chapter 'Future perspectives').

10.2 Manuscripts and methods focusing on lipid metabolism

As already stated, inflammation and lipid metabolism are connected in the pathology of several diseases. However, for the sake of clarity, the manuscripts included in this thesis are here discussed with respect to their main focus, either inflammatory processes or lipid metabolism. Knowledge on the lipid metabolism was gained using two distinct *in vitro* cell models: human THP-1 macrophages derived by phorbol 12-myristate 13-acetate (PMA) treatment of THP-1 monocytes (Manuscript I: Schubert et al. 2020; Manuscript IX: Kluge et al. 2021; Manuscript VII: Schmölz et al. 2018; Manuscript VIII: Kluge et al. 2020) and HepG2 human liver cells (Manuscript AI: Hoang et al. 2019). Both represent well-characterized and commonly used immortalized cancer cell lines (Tsuchiya et al. 1980; Knowles et al. 1980; López-Terrada et al. 2009). Since THP-1 macrophages are used as model system for foam cell formation for decades (Banka et al. 1991), we chose this accepted model for our experiments on

macrophage lipid metabolism. The transferability of lipid metabolism-related findings from THP-1 macrophages to other human macrophages, most strikingly to PBMCs derived from blood of human donors, and murine macrophage cell lines was demonstrated in manuscript I (Manuscript I: Schubert et al. 2020), underlining their suitability as model system.

The same study is central to the work on macrophage lipid metabolism in this thesis. A wealth of methods and setups (the Supplementary Material to Manuscript I contains additional 17 figures and 6 tables) was applied to THP-1 macrophages to characterize the response to PPARγ agonists and the PPARγ antagonist GW9662, which ultimately turned out to act as a PPARδ agonist. This unexpected mechanism of action was thoroughly examined and confirmed using chemical antagonists for all PPAR subtypes as well as small interfering RNA (siRNA)-mediated knockdown of all PPAR subtypes in THP-1 macrophages (Manuscript I: Schubert et al. 2020). In the following, the central position of Manuscript I will briefly be outlined.

First, PLIN2 and CD36 were confirmed as conserved PPAR targets across various human and murine macrophage cell lines. This has implications for a previous work of Wallert et al., demonstrating that LCM induce CD36 in THP-1 macrophages, thus affecting foam cell formation (Wallert et al. 2014). Further, LCM were shown to suppress lipotoxicity by up-regulation of PLIN2 mRNA and protein and concomitantly of neutral lipids and lipid droplet formation in macrophages (Manuscript VII: Schmölz et al. 2018). The same effects were observed with GW9662 and thus PPARδ activation (Manuscript I: Schubert et al. 2020), strongly suggesting an involvement of PPARδ in the effects of LCM in macrophages. Manuscript I not only provides explanatory approaches for the mechanisms behind previous findings (Manuscript VII: Schmölz et al. 2018; Wallert et al. 2014), but also a solid basis for following studies. In this context, it should be emphasized that GW9662 was proven to be useless when trying to examine PPARγ function in macrophages. Further, PPARδ was shown to be the predominant subtype in macrophages and the consequences of (arbitrary) PPARδ activation on THP-1 lipid metabolism and gene expression (the expression of 384 PPAR-related genes was measured by an array approach) were thoroughly examined. The finding of the predominant role of PPARδ and the implications for the mechanisms of action of LCM in macrophage lipid metabolism were seized in subsequent studies (Manuscript VIII: Kluge et al. 2020; Manuscript IX: Kluge et al. 2021).

The knowledge on PPARδ function in macrophages was used for the establishment of a new rapid and simple *in vitro* LPL activity assay (Manuscript VIII: Kluge et al. 2020). This new method circumvents the drawbacks of currently available commercial kits, most prominently the optimization for *ex vivo* (post-heparin plasma) samples or the need for harvesting and homogenization of cell samples. The new method is applicable to living cells for at least 24 h and subsequent analyses by other assays are possible. During the course of our work on PPAR (refer to the chapters ‘Contribution to the development of the field’ and ‘Future perspectives’), we observed that PPARδ activation strongly induces an endogenous inhibitor (ANGPTL4) of LPL activity in THP-1 macrophages, which opened up a new opportunity in the validation of the assay. Hence, the chemical PPARδ agonist GW0742 could be used in addition to the well-known inhibitor orlistat as control in respective experiments. With the establishment of this *in vitro* assay of LPL activity, we created a new opportunity to thoroughly examine the ability of compounds to influence the LPL system of macrophages and most likely of other cell types in the future. Since LPL is the pivotal mediator of triglyceride hydrolysis from triglyceride-rich lipoproteins, global energy balance, insulin action, body weight and CVD risk are associated with the activity of this enzyme (Hegele 2016). Thus, LPL has implications in most

if not all NCD. The new *in vitro* LPL activity assay may need some improvements in the future like the demonstration of its applicability with different and variably composed lipoproteins or the opportunity to absolutely quantify the release of substrate to create more meaningful results. However, the assay in its current form represents a suitable tool for hypothesis generation and verification on the level of molecular regulatory mechanisms.

The hypothesis of a LPL activity regulation by the LCM α-13'-COOH was already examined using the new LPL activity assay (Manuscript IX: Kluge et al. 2021). This presumption is largely based on the striking finding that α-13'-COOH strongly induces ANGPTL4, a well-known PPAR target gene (Georgiadi et al. 2010; Dijk and Kersten 2014) and endogenous suppressor of LPL activity (Makoveichuk et al. 2012; Berg et al. 2017). The LCM α-13'-COOH indeed reduces the LPL activity induced by VLDL treatment of THP-1 macrophages. In combination with gene and protein expression studies of LPL and ANGPTL4 as well as of neutral lipid accumulation in response to VLDL, a distinct mechanism of α-13'-COOH action compared to the precursor α-TOH was found. This further supports the hypothesis of the LCM as regulatory metabolites of α-TOH (Manuscript III: Schubert et al. 2018). However, albeit evidence for an involvement of PPARδ in the regulation of ANGPTL4 gene expression by α-13'-COOH was provided, this could not be confirmed as being the central regulatory aspect in this study. Post-translational regulatory mechanisms by α-13'-COOH may thus be of greater relevance. The limitations of this study represent some general limitations still existing in LCM research. These comprise among others that there is no data reported regarding the uptake and cellular distribution of α-13'-COOH in macrophages and other cell types that might serve to explain post-translational actions. Further, it is currently unknown, whether α-13'-COOH is able to act on membrane-bound receptors or ion channels which might explain the regulation of the LPL system and other effects on cellular lipid metabolism. Notwithstanding this, this work (Manuscript IX: Kluge et al. 2021) confirms the applicability of the LPL assay to examine the ability of compounds of interest to modulate the LPL system. Further, a complex *in vitro* testing system for the axis of ANGPTL4, LPL, and lipoprotein-induced lipid accumulation was established. This can likely be transferred to other cell types and used for different promising compounds like vitamin E-related substances (GA, (Manuscript V: Kluge et al. 2016)) or emerging pharmacological drugs (Geldenhuys et al. 2017). Hence, a new tool to examine molecular regulatory mechanisms of a central process in CVD (and NCD) development is at hand.

A further *in vitro* testing system for basic research in the field of NCD was established with focus on liver metabolism (Manuscript AI: Hoang et al. 2019). Here, examination of neutral lipid accumulation, a hallmark of NAFLD, was optimized and molecular markers of hepatic steatogenesis were identified. This model currently focusses on the stimulation of lipid accumulation and was shown to work with a variety of different stimuli (carbohydrates as well as fatty acids). In addition, first attempts to integrate an inflammatory stimulus (TNF) were made. A limitation of this model is that it is not sufficiently complex to resemble the cellular structure of the liver and this may bias the translation of respective findings to the *in vivo* situation. A more complex *in vitro* test system could be obtained by co-culturing the HepG2 cells from this model with stellate cells and/or Kupffer cells ('liver macrophages'). Respective approaches using macrophages have already been reported (Kanuri and Bergheim 2013; Granitzny et al. 2017; Wewering et al. 2017). In combination with our knowledge on the inflammatory response of macrophages (refer to the chapter 'Manuscripts and methods focusing on inflammatory processes'), a more accurate model for the interplay of inflammation and metabolism in liver tissue may be created.

10.3 Concluding remarks on methods to examine lipid metabolism

The thorough investigation of the lipid metabolism of PMA-derived THP-1 macrophages renders these cells a good model for understanding the general mechanisms of the regulation of macrophage lipid metabolism. Based on this knowledge, hypotheses for the modes of action of the LCM, GA and other compounds can be formulated and examined. Notwithstanding this, this model is suitable for *in vitro* research on basic mechanisms and translation to the *in vivo* situation must not necessarily be possible. The main aim of this thesis, the understanding of basic principles of the lipid metabolism of macrophages and their modulation by the LCM and GA, can definitely be achieved using this test system. However, first *in vivo* trials using LCM and GA focussed on the anti-inflammatory component of their effects and trials specifically aiming on the modulation of the lipid metabolism are pending. These future trials focusing on lipid metabolism modulation should be carefully planned based on the profound and growing knowledge obtained from *in vitro* studies.

10.4 Manuscripts and methods focusing on inflammatory processes

Knowledge on the modulation of inflammatory responses was mainly gained using the murine RAW264.7 macrophage model. We chose this model based on several considerations. First, human THP-1 cells, especially when differentiated using our standard compound PMA, show some drawbacks regarding the use as model for inflammatory responses. These include, depending on the used concentration of PMA, amongst others a strong activation in terms of cytokine expression, a lack of cluster of differentiation 14 expression (both leading to insufficient responses to LPS), incomplete adherence or a general instability of the model system (Park et al. 2007; Daigneault et al. 2010; Berg-Rolness 2014). Second, the inflammatory response of RAW264.7 to LPS is well-characterized by our group (Wallert et al. 2015; Schmölz et al. 2017a; Schmölz et al. 2017b; Manuscript II: Schubert et al. 2020; Manuscript X: Wallert et al. 2019; Manuscript AII: Müller et al. 2019). Third, basic research on inflammatory responses in *in vitro* models aims at providing reliable data for the conception of trials using mouse models with inflammatory background. In addition, RAW264.7 macrophages represent an established and commonly used cell line for examination of anti-inflammatory effects as more than 3,000 results for the search term ‘raw264.7 [AND] anti-inflammatory’ on PubMed (as of June 2020) show (*www.PubMed.gov)*.

