Metabolism of Polyunsaturated Fatty Acids and their Oxylipins

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation von

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2019

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Tag der Promotion: 08.02.2019

Abstract

Background and aim: The beneficial health effects of omega (n)3 polyunsaturated fatty acids (PUFAs) have been mainly attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Many of the positive health effects are believed to be mediated via their oxidized metabolites called oxylipins. The intake of EPA and DHA is low in most countries due to low fish intake. Consequently, the omega-3 index, which is defined as relative weight percent of EPA+DHA in red blood cells (RBCs), is low. Furthermore, PUFAs are also subject to a complex metabolism that is not fully understood yet. However, there is consensus that human enzymes can synthesize EPA and DHA from their essential precursor PUFA alpha-linolenic acid (ALA). However, ALA intake and conversion rates have been shown to be quite low. It is currently being discussed that the high intake of the n6 PUFA linoleic acid (LA) might be one reason for low conversion rates. Furthermore, retroconversion of DHA to EPA has been shown to occur upon DHA supplementation. Methodologically, the investigation of the metabolism of PUFAs and their oxylipins in humans is challenging. Previous studies were limited by inhomogeneous study collectives, suboptimal designs and analyses solely based on relative fatty acid blood ratios (instead of absolute concentrations). Moreover, analytical-, inter- and intra-day fluctuations of oxylipin patterns are unknown. The aim of the present thesis was therefore to (i) investigate the metabolism of PUFAs and their oxylipins in human blood with homogeneous study collectives and under controlled conditions, using defined PUFA administrations and (ii) determine analytical-, inter- and intra-day variations of oxylipin patterns.

Methods: Four separate human studies were carried out. All studies were conducted with homogenous collectives (n=12-19) regarding dietary habits (especially fish and PUFA intake), sex, age, smoking status, BMI and EPA and DHA concentrations in blood. In two separate studies, the effects of a 12-week ALA and DHA supplementation, respectively, on PUFA and oxylipin patterns were investigated. Several intermediate timepoints (1, 3 and 6 weeks) allowed for the analysis of PUFA and oxylipin concentrations over the course of time. In a further two-week cross-over study, the effects of a low-LA/high-ALA (0.56:1) and a high-LA/low-ALA diet (25.6:1) on PUFA metabolism were compared. In all studies, PUFAs were quantified in RBCs, which have been shown to have the lowest variability compared to other blood sample types. The last study investigated analytical variance of oxylipin concentrations, the effect of standardized and non-standardized diet on fasting oxylipins and inter- as well as intra-day fluctuations of the oxylipin pattern.

Results: In all studies, the variability of PUFA and oxylipin data was significantly lower compared to previous studies. ALA supplementation increased ALA, EPA, DPAn3 and decreased DHA concentrations in RBCs, while direct DHA supplementation increased DHA as well as EPA concentrations. In both studies, changes in plasma oxylipin concentrations

generally reflected their precursor PUFAs in RBCs. The low-LA/high-ALA diet led to a faster increase of ALA and EPA concentrations compared to simple ALA supplementation while DPAn3 and DHA concentrations remained constant. Analytical variance of oxylipins was comparable to those of other LC-MS based oxylipin quantification methods and for 84 % of analytes between 5-20 %. Fasting plasma oxylipin samples were subject to minor fluctuations between different days irrespective from a standardization of the diet, while oxylipin concentrations during the day fluctuated largely.

Conclusion: Modifications of dietary PUFA intakes lead to shifts in the entire PUFA profile in RBCs. An ALA intake 10-fold higher than common in Germany does not lead to an increase of the omega-3 index confirming the low conversion of ALA to EPA and DHA observed in other studies. In contrast, a concomitant reduction of LA intake slightly increases the omega-3 index supporting the inhibitory effect of LA on ALA conversion. Supplementation of preformed DHA leads to a large increase of DHA, but also increases EPA possibly due to retroconversion of DHA to EPA. From a dietary point of view, supplementation of preformed DHA should be preferred over ALA supplementation to significantly increase the status of EPA and DHA. DHA and ALA supplementation causes a shift in the whole oxylipin pattern, which indicates a complex interplay between PUFA and oxylipin metabolism. Analytical variance of oxylipin concentrations of the method used here is low and fasting plasma seems to be suitable for the investigation of oxylipin biology.

Keywords: metabolism, polyunsaturated fatty acids, oxylipins

Zusammenfassung

Hintergrund und Ziel: Die gesundheitsfördernde Wirkung von mehrfach ungesättigten omega-3 Fett-säuren (n3 PUFAs) wird hauptsächlich der Eicosapentaensäure (EPA) und Docosahexaensäure (DHA) zugeschrieben. Es wird angenommen, dass n3 PUFAs viele ihrer Effekte über deren oxidierte Meta-boliten, die Oxylipine, vermitteln. Aufgrund des geringen Fischverzehrs ist die Zufuhr von EPA und DHA in den meisten Ländern zu gering. Entsprechend ist der Omega-3-Index (relativer Gewichtsanteil von EPA+DHA in roten Blutzellen (RBCs)) zu niedrig. Zudem unterliegen PUFAs einem noch nicht vollständig verstandenen komplexen Metabolismus. Bekannt ist, dass EPA und DHA aus ihrer essentiellen Vorläufer-Fettsäure alpha-Linolensäure (ALA) synthetisiert werden können. Die ALA-Zufuhr und -konversionsrate ist jedoch sehr gering. Aktuell wird diskutiert, ob die hohe Zufuhr der omega-6 (n6) PUFA Linolsäure (LA) ein Grund für die niedrige Konversionsrate sein könnte. Darüber ist eine Retrokonversion von DHA zu EPA beschrieben worden. Die Erforschung des Stoffwechsels von PUFAs und deren Oxylipinen am Menschen ist methodisch anspruchsvoll. Frühere Studien waren durch inhomogene Studienkollektive, suboptimale Studiendesigns sowie Analysen, die ausschließlich auf relativen Fettsäure-blutverhältnissen (anstelle von Konzentrationen) beruhten, limitiert. Analytische, inter- und intra-Tagesschwankungen der Oxylipine sind bisher auch kaum untersucht worden. Das Ziel der vorliegenden Arbeit war daher, (i) den Metabolismus von PUFAs und deren Oxylipinen im menschlichen Blut mit homogenen Studienkollektiven, unter kontrollierten Bedingungen und Verwendung definierter PUFA-Verabreichungen zu untersuchen und ii) analytische, inter- und intra-Tagesschwankungen von Oxylipinmustern bestimmen.

Methodik: Es wurden vier separate Humanstudien durchgeführt. Alle Studien wurden mit homogenen Kollektiven (n=12-19) hinsichtlich Ernährungsgewohnheiten (insbesondere Fisch- und PUFA-Aufnahme), Geschlecht, Alter, Raucherstatus, BMI und EPA- und DHA-Konzentrationen im Blut durchgeführt. In zwei Studien wurden jeweils die Effekte einer 12-wöchigen ALA- bzw. DHA-Supplementation auf PUFA- und Oxylipinmuster untersucht. Mehrere Zwischenzeitpunkte (nach 1, 3 und 6 Wochen) ermöglichten die Analyse von PUFA- und Oxylipin-Konzentrationen im Zeitverlauf. In einer weiteren zweiwöchigen Cross-Over-Studie wurden die Effekte einer Ernährung mit niedrigem LA/ALA- (0,56:1) bzw. hohem LA/ALA-Verhältnis (25,6:1) auf den PUFA-Metabolismus verglichen. In allen Studien wurden PUFAs in RBCs gemessen, welche im Vergleich zu anderen Blutprobentypen die geringste Variabilität aufweisen. Die letzte Studie untersuchte die analytische Varianz der Oxylipin-Konzentration, den Effekt einer standardisierten und nicht-standardisierten Ernährung auf im Nüchternzustand gemessene Oxylipin-Konzentrationen sowie inter- und intra-Tagesschwankungen des Oxylipinmusters.

Ergebnisse: In allen Studien war die Variabilität der PUFA- und Oxylipin-Daten im Vergleich zu früheren Studien signifikant niedriger. ALA-Supplementation führte zu einer Erhöhung der Konzentration an ALA, EPA und DPAn3 und einer Senkung der DHA-Konzentrationen in RBCs, während direkte DHA-Supplementation die DHA sowie auch EPA-Konzentrationen in RBCs erhöhte. In beiden Studien spiegelten Veränderungen der Plasma-Oxylipin-Konzentrationen im Allgemeinen ihre Vorläufer-PUFAs in RBCs wider. Die Ernährung mit niedrigem LA/ALA-Verhältnis führte im Vergleich zu einer einfachen ALA-Gabe zu einem schnelleren Anstieg der ALA- und EPA-Konzentrationen, während die Konzentrationen von DPAn3 und DHA konstant blieben. Die analytische Varianz der Oxylipine war vergleichbar mit denen anderer LC-MS-basierter Oxylipin-Quantifizierungsmethoden und lag bei 84 % der Analyten zwischen 5-20 %. Nüchtern-Plasma Oxylipin-Proben unterlagen im Vergleich verschiedener Tage geringen Schwankungen, unabhängig von einer Standardisierung der Ernährung, während die Oxylipin-Konzentrationen im Laufe eines einzelnen Tages stark schwankten.

Schlussfolgerung: Veränderungen der PUFA-Zufuhr in der Nahrung führen zu Verschiebungen des gesamten RBC-PUFA-Profils. Eine zehnfach höhere ALA-Zufuhr als in Deutschland üblich führt nicht zu einer Erhöhung des Omega-3-Indexes, was die in anderen Studien beobachtete niedrige Umwandlung von ALA zu EPA und DHA bestätigt. Im Gegensatz dazu erhöht eine gleichzeitige Reduktion der LA-Zufuhr den Omega-3-Index geringfügig, was die inhibitorische Wirkung von LA auf die ALA-Konversion bekräftigt. Die Supplementation von DHA führt zu einem hohen Anstieg von DHA, aber auch zu einer Erhöhung von EPA, möglicherweise aufgrund von Retrokonversion von DHA zu EPA. Aus ernährungsphysiologischer Sicht sollte die Supplementation von DHA gegenüber ALA bevorzugt werden, um den Status von EPA und DHA signifikant zu erhöhen. DHA- und ALA-Supplementation bewirkt eine Verschiebung des gesamten Oxylipinmusters, was auf ein komplexes Wechselspiel zwischen PUFA- und Oxylipin-Metabolismus hindeutet. Die analytische Varianz der Oxylipin-Konzentration der hier verwendeten Methodik ist gering und Nüchtern-Plasma scheint für die Untersuchung der Oxylipine geeignet zu sein.

Schlagworte: Metabolismus, mehrfach ungesättigte Fettsäuren, Oxylipine

Scientific publications derived from this thesis

Original research papers (peer-review)

I. **Greupner T***, Kutzner L*, Nolte F, Strangmann A, Kohrs H, Hahn A, Schebb NH, Schuchardt JP (2018): Effects of a 12-week high-α-linolenic acid intervention on EPA and DHA concentrations in red blood cells and plasma oxylipin pattern in subjects with a low EPA and DHA status. *Food Funct* 9(3), 1587-1600.

II. **Greupner T**, Kutzner L, Pagenkopf S, Kohrs H, Hahn A, Schebb NH, Schuchardt JP (2018): Effects of a low and a high dietary LA/ALA ratio on long-chain PUFA concentrations in red blood cells. *Food Funct* 9(9), 4742-4754.

III. Schuchardt JP, Ostermann AI, Stork L, Kutzner L, Kohrs H, **Greupner T**, Hahn A, Schebb NH (2016): Effects of docosahexaenoic acid supplementation on PUFA levels in red blood cells and plasma. *Prostaglandins Leukot Essent Fatty Acids* 115(12), 12-23.

IV. Schuchardt JP, Ostermann AI, Stork L, Fritzsch S, Kohrs H, **Greupner T**, Hahn A, Schebb NH (2017): Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood. *Prostaglandins Leukot Essent Fatty Acids* 121(6), 76-87.

V. Ostermann AI*, **Greupner T***, Kutzner L, Hartung NM, Hahn A, Schuchardt JP, Schebb NH (2018): Intra-individual variance of human plasma oxylipin pattern: Low inter-day variability in fasting blood samples versus high variability during the day. *Anal. Methods* 10(40), 4935-4944.

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Conference contributions

Posters

Greupner T, Kutzner L, Kohrs H, Pagenkopf S, Konrad T, Hahn A, Schebb NH, Schuchardt JP (2018): Effects of a high and a low dietary LA/ALA ratio on fatty acid concentrations in red blood cells, ISSFAL, Las Vegas, United States, 27.-31.05.2018.

Greupner T, Kutzner L, Nolte F, Kohrs H, Hahn A, Schebb NH, Schuchardt JP (2018): A high-ALA diet does not increase ΣEPA+DHA in red blood cells, ISSFAL, Las Vegas, United States, 27.-31.05.2018.

Greupner T, Pagenkopf S, Schebb NH, Hahn A, Schuchardt JP (2018): Einstellung eines definierten Fettsäuremusters in experimentellen Ernährungsstudien – eine Methodenevaluierung, Regionalverband Nord, Lebensmittelchemische Gesellschaft, Fachgruppe in der GDCh, Hannover, Arbeitstagung, 7.-8.03.2018.

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List of abbreviations

AA / ARA	Arachidonic acid
AdA	Adrenic acid
ALA	Alpha-linolenic acid
BMI	Body mass index
COX	Cyclooxygenase
cPL	Cytosolic phospholipase
CYP	Cytochrome P450 monooxygenases
DGE	German Nutrition Society (Deutsche Gesellschaft für Ernährung)
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DiHDPE	Dihydroxy docosapentaenoic acid
DiHETE	Dihydroxy eicosatetraenoic acid
DiHETrE	Dihydroxy eicosatrienoic acid
DiHODE	Dihydroxy octadecadienoic acid
DiHOME	Dihydroxy octadecenoic acid
DPAn3	Omega-3 docosapentaenoic acid
DPAn6	Omega-6 docosapentaenoic acid
D5D	Delta-5 desaturase
D6D	Delta-6 desaturase
EFSA	European Food Safety Authority
ELOVL	Elongation of very long chain fatty acids
en%	Percent of total energy
EPA	Eicosapentaenoic acid
EpDPE	Epoxy docosapentaenoic acid
EpETE	Epoxy eicosatetraenoic acid
EpETrE	Epoxy eicosatrienoic acid
EpODE	Epoxy octadecadienoic acid
EpOME	Epoxy octadecenoic acid
FA(s)	Fatty acid(s)
FADS	Fatty acid desaturases
FAME(s)	Fatty acid methyl ester(s)
GC-FID	Gas chromatography with flame ionization detection
GLA	Gamma-linolenic acid
GPCR	G-protein coupled receptor
HDHA	Hydroxy docosahexaenoic acid

HDL	High density lipoprotein
HEPE	Hydroxy eicosapentaenoic acid
HETE	Hydroxy eicosatetraenoic acid
HODE	Hydroxy octadecadienoic acid
HOTrE	Hydroxy octadecatrienoic acid
HUFA	Highly unsaturated fatty acids
HpDHA	Hydroperoxy docosahexaenoic acid
HpEPE	Hydroperoxy eicosapentaenoic acid
HpETE	Hydroperoxy arachidonic acid
iNOS	Inducible nitric oxide synthase
IoM	Institute of Medicine
IS	Internal standard
IsoP	Isoprostanes
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	Linoleic acid
LC	Long-chain
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LLOQ	Lower limit of quantification
LOX	Lipoxygenases
LT	Leukotrienes
Lx	Lipoxin
Mar	Maresin
MEFAs	CYP-generated mono-epoxides
MMPs	Matrix metalloproteinases
MUFA(s)	Monounsaturated fatty acid(s)
NFkB	Nuclear factor κ B
n.s.	Not significant
n	Omega
PD(s)	Protectins
PG(s)	Prostaglandin(s)
PPAR	Proliferator activated receptor
PUFA(s)	Polyunsaturated fatty acid(s)
QC	Quality control
RBC(s)	Red blood cell(s)
RvD	D series resolvins
SD	Standard deviation

SDA	Stearidonic acid
SE	Standard error
sEH	Soluble epoxide hydrolase
SFA(s)	Saturated fatty acid(s)
SNPs	Single nucleotide polymorphisms
TC	Total cholesterol
TG	Triglycerides
TLR(s)	Toll-like receptor(s)
Tx(s)	Thromboxane(s)
UL	Tolerable Upper Intake Level
U.S.	United States
wk(s)	Week(s)

1. Background

1.1. Definitions, properties and functions of polyunsaturated fatty acids

Fatty acids (FAs) consist of a hydrocarbon chain and a carboxylic acid group. FAs without double bonds in the hydrocarbon chain are saturated fatty acids (SFAs). Monounsaturated fatty acids (MUFAs) have one double bond, whereas polyunsaturated fatty acids (PUFAs) have two or more double bonds. Long-chain (LC) PUFAs are defined here as having more than 18 carbon atoms – other definitions might be used in different sources. Substituents with double bonds are attributed to either *cis* or *trans* isomer categories depending on their position in relation to a reference line. *Cis* configuration is the most common natural form of double bonds, whereas *trans* configuration can arise during food processing or by bacterial conversion of ruminants (e.g. conjugated linoleic acids). In most dietary fats, PUFAs contain up to four double bonds. Only marine sources like fish contain PUFAs with up to six double bonds. The position of the first double bond counted from the methyl end of the hydrocarbon chain, also called the omega (n)-end, is decisive about whether the PUFA (or MUFA) belongs to the n3, n6 (**Fig. 1**) or n9 family; the n9 FAs are not subject of this thesis.

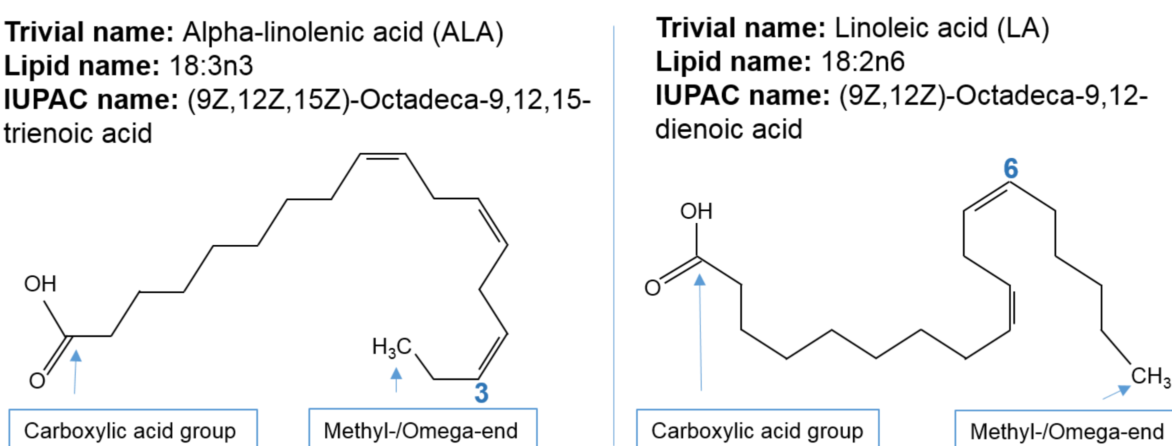

Fig. 1: Exemplary structural formula of an omega-3 (alpha-linolenic acid) and an omega-6 (linolenic acid) polyunsaturated fatty acid.

Like all chemical molecules, FAs are clearly named using the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC). Alternatively, commonly used trivial names and lipid names exist. The lipid name is given in the form X:Y(nZ), where the X stands for the number of C-atoms, Y for the number of double bonds and Z for the position of the first double bond relative to the n-end (**Fig. 1**).

Due to the double bonds in the molecules of PUFAs, these FAs have a variety of unique features regarding their chemical and physical properties. For example, the melting point of

PUFAs decreases with increasing number of double bonds. This causes the LC n3 PUFAs eicosapentaenoic acid (EPA; 20:5n3) with five double bonds and docosahexaenoic acid (DHA, 22:6n3) with six double bonds to have a very low melting point of -54 and -44°C, respectively. The resulting retention of the flexibility of biological structures is – especially for organisms that live in cold environments – indispensable for maintaining life processes.

Additionally, PUFAs have a higher reactivity compared to SFAs and thus, they are for example susceptible to oxidation, which occurs frequently during food processing, storage and cooking. In this case, oxidation goes in hand with negative effects on food quality by producing off-flavors and shortened shelf life. Oxidation of PUFAs also occurs after ingestion in the human metabolism and leads to the formation of eicosanoids and other oxylipins. These oxylipins are comprised of a large variety of lipid mediators with functions in the human body many of which are distinct and have already been described in detail [7] (see chapter 1.4). Besides formation of oxylipins, the main function of n3 and n6 PUFAs is located in cell membranes, wherein they are structurally and functionally integrated via phospholipids. They contribute to the membrane fluidity and permeability, activity of membrane-bound enzymes and receptors and signal transduction of membranes [8] (see chapter 1.6). Lastly, PUFAs also provide energy for the body as they are substrates for beta-oxidation [9,10].

1.2. Dietary PUFA sources, intake recommendations and supply situation

Sources

The essential PUFAs linoleic acid (LA; 18:2n6) and alpha-linolenic acid (ALA; 18:3n3) are mainly present in vegetable foods. Rich sources are nuts, seeds, like chia and linseeds and vegetable oils made thereof. LA is ubiquitous and large amounts can be found in all liquid vegetable fats; sunflower oil, for example, contains about 63 % of LA [11]. ALA is only present in a few rarely consumed plant oils, particularly linseed- (about 53 %) [11] and chia oil (50-57 %) [12] and in smaller quantities of 10-12 % also in rapeseed- and walnut oil. Of note, LA predominates also in the two latter named oils [11].