10.4.1 A basic concept – Fatty acids suppress the inflammatory response to LPS

The usability of the RAW264.7 macrophage model for investigation of the anti-inflammatory capacity of compounds *versus* a LPS stimulus was further confirmed by our work on nut oils (Manuscript AII: Müller et al. 2019). It was shown that lipids (either mixtures of nut-derived FA or single FA) attenuate the response to LPS with respect to the gene expression of the inflammatory markers *Cox2 (Ptgs2), Tnf, Il1b, Il6* and *Nos2*. Concomitantly, protein expression of NOS2 and NO release were diminished. This mechanism represents a hallmark of the murine inflammatory response and attenuation of this process is thus a marker for the anti-inflammatory capacity albeit its significance for human physiology is questionable (Gross et al. 2014). However, this study has also implications for the research on α-13’-COOH and GA, since they carry a carboxyl moiety and are thus structurally related to FA. Confirmation of this *in vitro* model as being suitable to examine anti-inflammatory properties using well-known anti-inflammatory substances like unsaturated fatty acids renders the model a good tool to examine respective properties of GA and the LCM.

10.4.2 Long-chain metabolites versus LPS – Elucidation of mechanistic aspects and a reliable target

The most promising LCM, α-13'-COOH (based on the reported potency, occurrence in human blood and accumulation in blood cells), was already reported to modulate the inflammatory response of the above mentioned RAW264.7 macrophage model in late 2015 (Wallert et al. 2015). However, this study basically provided information on the outcome (*i.e.* cytokine gene expression, PTGS2 and NOS2 expression and release of prostaglandins as well as NO) but not on the underlying regulatory mechanism. Unfortunately, this was a substantial limitation of this study. Understanding the molecular mechanism of action of α-13'-COOH is crucial for better understanding the physiologic effects as well as to extrapolate possible pharmacologic applications. We thus undertook a further study in the RAW264.7 macrophage model trying to dissect the underlying mechanism of action (Manuscript II: Schubert et al. 2020). With this work, first evidence on the cellular response to α-13'-COOH subsequently leading to a diminished response to LPS was provided. Benefits of this work were the thorough characterization of the genetic response to α-13'-COOH with a focus on markers of endotoxin (cross-) tolerance. Further, the initial incubation regime comprised of a 24 h pre-treatment and an additional co-treatment of α-13'-COOH with LPS (Wallert et al. 2015) was subdivided, leading to the conclusion that an adaption within 24 h is necessary for most of the reported effects. First data on the cytokine secretion (and not only the gene expression) as well as pathways implicated in the adaption process (MAPK and NFκB) complete the picture of this work (Manuscript II: Schubert et al. 2020). Taken together, this study provides valuable information on the cellular response of macrophages to α-13'-COOH, which may not only account for the anti-inflammatory properties but also for other known functions. However, with respect to the molecular regulatory mechanism, the study is limited by its focus on gene expression data. While the strong and prolonged induction of certain feedback regulators of MAPK and NFκB signaling on the level of gene expression suggest an induction of these mediators on protein level, this was not confirmed (partly due to the lack of specific antibodies for immunoblots) experimentally. Hence, the pathway starting from α-13'-COOH recognition by the cells is not completely proven, albeit available data and knowledge from the literature support the formulated hypotheses. Thus there are open questions remaining for future studies. Notwithstanding this, this study provides valuable information for the interpretation of the results of an *in vivo* study using a rat model of endotoxemia, carried out during the work on this thesis (unfortunately before final results of Manuscript II were available). The model was set up with a pre-treatment period of 1 h with α-13'-COOH before challenging rats with LPS. Cytokine levels in blood were determined after 5 h, however, the anticipated clear suppressing effect of α-13'-COOH failed to appear (unpublished data). In the light of the *in vitro* findings from Manuscript II (Manuscript II: Schubert et al. 2020), this outcome does not appear surprising, mainly for two reasons. First, 1 h pre-treatment with α-13'-COOH is likely not sufficiently long to induce a profound adaption of immune cells to the stimulus and second, the *in vitro* studies showed that cytokine gene expression levels are not necessarily a meaningful predictor of the release and accumulation of cytokines (protein) in surrounding fluids. Taken together, these findings clearly underline the importance of thorough *in vitro* investigations of the effects of compounds of interest. This especially applies if interest in these is emerging, but knowledge is still sparse. Notwithstanding this, the *in vitro* studies (Manuscript II: Schubert et al. 2020) yielded a promising result which is in line with the results of the above delineated *in vivo* trial. The key regulator of monocyte migration and infiltration, CCL2 (Deshmane et al. 2009), was found strongly suppressed with respect to protein release *and* gene expression by α-13'-COOH pre-treatment before LPS challenge in the *in vitro* study. This appeared irrespective of the time of pre-treatment and was unique among the observed cytokines

(Manuscript II: Schubert et al. 2020). Interestingly, a significantly suppressed release of CCL2 (by about 40 %) was also found in male rats treated with 1 mg/kg body weight α-13'-COOH in the *in vivo* endotoxemia model (Figure 5, unpublished data). This indicates that regulation of CCL2 may be a conserved and reliable function of the vitamin E-derived LCM α-13'-COOH and thus represents a highly interesting topic for future LCM research.

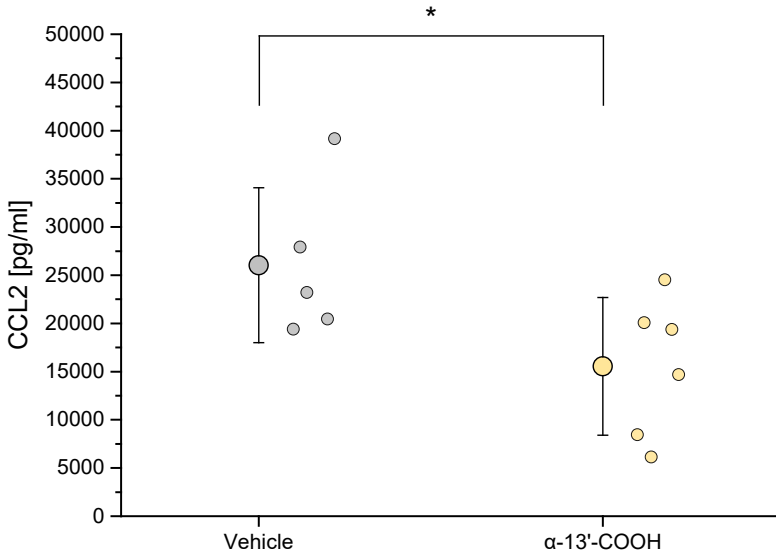

Figure 5: Plasma CCL2 levels are suppressed by α-13'-COOH in a rat endotoxemia model.
Male Wistar rats were injected with 1 mg/kg body weight of α-13'-COOH or vehicle 1 h prior to endotoxemia induction by LPS injection. Rats were sacrificed 5 h after LPS application and blood was collected by cardiac puncture. CCL2 levels were determined using a bead-based immunoassay and flow cytometry. Data from individual animals is represented by small dots and the mean of the group by the large dot. Standard deviation is given as error bar. The star indicates a significant difference in the mean of the groups calculated using the student's t-test in Microsoft Excel ($p < 0.05$).

10.4.3 Garcinoic acid versus LPS – *In vitro* versus *in vivo* situation and pitfalls in setting up animal models

The anti-inflammatory potential of the phytomedicine *Garcinia kola* seed extract as well as of one lead anti-inflammatory compound, GA (Manuscript V: Kluge et al. 2016), was examined in the *in vitro* model of RAW264.7 macrophages (Manuscript X: Wallert et al. 2019). Promising results were obtained regarding the suppression of PTGS2 and NOS2 protein and gene expression as well as the release of respective effector molecules. In line with the findings from nut extracts, fatty acids (Manuscript AII: Müller et al. 2019) and LCM (Wallert et al. 2015), the *Garcinia kola* seed extract and GA suppressed the gene expression of several cytokines (*Tnf, Il6, Il1b)* in the *in vitro* model of LPS-stimulated RAW264.7 macrophages (Manuscript X: Wallert et al. 2019). Based on the central role of macrophages in atherosclerosis and the inflammatory character of this disease, the atherosclerosis model of ApoE knockout in combination with a high-fat diet was chosen to translate these findings to the *in vivo* situation. Mice received 1 mg/kg body weight GA once per week intraperitoneally and were fed *ad libitum* for 8 weeks. Unexpectedly, merely one property of GA's delineated *in vitro* effects could be linked to the *in vivo* findings: Nitrotyrosinylation was suppressed in atherosclerotic plaques of GA-treated mice. This represents an indirect marker of NO production, which is strongly suppressed in the *in vitro* model. The absence of other *in vivo* effects emphasizes the difficulties in translating *in vitro* findings to *in vivo* models. With respect to cytokine levels, the pitfall may be the attempt to directly translate from gene expression data to circulating cytokines, as discussed above. Additionally, albeit the choice of the model system appears

reasonable as a low-grade inflammation is induced by the diet, it may not be entirely appropriate to investigate the effects of GA. Aspects to consider in this regard include that the ApoE knockout model itself is not the most appropriate model when focussing on inflammation (Oppi et al. 2019). Especially in combination with a high-fat diet, metabolism of the mice is highly disturbed and they show properties of a diabetes (or metabolic syndrome) model (Oppi et al. 2019) and the lipid overload may interfere with the effects of the lipid-soluble GA (as discussed in the chapter 'Integrating metabolism and inflammation'). Further, the distribution of GA in the organism after i.p. injection is unknown and it might thus be possible that GA is rapidly excreted. Consequently, immune cells (macrophages) may not face effective concentrations. However, an accumulation of GA, unlike that of the related α-13'-COOH, was not found in immune cells (Pein et al. 2018). Taken together, the basic knowledge on the regulatory properties *in vitro* as well as the fate of GA *in vivo* is not sufficient to predict *in vivo* functions. A more thorough investigation of this promising anti-inflammatory lead compound is thus needed.

10.4.4 Concluding remarks on the models used to investigate anti-inflammatory effects

The RAW264.7 macrophage cell line represents a widely used *in vitro* model to study the inflammatory response and the response of these cells to LPS is well-known. Using this model to examine the anti-inflammatory capacity of LCM and GA is thus logical. The model of human THP-1 macrophages which may better resemble the response of human macrophages has the clear drawback that a stimulus is needed to transform the monocytes into macrophages. This stimulus-induced differentiation and concomitant activation has several disadvantages regarding the examination of the inflammatory response (Chanput et al. 2014) (*vide supra*). Given that the first step in translation of *in vitro* findings to the *in vivo* situation is usually the use of rodent models, the use of mouse macrophages for *in vivo* testing can be regarded as an advantage. However, as outlined above, translating findings from mouse cells to the whole organism is difficult and appropriate models must be chosen carefully based on the available *in vitro* data.

'The most brilliant design, the most elegant procedures, the purest reagents, along with investigator talent, public money, and animal life are all wasted if the choice of animal is incorrect.'

Bernard Rollin (Rollin and Kesel 1990)

There are still great efforts to be made to unequivocally demonstrate the *in vivo* functions of GA and the LCM. Notwithstanding this, there are also promising results from *in vivo* trials available for LCM. Proof of principle studies carried out in a wound healing mice model using an innovative carrier material showed that α-13'-COOH can significantly accelerate the wound healing process (unpublished data). The great pharmacological potential of the substance class of LCM and related compounds was thus emphasized once again.