Sources that are rich in the LC n3 PUFAs EPA, n3-docosapentaenoic acid (DPAn3, 22:5n3) and DHA are primarily oily cold-water fish like salmon, tuna, herring and mackerel, fish oils and krill oils. High variability in EPA and DHA content within fish (oils) has been observed, which was indicated to be influenced by geographical and seasonal differences [13]. It is important to state that not all fish species contain LC n3 PUFA quantities as high as the abovementioned. For example, salmon provides about 2.1 g of LC n3 PUFAs per 100 g portion (0.6 g of EPA, 0.3 g of DPAn3 and 1.2 g of DHA), whereas cod contains only 0.35 g (0.1 g of EPA, 0.25 g of DHA and almost no DPAn3) [11]. Indeed, the LC n3 PUFAs are not synthesized

by fish – they are originally synthesized by microalgae and when fish ingest phytoplankton that consumed microalgae, they accumulate the LC n3 PUFAs in their tissues [14]. This is why EPA and DHA contents in fish depend on their feed and farmed fish stemming from aquaculture show more constant rates of LC n3 PUFAs compared to wild-caught fish [15]. One ingredient of the feed of farmed fish is fish oil which is increasingly often replaced by vegetable oils (e.g. rapeseed oil) leading to lower EPA and DHA contents in farmed fish [15].

The LC n6 PUFA arachidonic acid (AA; 20:4n6) is only found in animal fats and usually only in very small amounts, which is why the supply is about 200 times lower than that of the essential n6 PUFA LA [16]. Hence, LA is the main dietary n6 PUFA.

Recommendations

Several organizations have set up recommendations for an adequate intake of the essential PUFAs LA and ALA. Some organizations also established recommendations regarding the intake of EPA and DHA. The recommendations of a selection of organizations are shown in **Tab. 1**. They differ substantially regarding the recommended amount of LA and ALA, ranging from 2-10 en% and 0.5-1.2 en%, respectively. This corresponds to 5.2-26.2 g LA and 1.3-3.1 g of ALA per day assuming a daily energy intake of 10.2 MJ. The International Society for the Studies of Fatty acids and Lipids (ISSFAL) is the only organization stating that a healthy upper limit to the intake of LA might exist [17].

Tab. 1: PUFA intake recommendations for adults from a selection of organizations.

Country/Region	Organization	Recommendations			Ref.
		LA	ALA	EPA+DHA	
Global	ISSFAL	2 en%[a]	0.7 en%	500 mg/day	[17]
Europe	EFSA	4 en%	0.5 en%	250 mg/day[b]	[18]
United States	IoM	5-10 en%	0.6-1.2 en%	not established	[19]
Germany	DGE	2.5 en%	0.5 en%	not established[c]	[20]

Abbreviations: DGE, German Nutrition Society (Deutsche Gesellschaft für Ernährung); EFSA, European Food Safety Authority; en%, percent of total energy; IoM, Institute of Medicine; ISSFAL, International Society for the Studies of Fatty acids and Lipids.

[a] A note that “there may be a healthy upper limit to the intake of LA” [17] was added to the recommendation.

[b] pregnant and breastfeeding women: 100-200 mg DHA/day in addition

[c] pregnant and breastfeeding women: 200 mg of DHA/day

In contrast to the Institute of Medicine (IoM) and the German Nutrition Society (DGE; Deutsche Gesellschaft für Ernährung), the ISSFAL and European Food Safety Authority (EFSA) give recommendations for healthy adults with respect to the intake of EPA and DHA, not just of ALA

and LA. The ISSFAL recommendation is with 500 mg per day of EPA plus DHA twice as high as the recommendation of the EFSA. Neither the EFSA nor IoM established a Tolerable Upper Intake Level (UL) for EPA and DHA, because no adverse effects have been observed for various intake levels [19,21].

Supply situation

The supply situation of a nutrient can generally be assessed by two means: The first is to estimate the dietary intake via nutrition surveys like food frequency questionnaires or dietary protocols. The second way is to determine validated blood parameters.

Estimation of dietary nutrient intakes from dietary protocols has several limitations. One of them is that they rely on self-reported data and are potentially biased by food choice, memory of the subjects, estimation of portion sizes and incomplete reporting [22]. In addition, numerous absorption-inhibiting and -promoting dietary factors affect the bioavailability of nutrients, which are not considered in the evaluation of nutritional protocols [23]. Also methodological limitations associated with inaccurate FA composition in food databases are leading to potentially incorrect estimates of dietary intakes [24]. The inaccuracy of databases regarding the LA and ALA content was shown in one of the projects associated with this thesis for the estimation of the FA intake via the evaluation of nutritional protocols. The nutrition software PRODI® was used in comparison to the additional determination of the FA content of (a majority of) the consumed foods: there were significant differences regarding LA and ALA intake between the sole evaluation using PRODI® and the combination with FA analysis of meals [25]. This shows that there is an urgent need for improved food composition databases especially with regard to FA content. In particular, in intervention studies with modification of LA (and ALA) intakes, precise documentation of the FA intake is challenging since LA is ubiquitous in the Western diet. Although the weighed dietary protocol is regarded as the gold standard in assessing dietary intakes in humans [26] most dietary intervention studies with LA and ALA did not use it or applied it only for part of the intervention period (reviewed in [27]).

Keeping in mind the potential limitations of studies that assessed the FA intake based on dietary protocols, they are a relevant source for the assessment of the supply situation. The dietary intake of n6 and n3 PUFAs was recently investigated using 266 country-specific nutrition surveys [1]. It has been shown that seafood- (EPA, DPAn3, DHA) and plant (mainly ALA) n3 PUFA intake is too low around the world while n6 PUFA (mainly LA) intake is especially high in Western countries (**Fig. 2**) leading to high LA/ALA ratios that are

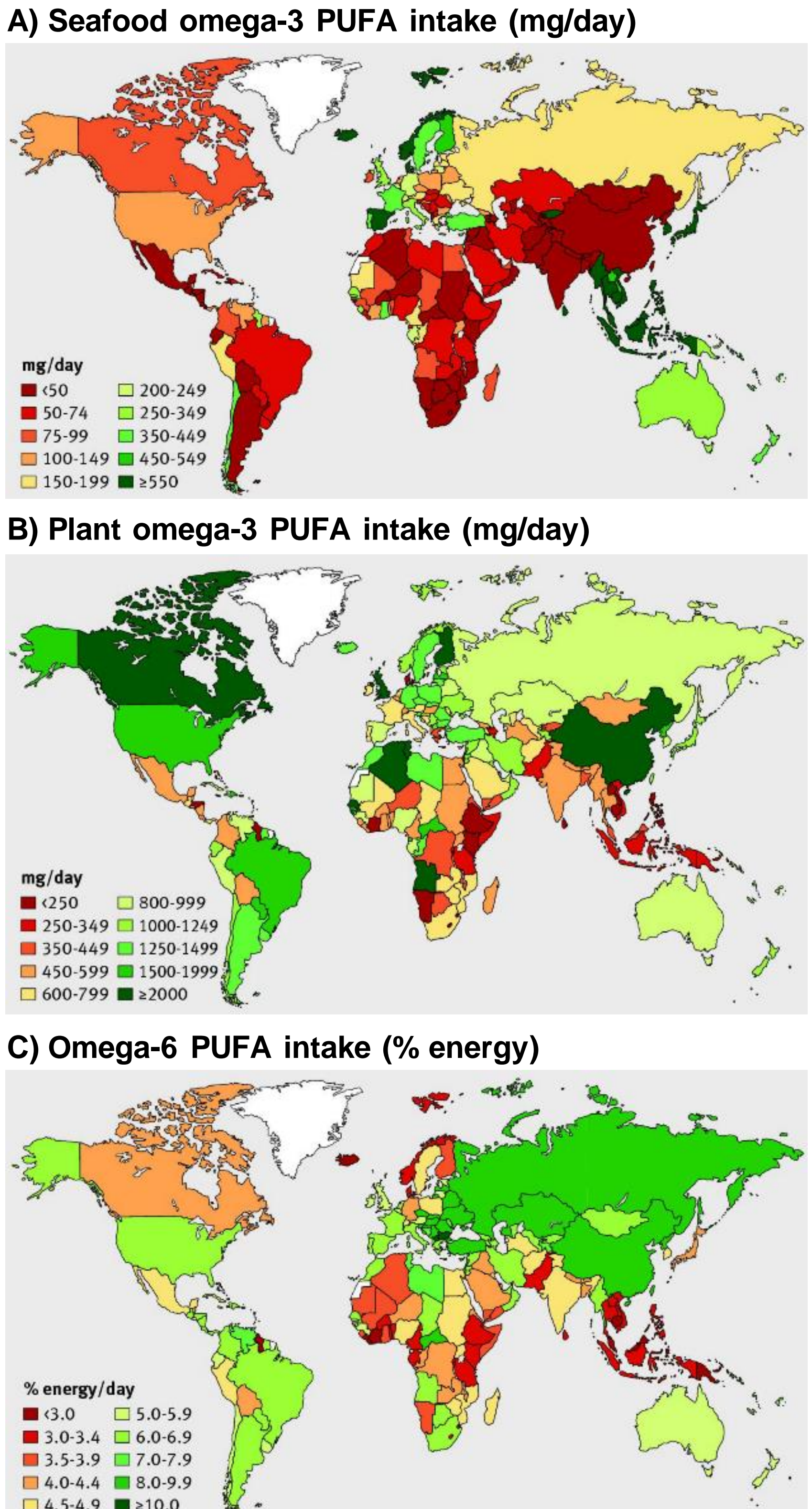

Fig. 2: Global and regional mean intake of (A) seafood and (B) plant omega-3 PUFAs and (C) omega-6 PUFAs in 2010 for adults aged ≥20 years according to [1].

Abbreviations: PUFAs, polyunsaturated fatty acids.

considered unfavorable [28–30]. In 142 countries, including nearly 80 % of the world's population, mean seafood n3 PUFA intake was below 250 mg per day. Therefore, the large majority does not meet the recommendations of the EFSA. The same holds for the recommendations of the ISSFAL since they are even higher (500 mg per day). Only in a few countries, seafood n3 PUFA intakes were above 550 mg per day among them the Pacific island nations, Spain, Iceland, Norway, Denmark, South Korea and Japan. The supply situation for plant n3 PUFAs (ALA) was slightly better than for seafood n3 PUFAs [1]. Mean plant n3 PUFA intake in Western Europe and the United States (U.S.) was 1120 mg and 1527 mg per day, respectively. Worldwide intake was on average 1371 mg per day indicating that about half of the world's population does not reach the recommendations of 1.3-3.1 g of ALA per day [1]. Intake of n6 PUFAs was on worldwide average 5.9 en%; in Western Europe 5.2 en% and in the U.S. 6.7 en% underlining the abovementioned oversupply especially in comparison to ALA intake. The intake of LA has increased substantially in many countries during the last century [31,32] due to the advice that originated in the 1960s: vegetable oils rich in PUFAs (but mainly LA) should be replaced by animal fats rich in SFAs [33]. Consequently, the dietary ratio of the n6 PUFA LA to the n3 PUFA ALA is in Western countries about 10-20:1 [34–36].

The second way to assess the supply situation of a nutrient is the determination of validated blood parameters. This way is more reliable compared to dietary protocol-based assessments since it overcomes all their abovementioned limitations. However, the lack of a gold standard for the measurement of FAs in human blood makes it difficult to compare the results of different studies with each other. The omega-3 index was proposed as a unit to assess the blood status of EPA and DHA. It is inversely associated with the risk for fatal coronary heart disease (CHD) and was proposed by Harris and von Schacky in 2004 [37]. The omega-3 index is calculated by the sum of EPA plus DHA in red blood cells (RBCs) expressed as a percentage of total RBC FAs [37,38]. Its classification uses the categories very low (≤4 % of EPA+DHA in RBCs), low (>4-6 %), moderate (>6-8 %) and high (>8 %) [37].

In a recent systematic review, EPA and DHA amounts in the blood stream were compared for different countries across the world (**Fig. 3**) [2]. However, the authors included not only data from EPA and DHA in RBCs, but also from whole blood, whole plasma and plasma phospholipids or cholesteryl esters and extrapolated RBC levels. Therefore, parts of the data are only an approximation and should be interpreted with caution, as only analysis based on RBCs best reflects long-term dietary fat intake [39]. The (partly extrapolated) omega-3 index can be predominantly categorized as 'very low' and 'low' (≤4-6 %) in Western countries like the U.S., Canada, Western Europe and Australia. Regions with high omega-3 index (>8 % of EPA+DHA in RBCs) include countries with traditionally high fish intake like Japan, Greenland

and Norway [2]. These results are basically in line with the seafood intake data shown above [1].

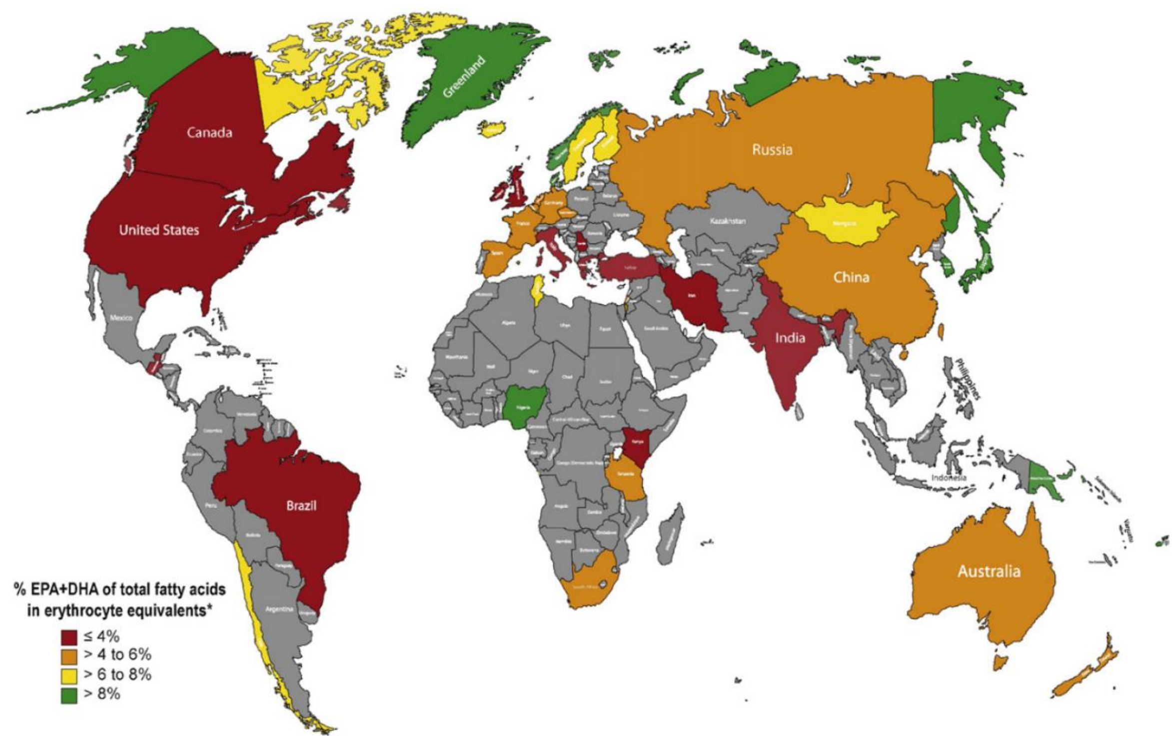

Fig. 3: Map of global blood levels of EPA+DHA in percent of total fatty acids according to [2]. Grey color code: No data available.

A few cohort studies measured the omega-3 index with the patent-pending HS-Omega-3 Index® methodology of Harris and von Schacky as described [37] (**Tab. 2**). Only these data can be directly compared with one another and indicate a slightly better supply situation of the U.S. population (4.5-5.6 %) than Stark et al. [2] extrapolated (≤4 %). The same indication holds for Italy, Spain and Canada. However, it is still in the low range for Western countries like U.S., Canada, Italy and Germany.

To conclude, even though reliable worldwide data are missing, the existing data indicate uniformly that the supply situation with EPA and DHA of the vast majority of the world population is far away from the consensus range of a potential optimum.

Tab. 2: Omega-3 index measured by HS-Omega-3 Index methodology of adult cohorts from different countries.

Country	n	Males %	Mean/median Omega-3 index %	Data source	Ref.
United States	8659	0	4.7	Women's Health Initiative Memory Study cohort[1]	[40]
United States	3196	45	5.6	Framingham Gen3 cohort[1]	[41]
Italy	461	77	4.8	Selected participants from GISSI-HF cohort[1]	[42]
Spain	198	52	7.1	Selected participants from PREDIMED trial[1]	[43]
Canada	1984	50	4.5	National Survey	[44]
Germany	446	0	5.5	VitaMinFemin Study	[45]
United States	86012	45	4.5	Clinical Lab of W.S. Harris	[46]

Abbreviations: n, number of subjects.
[1] baseline data

1.3. Metabolism of PUFAs

Humans and other mammals are not able to synthesize the parent PUFAs LA and ALA because they lack the specific desaturases, namely delta-12 and delta-15 desaturase. LA and ALA are therefore essential and must be obtained through the diet. Simultaneously, a conversion of n3 to n6 PUFAs is not possible, since the introduction of new double bonds by mammal desaturases is only possible between already existing double bonds and the carboxylic acid group. Therefore, humans are only able to form the n6 PUFA AA from the parent n6 PUFA LA, while the n3 PUFAs EPA and DHA can only be synthesized from the parent n3 PUFA ALA. Unlike humans, plants have the enzymes delta-12 and delta-15 desaturase and are therefore able to form LA and ALA and interconvert between n3 and n6 PUFAs. Humans and other mammals have all enzymes that are necessary to synthesize longer-chain and higher unsaturated PUFAs from their precursors – the n6 PUFA LA and the n3 PUFA ALA. The conversion of LA and ALA to their respective LC derivatives is a multistage desaturation and chain-elongation process [47–49] (**Fig. 4**), which is – except for the final step – located in the endoplasmic reticulum.

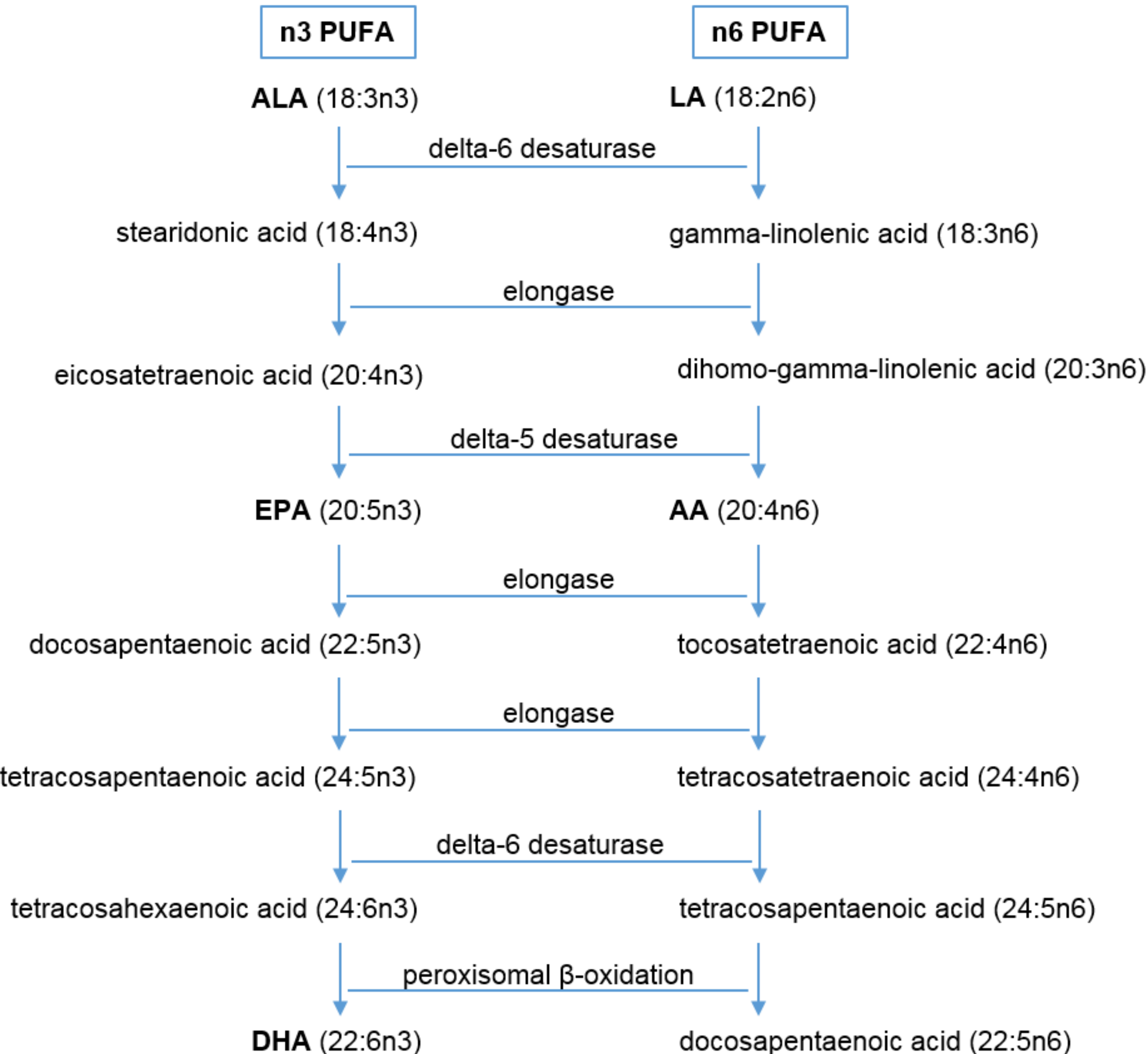

Fig. 4: Synthesis of long-chain omega-3 and omega-6 polyunsaturated fatty acids from their precursor fatty acids alpha-linolenic acid and linoleic acid modified from [3].

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; n3, omega-3; n6, omega-6; PUFA, polyunsaturated fatty acid.

All enzymes that are necessary for the conversion are shared between the n3 and n6 PUFAs. In the first step, the precursor PUFAs LA and ALA are further desaturated by the action of delta-6 desaturase (D6D), which means that another double bond is introduced into the molecule. This results in the formation of stearidonic acid (SDA, 18:4n3) on the n3 side and gamma-linolenic acid (GLA, 18:3n6) on the n6 side. By elongation with a C-2 unit catalyzed by an elongase, the n3 PUFA eicosatetraenoic acid (20:4n3) and the n6 PUFA dihomo-gamma-linolenic acid (DGLA; 20:3n6) are formed. Insertion of another double bond by delta-5 desaturase (D5D) leads to the formation of the n3 PUFA EPA (20:5n3) and the n6 PUFA AA.

Two further elongation steps lead to the formation of DPAn3 and tetracosapentaenoic acid (24:5n3) on the n3 side and docosatetraenoic acid (22:4n6) and tetracosatetraenoic acid (24:4n6) on the n6 side. Another double bond, catalyzed by D6D, is introduced into the molecule, producing the FAs tetracosahexaenoic acid (24:6n3) and tetracosapentaenoic acid (24:5n6). The consequent peroxisomal beta-oxidation, i.e. cleavage of a C-2 unit, leads to the formation of DHA (22:6n3) and n6-docosapentaenoic acid (DPAn6, 22:5n6). Only the last step of the conversion is located in the peroxisome.

Several human studies have been shown that EPA increases upon DHA supplementation [50–54], which can metabolically result from either increased synthesis from other n3 PUFAs or decreased degradation of EPA. Nonetheless, the result of both potential causes is commonly referred to as (apparent) retroconversion from DHA to EPA [54]. However, as the term might suggest, retroconversion is not a reversal of the conversion pathway. Unlike conversion, retroconversion takes place in mitochondria and/or peroxisomes and is mediated by different enzymes [55]. From DHA to EPA, retroconversion appears to proceed via β-oxidation and requires the participation of specific enzymes (Δ3,Δ2-enoyl-CoA isomerase, Δ3,5,Δ2,4-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase) to remove the double bond [56–59]. The retroconversion rate of DHA to EPA has been suggested to be between 7-14 % [51,60,61]. However, these studies determined only relative weight percentages of total FAs, which might lead to calculation of falsified retroconversion rates because changes of EPA and DHA cannot be evaluated independent from one another. The calculation of retroconversion rates based on concentrations would be more accurate.

Several **influencing factors** have been shown to alter the PUFA metabolism and/or PUFA profile. As n3 and n6 PUFAs compete for the same enzymes, **LA is discussed to act as a competitive inhibitor of ALA** metabolism and vice versa [28]. The efficiency of the conversion process is with 5-8 % from ALA to EPA and 0.5-5 % from EPA to DHA generally very low in adult humans on a Western diet [29,47,62,63]. The rate limiting enzyme in the conversion process is the D6D [64,65], which is utilized twice: It converts LA to GLA, ALA to SDA and with lower affinity than the first conversion step in both reaction chains also tetracosatetraenoic acid (24:4n6) to tetracosapentanoic acid (24:5n6) and tetracosapentaenoic acid (24:5n3) to tetracosahexaenoic acid (24:6n3) (**Fig. 4**). The affinity of the D6D is 2-3-times higher for the n3 FA ALA than for the n6 FA LA [66–69], but LA is more abundant in the Western diet compared to ALA. Due to the high dietary intake of the n6 FA LA compared to the n3 FA ALA, the higher D6D affinity to ALA is overcompensated by its lower relative concentration within the body. The competition for conversion enzymes between the n3 and n6 PUFAs might be one possible explanation for the inefficient conversion of the n3 PUFA ALA to EPA and especially DHA [27,30,47,53].

Further individual influencing factors are diet, age, sex, body weight and body mass index (BMI), genetics and smoking status. Thus, the FA profile is subject to high biological variability, which impedes the interpretation of biological data.

Diet has been shown to be the most important determinant of EPA and DHA status (i.e. omega-3 index) [43,70–72]. The number of fish servings [44,70,73] and the intake of (supplemental or dietary) EPA and DHA [41,43,70,72] were positively associated with blood levels of EPA and DHA. In one study, about half of the variability could be explained by the diet (fish intake frequency and/or EPA and DHA supplementation) [70], whereas in another study with a population of increased risk for CHD only 12 % of the variability could be explained by EPA and DHA intake [43]. Moreover, the dietary amount and ratio of the competing precursor PUFAs LA and ALA have been shown to have an influence on the LC n3 PUFA status [27].

Age has been shown to influence the PUFA profile [41,45,54,70–73]. With increasing age higher amounts of EPA and DHA in phospholipids [71] and RBCs [70,72] have been observed. A higher retroconversion rate from DHA to EPA has been observed among old (mean age: 77 years) compared to young (mean age: 27 years) individuals [54].

Women tend to have a higher omega-3 index compared to men [41,44,72,74] with higher DHA RBC amounts regardless of dietary intake [52,71,75–78], but lower EPA amounts [71]. The **sex** differences in EPA and DHA were similar at all ages [71]. The reason for sex differences may be a greater capacity for ALA conversion into LC n3 PUFAs in women compared to men [74,79,80]. The conversion rate of ALA to DHA in women amounts to 9 % and may be higher compared to men partly due to a lower rate of beta-oxidation of ALA [81] and the influence of estrogen and other sex hormones on PUFA metabolism [74,82], more precisely D6D [81]. The authors hypothesized that higher DHA amounts are of particular importance in maintaining adequate provision in pregnancy [81]. Moreover, hormonal contraceptives have been shown in women [83,84] and rats [85] to enhance the conversion rate of ALA to DHA, which underlines the effect of estrogen on the desaturation and elongation pathway of ALA [79]. Furthermore, a lower retroconversion rate of DHA to EPA has been observed among post-menopausal women receiving hormone replacement therapy compared to post-menopausal women receiving no hormones [52].

Body weight and **BMI** have been shown to be inversely associated with EPA and DHA concentrations [44,72,73]. In other words, lower levels were observed in obese subjects compared to subjects of normal weight [44,73] independent of reported frequency of fish intake [73]. Individuals with lower body weight tend to have a greater response to a given EPA and DHA intake compared to individuals with higher body weight [72].

Genetic variants in PUFA metabolizing enzymes have been linked to altered PUFA profiles [86–93]. Thus, for example single nucleotide polymorphisms (SNPs) of fatty acid desaturases (FADS) 1, FADS2, FADS3 and elongation of very long chain fatty acids (ELOVL)2 genes have been linked to slightly increased levels of EPA and DHA [91–93], whereas other SNPs of FADS genes were associated with higher LA and ALA and slightly lower LC n3 PUFA concentrations [90].

The majority of studies suggest that **smoking** has an effect on LC n3 PUFA levels [41,44,45,70,94,95], whereas only one study found no effect of smoking [73]. All studies that observed an effect of smoking found lower amounts of EPA and DHA in smokers compared to non-smokers [41,44,45,70,94].

Other factors that have been shown to be associated with the PUFA profile are **physical exercise** [72], **type 2 diabetes** [73] and **education** [41].

Analytics and **pre-analytics**, i.e. the storage temperature of the samples has been shown to have an influence on the FA content in whole blood [96] and RBCs [40]. High degradation of EPA and DHA occurs predominantly at -20°C compared to -75 and room temperature [96]. Ideal storage temperature is indicated to be -80°C [40].

Moreover, it is important to carefully select the **sample type** in blood in which the FA profile should be measured. The most commonly used blood sample type for the investigation of PUFA status are RBCs, plasma, and plasma phospholipids [97]. Plasma and plasma phospholipid concentrations show higher biological variability compared to RBCs and plasma concentrations are in comparison to RBCs and plasma phospholipids altered by meal consumption [97]. Thus, RBCs are the blood sample type with the lowest variability and no influence of fasting or fed state on its FA profile [97]. Further advantages of determining the FA profile in RBCs are the reflection of tissue/organ FA composition and high pre-analytical stability [98–102]. However, the determination of RBC FA composition does not necessarily allow to draw conclusions about the profile in all organs whose determination in humans is not possible for ethical reasons. For example, DHA amounts in RBCs range from 1.87 to 8.3 % [46], whereas the brain DHA content is with 40 % of total FAs much higher [103]. As the analysis of RBC FAs reflects longer-term intakes (~ previous 120 days) [39], Harris and von Schacky suggest the RBC content of EPA and DHA (i.e. omega-3 index) as sample type for the assessment of the n3 PUFA status [37,38]. Although RBCs were identified as the potential standard of the future, a widespread shift to RBC FA analysis has not occurred.

Previous (intervention) studies on the effect of dietary PUFA modification on PUFA profiles in blood had issues controlling variability stemming from mostly several of the abovementioned influencing factors. They either did not use RBCs as blood sample type to evaluate changes

in PUFA concentrations [104–109], had no homogeneous study collective with regards to, age [110–114], sex [105,106,110–118], basal status of EPA and DHA [110,114,115], weight or BMI [107,114] and smoking status [110], or used suboptimal storage temperatures [119]. Most studies had no (appropriate) run-in and/or wash-out phase [106,107,109,110,112,113, 115,116,120–123]. Further, the majority of studies analyzed solely relative weight percent of total FAs and did not determine concentrations of FAs.

All those downsides sum up to a high variability and potentially misleading data interpretation. They also do not allow separating different influencing factors and their effects from each other. Therefore, factors that could compromise data in a way that interferes with the study goal at hand need to be minimized when planning a study on the effects of an intervention on the PUFA profile: Blood sample type needs to match the study design, appropriate run-in and wash-out phases should be included and the study collective should be homogenous with regard to the abovementioned influencing factors of the PUFA profile.

1.4. Metabolism of oxylipins

PUFA effects are mainly mediated via their oxidized metabolites – called oxylipins [7]; even though also other PUFA-derived lipid mediators like endocannabinoids have been identified that contribute to the physiological effects of LC n3 PUFAs [124].

Oxylipins are formed endogenously from a number of PUFA precursors, such as the n6 PUFAs AA, LA, GLA, DGLA and adrenic acid (AdA; 22:4n6), and the n3 PUFAs ALA, SDA, EPA and DHA [7]. The most well-known oxylipins are the eicosanoids formed from the n6 PUFA AA [125]. In a variety of enzymatic and non-enzymatic reactions a plethora of different oxylipins are produced, some of which can be formed through more than one pathway [126]. Enzymatically, PUFAs are converted to oxylipins by three main pathways involving cyclooxygenases (COX), lipoxygenases (LOX) [127] and cytochrome P450 monooxygenases (CYP) [128] (**Fig. 5**). Non-enzymatical conversion happens via autoxidation [129]. COX enzymes (COX-1 and COX-2) convert PUFAs into prostanoids, i.e. prostaglandins (PGs) like PGE_2 and thromboxanes (Txs) like TxA_2 [130–132]. LOX (5-LOX, 12-LOX and 15-LOX) catalyze the formation of hydroperoxy-PUFAs which can either be reduced to hydroxy-PUFAs or further metabolized, e.g. by LOX, to multiply hydroxylated PUFAs [133]. Multiple hydroxylation of EPA, DHA and AA leads to potent inflammation resolving molecules, called specialized pro-resolving mediators (SPM), like lipoxins, resolvins, proctectins and maresins [133]. CYP enzymes (e.g., CYP4 and CYP2 family) can act as epoxygenases or ω-hydroxylases resulting mostly in epoxy-PUFAs or terminally (ω and ω-n) hydroxylated PUFAs

[7,134]. Epoxy-PUFAs of CYP action are rapidly metabolized by sEH [135] into dihydroxy-PUFAs which have a lower biological activity [136].

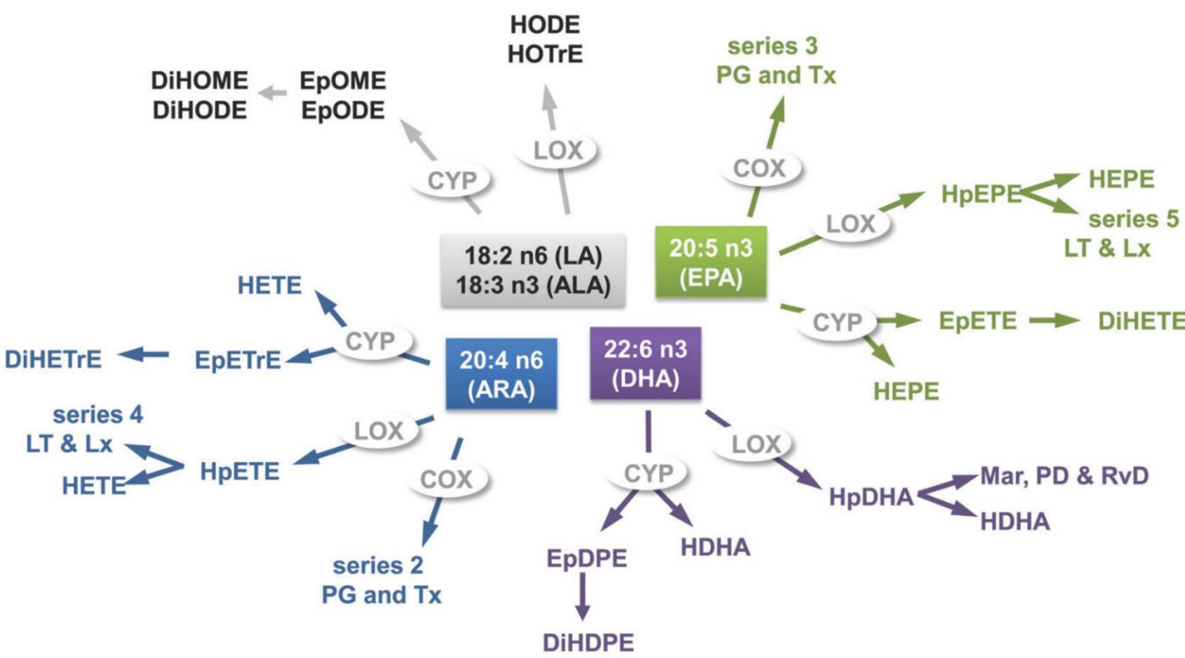

Fig. 5: Enzymatic oxylipin formation: overview of selected CYP, LOX and COX pathways of LA, ALA, ARA, EPA and DHA in the ARA cascade according to [4].

Abbreviations: ALA, alpha-linolenic acid; ARA, arachidonic acid; COX, cyclooxygenases; CYP, cytochrome P450 monooxygenases; LOX, lipoxygenases.

Oxylipins can be formed by autoxidation of PUFAs giving rise to e.g. hydro(pero)xy-PUFAs or PG-like structures such as isoprostanes [137,138]. The hydroxy-PUFA 15-HETE is one example for overlapping formation routes, as it can either be generated by 15-LOX, by COX enzymes or by autoxidation. Overlapping formation routes lead to molecules with differences in stereochemistry [139]. For several oxylipins formation routes have not yet been discovered, e.g. that of 18-HEPE which is the major metabolite in several cultured cells that were incubated with EPA [140].

The major amount of circulating oxylipins – especially epoxy- and hydroxy-PUFAs – is esterified to phospholipids [141,142]. However, the functions of esterified oxylipins in (patho)physiology are largely unknown [7]. Esterified and free oxylipins both have been shown to be biologically active, with the latter being indicated as the more active form [143]. Thus, the task of analyzing free vs. esterified oxylipins is important for the interpretation of the results in a biological context. Liberation of oxylipins from their esterified form during analysis is often achieved using base hydrolysis, leading to a sum parameter of free and esterified oxylipins

called total oxylipins [126]. Indeed, similar trends were observed for free and total oxylipin patterns when comparing relative changes induced by dietary n3 PUFA supplementation [141].

Oxylipin formation is dependent on the amount of dietary PUFAs including competing PUFAs for conversion enzymes and release from membrane phospholipids [7]. Therefore, most factors that have been shown to influence the PUFA profile may also impact the oxylipin pattern, either directly or indirectly via an influence on PUFA metabolism. In addition, a direct relationship to the oxylipin pattern was found for some factors: **Diet** has been shown to modulate the pattern of oxylipins [144–146]. **Age** has been shown to be an influencing factor of the oxylipin profile [147,148]. In older individuals the concentration of 5-LOX products (5-HETE, 5-HEPE, 7-HDHA, 20-HETE) has been shown to be elevated compared to younger individuals [147]. Moreover, **genetic variants** in PUFA metabolizing enzymes have been linked to altered oxylipin profiles [86,149]. Other factors that have been shown to be associated with the oxylipin profile are **physical exercise** [150], **drugs** [151] and **health status** [152–154]. Also (**pre-)analytics**, i.e. the time between blood collection and plasma preparation, is a factor that is known to have an influence on the oxylipin concentration in the sample [126]. In previous studies, variations of oxylipin concentrations were high [141,155–157] probably due to inhomogeneous study collectives with respect to the abovementioned influencing factors. Thus, a homogeneous study collective regarding influencing factors of both PUFAs and oxylipins is essential in order to generate data with low variability.

In the blood of humans the oxylipin profile is mostly determined in plasma [4]. However, plasma oxylipin concentrations vary substantially in the postprandial state based on an individual's most recent meal, so they do not reflect long-term dietary consumption [158]. Data about the extent of variation following (standardized) meal ingestion are sparse [146,158,159]. Moreover, blood for oxylipin analyses is usually collected in the morning following overnight fasting [144,160–162] and so far, no data exist on the extent of inter-day variation of oxylipin patterns in fasting plasma samples. Lastly, data about precision of the respective analytical method are crucial in order to evaluate if changes of oxylipin concentrations are within the analytical variation or interpretable biological effects.

1.5. Omega-3 PUFAs in health and disease

Most research on the effects of PUFAs on health outcomes was conducted on the LC n3 PUFAs EPA and DHA whose interest increased rapidly in the 1970s. At this time the Danish researchers Bang and Dyerberg associated the consumption of large amounts of fish and marine mammals rich in EPA and DHA among Greenland Eskimos with a lower incidence of cardiovascular disease (CVD) compared to the Danish population [163–165] and

demonstrated blood-thinning effects of LC n3 PUFAs [165]. Since then, beneficial effects of dietary LC n3 PUFAs have been found for a variety of pathological conditions. First and foremost are protective and risk reducing effects that were observed for overall CVD [41,166–170] and mortality thereof [168,171,172], (fatal) CHD [173,174], sudden cardiac death [168,174–176] and early- [177] and total mortality [168,171,172,175,178,179]. This applies both to the primary prevention of healthy subjects without risk factors [166,170] and subjects with risk factors [169] as well as to secondary prevention [167,168,170,172]. Coherently, low levels of EPA and DHA in RBCs, i.e. omega-3 index, have been associated to an increased risk of death from CVD [37]. This becomes particularly clear in an observational study in which the rates of cardiac death were compared between the Japanese and Italian population, which stand exemplary for a good and bad supply situation with EPA and DHA, respectively. In the Japanese, cardiac death rates were very low at 2.5 per 1000 person years, as compared to 17 cardiac deaths per 1000 person years in the Italian population [169].

Additionally, LC n3 PUFAs have been shown to be protective against CVD- and CHD associated risk factors like atherosclerosis and thrombosis [173,180], arrhythmia [181], elevated blood pressure [182,183], elevated triglyceride (TG) levels [148,184–187] and low high density lipoprotein (HDL) cholesterol levels [186]. This underlines the plausibility of the observed CVD and CHD protective effects of EPA and DHA. Overall, DHA seems to be more effective than EPA in reducing risk factors for CVD [188,189]. On the contrary, ALA supplementation has neither been shown to affect CVD risk factors [190] nor CVD outcomes [170], whereas LA substitution in place of SFAs led to increased rates of death from all causes, CHD and CVD in a cohort study [191].

While epidemiological studies such as the above-cited found predominantly protective effects of LC n3 PUFAs, some large clinical trials and meta-analyses thereof found no effect [192–197]. Reasons for lacking effects of LC n3 PUFAs on CVD outcomes of clinical studies may be an insufficiently high dose (of at least 500 mg of DHA per day [198]) and inclusion of participants with already high LC n3 PUFA intake respectively status [129,198,199]. Some of these studies did not even screen for (low) EPA and DHA status [193,195].

In addition to CVD – which also has an inflammatory component – it has been shown that EPA and DHA contribute to a reduction of inflammatory conditions [5,200,201]. Here again, DHA is discussed as being more effective than EPA in modulating specific markers of inflammation [186]. However, results regarding diseases with large inflammatory components are conflicting, e.g. in intervention studies on the effectivity of n3 PUFAs on the relapse in Crohn´s disease one study showed a significantly lower rate of relapse over 12 months [202], whereas two studies with similar design could not reproduce this finding [203].

Moreover, EPA and DHA have been shown to be cancer preventive, inhibit cancer growth and improve the outcome of cancer treatments – mainly for colorectal-, breast-, and prostatic cancer [204–207]. Epoxy metabolites of DHA have been shown to act antiangiogenic and anticancerogenic [208]. Conversely, a high intake of the n6 PUFA LA (>17.4 g per day) and a genetic variant (genotype AA) in the 5-LOX-activating Protein Gene was associated with a 80 % higher breast cancer risk compared to women with genotypes of AG or GG and same LA intake, whereas in women consuming less than 17.4 g of LA per day the same genotype (AA) was not associated with increased breast cancer risk [209].