11 Contribution to the development of the field

The history of science shows that assumptions made based on the current state of knowledge at a respective point in time repeatedly had to be corrected later. Consequently, generating new evidence that questions old views and paradigms is central to the scientific progress.

'The characteristic of scientific progress is our knowing that we did not know.'

Gaston Bachelard (Yomtovian and Jacobs 2010)

In addition, generating new hypotheses on what we still do *not* know based on new findings is another central aspect of scientific progress. These hypotheses may or may not be disproved in the future. However, both will contribute to the scientific progress. In the following, a subjective estimation of the contribution of this thesis and the included manuscripts to the development of the field is provided. This estimation is logically based on the past and present state of knowledge in the field and the real contribution to the development of the field will naturally only become apparent in the future.

11.1 GW9662 – Implications in PPAR research and LCM research

Since almost 40 years, PPARs are in the focus of scientific research. Consequently, several functions of this family of nuclear receptors have been reported including their participation in nutrient and energy metabolism and the regulation of cellular and whole-body energy homeostasis. In combination with their regulatory role in inflammatory processes, they have implications in a variety of diseases (Hong et al. 2019). Hence, there is a great interest in the elucidation of their molecular mechanisms of action in order to understand their physiologic and pathophysiologic role and to develop pharmacological strategies (refer to the chapter ‘The great framework: non-communicable diseases as consequence of meta-inflammation’).

The work on the PPARγ antagonist GW9662 (Manuscript I: Schubert et al. 2020) thus contributes for the reasons listed below to the field of PPAR research in general.

(I) Manuscript I clearly shows that the PPARγ antagonist GW9662 is not suitable for the investigation of PPARγ contribution to effects on macrophage (lipid) metabolism.
(II) Future studies in macrophages should thus avoid the use of GW9662 or at least consider the known and possible adverse effects. This might also be true for other cells and tissues where PPARγ is not the predominant subtype (*vide infra*).
(III) Previous studies in which GW9662 was applied to macrophages will need reconsideration in the light of the findings from Manuscript I, especially when focussed on lipid metabolism.
(IV) Manuscript I highlights the predominant role of PPARδ in macrophages and that PPAR subtype expression in individual cells and tissues needs thorough consideration in the setup and interpretation of studies.

Taken together, the work (Manuscript I: Schubert et al. 2020) illustrates that PPAR research needs well-characterized and functional tools and that the experimental model should be thoroughly characterized and the experimental setup carefully planned. Despite this, Manuscript I has also implications for the own future work and contributed to the field of research on vitamin E, the LCM and related compounds like GA for the reasons listed below.

(I) Macrophage models are to date central to our work on LCM and GA and will remain so in the (near) future (refer to the chapter ‘Future perspectives’). Manuscript I thoroughly characterizes the lipid metabolism of the THP-1 macrophage model and thus provides a solid basis for future studies.
(II) Manuscript I provides explanatory approaches for the findings from previous studies (Wallert et al. 2014; Manuscript VII: Schmölz et al. 2018) on the modulation of central aspects of macrophage lipid metabolism by the LCM (refer to the chapters ‘Integrating metabolism and inflammation’ and ‘Review of the studies and applied methods’).

(III) The LCM and GA are lipid-soluble compounds and PPAR are thus likely involved in their effects. First studies implicate a respective relationship (refer to the chapter 'Review of the studies and applied methods'). Showing the predominance of PPARδ in THP-1 macrophages has thus implications in future studies on LCM and GA in macrophages.

(IV) The establishment of effective siRNA-mediated knockdowns of all PPAR subtypes in the course of the work on manuscript I opens new possibilities for the future research on LCM and GA.

11.2 Garcinoic Acid – Promising lead compound and centrality in LCM research

Garcinoic acid represents a main anti-inflammatory constituent of the African ethnomedicine *Garcinia kola*. Structurally, this compound represents δ-tocotrienol carrying a carboxyl moiety (refer to the chapter 'Long-chain metabolites of vitamin E') and can thus serve as basis for the research on T3 functions, on LCM functions (as basis for their semi-synthesis) and for the development of pharmacologically active substances (as lead compound). The work comprised in this manuscript contributed to the field of GA research by means of the following aspects.

(I) The bioactivity of GA as well as the implications in vitamin E research and especially LCM research were extensively reviewed (Manuscript V: Kluge et al. 2016). With this work, an understanding of the relevance of GA was given to a broad audience. Moreover, this work also set the direction of future research.

(II) The anti-inflammatory potential of GA was shown in an *in vitro* model of mouse macrophages (Manuscript X: Wallert et al. 2019). This, on the one hand, confirms the assumptions from the previously published review (Manuscript V: Kluge et al. 2016) and on the other hand provides an interesting basis for future research on the anti-inflammatory properties of GA.

(III) The *in vivo* mouse model trial on GA illustrates the difficulties in the translation of *in vitro* results to the *in vivo* situation (Manuscript X: Wallert et al. 2019). However, suppression of NO formation may represent a strong and conserved function of GA. The results of this work are moreover of great value as they reveal the gaps in current knowledge and set the direction for future research (refer to the chapter 'Garcinoic acid versus LPS – In vitro versus in vivo situation and pitfalls in setting up animal models').

11.3 Long-chain metabolites – Potential bioactive metabolites mediating vitamin E functions

The main focus of this thesis is on LCM research and thus, the majority of the articles and contributions relate to this field. As outlined in the chapter 'Aim of the work', knowledge was sparse when the work on this thesis began. Hence, the work comprised in this thesis contributed to the development of the field in many facets. The contributions subjectively judged to be most significant are listed below.

(I) The knowledge on LCM was repeatedly summarized (*i.e.* updated) with focus on varying aspects, like the relevance of GA as structural precursor (Manuscript V: Kluge et al. 2016), the metabolic fate of the parent substance α-TOH (Manuscript VI: Schmölz et al. 2018), functions of the substance class in general (Manuscript XI: Wallert et al. 2020) and the bioactivity of LCM (Manuscript IV: Kluge et al. 2019).

With these publications, the emerging class of the regulatory LCM was further popularized among vitamin E researchers. Moreover, with placing respective articles in *Studies in Natural Products Chemistry* (Manuscript V: Kluge et al. 2016) and *Frontiers in Pharmacology* (Manuscript XI: Wallert et al. 2020), the pharmacologically interesting LCM and related compounds were also made known to the relevant target group.

(II) The general concept of a metabolic activation of lipid-soluble vitamins was created based on the extension of knowledge on the lipid-soluble vitamins A, D and K to vitamin E and the LCM. This concept was then provided to the scientific community (Manuscript III: Schubert et al. 2018). The hypotheses formulated in this manuscript set the direction for the following research on the physiologic functions.

(III) The knowledge on mechanistic aspects of the anti-inflammatory action of the LCM α-13'-COOH was extended (Manuscript II: Schubert et al. 2020). This is of particular importance as all *in vivo* models performed so far with the LCM were aimed at the attenuation of inflammatory processes. To be able to interpret the promising as well as the disappointing results of these trials correctly, a profound knowledge of the underlying regulatory mechanisms is crucial (refer to the chapter 'Review of the studies and applied methods').

(IV) The knowledge on regulatory aspects of the LCM on the lipid metabolism of macrophages was extended. Interestingly, α-13'COOH was shown to suppress the adverse process and consequences of foam cell formation via multiple ways (Manuscript IX: Kluge et al. 2021; Manuscript VII: Schmölz et al. 2018; Wallert et al. 2014) *in vitro*. This suggests a beneficial role in the pathology of atherosclerosis and consequently CVD (as well as other NCD; refer to the chapters 'The great framework: non-communicable diseases as consequence of meta-inflammation' and 'Vitamin E and non-communicable diseases'). Respective *in vivo* trials are pending, however, a solid knowledge base for careful planning is provided.

(V) Different *in vitro* methods for elucidating the functions of the LCM (and other compounds) have been validated, developed or refined (Manuscript VIII: Kluge et al. 2020; Manuscript IX: Kluge et al. 2021; Manuscript AI: Hoang et al. 2019). This opens new possibilities to examine certain regulatory aspects of LCM with more precise focus on the lipid metabolism and to investigate completely new aspects. This lays the foundation for the elucidation of further regulatory properties of the LCM in the future.

12 Future perspectives

Substantial progress has been made in elucidating biological functions of the vitamin E-derived LCM and related compounds within the last ten years (refer to the chapters 'Biological activity' and 'Contribution to the development of the field'). However, the field of LCM research can still be regarded as being in its infancy and the road is still a long one to finally prove the biological relevance of the LCM for human health. Albeit achieving this aim requires complex strategies and probably several years of experimental work, the overall strategic approach can be roughly broken down to the following three levels of research.

(I) Identifying **molecular mechanisms**.
This includes the identification of a specific receptor or uptake mechanism, elucidation of involved signaling mechanisms and characterization of the cellular response to LCM.

(II) Elucidation of the effects on the level of the **whole organism**.

This comprises the characterization of the formation of LCM (hepatic and extrahepatic metabolism), transport and tissue distribution, synthesis rate and depletion rate as well as the definition of physiologic and pathophysiologic concentrations of the LCM.

(III) Extrapolation of the knowledge to **human physiology**.
As intervention studies in humans using LCM directly will probably not be ethically acceptable in the short and middle-term, LCM functions need to be extrapolated from studies using the safe parent compound TOH. This approach requires thorough investigation of tissue distribution of TOH and LCM in animal models and the extrapolation of a reliable correlation of LCM levels in easily accessible tissues like blood with the levels in inaccessible tissues in living humans.

The following section will provide a brief discussion of a personal selection of possible approaches and strategies for expanding the knowledge at the three levels mentioned above. Certainly this is far from covering all possible strategies and should therefore be seen as the personal focus of the author of this thesis.

12.1 Identifying molecular mechanisms

A lot of efforts have been made to characterize the cellular response to the LCM and related substances in the work comprised in this thesis as well as in yet unpublished studies carried out during the work on this thesis and, not less important, in other works in the field of LCM research. Albeit striking and reproducible characteristics, like the modulation of the lipid metabolism and anti-inflammatory properties (refer to the chapter ‘Contribution to the development of the field’) were found, the underlying molecular regulatory mechanisms could hardly be clarified so far. The best characterized mechanism of action of the LCM is the inhibition of 5-lipoxygenase activity, with strong evidence for an allosteric mode of inhibition with high affinity of the carboxychromanol to the enzyme (Jiang et al. 2011; Jang et al. 2016; Pein et al. 2018). However, the underlying mechanisms of several other cellular responses like the induction of cellular lipid storage mechanisms and the prevention of lipid overload and lipotoxicity and the suppression of the release of inflammatory mediators like prostaglandins, NO and certain cytokines are less well understood (Wallert et al. 2014; Wallert et al. 2015; Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021). Basically, the transcriptional regulation of key enzymes and proteins in these processes like PLIN2, ANGPTL4, PTGS2 and NOS2 have been reported. Yet, the exact mechanism of transcriptional regulation by the LCM remains elusive.