In addition, a low omega-3 index has been associated with neurodegeneration and cognitive impairment [205,210–217]. Further research areas with potential benefits of LC n3 PUFAs are cellular and telomeric aging [218], attention deficit hyperactivity disorder [219], visual and neurological development [220] and (postpartum) depression [221–224].

1.6. Mechanisms of action of PUFAs

The molecular mechanisms of how PUFAs in general elicit their physiological effects remain controversial and their mode of action is still not fully understood. It is assumed that the biological effects of PUFAs are mediated through several mechanisms many of which are interlinked (**Fig. 6**) [176]. Among the mechanisms are alteration of the membrane composition and the modulation of gene expression [176]. A relevant part of PUFA related effects has been attributed to their oxylipins [7]. A key aspect appears to be the incorporation of the FAs into cell membrane phospholipids. PUFAs are integral parts of the lipid bilayer of biological membranes and their physicochemical properties are influenced by their lipid composition [176]. An increased intake of n3 PUFAs results in a dose-dependent incorporation of these FAs into membrane phospholipids and a concomitant displacement of n6 PUFAs [225]. Within the membrane itself, it has been shown that the enhanced incorporation of n3 PUFAs leads to altered membrane fluidity and raft assembly of lipid structures [226–230]. Moreover, an altered membrane composition affects protein function and signaling events as PUFAs directly regulate gene expression via nuclear receptors and transcription factors [176].

After hydrolysis of PUFAs from membrane phospholipids by the cytosolic phospholipase (cPL)A_2 anti-inflammatory actions of the generated free intracellular LC n3 PUFAs have been shown to be at least partly mediated by cell surface and intracellular receptors, G-protein coupled receptor (GPCR)120 and peroxisome proliferator activated receptor (PPAR)-γ, respectively. Both these receptors appear to be involved in the inhibiting activation of nuclear factor κB (NFkB), which is the prototypical pro-inflammatory transcription factor. NFkB controls the expression of several pro-inflammatory genes, such as cytokines, adhesion molecules,

inducible nitric oxide synthase (iNOS), COX-2 and matrix metalloproteinases (MMPs) (**Fig. 6A**). Studies suggest three mechanisms that underlie the suppression of inflammatory signaling via NFκB: First, activation of PPAR-γ by free EPA and DHA which suppresses nuclear translocation of NFκB [5,176], second, interfering with early membrane events involved in activation of NFκB via toll-like receptor (TLR)4 and third, action via GPCR120, which initiates an anti-inflammatory signaling cascade that inhibits signaling leading to NFκB activation (**Fig. 6A**) [5].

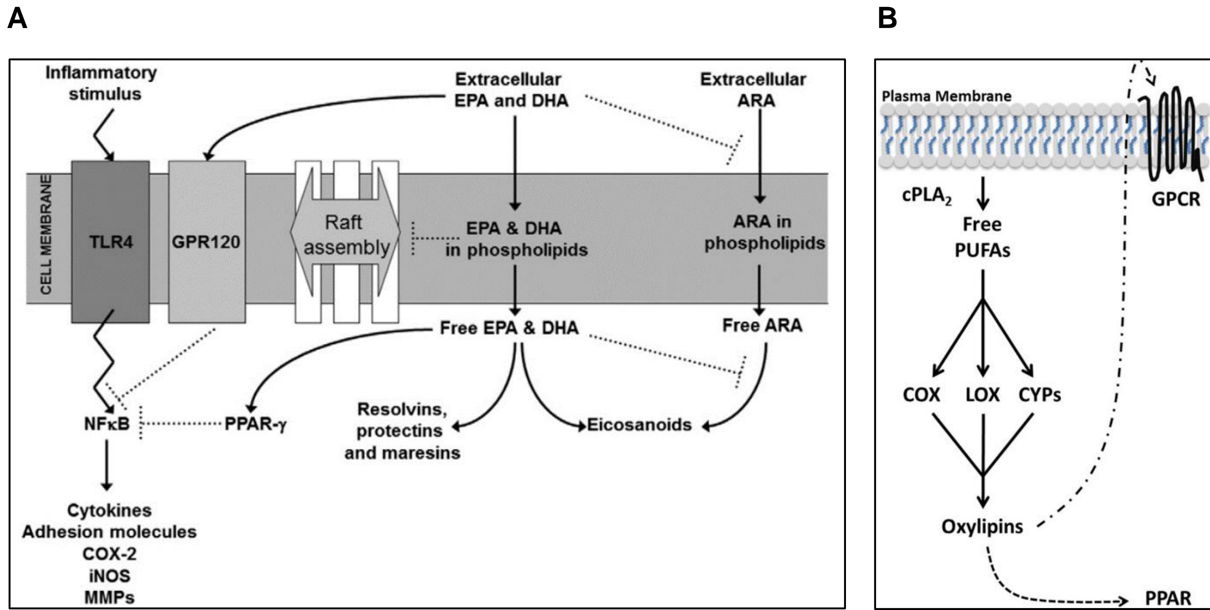

Fig. 6: Signaling pathways of polyunsaturated fatty acids and their oxylipins according to [5] and [6]. For details see text.

Abbreviations: ARA, arachidonic acid; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GP(C)R, G-protein coupled receptor; iNOS, inducible nitric oxide synthase; LOX, lipoxygenases; MMPs, matrix metalloproteinases; NFκB, nuclear factor κ B, PUFAs, polyunsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; TLR4, toll-like receptor 4.

After hydrolysis of PUFAs from the phospholipid membrane by $cPLA_2$ the generated free PUFAs are metabolized into distinct oxylipins (see chapter 1.4). Oxylipins can mediate their effects by binding to GPCRs on the surface of cells after diffusion through the plasma. Additionally, select oxylipins can bind to intracellular effectors like PPARs [231] (**Fig. 6B**). Oxylipin functions can be similar or opposing regardless of which PUFA and enzymatic pathway they derive from [7]. Epoxy FAs of EPA and DHA, which are the main products of EPA and DHA, have been shown in animal and cell studies to be potent vasodilators [232–235], counteract inflammatory conditions [232] and modulate ion channels [233,235–237]. While 17(18)-EpETE and 19(20)-EpDPE are potent anti-arrhythmic acting mediators [98] and 19(20)-EpDPE has been shown to inhibit angiogenesis, epoxy-FAs deriving from AA promote angiogenesis [208].

For a long time it was assumed that the breakdown of pro-inflammatory mediators (e.g. PGs and Txs) was sufficient to end the inflammatory response [238]. This assumption led to the explanation that n3 PUFA effects were mediated by suppressing the oxidative metabolism due to competition for the same enzymes with AA, which gives rise to mainly pro-inflammatory and pro-angiogenic eicosanoids [239]. However, it has been shown that this hypothesis is too simplistic and outdated [176]. Next to anti-inflammatory effects of n3 PUFAs that are independent of AA (e.g. via GPCRs as aforementioned), resolution of inflammation is the key to end inflammatory processes. Emerging evidence suggests that EPA and DHA play an important role in inflammation resolution via SPMs such as resolvins, protectins, and maresins [240] and CYP-generated mono-epoxides (MEFAs) (e.g. EpETE, EpDPE) [128]. Moreover, AA itself not only gives rise to pro-inflammatory mediators, but some AA-derived eicosanoids (e.g. EpETrEs and lipoxins) also have anti-inflammatory and pro-resolving effects [240]. Both, SPMs and lipoxins are key drivers of the inflammation resolution programs [176].

1.7. Objectives

Due to the high variability found in previous studies, the general aim of this thesis was to investigate the effects of modified PUFA intakes on the metabolism of PUFAs and their oxylipins in homogeneous study collectives and under tightly controlled conditions. To minimize the variability in the generated data, the studies in this thesis were carried out with homogenous study collectives with regard to dietary habits (especially fish and PUFA intake), age, sex, smoking status and BMI. Moreover, subjects were pre-screened for their endogenous PUFA status and only those subjects with low EPA and DHA concentrations in RBCs were included. Appropriate run-in and/or wash-out phases were also included in the study designs. These methodological aspects were considered to generate more reliable data less prone to biases and lead to a higher level of detail regarding the interpretation of effects.

Four different human studies were carried out, which are summarized in **Tab. 3**. The aim of the **ALA study** (Paper I) was to investigate the effects of a 12-week ALA supplementation from linseed oil on the PUFA profile in RBCs and free oxylipin patterns in plasma. The cross-over study, **LA/ALA** (Paper II), was aimed at comparing the effects of 2-week interventions of 2 extreme dietary ratios (low-LA/high-ALA: 0.56:1, high-LA/low-ALA diet: 25.6:1) of the competing essential PUFAs LA and ALA on the PUFA profile in RBCs. Dietary conditions were strictly controlled in the LA/ALA study and data on nutrient intake, especially fat intake, were collected over the entire duration of the intervention periods using (partly) weighed dietary protocols and additional FA analysis of the daily provided lunch meals. In the **DHA study** the effects of a 12-week DHA supplementation on the profile of plasma and RBC PUFAs were

compared (Paper III). Moreover, the effects of the 12-week DHA supplementation on free oxylipins in plasma as well as oxylipin formation in response to an ex-vivo induced inflammatory response were investigated (Paper IV). Three intermediate measurement timepoints in the 12-week **ALA** and **DHA study** and one in the 2-week intervention periods of the **LA/ALA study** allowed for analyzing concentrations over the course of time. The **Oxylipin study** aimed at investigating (i) analytical variation, (ii) inter-day variation in plasma samples collected 48 hours apart (with and without standardized nutrition) and (iii) intra-day variation on a standardized diet (Paper V).

Tab. 3: Overview of the studies carried out.

Study name	Intervention	Measurement timepoints	Analysis of
ALA	ALA supplementation	0[a], 1, 3, 6, 12 weeks	FAs in RBCs Free oxylipins in plasma
LA/ALA	1) high-ALA/low-LA diet 2) low-ALA/high-LA diet	0[a], 1, 2 weeks	FAs in RBCs
DHA	DHA supplementation	0[a], 1, 3, 6, 12 weeks	FAs in RBCs and plasma Free oxylipins in plasma
Oxylipin	non-standardized diet standardized diet (inter-day) standardized diet (intra-day)	0[a], 48 hours 0[a], 24, 48, 72 hours 0[a], 2, 4, 6, 8 hours	Free oxylipins in plasma

Abbreviations: ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; FAs, fatty acids; LA, linoleic acid; n.a., not applicable; RBCs, red blood cells.
[a] baseline timepoint

2. Paper I

Effects of a 12-week high-α-linolenic acid intervention on EPA and DHA concentrations in red blood cells and plasma oxylipin pattern in subjects with a low EPA and DHA status

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Published in: *Food & Function*, 9 (2018), 1587-1600.

Link: *https://pubs.rsc.org/en/content/articlehtml/2018/fo/c7fo01809f*

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Food &
Function

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Cite this: DOI: 10.1039/c7fo01809f

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Effects of a 12-week high-α-linolenic acid intervention on EPA and DHA concentrations in red blood cells and plasma oxylipin pattern in subjects with a low EPA and DHA status†

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The essential omega-3 fatty acid alpha-linolenic acid (ALA, 18:3n3) can be converted into EPA and DHA. The aim of the present study was to determine the effect of a high-ALA diet on EPA and DHA levels in red blood cells (RBCs) and their oxylipins in the plasma of subjects with a low EPA and DHA status. Fatty acid concentrations [μg mL^{-1}] and relative amounts [% of total fatty acids] in the RBCs of 19 healthy men (mean age 26.4 ± 4.6 years) were analyzed by means of GC-FID. Free plasma oxylipin concentrations were determined by LC-MS based targeted metabolomics. Samples were collected and analyzed at baseline (week 0) and after 1 (week 1), 3 (week 3), 6 (week 6), and 12 (week 12) weeks of high dietary ALA intake (14.0 ± 0.45 g day^{-1}). ALA concentrations significantly ($p < 0.001$) increased from 1.44 ± 0.10 (week 0) to 4.65 ± 0.22 (week 1), 5.47 ± 0.23 (week 3), 6.25 ± 0.24 (week 6), and 5.80 ± 0.28 (week 12) μg mL^{-1}. EPA concentrations increased from 6.13 ± 0.51 (week 0) to 7.33 ± 0.33 (week 1), 8.38 ± 0.42 ($p = 0.021$, week 3), 10.9 ± 0.67 ($p < 0.001$, week 6), and 11.0 ± 0.64 ($p < 0.001$, week 12) μg mL^{-1}. DHA concentrations unexpectedly decreased from 41.0 ± 1.93 (week 0) to 37.0 ± 1.32 (week 1), 36.1 ± 1.37 (week 3), 35.1 ± 1.06 ($p = 0.010$, week 6), and 30.4 ± 1.09 ($p < 0.001$, week 12) μg mL^{-1}. Relative ΣEPA + DHA amounts were unchanged during the intervention (week 0: 4.63 ± 0.19, week 1: 4.67 ± 0.16, week 3: 4.61 ± 0.13, week 6: 4.73 ± 0.15, week 12: 4.52 ± 0.11). ALA- and EPA-derived hydroxy- and dihydroxy-PUFA increased similarly to their PUFA precursors, although in the case of ALA-derived oxylipins, the concentrations increased less rapidly and to a lesser extent compared to the concentrations of their precursor FA. LA-derived oxylipins remained unchanged and arachidonic acid and DHA oxylipin concentrations were not significantly changed. Our results confirm that the intake of ALA is not a sufficient source for the increase of EPA + DHA in subjects on a Western diet. Specifically, a high-ALA diet results in increased EPA and declined DHA concentrations. However, the changes effectively balance each other out so that ΣEPA + DHA in RBCs – which is an established marker for health protective effects of omega-3-PUFA – remains constant. The PUFA levels in RBCs reflect the concentration and its changes in plasma hydroxy- and dihydroxy-PUFA concentrations for ALA and EPA.

Received 16th November 2017,
Accepted 26th January 2018

DOI: 10.1039/c7fo01809f

rsc.li/food-function

Introduction

Health benefits including lower risk of cardiovascular disease and mortality,[1–5] reduction of inflammatory conditions,[2,6] angiogenesis, tumor growth and metastasis[2,7] and a better visual and neurological development[8] are associated with long chain (LC) omega-3 (n3) polyunsaturated fatty acids (PUFAs), namely eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3). The positive health effects are believed to be mediated in part by the oxylipins formed from EPA and DHA in the arachidonic acid (AA, C20:4n6) cascade *via* different enzymatic pathways: cyclooxygenase (COX) action leads to prostanoid formation, lipoxygenases (LOX) give rise to

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c7fo01809f

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hydroperoxy-PUFA which can be reduced to hydroxy-PUFA, whereas cytochrome P450 (CYP) enzymes act as epoxygenases and ω-hydroxylases.[9,10] Epoxy-PUFA can be hydrolyzed to the corresponding diols by soluble epoxide hydrolase (sEH).[11] Additionally, EPA and DHA can also be converted to oxidative products by autoxidation.[10]

The primary dietary source of EPA and DHA is oily cold water fish such as salmon, tuna, herring and mackerel. In most Western diets, the intake of EPA and DHA is low due to low fish consumption. In the U.S. the daily mean EPA and DHA intake of adults is 30–40 mg and 70–80 mg, respectively.[12] In Germany, the median EPA and DHA intake is 65–78 mg and 107–135 mg, respectively.[13] Thus, the intake of EPA and DHA is about 2.5- to 5-fold lower compared to the recommendation of a minimum intake of 500 mg EPA and DHA per day for cardiovascular health.[14] Another source of LC n3 PUFAs is the essential fatty acid alpha-linolenic acid (ALA, C18:3n3), which is present in high amounts in plant oils, particularly linseed, chia, perilla and walnut oil. The essential n3 fatty acid ALA can be converted into EPA, docosapentaenoic acid (DPAn3, C22:5n3) and DHA in a multistep elongation and desaturation reaction.[15] However, several studies, including studies with stable isotopes, suggest that the conversion rate from ALA to EPA (5–8%), to DPAn3 (5–8%) and especially to DHA (0.5–5%) is low in subjects on a Western diet[16–18] as reviewed in ref. 19–21. One reason is the high intake of the essential omega-6 (n6) PUFA linoleic acid (LA, C18:2n6), which competes with ALA for the rate-limiting enzyme Δ6-desaturase transforming LA into gamma-linolenic acid (GLA, C18:3n6) while blocking ALA into stearidonic acid (C18:4 n3).[22–25]

The mean intake of ALA and LA in the U.S. is 1.5–1.6 g d^{-1} and 15.1–15.9 g d^{-1},[12] respectively, while the median intake of ALA and LA in Germany is 0.9–1.3 g d^{-1} and 7.3–10.1 g d^{-1}, respectively.[13] The LA intake is particularly high in the U.S. due to the high consumption of LA-rich plant oils such as corn, sunflower, and soybean oil.[26] Although the intake recommendations for ALA (1.1–1.6 g d^{-1}) and LA (12–17 g d^{-1}) in the U.S.[27] and Germany (ALA: 1.1 g d^{-1}, LA: 5.4 g d^{-1} calculated on the basis of 0.5% of total energy (en%) and 2.5 en%, respectively, and 2000 kcal d^{-1} (ref. 28)) are basically met, the blood levels of EPA and DHA in the general population of both countries are low[29] probably due to the overall low n3 PUFA intake and the resulting high LA/ALA ratio (about 11 : 1 in the U.S. and 8–10 : 1 in Germany). The low levels of EPA and DHA in RBCs, *i.e.* omega-3 index, are associated with an increased risk of death from cardiovascular disease,[30] neurodegeneration and cognitive impairment[31,32] and total mortality.[33,34]

The question whether low LC n3 PUFA blood levels can be increased by supplementing ALA-rich oils remains controversial. Few studies have investigated the effect of supplementing ALA on blood EPA and DHA content in subjects with a low LC n3 PUFA status on a Western diet. Li *et al.*[35] studied the effect of moderate-ALA diet (3.7 ± 1.4 g d^{-1}) and high-ALA diet (15.4 ± 7.5 g d^{-1}) in vegetarians, while Fokkema *et al.*[36] observed the effect of a short-term low-dose ALA-enriched diet (2.01 g d^{-1}) in vegans. The effect of a high-ALA diet on absolute LC PUFA concentrations in red blood cells (RBCs) has not been studied in a long-term study with healthy omnivores.

The aim of the present study was therefore to determine the short (1 and 3 weeks) and long term (6 and 12 weeks) effect of a high-ALA diet providing a daily ALA dose of 14.0 ± 0.45 g on EPA and DHA concentrations and relative EPA and DHA amounts in RBCs and their oxylipins in the plasma of subjects with a low EPA and DHA status consuming a Western (German) diet.

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Materials and methods

This investigator initiated study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee at the medical chamber of Lower Saxony (Hannover, Germany). Written informed consent was obtained from all subjects. The study is registered in the German clinical trial register (no. DRKS00006765).

Study design

The study was conducted at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany and consisted of a screening phase, a 12-week lasting intervention period and an 8-week lasting follow-up period. During the intervention period, five examinations were carried out, at the beginning (week 0) and after one (week 1), three (week 3), six (week 6) and twelve (week 12) weeks. Two further examinations were carried out in the follow-up period (week 14 and week 20). During the intervention period, the subjects daily ingested 22.3 g of linseed oil (All Organic Trading GmbH, Kempten, Germany) with an ALA content of 58% of total fatty acids (Table S1†). Hence, the ALA intake from linseed oil was 12.9 g day^{-1}. The peroxide value of the linseed oil was 1.0 meq kg^{-1} and the acid value was 0.28 mg KOH per g.

During each visit, fasting blood was collected, blood pressure was measured and the subjects completed a questionnaire to obtain information about changes in medication, lifestyle habits (*e.g.* physical activity), and tolerability of linseed oil. The questionnaire additionally included questions about dietary changes during the study. Moreover, the participants were requested to restrict their dietary n3 PUFA intake four weeks before and throughout the study, *e.g.* abstain from fatty fish (salmon, herring, tuna and mackerel) and ALA-rich vegetable foodstuffs such as linseeds, chia or walnuts (including oils), to minimize the effects on the variability of ALA and LC PUFA intake and blood levels. Prior to visit week 0, week 6, and week 12, the subjects completed a 3-day dietary questionnaire including two working days and one weekend day. The dietary questionnaires were analyzed using PRODI (Nutri-Science GmbH, Freiburg, Germany) to obtain the data on energy and nutrient intake. The total ALA intake was calculated retrospectively by adding the ALA intake from the background diet to the ALA dose from the linseed oil intake.

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Blood samples were collected in the morning between 6:30 and 11:00 a.m. after overnight fasting. The examinations were scheduled at the same time for each subject. The samples were obtained by venipuncture of an arm vein using Multiflyneedles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt). For the analysis of fatty acids in the plasma, EDTA blood monovettes were centrifuged for 10 min at 1500*g* and 4 °C and the plasma was transferred into 1.5 mL plastic tubes (Sarstedt) and immediately frozen and stored at −80 °C until extraction and analysis. For the analysis of fatty acids in RBCs, the cell sediment after centrifugation for 10 min at 1500*g* and 4 °C and removal of the plasma was washed twice with PBS (containing 1.5 mg mL^{-1} EDTA). Finally, the RBCs were reconstituted in PBS to the initial blood volume, transferred into 1.5 mL Eppendorf tubes and immediately frozen and stored at −80 °C until extraction and analysis. All transfer steps were carried out on ice. Other sets of blood samples (serum and EDTA monovettes) were sent to external laboratories for the measurement of clinical parameters. Serum lipid levels, liver enzymes and small blood picture were determined in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany.