12.1.1 Are PPAR mediating the effects of LCM?

This thesis makes significant contributions to two lines of evidence for possible regulatory mechanisms exerted by the LCM. The first line of evidence relates to the regulation of macrophage lipid metabolism by the LCM and most prominently α-13’-COOH. The prominent reported target genes of α-13’-COOH, CD36 (Wallert et al. 2014), PLIN2 (Manuscript VII: Schmölz et al. 2018) and ANGPTL4 (Manuscript IX: Kluge et al. 2021) all represent well-known PPAR target genes. In one of the central works of this thesis the role of PPAR in the lipid metabolism of the THP-1 macrophage model was thoroughly examined (Manuscript I: Schubert et al. 2020). This work thus strengthens the hypothesis that PPAR are involved in the regulatory mechanism exerted by the LCM and provides a profound understanding of the effects of PPAR agonists and antagonists on macrophage lipid metabolism (refer to the chapter ‘Integrating metabolism and inflammation’). Further, hitherto unpublished data support this hypothesis. The currently accepted work on the modulation of the LPL system (Manuscript IX:

Kluge et al. 2021) indicates an involvement of PPARδ in the regulation of ANGPTL4. This finding is supported by yet unpublished work of the author of this thesis, showing that the PPARδ antagonist GSK3787 (4-chloro-N-(2-{[5-trifluoromethyl)-2-pyridyl]sulfonyl}ethyl) benzamide) efficiently and significantly suppresses the induction of the gene expression of ANGPTL4 and PLIN2 and in tendency that of CD36 by α-13'-COOH in THP-1 macrophages (Figure 6). It should be noted that chemical antagonists designated as specific for PPARα and PPARγ show less pronounced effects (Figure 6), albeit this may be biased by off-target action on the prominent subtype PPARδ in macrophages as reported for GW9662 (Manuscript I: Schubert et al. 2020).

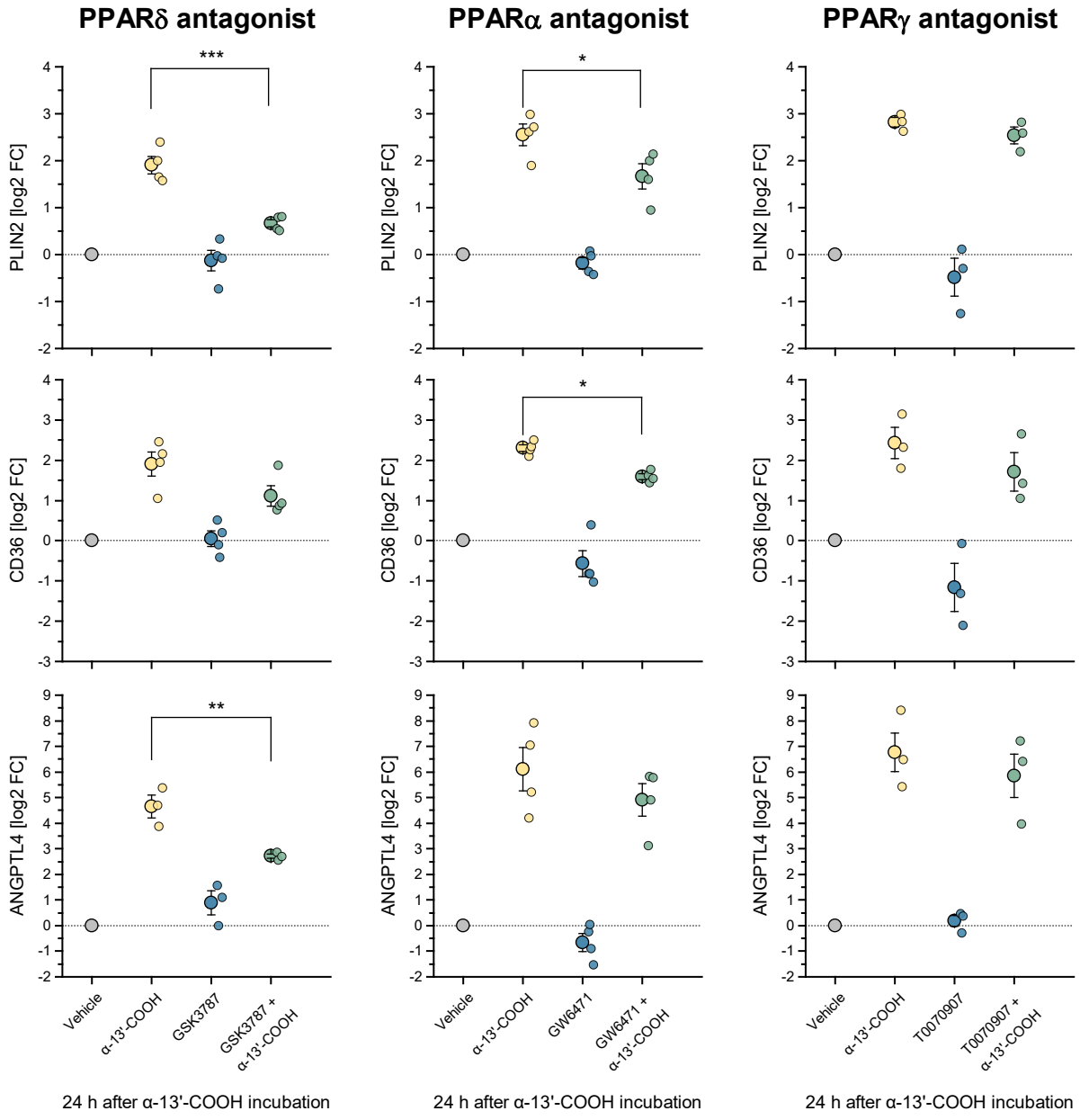

Figure 6: The PPARδ antagonist GSK3787 significantly blocks the induction of the expression of lipid metabolism-related genes by α-13'-COOH in THP-1 macrophages.
THP-1 monocytes were differentiated by PMA treatment for 24 h and subsequently pre-treated with the indicated PPAR antagonists for 1 h prior to addition of 5 µM α-13'-COOH. Cells were harvested 24 h later and mRNA abundance of PLIN2, CD36 and ANGPTL4 was examined using RT-qPCR. Small dots represent individual biological replicates and the large dot represents the mean. Error bars represent the calculated RQmin/max based on the standard error of mean. Statistical significance was calculated by Tukey's test following a significant repeated measures ANOVA using OriginLab's Origin 2016G.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Supporting the hypothesis of an involvement of PPAR, studies using a PPAR-Gal4 hybrid reporter gene assay based on human embryonic kidney cells (HEK293) revealed that α-13'-COOH treatment leads to an activation of all PPAR subtype constructs (personal communication, Dr. Daniel Merk, Frankfurt). However, experiments using the reported siRNA mediated knockdown of PPAR gene expression (Manuscript I: Schubert et al. 2020) as well as efficient knockdown of the heterodimer partner RXRA (retinoid X receptor (RXR)α) and RXRB (RXRβ) gene expression revealed that the target genes PLIN2, CD36 and ANGPTL4 are under control of PPARδ and PPARγ in THP-1 macrophages, yet, the inductive capacity of α-13'-COOH with respect to these genes was not diminished (unpublished data from experiments performed by the author of this thesis). Taken together, the data strongly suggest that PPAR affect the regulatory mechanism of LCM, however, validation of PPAR as *direct* mediators of LCM signaling is pending. Based on the available data, PPAR activation and subsequent gene regulation may represent a secondary effect in response to LCM (potentially due to the alterations in the lipid metabolism as outlined in the chapter 'Lipid droplets – Regulatory cellular substructures in lipid metabolism and inflammation'). Thus, although promising evidence has been generated largely by the author of this thesis' work, additional efforts must be made to prove or disprove a *direct* mediation of the LCM effects by PPAR. In this respect, three central aspects need to be clarified.

First, do LCM reach PPAR-relevant cell compartments and if so, in which structural form? Central for answering this question is the sensitive and reliable analytical measurement of the LCM. Respective techniques have recently been reported and a method for calculating intracellular vitamin E metabolite concentrations was provided (Pein et al. 2018). Promising first data indicate that certain cells metabolize or selectively enrich the carboxychromanol form of the LCM (Schmölz 2018). Fractionation of the cells to determine the subcellular localization of the LCM would be the next step. Respective approaches have been repeatedly reported for TOH as well as T3 (Guarnieri et al. 1980; Traber and Kayden 1987; Qian et al. 2005; Irías-Mata et al. 2018). While α-13'-COOH is known to reach relevant concentrations in leukocytes ((Pein et al. 2018) and unpublished data), spatial proximity or a targeted shuttling of α-13'-COOH allowing a direct interaction of LCM and PPAR needs to be proven by suitable approaches.

Second, the ability of PPAR to bind LCM needs to be proven. Scientific progress lead to a multitude of approaches to assess the binding of ligands to PPAR. Initial information on the potential of LCM to bind PPAR could be derived from *in silico* methods like computational molecular docking studies (El-Houri et al. 2015; Encinar et al. 2015). Since significance of these approaches is limited, results should be proven using respective biophysical and cell-based approaches. The above outlined reporter gene assay is not sufficient, since it allows the theoretical possibility of the release of a PPAR ligand in response to the LCM, potentially biasing the results. Appropriate approaches comprise among others competition with radiolabelled ligands or crystallographic approaches. However, these methods are time-consuming and require special expertise. Hence, methods based on fluorescence spectroscopy have been developed. Approaches using purified PPAR ligand binding domains to examine direct binding capacity and affinity of ligands comprise anilinonaphthalene-8-sulfonic acid (ANS) binding assays and gel-based competition assays (Zorrilla and Pérez-Sala 2013; Videira et al. 2018). Ideally, several of these methods should be performed to confirm the binding ability, starting with the most easily feasible and reproducible approach.

Third, direct relevance of PPAR for LCM effects needs to be proven. This probably represents the most challenging task. Albeit work on chemical PPAR agonists and antagonists

(Manuscript I: Schubert et al. 2020) showed that these compounds lead to the very same cellular response like the LCM α-13'-COOH with respect to macrophage lipid metabolism (Manuscript VII: Schmölz et al. 2018; Manuscript IX: Kluge et al. 2021; Wallert et al. 2014), this must not necessarily be directly related to PPAR activation by the LCM. Final proof should be obtained by removing PPAR from the experimental system and subsequently observing a loss of the function of the LCM. To achieve a system with low PPAR expression, siRNA-mediated knockdown was performed as indicated above. However, it is in the nature of the siRNA approach that residual activity of the targeted protein cannot be completely excluded. To avoid this uncertainty, cells with a complete loss of PPAR expression should be used for respective experiments. However, to the best of the author's knowledge, PPAR-deficient THP-1 or RAW264.7 cells (the models used in preliminary studies) are not commercially available. Alternatively, deficient macrophages could be isolated from PPAR knockout mice (Babaev et al. 2005). However, this approach is very complex and time-consuming and thus poorly suited to thoroughly study the molecular mechanisms. In this regard, the new promising technique of clustered regularly interspaced short palindromic repeats (CRISPR) gene editing in commonly used cell lines may be more favorable. Knockout of PPARγ in THP-1 cells using this technique has already been reported (Majithia et al. 2016; Niu et al. 2017). However, due to the central role of PPAR in the regulation of cellular processes, deletion of these will likely disturb the cellular homeostasis and might affect the response to LCM. Further, deletion of one PPAR subtype might be compensated by the other subtypes since their ligand and target patterns overlap. This would severely exacerbate the elucidation of a PPAR subtype as receptor for the LCM unless only one subtype binds with very high affinity (for which there is currently no evidence). Respective experiments are therefore highly complex and entail some pitfalls that must be avoided.