Study population

Participants were recruited from the general population in Hannover, Germany through advertisements. Several selection criteria were defined to assemble a homogeneous study collective. In particular, only men within a narrow age range from 20 to 40 years were included to minimize potential fluctuations in lipid profiles due to age or hormonal influence. Subjects were preselected *via* screening questionnaires according to the following inclusion criteria: male sex, age between 20 and 40 years, body mass index (BMI) between 20 and 27 kg m^{-2}, and mixed diet with low meat and fish consumption. The exclusion criteria were defined as follows: smoking, serum triglyceride levels ≥150 mg dl^{-1} (≥1.7 mmol l^{-1}), serum total cholesterol levels ≥200 mg dl^{-1} (≥5.2 mmol l^{-1}), a relative amount of ΣEPA + DHA in whole blood ≤3 and ≥6%, intake of fish (>2 times per week) as well as addiction to alcohol, drugs and/or medications and diseases: chronic diseases (*e.g.* malignant tumors, manifest cardiovascular disease, insulin-dependent type 1 and 2 diabetes, and severe renal or liver diseases); chronic gastrointestinal disorders (especially small intestine, pancreas, and liver) as well as prior gastrointestinal surgical procedures (*e.g.* gastrectomy); hormonal disorders (*e.g.* Cushing's syndrome and untreated hyperthyroidism); uncontrolled hypertension; blood coagulation disorders and intake of coagulation-inhibiting drugs; periodic intake of laxatives; intake of anti-inflammatory drugs (including acetylsalicylic acid); and intake of lipid lowering drugs or supplements during the last 3 months before baseline examination. The inclusion and exclusion criteria were assessed *via* questionnaires. The pre-selected subjects were invited for a screening examination to collect fasting blood for the analysis of serum lipid levels, liver enzymes and fatty acid profiles in whole blood.

Fatty acid analysis

The concentrations of fatty acids were determined by means of gas chromatography (GC) with flame ionization detection as described[37] with slight modifications. In brief, lipids were extracted with MTBE/MeOH and derivatized with methanolic hydrogen chloride, and the resulting fatty acid methyl esters (FAME) were quantified using methyl pentacosanoate (C25:0 FAME) as the internal standard (IS). In addition to the determined concentration, reported as µg fatty acid per mL blood, the relative amount (% of total fatty acids) of each fatty acid was calculated directly based on peak areas as described.[37]

Oxylipin analysis

The concentrations of oxylipins in the plasma were determined by means of an established liquid chromatography-mass spectrometry (LC-MS) based targeted metabolomics platform (Table S2†) as described[38–40] with slight modifications. In brief, after the addition of the IS and antioxidant solution, the plasma samples were diluted with 1 M sodium acetate in water : MeOH (95 : 5 v : v) adjusted to pH 6.0 with acetic acid. Additionally, 10 µL of a solution of the LOX inhibitor 2-(1-thienyl)ethyl 3,4-dihydroxybenzylidenecyanoacetate (2 µM) and the protease inhibitor phenylmethylsulfonyl fluoride (5 mM) was added. The extraction was carried out on a nonpolar (C8)/anion exchange mixed mode material (Bond Elut Certify II, 200 mg, Agilent) utilizing ethyl acetate/*n*-hexane (75 : 25, v : v) with 1% acetic acid as the eluent. The LC-MS measurement was carried out in scheduled selected reaction monitoring following negative electrospray ionization and the quantification of oxylipins was performed by external calibration utilizing 13 deuterated IS.[38–40]

Calculations and statistics

The results of anthropometrical measures, serum lipid levels, dietary energy and fat intake are presented as mean ± standard deviation (SD), while the PUFA levels in RBC membranes and their relative change (%) are presented as mean ± standard error (SE). If the concentration of an analyte was below the lower limit of quantification (LLOQ) in more than 50% of the samples at one time point, the LLOQ is given for this analyte. Relative changes in the variables (v) were calculated individually for each subject at each time point (x) as $\Delta\%$, calculated by: $\Delta\% = 100 \times (v_{tx} - v_{\text{week }0})/v_{\text{week }0}$.

The activities of delta-5 desaturase (D5D) and delta-6 desaturase (D6D) were calculated using product-to-precursor ratios: (C20:4n6/C20:3n6) and (C20:3n6/C18:2n6), respectively, as previously described.[41] The indices of highly unsaturated fatty acids (HUFA) were calculated as follows: % n3 in HUFA = 100 × (C20:5n3 + C22:5n3 + C22:6n3)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3); % n6 in HUFA = 100 × (C20:3n6 + C20:4n6 + C22:4n6)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3), modified from ref. 42.

The distributions of the sample sets were analyzed by means of the Kolmogorov–Smirnov test. The differences between baseline (week 0) levels and different time points

after high-ALA diet (week 1, week 3, week 6, week 12) as well as between week 12 and week 14 and week 20 were analyzed by ANOVA for repeated measurements with the acceptance of statistical significance at $p \leq 0.05$. To determine the statistical significance between baseline levels and each time point (as well as between week 12 and week 14 and week 20), *t*-tests for paired samples were carried out. For *t*-tests, the significance levels were adjusted according to the Holm–Bonferroni method. All statistical analyses were carried out with the SPSS software (Version 24, SPSS Inc., Chicago, IL, USA).

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Results

Study population

Twenty male subjects met the criteria and thus were included in the study. All participants (mean age 26.2 ± 4.53 years) were healthy and had a normal BMI (24.9 ± 2.0 kg m^{-2}) and serum lipid profile (Table 1). The eating habits (especially fish consumption) and the physical activity of the probands did not change during the intervention period as investigated by dietary questionnaires. The study collective consumed a normal mixed diet (including meat) with low fish consumption (≤1 fish serving per week) and low fruit and vegetable consumption (1–2 portions per day) and had a medium physical activity status (3–5 hours of sports per week) and a high education level (advanced technical college certificate). One subject withdrew his consent after 1 week of the intervention. All other 19 participants completed the 12-week intervention period and attended at all five intervention time points. At week 14 of the follow-up period, only 17, and at week 20, 13 participants attended the examinations. Linseed oil was well tolerated and no adverse effects were reported during the intervention period.

The evaluation of the 3-day dietary questionnaires showed that the variability of PUFA intake other than ALA was minimal (Table 2). For LA, AA, EPA, DPAn3 and DHA intake, no significant changes occurred during the intervention period. The intake of ALA from the background diet was low with minimal variability. The total ALA intake was 1.39 ± 1.31 g d^{-1} at week 0, 13.9 ± 0.34 g d^{-1} at week 6 and 14.0 ± 0.53 g d^{-1} at week 12. Due to the high-ALA diet, the total PUFA intake significantly ($p < 0.001$) increased from week 0 (11.2 ± 7.02 g d^{-1}) to week 6 (23.3 ± 3.14 g d^{-1}) and week 12 (22.9 ± 5.87 g d^{-1}). The energy and total fat intake did not change significantly; however, the energy intake was lower at week 12 (2403 ± 524 kcal d^{-1}) compared to week 0 (2910 ± 1181 kcal d^{-1}) and week 6 (2924 ± 820 kcal d^{-1}) (Table 2). In addition, for saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) intake, no significant changes were detected during the intervention period. The intake of SFA decreased (n.s.) from week 0 (31.1 ± 15.5 g d^{-1}) to week 6 (26.9 ± 11.0 g d^{-1}) and week 12 (24.4 ± 9.83 g d^{-1}). Although not significant, the intake of EPA, DPAn3 and DHA slightly decreased during the intervention period (Table 2).

Body weight, BMI, blood pressure and total cholesterol (TC) levels were unchanged during the intervention (Table 1). Also,

Table 1 Clinical, biochemical and anthropometric parameters of the study population

	Week 0 Mean ± SD	Week 1 Mean ± SD	*t*-Test[a] *p* (week 1–week 0)	Week 3 Mean ± SD	*t*-Test[a] *p* (week 3–week 0)	Week 6 Mean ± SD	*t*-Test[a] *p* (week 6–week 0)	Week 12 Mean ± SD	*t*-Test[a] *p* (week 12–week 0)	An reM[b] *p*
Age (years)	26.2 ± 4.53									
Weight (kg)	82.7 ± 8.25							84.4 ± 7.83	—	n.s.
BMI (kg m^{-2})	24.7 ± 2.08							25.3 ± 1.87	—	n.s.
Sys BP (mmHg)	128 ± 12.0	125 ± 12.1	—	128 ± 9.27	—	131 ± 10.9	—	131 ± 14.9	—	n.s.
Dias BP (mmHg)	77.8 ± 9.39	77.0 ± 6.37	—	77.2 ± 5.21	—	78.2 ± 5.06	—	76.3 ± 5.23	—	n.s.
TC (mg dl^{-1})	168 ± 22.9	162 ± 23.2	—	159 ± 22.9	—	167 ± 25.8	—	167 ± 26.3	—	n.s.
LDL (mg dl^{-1})	108 ± 18.5	96.9 ± 16.6	**0.010**	102 ± 17.9	n.s.	105 ± 19.5	n.s.	101 ± 17.0	n.s.	**0.022**
HDL (mg dl^{-1})	54.1 ± 7.88	52.4 ± 8.28	n.s.	50.6 ± 8.47	n.s.	55.4 ± 7.8	n.s.	54.8 ± 10.9	n.s.	**0.002**
TG (mg dl^{-1})	77.1 ± 20.4	65.9 ± 18.8	n.s.	76.1 ± 27.7	n.s.	86.0 ± 35.4	n.s.	87.1 ± 36.4	n.s.	**0.026**

The levels are shown at week 0, 1, 3, 6, and 12 of the high-ALA diet (14.0 ± 0.45 g d^{-1}). BMI: body mass index; dias BP: diastolic blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; n.s.: not significant; SD: standard deviation; sys BP: systolic blood pressure; TC: total cholesterol; TG: triglycerides, wk: week. [a] *t*-Test for paired samples with Holm–Bonferroni correction; significance level $p \leq 0.05$. [b] ANOVA for repeated measures (An reM); significance level $p \leq 0.05$.

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Table 2 Daily energy and fat intake from 3-day dietary questionnaires

	Week 0 Mean ± SD	Week 6 Mean ± SD	*t*-Test[a]	Week 12 Mean ± SD	*t*-Test[a]	An reM[b] *p*
Energy intake (kcal)	2910 ± 1181	2924 ± 820		2403 ± 524		n.s.
Total fat intake (g)	113 ± 40.3	128 ± 42.0		108 ± 24.3		n.s.
SFA (g)	31.1 ± 15.5	26.9 ± 11.0	n.s.	24.4 ± 9.83	n.s.	**0.026**
MUFA (g)	14.3 ± 6.88	14.6 ± 8.44		13.9 ± 6.14		n.s.
PUFA (g)	11.2 ± 7.02	23.3 ± 3.14	**0.002**	22.9 ± 5.87	**0.004**	**<0.001**
LA (g)	9.25 ± 5.93	9.14 ± 2.87		9.58 ± 3.34		n.s.
ALA (g)	1.39 ± 1.31	13.9 ± 0.34	**<0.001**	14.0 ± 0.53	**<0.001**	**<0.001**
AA (g)	0.09 ± 0.07	0.16 ± 0.18		0.10 ± 0.07		n.s.
EPA (g)	0.19 ± 0.52	0.02 ± 0.03		0.01 ± 0.03		n.s.
DPAn3 (g)	0.06 ± 0.06	0.04 ± 0.06		0.03 ± 0.06		n.s.
DHA (g)	0.15 ± 0.18	0.06 ± 0.06		0.05 ± 0.06		n.s.

The levels are shown at week 0, 6 and 12 of the high-ALA diet (14.0 ± 0.45 g d^{-1}). AA: arachidonic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; DPAn3: docosapentaenoic acid; LA: linoleic acid; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; wk: week. [a] *t*-Test for paired samples with Holm–Bonferroni correction; significance level $p \leq 0.05$. [b] ANOVA for repeated measures (An reM); significance level $p \leq 0.05$.

low density lipoprotein (LDL), high density lipoprotein (HDL) and triglyceride (TG) levels did not change during the intervention period even though statistically significant lower LDL levels (108 ± 18.5 to 96.9 ± 16.6 mg dl^{-1}) were detected after 1 week (no significant differences after 3, 6 and 12 weeks).

Changes of fatty acid profile in RBCs

At baseline, the relative amount of ΣEPA + DHA in the RBCs of the study collective was 4.63 ± 0.19% of total fatty acids (Table 3). Thus, all subjects had a low ΣEPA + DHA status within a narrow range.

Prior to the intervention, AA was present in the highest concentrations in the RBCs (150 ± 3.90 μg mL^{-1}) among all PUFAs, followed by LA (104 ± 3.60 μg mL^{-1}), DHA (41.0 ± 1.93 μg mL^{-1}), C22:4n6 (31.6 ± 1.01 μg mL^{-1}), DPAn3 (25.9 ± 0.82 μg mL^{-1}) and EPA (6.13 ± 0.51 μg mL^{-1}) (Table 3). The ALA concentrations in the RBCs were low with 1.44 ± 0.10 μg mL^{-1} at week 0. In the course of the high-ALA diet, the concentrations and relative amount of ALA, EPA and DPAn3 in the RBCs (Fig. 1A–C) increased significantly and decreased again in the follow-up period, whereas the DHA concentrations decreased in response to the high-ALA diet (Fig. 1D). In the following, only the concentrations are discussed unless the relative fatty acid distribution showed a different trend. It is noteworthy that the relative amount of ΣEPA + DHA in the RBCs did not change in response to the high-ALA diet (Table 3).

The ALA concentrations in the RBCs increased time-dependently ($p < 0.001$) from 1.44 ± 0.10 μg mL^{-1} (week 0) to 4.65 ± 0.22 μg mL^{-1} (week 1) and 5.47 ± 0.23 μg mL^{-1} (week 3) corresponding to a mean change of 238 ± 24% and 294 ± 23%, respectively (Table 3, Fig. 1A). The highest ALA levels (6.25 ± 0.24 μg mL^{-1}) were observed after 6 weeks of the high-ALA diet. In the follow-up period, the ALA concentrations dropped ($p < 0.001$) rapidly from 5.80 ± 0.28 μg mL^{-1} at week 12 to 2.62 ± 0.16 μg mL^{-1} after 2 weeks (week 14) and 2.27 ± 0.21 μg mL^{-1} after 8 weeks (week 20) (Table S3†).

The EPA levels in the RBCs increased linearly and almost doubled ($p < 0.001$) in concentration from baseline (6.13 ± 0.51 μg mL^{-1}) to 10.9 ± 0.67 μg mL^{-1} at week 6 and 11.0 ± 0.64 μg mL^{-1} at week 12 (Table 3, Fig. 1B). In the follow-up period, the concentrations decreased more slowly compared to ALA with 10.0 ± 0.52 μg mL^{-1} after 2 weeks (week 14) and 8.53 ± 0.69 μg mL^{-1} after 8 weeks (week 20) (Table S3†).

The changes in DPAn3 concentrations were smaller and only significant after 6 ($p = 0.033$) and 12 weeks ($p = 0.014$) of the high-ALA diet, whereas the changes of the relative amounts were statistically significant ($p < 0.001$) after 3, 6 and 12 weeks (Table 3, Fig. 1C). From baseline to week 12, the DPAn3 concentrations increased from 25.9 ± 0.51 μg mL^{-1} to 32.3 ± 1.35 μg mL^{-1} and remained high over the follow-up period (35.3 ± 1.63 μg mL^{-1} at week 14 and 32.6 ± 1.49 at week 20) (Table S3†).

A linear and significant ($p < 0.001$) reduction of DHA concentrations was observed between baseline (41.0 ± 1.93 μg mL^{-1}) and week 12 (30.4 ± 1.09 μg mL^{-1}) (Table 3, Fig. 1D). After 2 and 8 weeks of follow-up, the DHA concentrations slightly increased to 33.8 ± 1.57 μg mL^{-1} (n.s.) (Table S3†).

The initial AA concentrations in the RBCs (150 ± 3.90 μg mL^{-1}) were only marginally reduced (136–138 μg mL^{-1}) in the first 6 weeks and then significantly ($p = 0.001$) dropped to 124 ± 3.53 μg mL^{-1} at week 12 (Table 3, Fig. 2), corresponding to a mean decrease of 16.6 ± 3.31%. The relative AA amount was statistically significantly reduced at all time points of the intervention. In the follow-up period, the AA concentrations increased, although not significantly, whereas the relative increase was significant ($p = 0.012$ (week 14) and $p = 0.001$ (week 20)) (Table S3†).

Both the absolute concentrations and relative amount of LA in the RBCs did not change in the course of the high-ALA diet and follow-up period (Tables 3 and S3†). The concentration of SFA, PUFA and Σn3 PUFA in the RBCs remained constant during the study. However, the concentrations of MUFA and Σn6 PUFA in the RBCs were significantly decreased after 12 weeks of the high-ALA diet (Table 3). The MUFA concentrations decreased ($p = 0.019$) from 210 ± 6.24 μg mL^{-1} at week 0 to 179 ± 5.89 μg mL^{-1} at week 12, while the Σn6 PUFA con-

Table 3 Concentration and relative amount of fatty acids in red blood cells

	Week 0 Mean ± SE	Week 1 Mean ± SE	*t*-Test[a] *p* (week 1–week 0)	Week 3 Mean ± SE	*t*-Test[a] *p* (week 3–week 0)	Week 6 Mean ± SE	*t*-Test[a] *p* (week 6–week 0)	Week 12 Mean ± SE	*t*-Test[a] *p* (week 12–week 0)	An reM[b] *p*
C12:0 μg mL^{-1}	<0.25	<0.25	—	<0.25	—	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—	—	—	—	—
C14:0 μg mL^{-1}	3.74 ± 0.10	3.02 ± 0.15	**0.001**	2.73 ± 0.16	**<0.001**	3.03 ± 0.15	**0.026**	3.09 ± 0.17	**n.s.**	**<0.001**
% of total FA	0.37 ± 0.01	0.32 ± 0.01	n.s.	0.28 ± 0.01	n.s.	0.31 ± 0.01	n.s.	0.34 ± 0.02	n.s.	**<0.001**
C14:1n5 μg mL^{-1}	<0.25	<0.25	—	<0.25	—	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—	—	—	—	—
C15:0 μg mL^{-1}	1.75 ± 0.05	1.62 ± 0.06	—	1.67 ± 0.09	—	1.61 ± 0.07	—	1.68 ± 0.19	—	n.s.
% of total FA	0.17 ± 0.01	0.17 ± 0.01	—	0.17 ± 0.01	—	0.16 ± 0.01	—	0.18 ± 0.02	—	n.s.
C16:0 μg mL^{-1}	196 ± 4.60	188 ± 3.00	—	187 ± 3.86	—	191 ± 5.60	—	184 ± 5.58	—	n.s.
% of total FA	19.3 ± 0.08	19.7 ± 0.15	—	19.4 ± 0.16	—	19.5 ± 0.19	—	20.1 ± 0.15	**0.002**	**0.029**
C16:1n7 μg mL^{-1}	2.65 ± 0.15	2.15 ± 0.12	—	2.30 ± 0.15	n.s.	2.44 ± 0.17	n.s.	2.35 ± 0.18	n.s.	**0.032**
% of total FA	0.26 ± 0.01	0.22 ± 0.01	—	0.24 ± 0.01	—	0.25 ± 0.01	—	0.25 ± 0.01	—	n.s.
C17:0 μg mL^{-1}	3.27 ± 0.07	3.03 ± 0.07	—	3.01 ± 0.12	n.s.	3.02 ± 0.09	n.s.	2.86 ± 0.10	**0.039**	**0.012**
% of total FA	0.32 ± 0.01	0.32 ± 0.01	—	0.31 ± 0.01	—	0.31 ± 0.01	—	0.31 ± 0.01	—	—
C18:0 μg mL^{-1}	154 ± 2.63	147 ± 1.92	—	148 ± 2.91	—	150 ± 3.41	—	143 ± 4.19	—	n.s.
% of total FA	15.1 ± 0.11	15.4 ± 0.13	—	15.3 ± 0.08	—	15.4 ± 0.10	**0.004**	15.6 ± 0.13	**<0.001**	**<0.001**
C18:1n9 μg mL^{-1}	135 ± 3.91	120 ± 2.44	**0.036**	121 ± 2.56	n.s.	122 ± 3.69	n.s.	115 ± 4.00	**0.035**	**0.001**
% of total FA	13.3 ± 0.18	12.5 ± 0.15	**0.013**	12.5 ± 0.16	**0.014**	12.5 ± 0.16	**0.017**	12.5 ± 0.16	**0.023**	**<0.001**
C18:1n7 μg mL^{-1}	14.5 ± 0.46	12.9 ± 0.28	**0.044**	12.6 ± 0.34	**0.016**	12.4 ± 0.34	**0.008**	11.9 ± 0.42	**0.010**	**<0.001**
% of total FA	1.43 ± 0.02	1.35 ± 0.02	**0.001**	1.30 ± 0.02	**<0.001**	1.27 ± 0.02	**<0.001**	1.30 ± 0.02	**0.002**	**<0.001**
C18:2n6 μg mL^{-1}	104 ± 3.60	97.9 ± 2.94	—	102 ± 3.60	—	103 ± 3.10	—	97.9 ± 3.69	—	n.s.
% of total FA	10.3 ± 0.32	10.3 ± 0.29	—	10.5 ± 0.29	—	10.6 ± 0.30	—	10.7 ± 0.28	—	n.s.
C18:3n6 μg mL^{-1}	<0.25	<0.25	—	<0.25	—	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—	—	—	—	—
C19:0 μg mL^{-1}	<0.25	<0.25	—	<0.25	—	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—	—	—	—	—
C18:3n3 μg mL^{-1}	1.44 ± 0.10	4.65 ± 0.22	**<0.001**	5.47 ± 0.23	**<0.001**	6.25 ± 0.24	**<0.001**	5.80 ± 0.28	**<0.001**	**<0.001**
% of total FA	0.14 ± 0.01	0.49 ± 0.02	**<0.001**	0.57 ± 0.03	**<0.001**	0.64 ± 0.02	**<0.001**	0.63 ± 0.03	**<0.001**	**<0.001**
C20:0 μg mL^{-1}	5.44 ± 0.12	4.41 ± 0.12	**<0.001**	4.34 ± 0.10	**<0.001**	4.30 ± 0.12	**<0.001**	3.91 ± 0.12	**<0.001**	**<0.001**
% of total FA	0.54 ± 0.01	0.46 ± 0.01	**0.003**	0.45 ± 0.01	**<0.001**	0.44 ± 0.01	**<0.001**	0.43 ± 0.01	**<0.001**	**<0.001**
C20:1n9 μg mL^{-1}	3.14 ± 0.11	2.90 ± 0.07	n.s.	2.98 ± 0.09	n.s.	2.85 ± 0.11	**0.003**	2.67 ± 0.10	**0.003**	**0.002**
% of total FA	0.31 ± 0.01	0.30 ± 0.01	—	0.31 ± 0.01	—	0.29 ± 0.01	—	0.29 ± 0.01	—	n.s.
C20:2n6 μg mL^{-1}	2.04 ± 0.09	1.92 ± 0.09	—	2.00 ± 0.12	—	1.87 ± 0.10	—	1.83 ± 0.12	—	n.s.
% of total FA	0.20 ± 0.01	0.20 ± 0.01	—	0.21 ± 0.01	—	0.19 ± 0.01	—	0.20 ± 0.01	—	n.s.
C20:3n6 μg mL^{-1}	15.5 ± 0.81	13.7 ± 0.68	**0.014**	13.1 ± 0.71	**0.024**	13.3 ± 0.71	**0.009**	12.2 ± 0.86	**0.001**	**<0.001**
% of total FA	1.53 ± 0.08	1.43 ± 0.07	n.s.	1.35 ± 0.06	n.s.	1.36 ± 0.06	n.s.	1.32 ± 0.07	**0.035**	**<0.001**
C20:4n6 μg mL^{-1}	150 ± 3.90	136 ± 2.33	n.s.	138 ± 2.69	n.s.	137 ± 3.24	**0.043**	124 ± 3.53	**0.001**	**<0.001**
% of total FA	14.8 ± 0.14	14.3 ± 0.14	**0.042**	14.3 ± 0.12	**0.035**	14.0 ± 0.12	**<0.001**	13.5 ± 0.14	**<0.001**	**<0.001**
C20:5n3 μg mL^{-1}	6.13 ± 0.51	7.33 ± 0.33	n.s.	8.38 ± 0.42	**0.021**	10.9 ± 0.67	**<0.001**	11.0 ± 0.64	**<0.001**	**<0.001**
% of total FA	0.60 ± 0.04	0.77 ± 0.03	**0.006**	0.87 ± 0.04	**0.002**	1.12 ± 0.06	**<0.001**	1.20 ± 0.06	**<0.001**	**<0.001**
C22:0 μg mL^{-1}	17.7 ± 0.56	16.6 ± 0.39	n.s.	16.9 ± 0.39	n.s.	16.9 ± 0.54	n.s.	15.3 ± 0.51	**0.026**	**<0.001**
% of total FA	1.75 ± 0.04	1.75 ± 0.04	n.s.	1.75 ± 0.04	n.s.	1.73 ± 0.04	n.s.	1.67 ± 0.03	n.s.	**0.020**
C22:1n9 μg mL^{-1}	2.42 ± 0.16	2.81 ± 0.30	n.s.	2.41 ± 0.16	n.s.	2.34 ± 0.23	n.s.	1.90 ± 0.20	n.s.	**0.016**
% of total FA	0.24 ± 0.02	0.29 ± 0.03	n.s.	0.25 ± 0.02	n.s.	0.25 ± 0.03	n.s.	0.21 ± 0.02	n.s.	**0.042**
C22:4n6 μg mL^{-1}	31.6 ± 1.01	31.6 ± 1.14	n.s.	30.7 ± 1.02	n.s.	29.3 ± 1.15	n.s.	25.1 ± 1.07	**<0.001**	**<0.001**
% of total FA	3.13 ± 0.11	3.20 ± 0.10	n.s.	3.18 ± 0.09	n.s.	2.99 ± 0.08	n.s.	2.73 ± 0.07	**<0.001**	**<0.001**
C22:5n3 μg mL^{-1}	25.9 ± 0.82	25.2 ± 0.59	n.s.	28.2 ± 0.80	n.s.	30.2 ± 1.03	**0.033**	32.3 ± 1.35	**0.014**	**<0.001**
% of total FA	2.55 ± 0.05	2.64 ± 0.05	n.s.	2.92 ± 0.06	**<0.001**	3.09 ± 0.07	**<0.001**	3.52 ± 0.10	**<0.001**	**<0.001**
C24:0 μg mL^{-1}	45.6 ± 1.25	45.3 ± 0.81	—	46.6 ± 1.04	—	47.1 ± 1.15	—	44.0 ± 1.34	—	n.s.
% of total FA	4.50 ± 0.08	4.75 ± 0.08	**0.001**	4.83 ± 0.10	**<0.001**	4.83 ± 0.08	**0.007**	4.81 ± 0.10	**0.004**	**<0.001**

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Table 3 (*Contd.*)

	Week 0 Mean ± SE	Week 1 Mean ± SE	*t*-Test[a] *p* (week 1–week 0)	Week 3 Mean ± SE	*t*-Test[a] *p* (week 3–week 0)	Week 6 Mean ± SE	*t*-Test[a] *p* (week 6–week 0)	Week 12 Mean ± SE	*t*-Test[a] *p* (week 12–week 0)	An reM[b] *p*
C22:6n3 μg mL^{-1}	41.0 ± 1.93	37.0 ± 1.32	n.s.	36.1 ± 1.37	n.s.	35.1 ± 1.06	**0.010**	30.4 ± 1.09	**<0.001**	**<0.001**
% of total FA	4.03 ± 0.16	3.90 ± 0.15	n.s.	3.74 ± 0.13	n.s.	3.62 ± 0.12	**0.017**	3.33 ± 0.11	**0.001**	**<0.001**
C24:1n9 μg mL^{-1}	51.8 ± 1.88	50.4 ± 1.13	n.s.	50.6 ± 1.03	n.s.	50.0 ± 1.65	n.s.	44.4 ± 1.52	**0.019**	**<0.001**
% of total FA	5.10 ± 0.11	5.28 ± 0.10	**0.003**	5.25 ± 0.09	**0.035**	5.11 ± 0.11	n.s.	4.84 ± 0.09	n.s.	**<0.001**
ΣTFA μg mL^{-1}	1016 ± 22.0	955 ± 12.1	n.s.	966 ± 17.3	n.s.	977 ± 22.2	n.s.	918 ± 26.1	n.s.	**0.015**
ΣSFA μg mL^{-1}	427 ± 8.49	408 ± 5.39	—	411 ± 7.59	—	418 ± 10.3	—	399 ± 11.3	—	n.s.
% of total FA	42.1 ± 0.16	42.8 ± 0.24	n.s.	42.5 ± 0.16	n.s.	42.7 ± 0.16	**0.001**	43.5 ± 0.23	**<0.001**	**<0.001**
ΣMUFA μg mL^{-1}	210 ± 6.24	191 ± 3.68	n.s.	192 ± 3.80	n.s.	193 ± 5.53	n.s.	179 ± 5.89	**0.019**	**<0.001**
% of total FA	20.7 ± 0.28	20.0 ± 0.23	n.s.	19.9 ± 0.24	n.s.	19.7 ± 0.24	**0.023**	19.4 ± 0.23	**0.003**	**<0.001**
ΣPUFA μg mL^{-1}	378 ± 8.35	355 ± 4.74	n.s.	364 ± 7.20	n.s.	367 ± 7.43	n.s.	340 ± 9.73	n.s.	**0.017**
% of total FA	37.3 ± 0.23	37.2 ± 0.24	—	37.7 ± 0.20	—	37.6 ± 0.26	—	37.1 ± 0.26	—	n.s.
Σn3 PUFA μg mL^{-1}	74.4 ± 2.83	74.2 ± 1.43	n.s.	78.1 ± 1.80	n.s.	82.4 ± 2.01	n.s.	79.4 ± 2.24	n.s.	**0.023**
% of total FA	7.32 ± 0.19	7.79 ± 0.15	**<0.001**	8.10 ± 0.13	**<0.001**	8.46 ± 0.17	**<0.001**	8.68 ± 0.13	**<0.001**	**<0.001**
Σn6 PUFA μg mL^{-1}	303 ± 6.55	280 ± 4.66	n.s.	286 ± 6.14	n.s.	285 ± 6.33	n.s.	261 ± 8.01	**0.004**	**<0.001**
% of total FA	29.9 ± 0.27	29.4 ± 0.29	n.s.	29.6 ± 0.25	n.s.	29.2 ± 0.26	n.s.	28.5 ± 0.25	**0.001**	**<0.001**
ΣEPA & DHA μg mL^{-1}	47.1 ± 2.36	46.4 ± 1.44	n.s.	44.5 ± 1.46	n.s.	46.0 ± 1.32	n.s.	41.3 ± 1.27	n.s.	**0.023**
% of total FA	4.63 ± 0.19	4.67 ± 0.16	—	4.61 ± 0.13	—	4.73 ± 0.15	—	4.52 ± 0.11	—	n.s.
Σn6/Σn3 PUFA	4.15 ± 0.13	3.81 ± 0.10	**<0.001**	3.68 ± 0.08	**0.002**	3.48 ± 0.09	**<0.001**	3.30 ± 0.07	**<0.001**	**<0.001**
AA/EPA	26.5 ± 1.55	19.3 ± 0.90	**<0.001**	17.0 ± 0.69	**<0.001**	13.3 ± 0.80	**<0.001**	11.8 ± 0.59	**<0.001**	**<0.001**
D5D index	10.1 ± 0.51	10.3 ± 0.46	—	10.9 ± 0.47	—	10.7 ± 0.52	—	10.8 ± 0.68	—	n.s.
D6D index	0.15 ± 0.01	0.14 ± 0.01	n.s.	0.13 ± 0.01	**<0.001**	0.13 ± 0.01	**<0.001**	0.12 ± 0.01	**<0.001**	**<0.001**
% n3 in HUFA	27.0 ± 0.64	27.9 ± 0.55	**0.010**	28.6 ± 0.47	**0.002**	29.8 ± 0.49	**<0.001**	31.4 ± 0.47	**<0.001**	**<0.001**
% n6 in HUFA	73.1 ± 0.64	72.2 ± 0.55	**0.010**	71.5 ± 0.47	**0.002**	70.2 ± 0.49	**<0.001**	68.6 ± 0.47	**<0.001**	**<0.001**

The levels are shown as concentration [μg mL^{-1}] in blood and as relative amount [%] of total fatty acids at week 0, 1, 3, 6, and 12 of the high-ALA diet (14.0 ± 0.45 g d^{-1}). AA: arachidonic acid; D5D/D6D index, delta-5/6 desaturase index: calculated according to ref. 41: D5D = C20:4n6/C20:3n6 and D6D = C20:3n6/C18:2n6; EPA: eicosapentaenoic acid; HUFA: highly unsaturated fatty acids; indices of HUFA calculated as follows, modified from ref. 42: % n3 in HUFA = 100 × (C20:5n3 + C22:5n3 + C22:6n3)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3); % n6 in HUFA = 100 × (C20:3n6 + C20:4n6 + C22:4n6)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3); MUFA: monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; n.s.: not significant; SFA: saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; PUFA: polyunsaturated fatty acids: C18:2n6, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:4n6, C20:5n3, C22:4n6, C22:5n3, C22:6n3; SE: standard error; TFA: total fatty acids; Σ n3 PUFA: C18:3n3, C20:3n3, C20:5n3, C22:5n3, C22:6n3; Σ n6 PUFA: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6; wk: week. [a] *t*-Test for paired samples with Holm–Bonferroni correction; significance level $p \leq 0.05$. [b] ANOVA for repeated measures (An reM); significance level $p \leq 0.05$.

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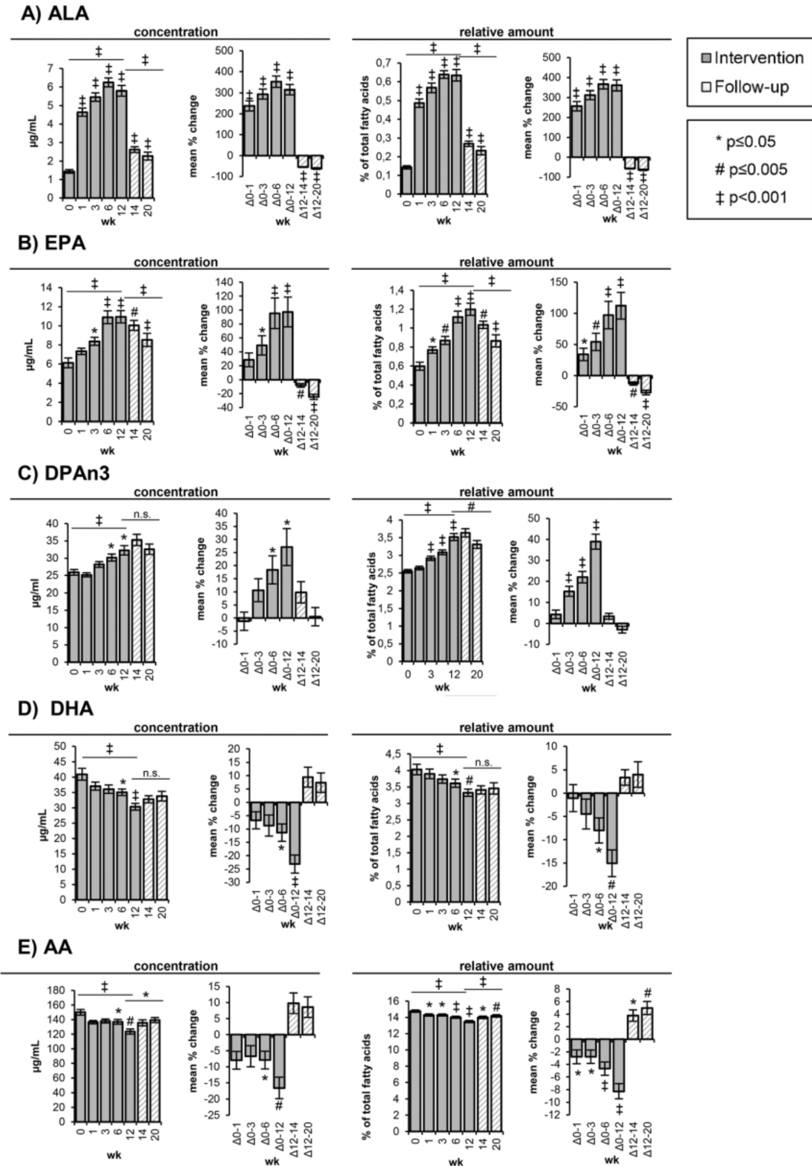

Fig. 1 Content of selected polyunsaturated fatty acids in red blood cells. Bars represent mean ± SE. The levels are shown as concentrations [μg mL^{-1}] and as relative amounts [%] of total fatty acids at week 0, 1, 3, 6, and 12 of the high-ALA diet (14.0 ± 0.45 g d^{-1}) and at week 14 and 20 of follow-up. AA: arachidonic acid (C20:4n6); ALA: alpha-linolenic acid (C18:3n3); DHA: docosahexaenoic acid (C22:6n3); DPAn3: docosapentaenoic acid (C22:5n3); EPA: eicosapentaenoic acid (C20:5n3); wk: week.

centrations decreased (p = 0.004) from 303 ± 6.55 μg mL^{-1} at week 0 to 261 ± 8.01 μg mL^{-1} at week 12 (Table 3).

The ratio of Σn6 to Σn3 PUFA significantly declined (p < 0.001) after 1 week of the high-ALA diet from 4.15 ± 0.13 (week 0) to 3.81 ± 0.10 (week 1) and further to 3.30 ± 0.07 after week 12 (Table 3). In the follow-up period, the Σn6/Σn3 PUFA ratio increased (p = 0.002) again to 3.73 ± 0.13 (week 20) (Table S3†).

Consistently, the ratio of AA to EPA dropped (p < 0.001) time-dependently from 26.5 ± 1.55 (week 0) to 11.8 ± 0.59 at week 12 and slowly increased (p < 0.001) in the follow-up period to 17.3 ± 1.06 (Table 3). The activity of D6D decreased (p < 0.001) in response to the high-ALA diet from 0.15 ± 0.01 (week 0) to 0.12 ± 0.01 at week 12 and increased (p < 0.001) again in the follow-up period (week 20: 0.17 ± 0.01) (Table 3). The percentage of n3 in HUFA increased from 27.0 ± 0.64

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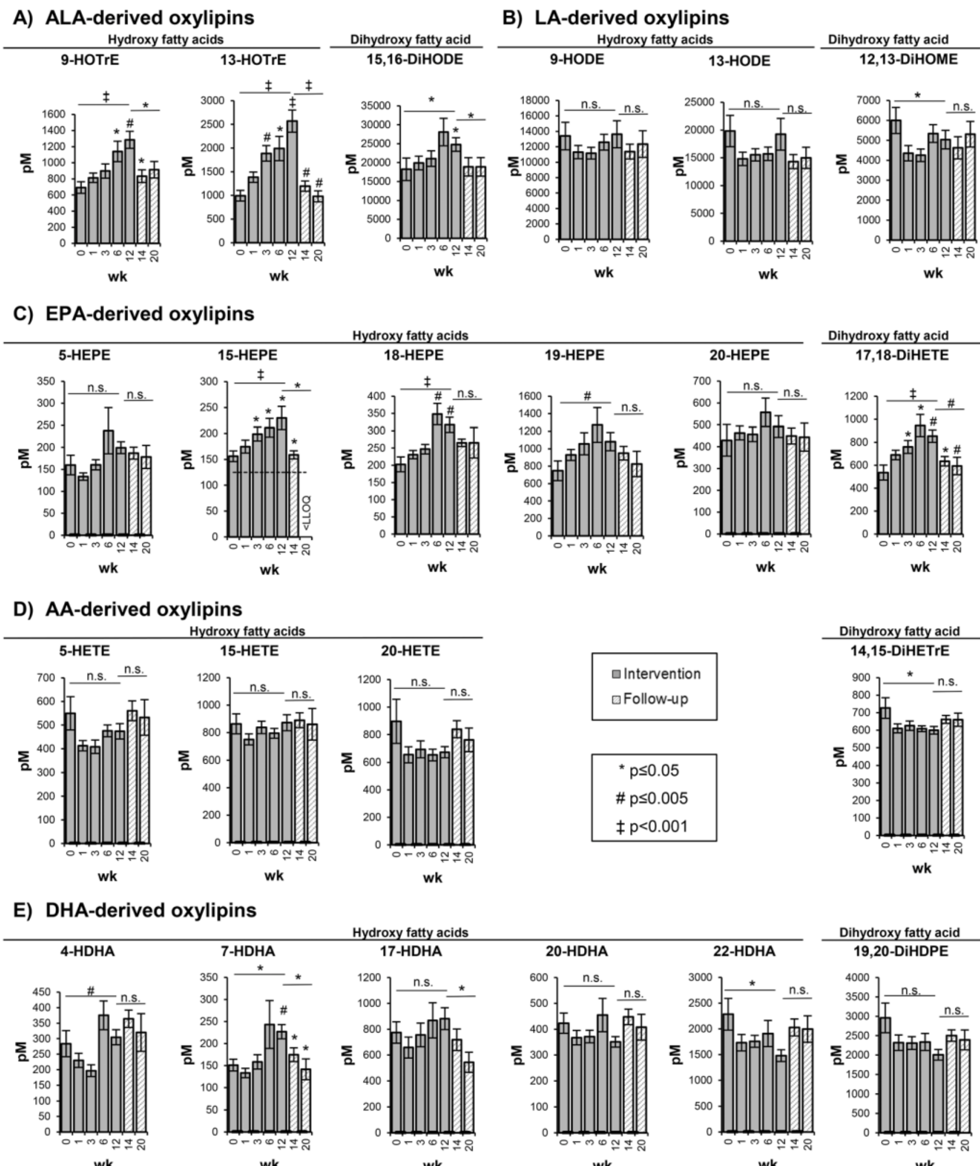

Fig. 2 Concentration of selected free oxylipins in the plasma. Bars represent mean ± SE [pM]. The plasma concentrations of free oxylipins are shown at week 0, 1, 3, 6, and 12 of the high-ALA diet (14.0 ± 0.45 g d^{-1}) and at week 14 and 20 of follow-up. AA: arachidonic acid (C20:4n6); ALA: alpha-linolenic acid (C18:3n3); DHA: docosahexaenoic acid (C22:6n3); DiHETE: dihydroxy eicosatetraenoic acid; DiHETrE: dihydroxy eicosatrienoic acid; DiHDPE: dihydroxy docosapentaenoic acid; DiHODE: dihydroxy octadecadienoic acid; DiHOME: dihydroxy octadecenoic acid; EPA: eicosapentaenoic acid (C20:5n3); HDHA: hydroxy docosahexaenoic acid; HEPE: hydroxy eicosapentaenoic acid; HETE: hydroxy eicosatetraenoic acid; HODE: hydroxy octadecadienoic acid; HOTrE: hydroxy octadecatrienoic acid; LA: linoleic acid (C18:2n6); LLOQ: lower limit of quantification; SE: standard error; wk: week.