12.1.2 Are surface receptors mediating the effects of LCM?

The second line of evidence relates to the possible existence of a cell surface receptor for the LCM. A key experiment supporting this hypothesis was conducted in the course of the work on the anti-inflammatory properties of α-13'-COOH (Manuscript II: Schubert et al. 2020). The LCM α-13'-COOH was shown to induce the phosphorylation of MAPK within 10 min. This fast induction of the MAPK signaling pathways suggests the activation of (a) receptor(s) subsequently activating the MAPK signaling cascade. Cell surface receptor-induced activation of MAPK pathways is a well-known signaling pathway (Cargnello and Roux 2011). A prominent role is played by G protein-coupled receptors (GPCR) in this context (Goldsmith and Dhanasekaran 2007). Interestingly, the first reported high-resolution GPCR crystal structure was that of the light receptor rhodopsin, stabilized by binding of 11-*cis*-retinal (Palczewski et al. 2000). The metabolite 11-*cis*-retinal is produced endogenously from dietary vitamin A and is essential for light perception in vertebrates and thus for vision in human (Sahu and Maeda 2016). The vitamin A metabolite 11-*cis*-retinal thus represents a prime example for metabolic functionalization of lipid soluble vitamins (refer to (Manuscript III: Schubert et al. 2018) for more detailed information on this hypothesis) and the distinct and essential role of these metabolites in human physiology, here mediated by interaction with a GPCR. Consequently, the author of this thesis hypothesizes that vitamin E functions might be mediated by the metabolic activation to LCM and their subsequent binding to yet unknown or possibly even already known (orphan) GPCR.

This hypothesis is supported by the growing understanding of GPCR structure and function and the discovery of several lipid-binding receptors, representing a subfamily of GPCR. Until 2018, crystal structures from eight unique lipid-binding GPCR were resolved in complex with

different ligands (Audet and Stevens 2019). The lipid-binding GPCR can be grouped according to their ligand type into receptors for leukotrienes, lysophospholipids, platelet-activating factor, prostanoids, free fatty acids, cannabinoids and other lipids (Audet and Stevens 2019). Since the most promising LCM α-13'-COOH (broadly speaking) structurally represents a branched long chain fatty acid connected to a chromane, the class of lipid-binding GPCR appear as obvious potential receptors for this compound. However, identification and structural characterization of a unique GPCR for α-13'-COOH would take several years of experimental work (Audet and Stevens 2019). Nevertheless, such efforts should be made, as they may lead to the discovery of unique physiological mechanisms, as the example of vitamin A shows. Notwithstanding this, α-13'-COOH might act on an already identified lipid-binding GPCR.

A first step in the identification of possible molecular targets and the generation of hypotheses can be the use of emerging, easily feasible methods. So, for example, the rapid development of computer science offers new opportunities, so-called *in silico* approaches. One of these approaches is represented by '*in silico* target fishing'. Here, the availability of large datasets of protein-small molecule interactions makes the virtual identification of probable targets of bioactive small molecules possible. The prediction of possible targets of LCM and related compounds was carried out by using 'SwissTargetPrediction' (Gfeller et al. 2014), however, several other prediction tools are available ('Similarity Ensemble Approach', 'SuperPred', 'HitPick', 'admetSAR', 'PASSonline') and should be used to support generated hypotheses or generate different hypotheses in the future. 'SwissTargetPrediction' currently predicts bioactivity based on searching similar molecules in 2D and 3D within a collection of more than 375,000 compounds known to be experimentally active on more than 3,000 macromolecular targets (Daina et al. 2019). In a reported validation process, the tool computed for 72 % of 500 test compounds at least one experimentally known target in the top-15 results (Daina et al. 2019). Predicting targets for α-13'-COOH using the 'SwissTargetPrediction' WebTool indeed lead to macromolecules that have experimentally shown to be related to this LCM (Figure 7). Most strikingly, 5-LOX (ALOX5) is found within the top-20[2] of the results and was recently experimentally verified to be inhibited by α-13'-COOH most probably by an allosteric mechanism (Pein et al. 2018). Besides, the cyclooxygenases PTGS1 and PTGS2 are among the top-10 results. Suppression of the formation of respective products by α-13'-COOH has also been reported (Wallert et al. 2015). Here, suppression of PTGS2 mRNA and protein expression was the first mechanism of action suggested (Wallert et al. 2015). However, later α-13'-COOH was reported to inhibit isolated bovine COX-1 (PTGS1) and human platelet COX-1 (Pein et al. 2018), while human recombinant COX-2 was not inhibited. Notwithstanding this, the prediction to target PTGS1 and PTGS2 is correct even if the action on PTGS2 may not be direct or may be with low affinity. Further, PPARγ and PPARα are predicted targets of α-13'-COOH and first experimental data suggest that these nuclear receptors are implicated in the effects of this LCM (refer to the chapter 'Contribution to the development of the field'). These results suggest that *in silico* target prediction can be a useful tool for hypothesis generation in LCM research.

[2] It should be noted that the computation lead to 42 results with a higher probability than zero and that these 42 results hardly differed in their computed probability.

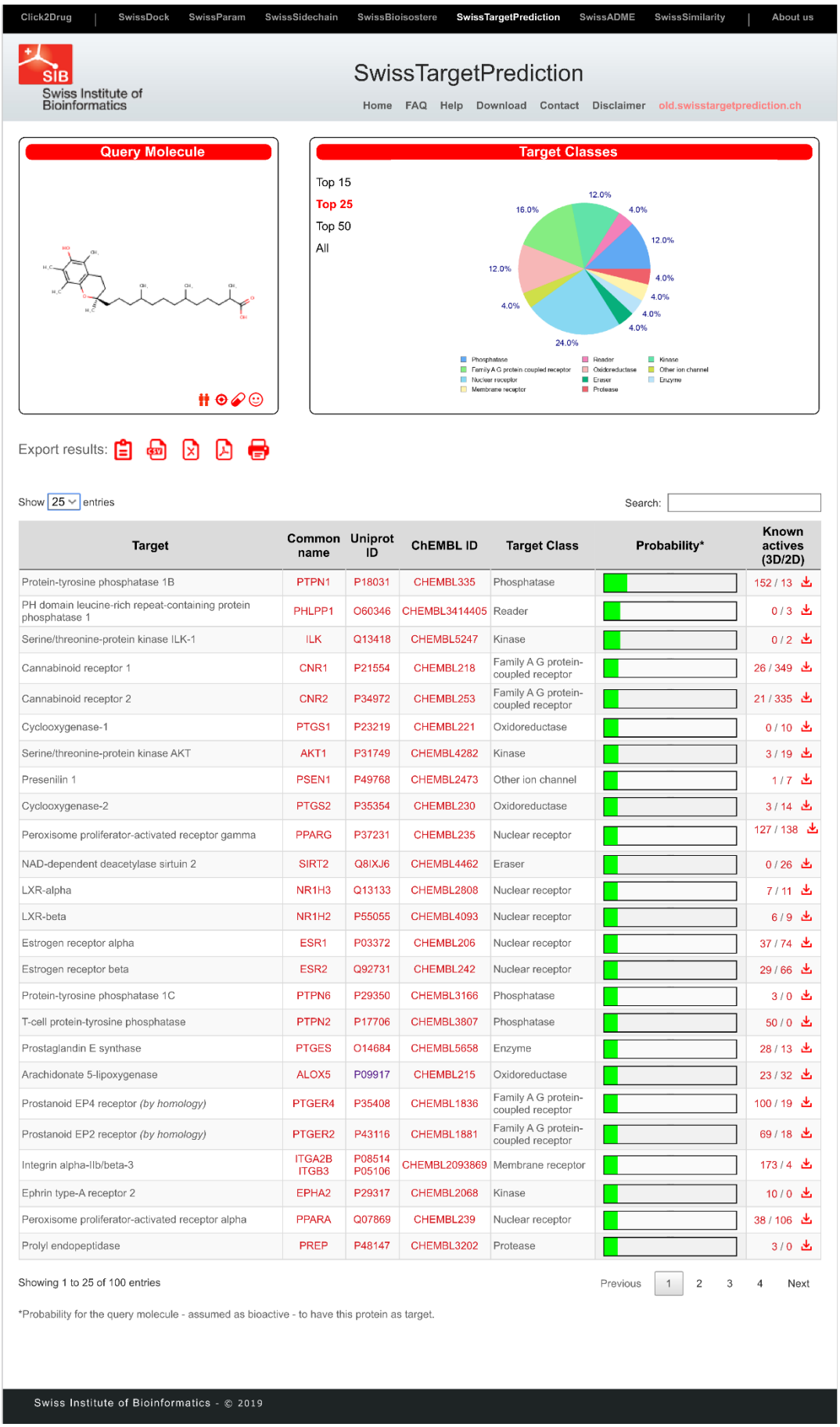

Figure 7: Results window of the *in silico* target fishing tool 'SwissTargetPrediction' after screening for potential molecular targets of α-13'-COOH.

With respect to GPCR, 'SwissTargetPrediction' yields the cannabinoid receptors 1 and 2 (CB1 and CB2) as well as the prostaglandin E_2 receptors EP4 and EP2 (PTGER4/2) as results within the top-25 for α-13'-COOH. This supports the hypothesis of an action on already identified GPCR. Strikingly, all of these receptors are expressed by macrophages (Katsuyama et al. 1998; Mai et al. 2015) and mRNA for cannabinoid receptor 2 (CNR2) is found in relevant abundance in our RAW264.7 macrophage model. Furthermore, the EP4 and EP2 receptors have implications in diseases, such as atherosclerosis (CVD), respiratory diseases, sepsis and diseases of the central nervous system like Alzheimer's disease and Parkinson's disease (Konya et al. 2013; Ganesh 2014), which have all been connected to vitamin E and consequently LCM (refer to the chapters 'Vitamin E and non-communicable diseases' and 'Biological activity'). Interestingly, EP2 and EP4 have been reported to modulate the inflammatory response of macrophages by a mechanism involving MAP kinase phosphatase 1 (MKP-1), p38 MAPK and COX-2 (Tang et al. 2017). All of these factors were found to be related to the inflammation-modulatory mechanisms of α-13'-COOH in RAW264.7 cells (Manuscript II: Schubert et al. 2020; Wallert et al. 2015). However, respective mediation of the effects of α-13'-COOH by prostanoid receptors must be proven experimentally. First easily feasible experiments could involve commercially available chemical agonists and antagonists for these receptors in combination with the LCM to get an impression of the significance of this hypothesis.