(week 0) to 31.4 ± 0.47 (week 12), whereas the percentage of n6 in HUFA decreased from 73.1 ± 0.64 (week 0) to 68.6 ± 0.47 (week 12) following the high-ALA diet (Table 3). A reversion of this change could be observed in the follow-up period: n3 in HUFA decreased again from 31.4 ± 0.47% (week 12) to 28.9 ± 0.63% (week 20) and n6 in HUFA increased from 68.6 ± 0.47% (week 12) to 71.1 ± 0.63 (Table S3†).

Changes of oxylipin concentrations in the plasma

The changes in the concentration of free hydroxy- and dihydroxy-PUFA in the plasma were represented by the shift in concentrations of their precursor PUFAs in the RBCs. As shown exemplarily for selected oxylipins, representing the metabolites of 5-LOX, 15-LOX and CYP catalyzed epoxygena-

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tion and hydrolysis by sEH, a similar but less pronounced trend compared to the precursor PUFA was observed (Fig. 2).

5- and 15-LOX-derived hydroxy-PUFA from ALA, *i.e.* 9-HOTrE and 13-HOTrE, increased time-dependently from 693 ± 71.7 pM (week 0) to 1285 ± 106 pM (week 12) ($p = 0.001$) and from 993 ± 113 pM (week 0) to 2569 ± 234 pM (week 12) ($p < 0.001$) (Fig. 2, Table S4†). The highest concentrations of 9- and 13-HOTrE were observed at week 12. The concentration of the terminal dihydroxy-PUFA 15,16-DiHODE also increased from 18 243 ± 2970 pM (week 0) to 24 795 ± 1802 pM (week 12) ($p = 0.033$); however, it was less pronounced compared to ALA-derived hydroxy-PUFA and, though not significant, the highest concentration was observed at week 6 (28 086 ± 3631 pM).

LA-derived oxylipins remained unchanged; however, the concentrations varied in the course of the trial (Fig. 2, Table S4†). This is consistent with the LA concentration in the RBCs that did not show significant changes in response to the high-ALA diet.

For EPA-derived oxylipins, as exemplarily shown in Fig. 2 for 5-, 15-, 18- and 20-HEPE and 17,18-DiHETE, an increase from week 0 to week 6 was observed followed by a slight decrease at week 12 (except 15-HEPE).

For most of the AA-derived hydroxy- and dihydroxy-PUFA, such as 5-, 15-, 20-HETE and 14,15-DiHETrE, a slight though statistically not significant decrease in concentration after 1 week of ALA supplementation was observed (Fig. 2). Similarly, no consistent shift of DHA-derived oxylipins was observed (Fig. 2, Table S4†). Overall, the oxylipin levels were reflected by the respective precursor fatty acid concentrations in the RBCs, though changes in the oxylipin levels occurred at later time points and were, due to higher SE, less pronounced compared to the precursor fatty acids.

Discussion

Factors like age,[43,44] BMI,[44] smoking[45] and genotype[46] showed an influence on the LC n3 PUFA status, especially on the conversion from ALA to DHA. Recently, it has been shown that the activity of endogenous EPA and DHA synthesis adapted during evolution in the presence of these fatty acids in the diet.[47] Several studies observed higher circulating relative DHA amounts in women compared to men independent of dietary intake[48–50] and higher conversion rates from ALA and EPA to DHA[51] possibly due to the influence of estrogen on the PUFA metabolism.[52] Consequently, to minimize the variability, a homogeneous collective of healthy, non-smoking men within a narrow range regarding age (mean age 26.2 ± 4.53 years) and BMI (24.9 ± 2.0 kg m^{-2}) was chosen to investigate the effect of ALA on the LC n3-PUFA levels.

In addition, to study the effect of a high daily ALA intake in a Western diet on the EPA and DHA blood levels, it is crucial to choose a study collective with low baseline EPA/DHA levels, since the expected conversion of ALA to EPA and DHA is the highest compared to subjects with a moderate or high EPA/DHA status.

We selected probands basically eating a mixed Western diet with a low meat and fish consumption and explicitly screened for low blood LC n3 PUFA status. The relative ΣEPA + DHA level in RBCs, similar to omega-3 index, was 4.15 ± 0.13% of total fatty acids and comparable to the (low) mean omega-3 index of men in the U.S. and Germany which is associated with the risk of cardiovascular disease.[53]

As expected, the high-ALA diet – providing an ALA amount of about 4.7 en% – resulted in a strong increase in the ALA levels in the RBCs. The incorporation of ALA (and other PUFAs) into the RBCs is determined by the blood cell turnover (the mean life span of a red blood cell is approximately 120 days in circulation) and thus the ALA and its bioconverted longer chain n3 PUFAs EPA and DHA do not fully reach the RBCs. Nevertheless, a 238 ± 24% increase of ALA concentration in the RBCs was observed after one week.

The ratio of LA to ALA in the RBCs (74.8 ± 3.54) at baseline is much higher than expected from the dietary supply of these C18 PUFAs (LA/ALA intake ratio: 7.70 ± 3.75). Even after a 12-week intake of similarly high amounts of LA (9.38 ± 3.08 g d^{-1}) and ALA (14.0 ± 0.45 g d^{-1}) with a ratio of 0.67 ± 0.21, the ratio of LA to ALA in the RBCs remained high (17.5 ± 0.97). The possible reasons for this could be that ALA significantly differs from LA in absorption, tissue distribution, membrane incorporation, and/or degradation. Most likely, a high percentage of 60–85% of ALA is rapidly degraded by beta-oxidation[54] before it becomes available for tissue distribution and membrane integration as well as elongation and desaturation to EPA.

The high-ALA diet also affected the concentrations of other PUFAs with significantly increased EPA and DPAn3 as well as significantly decreased DHA and AA concentrations in the RBCs. Of note, the concentration of EPA was with a change of about 5 μg mL^{-1} similarly elevated as the ALA concentration. The percentage increase in the concentrations and relative amounts was more pronounced for EPA (week 12: 97 ± 21% and 112 ± 17%, respectively) compared to DPAn3 (week 12: 27 ± 7% and 39 ± 4%, respectively). It is likely that increasing EPA levels are the result of a conversion from ALA; however, it cannot be excluded that the retroconversion of DHA to DPAn3 and EPA occurred, which would also (partly) explain decreasing DHA concentrations. These results are consistent with previous studies, where a dose-dependent and higher increase of EPA compared to DPAn3 was observed in the RBCs[55,56] and plasma and platelet phospholipids.[35] However, supplementing EPA is more efficacious compared to ALA in raising the EPA blood levels.[19] A six-week supplementation of 600 mg EPA per day, which corresponds to a ~10-fold increase compared to the median intake in Germany, resulted in a 138% increase of the relative serum phospholipid EPA amount.[57] A higher dose of 2.0 g EPA per day caused a 325% increase of the relative EPA amount in the RBCs.[58]

The initial ALA concentrations were also low (1.44 ± 0.10 μg mL^{-1}) compared to the EPA concentrations (6.13 ± 0.51 μg mL^{-1}) and the ratio of ALA to EPA increased in the first three weeks and slightly decreased thereafter. The high-ALA diet led to a strong increase of ALA in the RBCs. However, as

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discussed before, with increasing ALA concentrations, the rate of ALA catabolism increases[24] as observed in the moderate drop of the ALA levels between week 6 and week 12. This may also explain the steady state in the EPA concentrations between week 6 (10.9 ± 0.67 μg mL^{-1}) and week 12 (11.0 ± 0.64 μg mL^{-1}).

The observed reduction of the DHA levels is surprising and in contrast to earlier studies, which determined a conversion (rate) of ALA to DHA of 0.5–5%.[16–21] However, in these studies, the ingested ALA amount was low (1.0–3.5 g)[17,59] compared to ours. A study with a similar design as our study including a high daily ALA dose (15.4 ± 7.5 g) showed no significant effect on relative DHA amounts in plasma and platelet phospholipids,[35] possibly due to low sample size (n = 7), an inhomogeneous initial LC n3 PUFA status and a highly variable ALA intake (high SD). In contrast, we observed significantly decreased DHA concentrations and relative amounts in the RBCs in response to the high-ALA diet. It should be noted that the biological variability of fatty acid concentrations in the RBCs is small compared to plasma and plasma phospholipids.[60] Nonetheless, several studies indicated a tendency for declining DHA levels in response to high ALA consumption, which was in most cases marginal and not statistically significant.[35,61–69] One possible explanation for the decreasing DHA concentrations in the RBCs may be that DHA accumulates in the nerve cells of the brain.[70] While human studies are limited to blood as the medium of investigation, animal studies demonstrated that the conversion of ALA to EPA and DHA is tissue specific. In a rat study with high-ALA chia seed supplementation, the accumulation of DHA in the heart and liver was observed, while the plasma DHA concentrations remained constant.[71]

In our study, the ALA intake from the background diet was tightly controlled and hence the variability of total daily ALA intake in the intervention period was low (~1.9 g). Only a few other studies found a significant reduction of the relative DHA levels in mononuclear cell phospholipids[66] and platelet phosphatidylcholine[69] in response to an ALA enriched diet. However, the LA amount in the diet of the studies by Kew *et al.* (13.1 and 16.2 g d^{-1})[66] and Weaver *et al.* (22.5 g d^{-1})[69] was much higher than in our study (<10 g) based on food questionnaires (Table 2).

The conversion efficiency of ALA to DHA appears to be affected by a high LA, ALA and total PUFA intake. A rat study observed the highest conversion of ALA to DHA as a result of feeding a narrow dietary range of 1–2 en% LA and 1–3 en% ALA, while the DHA levels were suppressed to basal levels (~2% total fatty acids) with the total PUFA levels above 3 en%.[72] Excessive LA and ALA compete with LC n3 PUFAs for the rate-limiting enzyme Δ6-desaturase.[73] Δ6-desaturase catalyzes the desaturation of LA to GLA, of ALA to stearidonic acid (C18:4n3) and also of tetracosapentaenoic acid (C24:5n3) to tetracosahexaenoic acid (C24:6n3), which is finally shortened to form DHA by peroxisomal β-oxidation.[15]

The intake of LA, AA, EPA, DPAn3, and DHA did not significantly change during the intervention compared to baseline (3-day dietary questionnaires Table 2). In addition, the ALA intake from the background diet (minus the ALA intake *via* the daily linseed oil ingestion) did not change between week 0, week 6 and week 12. On the other hand, the intake of EPA, DPAn3, and DHA *via* the background diet was slightly decreased, possibly due to the advice to avoid oily fish meals during the intervention time. However, it seems unlikely that this statistically insignificant decline caused the observed decrease in the DHA concentrations in the RBCs.

However, it should be noted that estimates of dietary fat intake relied on self-reported data and are potentially biased by food choice, incomplete dietary protocols and methodological limitations associated with accurate fatty acid composition data in food databases.[74]

The beneficial health effects of LC n3 PUFA are believed to be (partly) mediated by oxidized mediators formed in the AA cascade.[10] A correlation between higher levels of precursor n3 PUFAs (*e.g.* EPA and DHA) and their oxylipins was demonstrated in different intervention studies.[75–79] Accordingly, in the present study, the changes of the oxylipin levels in the plasma are generally reflected by the changes of the respective precursor fatty acids in the RBCs. As expected, the levels of ALA-derived oxylipins increased in response to the higher dietary intake of ALA. In contrast to the ALA concentrations in the RBCs, which increased more than 3-fold after only 1 week of ALA supplementation, ALA-derived oxylipins were only slightly but not significantly elevated. Moreover, whilst the ALA concentration in the RBCs seemed to reach a steady state after 6 weeks of the high-ALA diet, ALA-derived oxylipins, such as hydroxy-PUFA 9- and 13-HOTrE, increased steadily until week 12 up to 1.9-fold and 2.6-fold, respectively. The slower and less pronounced rise of ALA-derived oxylipins compared to their precursor fatty acid was not observed for DHA-derived oxylipins compared to the blood cell concentrations of DHA in a similar study.[78,79] A possible explanation is the lower baseline concentration of ALA compared to DHA, which might have led to a more rapid increase and the higher supplemented dose.[77]

With regard to oxylipin formation, ALA is mostly discussed as a precursor of LC n3 PUFA;[80] therefore, the biological role of ALA-derived oxylipins is only poorly understood. Some studies demonstrated positive biological effects of ALA-derived oxylipins;[81,82] however, further investigation of these mediators needs to be carried out as ALA-derived oxylipins are present in relevant concentrations in humans on a Western diet.[10]

Consistent with the elevated levels of EPA in the RBCs (1.8-fold increase at week 6), a high-ALA diet leads to increasing concentrations of EPA-derived hydroxy- and dihydroxy-PUFA in the plasma (~1.3- to 1.8-fold at week 6). The higher levels of EPA-derived oxylipins upon a high-ALA diet may have beneficial health effects, *e.g.* 18-HEPE – a precursor of pro-resolving and anti-inflammatory E-series resolvins[83] – concentrations increased 1.7-fold at week 6. However, it has to be noted that direct supplementation with EPA raises the EPA-derived oxylipin levels more efficiently.[84]

Although the DHA concentrations in the RBCs were significantly lowered in response to the high-ALA diet, the DHA-derived oxylipin levels showed no consistent shift towards lower levels.

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The conversion rates of ALA to EPA and DHA as well as the formation of oxylipins from n3 PUFA are influenced by the presence of n6 PUFA competing for the same enzymes.[15,85] Several studies demonstrated that n3 PUFA supplementation leads to declining AA and AA-derived oxylipin concentration; however, the results were heterogeneous between different intervention studies.[84] Despite the significant decrease of AA in the RBCs in the present study, only a slight but not significant decline of AA-derived oxylipins, *e.g.* hydroxy-PUFA 5- and 20-HETE, was observed, while no effect was observed for, *e.g.*, the 15-LOX product 15-HETE.

Similar results were obtained for LA-derived oxylipins and no relevant reduction in response to high-ALA intake was observed. Most likely, the excess of LA in the diet (and consequently in the RBC membrane concentration) was too high to be modified by a high-ALA diet. A decrease of the LA/ALA ratio (from 74.8 ± 3.54 (week 0) to 17.5 ± 0.97 (week 12)) and the ratio of LOX-derived hydroxy-PUFA 9-HODE/9-HOTrE (19.4 ± 1.04 (week 0) to 10.6 ± 0.68 (week 12)) and 13-HODE/13-HOTrE (from 20.5 ± 1.39 (week 0) to 7.73 ± 0.78 (week 12)) results from elevated ALA and ALA-derived oxylipin concentrations with constant LA and LA-derived oxylipin concentrations. A reduction of LA and its oxylipins is assumed to be beneficial, as negative health effects were observed for LA metabolites such as sEH products of the CYP-derived epoxy-PUFA.[86–88] A reduction of the LA metabolite 9,10-DiHOME by supplementation with a lower (6 g d^{-1}) dose of ALA compared to our study was demonstrated by Caligiuri *et al.* in young individuals (19–28 years),[89] after only 4 weeks of the intervention. However, the participants had to abstain from dietary oils, which might have altered their normal eating habits, thus, leading to shifts in the fatty acid and oxylipin pattern.

Conclusions

Our results demonstrate that a high-ALA diet of 14.0 ± 0.45 g day^{-1} – which is 8–12 times higher than the common intake recommendation for this essential fatty acid – results in a significant increase in ALA, EPA and DPAn3 concentrations in RBCs and a significant decline in DHA concentrations. However, the ΣEPA + DHA concentration in RBCs – which is associated with cardiac, cerebral, and general health status – was not affected in response to a high-ALA diet. The changes in the plasma oxylipin levels were generally reflected by their precursor fatty acids in RBCs. The high-ALA diet failed to modulate LA and LA-derived oxylipins. Our results demonstrate on both the fatty acid as well as the oxylipin level that on a Western diet (with high LA intake), ALA is not a significant source for endogenous EPA and DHA levels.

Abbreviations

AA	Arachidonic acid
ALA	Alpha-linolenic acid
BMI	Body mass index
COX	Cyclooxygenase
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DiHETE	Dihydroxy eicosatetraenoic acid
DiHETrE	Dihydroxy eicosatrienoic acid
DiHODE	Dihydroxy octadecadienoic acid
DiHOME	Dihydroxy octadecenoic acid
DPAn3	n3 docosapentaenoic acid
D5D	Delta-5 desaturase
D6D	Delta-6 desaturase
en%	Percent of total energy
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
HDHA	Hydroxy docosahexaenoic acid
HDL	High density lipoprotein
HEPE	Hydroxy eicosapentaenoic acid
HETE	Hydroxy eicosatetraenoic acid
HODE	Hydroxy octadecadienoic acid
HOTrE	Hydroxy octadecatrienoic acid
HUFA	Highly unsaturated fatty acids
IS	Internal standard
LA	Linoleic acid
LC	Long chain
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LLOQ	Lower limit of quantification
LOX	Lipoxygenases
MUFA(s)	Monounsaturated fatty acid(s)
n.s.	Not significant
n3	Omega-3
n6	Omega-6
PUFA(s)	Polyunsaturated fatty acid(s)
RBCs	Red blood cells
SD	Standard deviation
SE	Standard error
sEH	Soluble epoxide hydrolase
SFA(s)	Saturated fatty acid(s)
TC	Total cholesterol
TG	Triglycerides
wk(s)	Week(s)

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This study was supported by grants from the German Research Foundation (Grant SCHE 1801 and SCHU 2516) to NHS and JPS.

We would like to thank the participants who contributed their time to this project.

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3. Paper II

Effects of a low and a high dietary LA/ALA ratio on long-chain PUFA concentrations in red blood cells

Authors: Greupner T, Kutzner L, Pagenkopf S, Kohrs H, Hahn A, Schebb NH, Schuchardt JP

Published in: *Food & Function*, 9 (2018), 4742-4754

Link: *https://pubs.rsc.org/en/content/articlehtml/2018/fo/c8fo00735g*

Food &
Function

PAPER

View Article Online
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Cite this: DOI: 10.1039/c8fo00735g

Effects of a low and a high dietary LA/ALA ratio on long-chain PUFA concentrations in red blood cells†

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Received 17th April 2018,
Accepted 7th August 2018

DOI: 10.1039/c8fo00735g

rsc.li/food-function

There is a debate about the optimal dietary ratio of the parent n6 fatty acid linoleic acid (LA) and n3 fatty acid alpha-linolenic acid (ALA) to promote an efficient conversion of ALA to EPA and DHA, which have implications for human health. The aim of the present study was to compare the effects of a low-LA/high-ALA ($_{lo}$LA/$_{hi}$ALA) diet with a high-LA/low-ALA ($_{hi}$LA/$_{lo}$ALA) diet on fatty acid concentrations in red blood cells (RBCs). Fifteen omnivore healthy men (mean age 26.1 ± 4.5 years) with a low initial EPA/DHA status (sum (∑) EPA + DHA% of total fatty acids in RBC at baseline: 4.03 ± 0.17) received both diets for two weeks with a nine-week wash-out phase in between. Fatty acid intake of the subjects was tightly controlled. Concentrations [µg mL^{-1}] and relative amounts [% of total fatty acids] of fatty acids in RBCs were analyzed at baseline (day 0), day 7 and 14 by means of GC-FID. The dietary LA/ALA ratios were 0.56 ± 0.27 : 1 and 25.6 ± 2.41 : 1 and led to significantly different changes of ALA, LA, EPA and ∑EPA + DHA concentrations in RBCs. In the course of the $_{lo}$LA/$_{hi}$ALA diet ALA and EPA concentrations and relative amounts of ∑EPA + DHA increased, whereas LA concentrations decreased. The DHA concentration was unaffected. The $_{hi}$LA/$_{lo}$ALA diet led to slightly decreased EPA concentrations, while all other fatty acid concentrations remained constant. Compared to our previous study, where we simply increased the ALA intake, our results show that ALA supplementation combined with a reduced LA intake ($_{lo}$LA/$_{hi}$ALA diet) more efficiently enhanced EPA blood concentrations. The absence of changes in the PUFA pattern in consequence of a LA/ALA ratio of 25.6 ± 2.41 : 1 suggests that the high LA/ALA ratio of the Western diet already leads to a saturation and a further increase of the ratio does not affect the PUFA pattern.