Cannabinoid receptors were initially described to occur in two different locations in complex organisms. The CB1 receptor is the principal receptor of the central nervous system, while the CB2 receptor is predominantly located on immune cells. However, this clear assignment is more and more questioned in the light of new findings (Barrie and Manolios 2017). Accordingly, the endocannabinoid system as whole is regarded as a promising target for diseases, such as neurodegenerative diseases, cardiovascular diseases as well as other inflammatory diseases and also for obesity and the metabolic syndrome as these have a clear inflammatory component (Pacher and Kunos 2013). As outlined in the chapters 'Vitamin E and non-communicable diseases' and 'Biological activity', vitamin E and the LCM have also implications for all of these diseases. Strikingly, the most severe and most straightforward result of very low vitamin E levels in human is AVED, a disease characterized by dysfunctionality in the central nervous system (refer to the chapter 'Ataxia with vitamin E deficiency'). This clearly demonstrates a significant role of vitamin E in the nervous system, a property it shares with endocannabinoids. Interestingly, the endocannabinoid system has already been discussed as therapeutic target for cerebellar ataxia (Stephens 2016) and vitamin E has been reported to interact with this system in rodent models (Crouzin et al. 2011). Despite the role of the endocannabinoid system in general brain functions like mood, learning and memory, pain-sensation and control of appetite (Irving et al. 2002), modulating this system can also have favorable effects on the control of neuroinflammation (Walter and Stella 2004). Moreover, the endocannabinoid system plays a role in systemic inflammatory processes, mainly due to the high abundance of CB2 in tissues relevant for immune function, such as tonsils, spleen, thymus and PBMC. However, CB2 is also found in lower abundance in other tissues like lung and pancreas (Turcotte et al. 2016). Strikingly, the CB2 is most abundant in cells of the macrophage lineage in healthy subjects (Dhopeshwarkar and Mackie 2014). Here, CB2R has been connected to extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and p38 MAPK activation (Dhopeshwarkar and Mackie 2014), resembling the effects we observed in RAW264.7 macrophages in response to α-13'-COOH (Manuscript II: Schubert et al. 2020). Interestingly, in microglial cells, a specialized macrophage population in the brain, cannabinoid receptor activation leads to a suppressed inflammatory response by inducing a negative

feedback loop involving ERK1/2 and an enhanced MKP-1 expression (Eljaschewitsch et al. 2006). Strikingly, the LCM α-13'-COOH leads to a similar feedback mechanism characterized by ERK1/2 activation and strong and sustained induction of *Dusp1* (MKP-1) gene expression (Manuscript II: Schubert et al. 2020). Interestingly, cannabinoids were reported to induce negative regulators of the MAPK and NFκB pathway in microglial cells which is linked to a diminished LPS response (Juknat et al. 2013). This essentially resembles what we hypothesize to be a major mechanism leading to the anti-inflammatory effects of α-13'-COOH (Manuscript II: Schubert et al. 2020). These lines of evidence suggest that a modulation of the endocannabinoid system by the vitamin E LCM α-13'-COOH, either directly by an action on the CB2 receptor, or indirectly via a yet unidentified related receptor, could account for the anti-inflammatory effects of this compound. Moreover, modulation of the endocannabinoid system could contribute to the known functions of vitamin E in central nervous system health. However, this hypothesis should be further substantiated with information from available literature and verified experimentally in future works.

12.1.3 Classical target fishing to identify receptors or mediators of LCM effects

In silico methods represent a helpful tool for hypothesis generation. However, they do not necessarily lead to the elucidation of the real biological targets. Hence, an 'analogous' experimental target-fishing could be applied to identify the receptor for the LCM. In brief, the LCM could be immobilized by covalent linking to appropriate beads and subsequently incubated with cell lysates. This allows for the binding of proteins to the LCM. After releasing the proteins, they can be separated by a two-dimensional gel electrophoresis. Promising protein spots can subsequently be isolated and the respective protein may be identified by mass spectrometry. However, albeit this method can reveal the true physiologic target(s) of the LCM, there are several pitfalls to avoid, especially when examining the LCM. The thorough comparison of the LCM with their substructures α-CEHC and pristanic acid lead to the conclusion that the entire molecule is required for the specific and potent effects of the LCM (Schmölz et al. 2017b). However, immobilization of the molecule requires the linking to a matrix and thus the modification of the structure. To the best of the author's knowledge this must be carried out either via the hydroxyl moiety at the chroman ring or via the carboxyl (or hydroxyl) moiety at the terminal position of the side chain. Both modifications may prevent the LCM from binding its true receptor and lead to biased results.

12.2 Elucidation of effects of the LCM at the level of whole organisms

In recent years, analytical methods for the LCM were refined (refer to the chapter 'Analytical approaches') to provide methods for the sensitive detection in various tissues. This provides the basis for elucidating the functions of the LCM at the level of complex organisms. A sensitive ultra-performance liquid chromatography ESI tandem mass spectrometry (UPLC-MS/MS)-based quantitation method allows a detection of the most promising LCM α-13'-COOH with a lower limit of detection of 1 nM (Pein et al. 2018). This method was successfully applied to human and mouse plasma samples, peritoneal exudates of mice and even to the respective peritoneal leukocytes (Pein et al. 2018). Thus, detection of the LCM is principally possible down to the level of distinct cell populations. This development allows for future strategic approaches to elucidate the role of the LCM in physiology and pathophysiology. From the author of this thesis' point of view, assessing the tissue distribution of LCM throughout an entire organism, preferably starting with rodent models, should be the central high priority aim. This is based on the following considerations. Knowing the tissues accumulating LCM would allow conclusions on their physiologic as well as pharmacologic functions and effects. Tissues with high concentrations of the LCM would likely represent their physiologic sites of formation

and/or action (except for adipose tissue, which might additionally represent a storage organ due to the lipophilicity of LCM). Further, knowing the sites of action would facilitate the development of more meaningful *in vitro* models. Combining knowledge on the sites of action and from respective *in vitro* experiments could lead to the elucidation and differentiation of the true physiologic and pharmacologic effects in complex organisms. Moreover, knowing the sites of accumulation could help to identify transport and storage mechanisms of LCM, for example by correlation with expression patterns of conceivable proteins like α-TTP, fatty-acid-binding proteins (FABP) or lipid droplet associated proteins. The most feasible approach to realize respective investigations would probably be to inject a defined amount of LCM into the bloodstream of healthy organisms and investigate the tissues after a defined time. This approach could be extended by taking samples at different time-points to get insights into the pharmacokinetics of the LCM. A further conceivable extension of these experiments would be long-term studies, providing information about tissue damage by prolonged administration of LCM. Respective approaches would allow for the estimation of physiologic and pathophysiologic concentrations. One should keep in mind that these experiments would not represent the physiological formation and distribution of the LCM, however, they would offer the possibility of a controlled experimental environment to get an understanding of the functions. An approach to examine the physiological formation of the LCM would be to provide a defined amount of the precursor (preferably α-TOH) as part of a controlled diet to subjects and examine the concentrations of the respective LCM in blood and tissues at multiple time-points. Moreover, studies using disease models as conducted in the past and currently underway, allow conclusions on physiologic functions (also refer to the chapter ‘Physiologic and pathophysiologic implications of the works in the context of NCD and meta-inflammation’).

12.3 Extrapolation of the knowledge to human physiology

As outlined before (refer to the chapters ‘Long-chain metabolites of vitamin E’ and ‘Aim of the work’), the major systemic LCM α-13’-COOH was repeatedly shown to occur in human blood (Wallert et al. 2014; Pein et al. 2018; Giusepponi et al. 2019; Giusepponi et al. 2017). However, Pein et al. provided the most detailed analysis and reported a concentration between 8 and 49 nM in the plasma of five healthy female and 5 healthy male humans (Pein et al. 2018). This is in accordance with a later report of 27 and 29 nM in two not supplemented subjects. Supplementation with 1000 IU *RRR*-α-TOH leads to an about tenfold increase of the blood levels (Giusepponi et al. 2017). However, these data to date do not allow any further conclusions, apart from a possible physiologic function of α-13’-COOH, since there is virtually no information about the formation and distribution of this compound in complex organisms available. Thus, studies on the tissue distribution (*vide supra*) are crucial. The knowledge about the tissue distribution combined with respective plasma levels in animals could be used to establish the (already determinable) levels of α-13’-COOH in human blood as marker for the ‘supply’ of certain tissues or the entire human. This would help to correlate certain physiologic or pathophysiologic conditions with the plasma levels and determine adequate ‘supply’ with α-13’-COOH. In support of this, the blood levels of LCM could be measured in healthy humans following a ‘healthy’ diet and in subjects with an unfavorable diet and possibly also in malnourished subjects. A comparison of the plasma levels and the correlation with the phenotype could probably allow conclusions about the role of the LCM in human physiology. However, such correlations might lead to misconceptions due to the complexity of human physiology and diet and hypotheses generated from these studies must be confirmed by the elucidation of definite regulatory mechanisms in adequate *in vivo* and *in vitro* models. A further interesting aspect would be data on the LCM levels in adipose subjects when distinct physiologic functions of the LCM are known. If the hypothesis of a loss of LCM function in

‘overfed’ cells and animals (refer to the chapter ‘Integrating metabolism and inflammation’) holds true, the severe disturbances of the metabolism in adipose subjects and the consequent resistance to α-13’-COOH may be related to pathophysiologic alterations (refer to the chapter ‘The great framework: non-communicable diseases as consequence of meta-inflammation’).

In the long run, the question as to whether the LCM (most probably α-13’-COOH) may represent the ‘true vitamin E’ should be answered, *i.e.* if the LCM represent the actual physiologically active and effective form of vitamin E. One central aspect in this regard is to prove whether the LCM can replace α-TOH, and thus the action as *vitamin*, in the treatment of the severe disorder AVED (refer to the chapter ‘Ataxia with vitamin E deficiency’). To date, lifelong high-dose supplementation of vitamin E represents the treatment of choice. This potentially leads to the formation of vitamin E metabolites, which could mediate or contribute to the beneficial effects of α-TOH. The key experiment to clarify the possibility of LCM as ‘true’ vitamin E could thus be the supplementation of AVED patients with α-13’-COOH instead of α-TOH. In case that α-13’-COOH supplementation leads to the same stabilization or improvement of the symptoms, strong evidence would be provided for its function as ‘true’ vitamin E. However, should α-13’-COOH supplementation have no effect in this regard, the LCM is not responsible for the *vitamin function* but may mediate other beneficial effects. However, as research on vitamin E-derived LCM is still in its infancy, human trials using these compounds are not tenable due to safety concerns. These concerns can be resolved by animal studies (outlined in the chapter ‘Elucidation of effects of the LCM at the level of whole organisms’) aiming at adverse effects of the LCM. As long as doubts regarding the safety remain, evidence for the LCM as the ‘true’ vitamin E should be collected in cell and animal models. Respective approaches and model systems like α-TTP-KO mice (Terasawa et al. 2000; Yokota et al. 2001) and generation and cultivation of Purkinje cells (Tao et al. 2010; Wang et al. 2015; Higuera et al. 2017; Watson et al. 2018) are already at hand.

Strikingly, a study in α-TTP-KO mice focusing on Purkinje cell integrity revealed that a supplementation with 600 mg α-tocopheryl acetate per kg diet leads to higher plasma TOH levels than that of wild-type mice fed with 34 mg α-tocopheryl acetate per kg diet (Ulatowski et al. 2014). In contrast, the supplementation of α-TTP-KO mice with vitamin E increases α-TOH levels only modestly in the brain, leading to about 30 % of the content of the brain of wild-type mice (Ulatowski et al. 2014). This confirms findings from a previous work reporting even lower levels (Yokota et al. 2001). Albeit obviously only a fraction of the plasma TOH is transferred to the brain, higher vitamin E levels are reported to positively affect the integrity of cerebellar Purkinje neurons (Ulatowski et al. 2014). This allows the author of this thesis to speculate that the LCM rather than the α-TOH itself could represent the crucial mediator of brain health (also refer to the chapter **‘**Are surface receptors mediating the effects of LCM?**’**). However, since vitamin E is predominantly known for its anti-oxidative functions (refer to the chapter ‘Vitamin E’), work has focussed on measuring makers of oxidative stress in α-TTP-KO mice. Consequently, elevated levels of thiobarbituric acid reactive substances (TBARS), 4-hydroxynonenal-modified protein (Yokota et al. 2001) and total 8-iso-prostaglandin F2α (t8-iso-PGF2α) (Yoshida et al. 2010) have been reported in the brain of α-TTP-KO mice. In accordance with the insight of Goethe (*vide infra*), the elevated levels of oxidative stress markers were ascribed to the loss of the protective anti-oxidative function of α-TOH in the brain, as this is the prominent function ascribed to α-TOH.

‘You only see what you already know and understand.’[3]
(*‘Man erblickt nur, was man schon weiß und versteht.’*)

Johann Wolfgang von Goethe (Goethe 1819)

What if it was not the lack of anti-oxidative protection of brain cells by TOH that was the cause of cell death but the lack of the preservative functions of the LCM on brain cells? Dying cells and clearance of dead material might be the cause of increased ROS (Galloway et al. 2019) and of consequently measured elevated markers of oxidative stress. Evidence for a role of the LCM in brain function is provided by their structural similarity to regulators of the endocannabinoid system (refer to the chapter ‘Are surface receptors mediating the effects of LCM?’). Another interesting aspect is the mantra of ‘α-TOH as the only TOH shown to prevent AVED’ and thus being the ‘one (true) vitamin E’ (Azzi 2018; Khadangi and Azzi 2019; Azzi 2019), as there are still many questions remaining regarding this issue. Why is α-TOH able to prevent AVED although there are low levels found in the brain of subjects with non-functional α-TTP, even with high supplementation of α-TOH (Yokota et al. 2001; Ulatowski et al. 2014)? Why is α-TOH the only cure for AVED if this effect should be merely based on its prominent anti-oxidative functions, especially as the sorting and transport function of α-TTP is missing? Shouldn’t the administration of other antioxidants capable of acting in the brain also be effective (to the best of the author’s knowledge, there are no studies on this issue available)? Further, studies suggest that vitamin E has no beneficial effect in other types of cerebellar ataxias with oxidative stress playing a role in their pathology (Barca et al. 2019), suggesting that the adverse effects of ROS are not sufficient to explain the pathology of ataxias. In conclusion, there are still major gaps in our knowledge and understanding of the functions of α-TOH and of vitamin E as whole. The LCM of α-TOH, especially the promising α-13’-COOH, may fill some of these gaps in the future.

Clearly, we must look behind what we already know and understand and try to pose the right questions. The LCM could be an eye-opener in vitamin E research and could significantly shape this field in the second 100 years of its research. However, the essential foundations must be laid in the (near) future and it will be exciting to see how the field of vitamin E research develops.

‘Nothing is less predictable than the development of an active scientific field.’

Charles Francis Richter (Spall 1980)

[3] Translation by the author of this dissertation. The quote is commonly translated as ‘We only see what we know.’.

13 Summary

Vitamin E was initially described as fertility factor in 1922 and has long been studied also for its beneficial properties in the prevention and treatment of all major non-communicable diseases (NCD). This group of diseases, whose main representatives include cardiovascular diseases, diabetes, chronic respiratory diseases and cancer, is responsible for around 70% of deaths worldwide. However, the results of the studies on the effect of vitamin E in these diseases are ambivalent and the effects found can no longer be explained solely by the prominent function of vitamin E as an antioxidant in the human body. For this reason, regulatory properties of vitamin E are becoming more and more the focus of interest. The long-chain metabolites of vitamin E (LCM), which are formed naturally in the body and were detected in the blood circulation, were proven to have potent biological effects. Accordingly, they form a class of regulatory molecules that could serve as an explanation for the ambivalent effects of vitamin E in human studies. Research on LCM represents a relatively young subfield within vitamin E research. Hence, merely three studies on biological effects of LCM formed from α-tocopherol were published when the work on this thesis started. These studies suggested that LCM may have desirable effects in terms of carcinogenesis, inflammatory response and lipid metabolism.

The aim of this work was therefore to extend and distribute the knowledge on the effects of LCM on lipid metabolism and the inflammatory response. Today there is general acceptance of the hypothesis that virtually all NCD are accompanied or triggered by a minor inflammation, often caused by a metabolic dysfunction. These diseases are therefore often summarized as ‘immunometabolic diseases’. Consequently, it is obvious that LCM could mediate the effect of vitamin E in these disease patterns and could potentially serve as possible therapeutic approaches in the future. Understanding the molecular effects of LCM is essential to establish plausibility between the observations from studies with vitamin E and the potential resulting effect of LCM.

The overall strategy of this work can basically be divided into three aspects. Firstly, the current state of knowledge on LCM and similar structures has been regularly summarized. This knowledge and the hypotheses generated on this basis were made available to a broad (professional) audience. Secondly, methods and models were developed, refined, characterized and validated, which are useful for research on LCM in particular and in principle in the overall context of immunometabolic diseases. Thirdly, investigations of further properties of LCM and similar structures with respect to the beneficial influence on lipid metabolism and the inflammatory response were performed.

The most important results of this strategy are:

- The publication of the review on the metabolic activation of lipid-soluble vitamins as a general (conserved) principle pointing the way for LCM research.
- The provision of extensive evidence that the widely used peroxisome proliferator-activated receptor (PPAR)γ antagonist GW9662 activates PPARδ in macrophages and leads to substantial changes in lipid metabolism and is therefore unsuitable for its intended use in this cell type.
- The first detailed study of the adaptive response of macrophages to LCM and the consequent alteration of the lipopolysaccharide (LPS) response, including the identification of the central chemotactic attractant chemokine (C-C motif) ligand 2 (CCL2) as a reliably suppressed target of LCM in the course of the initial description of the modulation of cytokine *secretion* by LCM.
- The elucidation of beneficial effects of LCM in the context of lipotoxicity and the lipoprotein lipase system, two key mechanisms in the pathogenesis of immunometabolic diseases.

- The presentation of the (anti-inflammatory) potential of the LCM-like substance δ-garcinoic acid by means of a comprehensive review and an experimental work.

The essential contributions of this thesis to the development of the research field thus include the suggestion of a direction for future LCM research with respect to the physiological function as a metabolically activated form of vitamin E by publishing several reviews. Furthermore, the demonstration of the unsuitability of GW9662 as tool for examination of PPARγ in macrophages necessitates the re-evaluation of some studies in the field and facilitates better setups of future studies. In addition, this study highlighted the predominance of PPARδ in macrophages, which currently has and prospectively will have a significant impact on the investigation of the effects of LCM in this model system. The characterization of the adaptive response of macrophages and the identification of CCL2 as a major target of LCM provide new promising starting points for future studies on the effects of LCM in macrophages. In addition, the LCM mediate protection against the harmful effects of lipid overload and could thus offer a positive effect in corresponding clinical pictures and unfavorable diets. Further, the LCM-like δ-garcinoic acid was highlighted as an interesting pharmacological lead substance for future research in the context of diseases with an inflammatory component.

These contributions are further steps on the long road to the superior aim of undoubted proof of LCM as active vitamin E metabolites. To achieve this, considerable efforts must be made in the future. These comprise the elucidation of molecular mechanisms of the regulation of cellular responses by LCM, as these are essential to explain the plausibility of effects attributed to vitamin E and to prove that LCM represent a crucial factor. Furthermore, the effects and properties of LCM must be elucidated at the level of the whole organism to understand physiological formation, transport and distribution as well as pharmacokinetics and to define physiological and pathophysiological concentrations. On this basis, the knowledge could finally be extrapolated to the situation in humans, where LCM has already been detected in the blood and could thus serve as a biomarker in easily accessible tissues.

The foundations were laid to open a new and exciting chapter in the second 100 years of vitamin E research. With sense and understanding must be built on these foundations to answer the right questions in the future and to clear up the remaining mysteries of vitamin E.

14 Zusammenfassung

Vitamin E wurde 1922 ursprünglich als Fertilitätsfaktor beschrieben, wird aber seit langem auch bezüglich seiner positiven Eigenschaften in der Prävention und der Behandlung aller wesentlichen nichtübertragbaren Krankheiten untersucht. Diese Gruppe von Erkrankungen, zu deren Hauptvertretern kardiovaskuläre Erkrankungen, Diabetes, chronische Atemwegserkrankungen und Krebs zählen, ist für rund 70 % der weltweiten Tode verantwortlich. Die Ergebnisse der Studien zur Wirkung von Vitamin E in diesen Krankheitsbildern sind allerdings ambivalent und die gefundenen Effekte können nicht mehr nur über die prominente Funktion von Vitamin E als Antioxidans im menschlichen Körper erklärt werden. Aus diesem Grund rücken regulatorische Eigenschaften von Vitamin E immer mehr in den Fokus des Interesses. Die langkettigen Vitamin-E-Metabolite (*long-chain metabolites*, LCM), die auf natürliche Weise im Körper gebildet werden und in der Blutzirkulation nachgewiesen wurden, besitzen nachweislich potente biologische Effekte. Dementsprechend bilden diese eine Klasse regulatorischer Moleküle, die als Erklärungsansatz für die ambivalenten Effekte von Vitamin E in Humanstudien dienen könnten. Die Forschung an LCM repräsentiert ein relativ junges Feld innerhalb der Vitamin-E-Forschung. Daher waren zu Beginn der Erarbeitung dieser Dissertation lediglich drei Studien zu biologischen Wirkungen der aus α-Tocopherol gebildeten α-LCM publiziert. Diese legten nahe, dass die LCM wünschenswerte Effekte im Hinblick auf Krebsentstehung, Entzündungsantwort und den Lipidmetabolismus entfalten können.

Ziel dieser Arbeit war es daher, das Wissen bezüglich der Effekte der α-LCM in Hinblick auf den Lipidmetabolismus und die Inflammationsantwort zu erweitern und verfügbar zu machen. Es ist mittlerweile für praktisch alle nichtübertragbaren Krankheiten belegt, dass die Entstehung dieser von einer geringgradigen Entzündung, oftmals durch eine metabolische Entgleisung verursacht, begleitet oder ausgelöst wird. Diese Erkrankungen werden daher oft als „immunometabolische Erkrankungen“ zusammengefasst. Folglich ist naheliegend, dass die LCM die Wirkung von Vitamin E in diesen Krankheitsbildern vermitteln könnten und potenziell zukünftig als mögliche Therapieansätze dienen könnten. Das Verständnis molekularer Effekte der LCM ist unerlässlich, um eine Plausibilität zwischen den Beobachtungen aus Studien mit Vitamin E und den LCM als mögliche vermittelnde Effektoren herzustellen.

Die Gesamtstrategie der Arbeit kann in drei grundsätzliche Aspekte untergliedert werden. Zum einen wurde der aktuelle Wissensstand zu LCM und ähnlichen Strukturen regelmäßig zusammengefasst. Dieses Wissen und die auf dieser Basis generierten Hypothesen wurden einem breiten (Fach-) Publikum zugänglich gemacht. Zum zweiten wurden Methoden und Modelle entwickelt, weiterentwickelt, charakterisiert und validiert, die der Forschung im Speziellen an LCM, grundsätzlich aber der Forschung im gesamten Kontext der immunometabolischen Erkrankungen zuträglich sind. Zum dritten wurden experimentelle Untersuchungen von weiteren Eigenschaften der LCM und ähnlicher Strukturen bezüglich der vorteilhaften Beeinflussung von Lipidmetabolismus und Inflammationsantwort durchgeführt.

Die wesentlichsten Resultate dieser Strategie sind:

- die Publikation der wegweisenden Übersichtsarbeit zur metabolischen Aktivierung lipidlöslicher Vitamine als generelles (konserviertes) Prinzip
- der umfangreiche Beleg der Wirkung des vielgenutzten Peroxisom-Proliferator-aktivierte-Rezeptoren (PPAR)γ-Antagonist GW9662 als PPARδ-Aktivator in Makrophagen und dessen substantiellen Effekten auf den Lipidmetabolismus und damit Nachweis der Untauglichkeit von GW9662 für seinen angedachten Einsatzzweck in diesem Zelltyp

- die erste detaillierte Untersuchung zur adaptiven Antwort von Makrophagen auf die LCM und deren Effekt auf die LPS-Antwort, inklusive der Identifizierung des zentralen chemotaktischen Lockstoffs CC-Chemokinligand 2 (CCL2) als verlässlich unterdrückten Ziels der LCM im Zuge der Erstbeschreibung der Modulation der Zytokinsekretion durch LCM
- die Aufklärung der vorteilhaften Effekte der LCM im Kontext von Lipotoxizität und des Lipoproteinlipase-Systems, zweier Schlüsselmechanismen in der Pathogenese immunometabolischer Erkrankungen
- die Darstellung des (anti-entzündlichen) Potentials der LCM-ähnlichen Substanz δ-Garciniasäure im Rahmen einer umfassenden Übersichtsarbeit und einer experimentellen Arbeit

Die wesentlichen Beiträge dieser Dissertation zur Entwicklung des Forschungsfeldes umfassen somit das Nahelegen einer Stoßrichtung für die zukünftige LCM-Forschung in Bezug auf die physiologische Funktion als metabolisch aktivierte Form des Vitamin E durch mehrere Übersichtsarbeiten. Weiterhin erfolgte der Nachweis, dass GW9662 für die Untersuchung von PPARγ in Makrophagen untauglich ist, was die Neubewertung einiger Studien im Feld notwendig macht und die bessere Planung zukünftiger Studien ermöglicht. Zudem wurde die herausragende Bedeutung von PPARδ in Makrophagen verdeutlicht, die einen wesentlichen Einfluss auf die Untersuchung der Effekte von LCM in diesem Modellsystem hat und in Zukunft haben wird. Die erstmalige Charakterisierung der adaptiven Antwort von Makrophagen auf LCM im Kontext der Inflammation und die Identifizierung von CCL2 als wesentlichem molekularem Ziel der LCM bieten neue vielversprechende Ansatzpunkte für weitere Studien der anti-entzündlichen Effekte der LCM. Zudem vermitteln die LCM einen Schutz vor den schädlichen Effekten einer Fettüberladung und könnten damit einen positiven Effekt in entsprechenden Krankheitsbildern, die durch ungünstige Ernährungsweisen bedingt sind, bieten. Weiterhin wurde die LCM-ähnliche δ-Garciniasäure als interessante pharmakologische Leitsubstanz für zukünftige Forschungen im Kontext von Erkrankungen mit entzündlicher Komponente herausgestellt.

Diese Beiträge sind weitere Schritte auf dem langen Weg bis zum übergeordneten Ziel des unzweifelhaften Nachweises der LCM als physiologisch aktive Metabolite des Vitamin E. Um dieses Ziel zu erreichen, müssen zukünftig umfangreiche Anstrengungen unternommen werden. Diese umfassen weitere Bestrebungen zur Aufklärung molekularer Mechanismen der Regulation zellulärer Antworten durch die LCM, da diese unerlässlich sind, um die biologische Plausibilität von Vitamin E zugeschriebenen Wirkungen zu erklären und die LCM als zu Grunde liegenden Faktor nachzuweisen. Weiterhin müssen die Effekte und Eigenschaften der LCM auf Ebene des gesamten Organismus aufgeklärt werden, um die physiologische Bildung, den Transport und die Verteilung sowie die Pharmakokinetik zu verstehen und physiologische und möglicherweise pathophysiologische Konzentrationen zu definieren. Auf dieser Basis kann schlussendlich die Extrapolation des Wissens auf die Situation im Menschen erfolgen, da hier die LCM bereits im Blut nachgewiesen wurden und somit in leicht zugänglichen Geweben als Biomarker dienen könnten.

Die Grundsteine wurden gelegt, um in den zweiten 100 Jahren der Vitamin-E-Forschung ein neues, spannendes Kapitel aufzuschlagen. Auf diesen muss mit Sinn und Verstand aufgebaut werden, um in Zukunft die richtigen Fragen zu beantworten und die verbleibenden Rätsel des Vitamin E aufzuklären.

In order to provide a concise overview of the manuscripts included in this thesis, all manuscripts are listed starting with ‘Manuscript’ and the respective Roman numeral in the publication bibliography.

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B Declaration of originality

Eidesstattliche Erklärung

Hiermit erkläre ich an Eidesstatt, dass:

(i) mir die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist.

(ii) ich die vorliegende Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe und alle benutzten Hilfsmittel, persönliche Mitteilungen und Quellen in der Arbeit angegeben habe.

(iii) mich lediglich die in den Manuskripten angegebenen Personen bei der Auswahl und der Auswertung des Materials sowie bei der Erstellung der Manuskripte unterstützt haben.

(iv) ich nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen habe.

(v) Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

(vi) ich diese Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe.

(vii) ich weder die gleiche oder eine in wesentlichen Teilen ähnliche, noch eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, Januar 2021 _____

Martin Schubert

C Curriculum vitae

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D Publications

Original contributions:

Schubert, Martin; Becher, Stefanie; Wallert, Maria; Maeß, Marten B.; Abhari, Masoumeh; Rennert, Knut; Mosig, Alexander S.; Große, Silke; Heller, Regine; Grün, Michael; Lorkowski, Stefan (2020): The peroxisome proliferator-activated receptor (PPAR)-γ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662) triggers perilipin 2 expression via PPARδ and induces lipogenesis and triglyceride accumulation in human THP-1 macrophages. In *Molecular Pharmacology* 97 (3), pp. 212–225. DOI: 10.1124/mol.119.117887.

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F Appendix

Manuscript AI

Differential capability of metabolic substrates to promote hepatocellular lipid accumulation

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Contribution to the manuscript:

- Contribution to the writing of the manuscript (critical evaluation)
- Guidance on and supervision of certain immunoblots

Personal contribution to the manuscript: 5-10%

Essence of Manuscript AI:

One of the most widespread metabolic disorders and thus an important NCD in developed societies is non-alcoholic fatty liver disease. This disease pattern is characterized by the excessive storage of triglycerides in lipid droplets of liver cells. With this work, a model system based on hepatocytes for comprehension of the potential of various substances to induce lipid accumulation was established and characterized. Various metabolic substrates as well as the pro-inflammatory cytokine TNF were examined with respect to their capacity of hepatocellular lipid accumulation induction. The model was consequently proven to be suitable to estimate the steatogenic potential of several treatment regimens of excessive fat and sugar supply. Further, PLIN2 was characterized as a molecular marker of sustained lipid accumulation in hepatocytes. With this work, a model for examining key pathological processes of NAFLD, a prime example of a NCD with strong inflammatory and metabolic background was established. This model represents a solid basis for future investigations on hypothetical beneficial effects of the vitamin E long-chain metabolites in the context of the immunometabolic disease NAFLD.

Manuscript AII

In vitro digested nut oils attenuate the lipopolysaccharide-induced inflammatory response in macrophages

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Contribution to the manuscript:

- Investigations and methodology

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Essence of Manuscript AII:

The fatty acid composition of the fat of nuts is regarded as a determinant of the health-beneficial effects of nut consumption, especially in the context of inflammatory diseases. In this work, the fatty acid composition of *in vitro* digested hazelnuts, almonds, walnuts, macadamias and pistachios was characterized and the anti-inflammatory capacity of the respective oily nut extracts was examined. For this, the murine macrophage cell line RAW264.7 was used. All examined nut extracts were able to suppress the gene expression of *Ptgs2, Nos2, Tnf, Il1β* and *Il6* and the formation of nitric oxide in response to LPS. In conclusion, evidence is provided for the health-beneficial effects of digested nuts with respect to inflammatory diseases. This work further contributes to LCM research since the above-mentioned effects have also been found for the LCM in macrophages and thus point to a mutual or comparable underlying molecular mechanism allowing hypothesis generation in this regard. Further, LPS-stimulation of RAW264.7 was again confirmed in the frame of this work as a suitable model for investigating the inflammatory response of macrophages. Insights from this work will thus help in future investigations on the anti-inflammatory potential of the vitamin E LCM and underlying mechanisms.