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Introduction

The long chain (LC) omega-3 (n3) polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3) are known for their beneficial health effects mainly with regard to cardiovascular[1–5] and cognitive health.[6–8] Dietary sources of EPA and DHA are limited and in the Western diet intake of these fatty acids is far below the recommendations. Accordingly, blood levels of EPA and DHA are low.[9]

The essential n3 precursor fatty acid alpha-linolenic acid (ALA, C18:3n3) is present in high amounts in some plant oils, particularly linseed-, chia-, perilla- and walnut oil and can be converted into EPA and DHA in a multistep elongation and desaturation reaction.[10] However, the efficiency of this process is generally low in adult humans.[11] It is, *inter alia*, influenced by the intake of the n6 precursor fatty acid linoleic acid (LA, C18:2n6) due to competition for the same desaturation and elongation enzymes[12,13] and for incorporation into cell membranes.[14] The intake of LA has increased substantially in Western diets during the last century.[15,16] Consequently, the dietary ratio of the n6 fatty acid LA to the n3 fatty acid ALA is about 10–20:1 (ref. 17–19) which is viewed as unfavorable and may result in an inefficient conversion of ALA to the physiologically important n3 PUFAs EPA and DHA.[12,13] A reduction of LA has been suggested to enhance the conversion of ALA to the longer chain n3 PUFAs EPA and DHA. Therefore, the biological efficacy of n3 PUFAs is improved and at the same time production of n6 derived pro-inflammatory mediators is decreased.[20]

The aim of our study was to compare two extreme ratios of LA to ALA involving a low-LA/high-ALA ($_{lo}$LA/$_{hi}$ALA) diet with a ratio of 0.5–1 : 1 and a high-LA/low-ALA ($_{hi}$LA/$_{lo}$ALA) diet with a

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ratio of LA 20–30 : 1 on fatty acid concentrations in red blood cells (RBCs) with special emphasis on EPA and DHA. To achieve the two dietary LA/ALA ratios, the fatty acid supply of the subjects was tightly controlled *via* a multistep method. A homogenous collective of healthy men in a narrow age class was chosen to minimize potential fluctuations due to age[21,22] and hormonal influences.[23]

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Material and methods

Study design

This investigator-initiated study was conducted according to the guidelines laid down in the Declaration of Helsinki. The ethic committee at the medical chamber of Lower Saxony (Hannover, Germany) approved all procedures. Written informed consent was obtained from all subjects. The study is registered in the German clinical trial register (no. DRKS00011199).

The study was conducted at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany. It consisted of a screening phase, two four-week run-in phases, a nine-week wash-out phase and two 14-day intervention phases (Fig. 1). In each intervention phase three examinations were carried out: at baseline (day 0), after 7 (day 7) and after 14 days (day 14). In the run-in phases the participants were requested to abstain from fish, seafood, and ALA-rich vegetable oils such as linseed oil or chia seeds. The aim of the intervention periods was to obtain two different dietary ratios of LA to ALA (0.5–1 : 1 and 20–30 : 1). Due to the cross-over design, each subject acted as its own control, which minimizes interindividual variability of blood fatty acid levels as well as potential fluctuations regarding dietary intakes between individuals.

The control of the fatty acid intake was achieved by the following measures:

(A) Daily provision of the lunch meal by the Institute of Food Science and Human Nutrition, Leibniz University Hannover. The aim was to provide the same quantities of PUFAs to the participants. The daily freshly cooked lunch was low in LA and practically free of n3 fatty acids including ALA, EPA and DHA. The lunch meals were identical in both intervention phases. Subjects were allowed to eat lunch meals *ad libitum*. The consumed portions were all weighed.

(B) Complete replacement and standardization of the spreadable fat. Participants had to consume 60 g of provided margarine per week in both intervention phases, which should replace other spreadable fat. The margarine had a fat content of 74.3 g per 100 g with a LA content of 15.2% and an ALA content of 6.8%. *Via* margarine, participants therefore consumed 0.97 g LA and 0.43 g ALA per day.

(C) Daily supplementation of an ALA- and a LA-rich vegetable oil to adjust the intake of LA and ALA of the participants. In the intervention period with the $_{lo}$LA/$_{hi}$ALA diet subjects daily ingested 22.3 g of linseed oil with an ALA content of 55.9% of total fatty acids (Table 1) resulting in a daily ALA intake from linseed oil of 12.5 g per day. In the intervention period with the $_{hi}$LA/$_{lo}$ALA diet, subjects ingested a daily dose of 22.3 g of sunflower oil with a LA content of 62.5% (Table 1). Hence, daily LA intake from sunflower oil was 13.9 g per day.

(D) Subjects were instructed to consume a low-fat and PUFA-poor diet containing no vegetable fats, but lots of fruit and vegetables, low-fat dairy products and white flour products. The subjects were provided with take-away foods (*e.g.*

Table 1 Fatty acid profile (% of total fatty acids) of the margarine, linseed and sunflower oil used in the study (own analysis)

Fatty acid	Common name	Margarine	Linseed oil	Sunflower oil
C12:0	Lauric acid	6.2	—	—
C14:0	Myristic acid	2.5	—	—
C16:0	Palmitic acid	22.1	6.0	6.1
C18:0	Stearic acid	2.4	4.9	3.2
C18:1n9	Oleic acid	44.8	19.3	28.2
C18:2n6	Linoleic acid	15.2	13.9	62.5
C18:3n3	α-Linolenic acid	6.8	55.9	—
C20:0	Arachidic acid	—	—	—
C22:0	Behenic acid	—	—	—
C24:0	Lignoceric acid	—	—	—

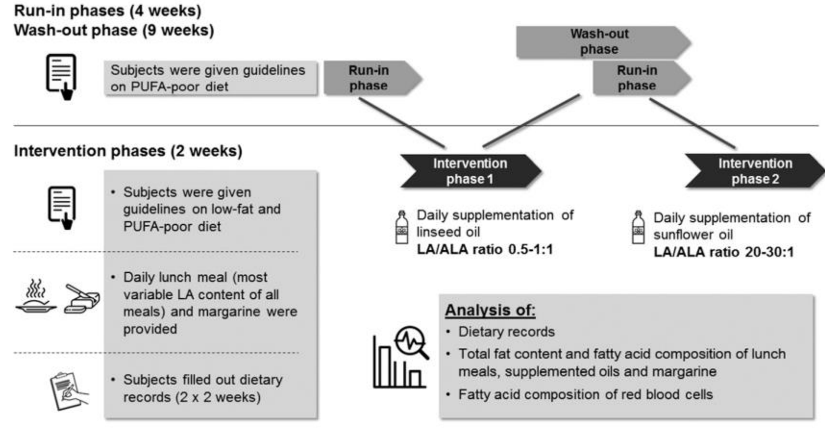

Fig. 1 Schematic presentation of the study methods. ALA: α-linolenic acid; LA: linoleic acid; PUFA: polyunsaturated fatty acids.

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fruits, vegetable-soups, white bread) to help them comply with the PUFA-poor diet. The participants were requested to maintain their diet unchanged during both intervention phases.

The subjects had to record the consumed foods and drinks during both intervention phases in (daily) nutritional records. The nutritional records were checked daily for the requested nutrition, completeness, readability and plausibility by nutritionists and, if necessary, ambiguities were clarified directly with the subjects. The analysis of energy and nutrient intake was implemented using PRODI® (Nutri-Science GmbH, Freiburg, Germany). In addition, the fat content and the fatty acid composition of the provided lunch was analyzed by GC and included in the PRODI® analysis.

Study population

Participants were recruited from the general population in Hannover, Germany by advertisements. Subjects were pre-selected *via* screening questionnaires according to the following inclusion criteria: Male sex, age between 20 and 40 years, body mass index (BMI) between 20 and 27 kg m^{-2}, mixed diet with low meat and fish consumption. Exclusion criteria were defined as followed: Smoking, serum triglyceride (TG) levels ≥150 mg dl^{-1} (≥1.7 mmol l^{-1}); serum total cholesterol levels ≥200 mg dl^{-1} (≥5.2 mmol l^{-1}); a relative amount of ∑EPA + DHA in red blood cells ≤3 and ≥6%, intake of fish (>2 times per week) as well as addiction to alcohol, drugs and/or medications and diseases: chronic diseases (*e.g.* malignant tumors, manifest cardiovascular disease, insulin-dependent type 1 and 2 diabetes, severe renal or liver diseases); chronic gastrointestinal disorders (especially small intestine, pancreas, liver) as well as prior gastrointestinal surgical procedures (*e.g.* gastrectomy); hormonal disorders (*e.g.* Cushing's syndrome and untreated hyperthyroidism); uncontrolled hypertension; blood coagulation disorders and intake of coagulation-inhibiting drugs; periodic intake of laxatives; intake of anti-inflammatory drugs (incl. acetylsalicylic acid); intake of lipid lowering drugs or supplements during the last 3 months before baseline examination. Inclusion and exclusion criteria were assessed *via* questionnaires. The pre-selected subjects were invited for a screening examination to collect fasting blood for the analysis of serum lipid levels, liver enzymes and fatty acid patterns in RBC.

Proband examination, blood sampling and pre-analytical procedures

During each examination, fasting blood was collected, blood pressure was measured and subjects completed a questionnaire to obtain information about changes in medication and lifestyle habits (*e.g.* physical activity), as well as the tolerability of linseed and sunflower oil. Blood samples were obtained by venipuncture of an arm vein using Multiflyneedles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt). All six examinations, including blood sampling, were performed at the same time for each subject. For analysis of fatty acids in RBCs, the cell sediment after centrifugation for 10 min at 1500*g* and 4 °C and removal of plasma was reconstituted in PBS to the initial blood volume, transferred into 1.5 mL Eppendorf tubes and immediately frozen and stored at −80 °C until extraction and analysis. All transfer steps were carried out on ice. Serum lipid levels, liver enzymes and small blood picture were determined in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany.

Fatty acid analyses

The total fat content of food samples was determined by gravimetry after lipid extraction according to Weibull-Stoldt performed as rapid microextraction.[24] Fatty acids in blood cells were analyzed as fatty acid methyl esters (FAME) by means of gas chromatography with flame ionization detection (GC-FID) on a 6890 series GC instrument (Agilent, Waldbronn, Germany) as described[25] with slight modifications. In brief, 10 µL internal standard (methyl pentacosanoate, FAME C25:0, 750 µM) was added to 100 µL of resuspended blood cells. Lipids were extracted with MTBE/MeOH and the lipid extract was derivatized with methanolic hydrogen chloride. The resulting FAMEs were separated on a FAMEWAX capillary column (30 m, 0.25 mm ID, 0.25 µm d_f; Restek, Bad Homburg, Germany) and quantification of FAMEs was based on response factors. Fatty acid concentrations in food samples were calculated as g fatty acid per 100 g fat. Fatty acid concentrations were quantified in whole blood cells, which are 99% RBCs. In RBC samples additionally to the concentration expressed as µg fatty acid per mL blood, the relative amount (% of total fatty acids) of each fatty acid was calculated directly based on peak areas.[25]

Calculations and statistics

Results of anthropometrical measures, serum lipid levels and dietary energy and fat intake are stated as mean ± standard deviation (SD), while PUFA levels in RBCs and its relative change (%) are stated as mean ± standard error (SE). If the concentration of an analyte was below the lower limit of quantification (LLOQ) in more than 50% of the samples at one time point, the LLOQ is given for this analyte. Relative changes of the variables (v) were calculated individually for each subject at each time point (x) as $\Delta\%$, calculated by: $\Delta\% = 100 \times (vt_x - vt_0)/vt_0$.

The distributions of the sample sets were analyzed by means of the Kolmogorov–Smirnov test. *t*-Tests for paired samples were used to determine statistical significance between the two interventions at baseline (day 0), after seven days (day 7) and after 14 days (day 14). To examine differences between the two interventions, two-factorial ANOVAs with repeated measurements of both factors were used. One-factorial ANOVAs with repeated measurements were carried out to examine the effect of time within the two interventions (day 0, day 7, day 14) separately for each intervention. *Post-hoc t*-tests for paired samples with Holm–Bonferroni-adjusted levels of significance were used to evaluate differences between the time points. Statistical significance was set at $p \leq 0.05$ for all analyses. All statistical analyses were carried out with SPSS software (Version 24, SPSS Inc., Chicago, IL, USA).

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Results

Study population

Fifteen male subjects met the criteria and thus were included in the study. All participants (mean age 26.1 ± 4.53 years) were healthy and had a normal BMI (24.0 ± 1.65 kg m^{-2}) and serum lipid pattern (Table 3). Before the beginning of the first four-week run-in phase, the study collective consumed a normal mixed diet (including 2–3 portions of meat per week) with low fish consumption (≤1 serving fish per week) and low fruit and vegetable consumption (1–2 portions per day) and had a medium physical activity status (3–5 hours of sports per week) and a high education level (all participants had the general matriculation standard). All 15 participants completed the two intervention periods and attended at all six examinations.

During the intervention periods, mean fruit and vegetable consumption increased to 5 portions per day, meat intake decreased to 2 portions per week and fish was not consumed during the intervention periods.

The examination of the dietary records combined with analysis of fatty acids in the lunch meal showed that the LA intake was 7.30 ± 0.37 g d^{-1} (2.78 en%) during $_{lo}$LA/$_{hi}$ALA diet and 18.2 ± 0.54 g d^{-1} (6.95 en%) during $_{hi}$LA/$_{lo}$ALA diet, while the ALA intake was 13.1 ± 0.22 g d^{-1} (4.98 en%) during $_{lo}$LA/$_{hi}$ALA diet and 0.71 ± 0.09 g d^{-1} (0.27 en%) during $_{hi}$LA/$_{lo}$ALA diet. The actual dietary LA/ALA ratios were therefore 0.56 ± 0.27 : 1 in the $_{lo}$LA/$_{hi}$ALA diet and 25.6 ± 2.41 : 1 in the $_{hi}$LA/$_{lo}$ALA diet (Table 2). Intake of arachidonic acid (AA), EPA, DPAn3 and DHA was very low and did not differ between both intervention periods. Total PUFA intake was significantly higher during $_{lo}$LA/$_{hi}$ALA diet (22.5 ± 0.64 g d^{-1}) compared to $_{hi}$LA/$_{lo}$ALA diet (18.5 ± 1.11 g d^{-1}), while saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) intake were marginally, but significantly, higher during $_{hi}$LA/$_{lo}$ALA diet. However, fat intake as well as energy, protein, and carbohydrate intake did not differ between both intervention periods (Table 2).

Table 2 Daily energy, macronutrient and fatty acid intake of the study participants during the $_{lo}$LA /$_{hi}$ALA diet and $_{hi}$LA/$_{lo}$ALA diet from 14-day dietary records

	$_{lo}$LA /$_{hi}$ALA Mean ± SD	$_{hi}$LA/$_{lo}$ALA Mean ± SD	*t*-Test[a]
Energy intake[b] (kcal)	2444 ± 327	2436 ± 340	n.s.
Protein[b] (g)	95.5 ± 16.1	95.9 ± 16.4	n.s.
Carbohydrates[b] (g)	325 ± 54.2	322 ± 54.2	n.s.
Total fat intake[b] (g)	67.6 ± 5.43	68.5 ± 6.93	n.s.
SFA[b] (g)	17.3 ± 2.23	19.4 ± 2.26	0.040
MUFA[b] (g)	10.2 ± 0.93	11.8 ± 1.41	<0.001
PUFA[b] (g)	22.5 ± 0.64	18.5 ± 1.11	<0.001
LA[c] (g)	7.30 ± 0.37	18.2 ± 0.54	<0.001
ALA[c] (g)	13.1 ± 0.22	0.71 ± 0.09	<0.001
LA/ALA[c]	0.56 ± 0.27 : 1	25.6 ± 2.41 : 1	—
AA[c] (g)	0.02 ± 0.01	0.02 ± 0.01	n.s.
EPA[c] (g)	0.00 ± 0.00	0.00 ± 0.00	n.s.
DPAn3[c] (g)	0.01 ± 0.01	0.01 ± 0.01	n.s.
DHA[c] (g)	0.03 ± 0.01	0.02 ± 0.01	n.s.

AA: arachidonic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; DPAn3: n3 docosapentaenoic acid; LA: linoleic acid; MUFA: monounsaturated fatty acids; n.s.: not significant; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids. [a] *t*-Test for paired samples; significance level $p \leq 0.05$ [b] Energy, protein, total fat, SFA, MUFA and PUFA intake were calculated from analyses of dietary records with PRODI® [c] LA, ALA, AA, EPA, DPAn3 and DHA intake were calculated from a combination of own analyses of meals that were provided by the Institute of Food Science and Human Nutrition and analyses of dietary records with PRODI®.

Clinical and anthropometric parameters of the participants were not significantly different between both interventions at baseline (Table 3). A slight decrease in body weight, BMI, total cholesterol (TC), low density lipoprotein (LDL) and high density lipoprotein (HDL) was observed in both intervention periods. During $_{hi}$LA/$_{lo}$ALA diet, but not during $_{lo}$LA/$_{hi}$ALA diet, the diastolic blood pressure decreased. All other parameters remained constant in both intervention periods (Table 3).

Changes of fatty acid patterns in RBCs

At baseline, there were only a few marginal differences in the fatty acid patterns of RBCs between both intervention phases (Table S1†). Prior interventions, AA was present in highest concentrations in RBCs ($_{lo}$LA/$_{hi}$ALA diet: 152 ± 4.08 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 145 ± 4.10 μg mL^{-1}) among all PUFAs, followed by LA ($_{lo}$LA/$_{hi}$ALA diet: 101 ± 3.78 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 99.1 ± 2.89 μg mL^{-1}), DHA ($_{lo}$LA/$_{hi}$ALA diet: 36.1 ± 1.75 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 34.3 ± 2.04 μg mL^{-1}), C22:4n6 ($_{lo}$LA/$_{hi}$ALA diet: 32.5 ± 1.58 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 29.5 ± 0.92 μg mL^{-1}), DPAn3 ($_{lo}$LA/$_{hi}$ALA diet: 27.3 ± 1.42 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 28.0 ± 0.98 μg mL^{-1}), DPAn6 ($_{lo}$LA/$_{hi}$ALA diet: 5.84 ± 0.35 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 5.12 ± 0.24 μg mL^{-1}) and EPA ($_{lo}$LA/$_{hi}$ALA diet: 5.49 ± 0.48 μg mL^{-1}; $_{hi}$LA/$_{lo}$ALA diet: 5.86 ± 0.41 μg mL^{-1}). ALA concentrations in RBCs were low at baseline of $_{lo}$LA/$_{hi}$ALA diet and $_{hi}$LA/$_{lo}$ALA diet with 1.44 ± 0.17 μg mL^{-1} and 1.47 ± 0.13 μg mL^{-1}, respectively (Table 4 & 5).

The two different dietary ratios of LA to ALA led to significantly different changes of the PUFA concentrations of ALA, LA, EPA and ∑EPA + DHA (Table S1†). In the following, the fatty acid concentrations are discussed unless the relative fatty acid distribution showed a different trend.

ALA

In the course of the $_{lo}$LA/$_{hi}$ALA diet ALA concentrations increased rapidly ($p < 0.001$) from 1.44 ± 0.17 μg mL^{-1} at baseline to 5.63 ± 0.45 μg mL^{-1} at day 7 and to 6.34 ± 0.63 μg mL^{-1} at day 14, corresponding to a mean change of 332 ± 40% and 354 ± 47% (Table 4 and Fig. 2A), whereas during $_{hi}$LA/$_{lo}$ALA diet ALA concentrations dropped after 7 days from 1.47 ± 0.13 μg mL^{-1} to 1.09 ± 0.09 μg mL^{-1} ($p = 0.011$) and increased again at day 14 to 1.41 ± 0.17 μg mL^{-1}, which is not significantly different from the baseline level (Table 5 and Fig. 2A).

LA

In the course of the $_{lo}$LA/$_{hi}$ALA diet a linear non-significant decrease of LA concentrations was observed between baseline (101 ± 3.78 μg mL^{-1}), day 7 (91.6 ± 2.59 μg mL^{-1}) and day 14 (82.8 ± 2.75 μg mL^{-1}) (Table 4 and Fig. 2F). Following the

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Table 3 Clinical, biochemical and anthropometric parameters of the study population during the $_{lo}$LA/$_{hi}$ALA diet and $_{hi}$LA/$_{lo}$ALA diet at baseline (day 0), after seven days (day 7) and after 14 days (day 14)

	$_{lo}$LA/$_{hi}$ALA				$_{hi}$LA/$_{lo}$ALA			
	Day 0 Mean ± SD	Day 7 Mean ± SD	Day 14 Mean ± SD	An reM	Day 0 Mean ± SD	Day 7 Mean ± SD	Day 14 Mean ± SD	An reM
Age (years)	26.1 ± 4.53							
Weight (kg)	81.4 ± 7.44		80.1 ± 7.49[d‡]		81.9 ± 8.11		80.9 ± 8.09[d‡]	
BMI (kg m^{-2})	24.0 ± 1.65		23.6 ± 1.70[d‡]		24.2 ± 1.81		23.9 ± 1.82[d‡]	
Sys BP (mmHg)	127 ± 7.99	124 ± 10.7	125 ± 12.0		129 ± 11.0	124 ± 11.8	126 ± 13.2	
Dias BP (mmHg)	77.7 ± 4.58	77.7 ± 5.30[b*]	76.0 ± 6.32		78.0 ± 6.21	73.7 ± 6.11[b*]	74.0 ± 5.73[k*]	[f*]
TC (mg dl^{-1})	177 ± 42.8	155 ± 32.4[g#]	150 ± 27.0[h#]	[e‡]	169 ± 34.8	148 ± 30.7[i‡]	152 ± 37.9[k*]	[f#]
HDL (mg dl^{-1})	56.5 ± 9.64	51.5 ± 9.53[g*]	49.7 ± 8.78[h*]	[e‡]	58.9 ± 9.77	51.5 ± 7.83[i‡]	53.0 ± 9.82	[f#]
LDL (mg dl^{-1})	110 ± 31.4	97.9 ± 26.4[b*, g*]	93.7 ± 18.9[h*]	[e#]	106 ± 28.5	91.0 ± 24.5[b*, i‡]	91.8 ± 27.5[k#]	[f‡]
TG (mg dl^{-1})	106 ± 50.8	100 ± 44.6	107 ± 46.7		108 ± 36.0	98.5 ± 31.3	99.9 ± 54.8	

Levels are shown at day 0, 7 and 14 of $_{lo}$LA/$_{hi}$ALA diet and $_{hi}$LA/$_{lo}$ALA diet. An reM: ANOVA for repeated measures; BMI: body mass index; dias BP: diastolic blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; SD: standard deviation; sys BP: systolic blood pressure; TC: total cholesterol; TG: triglycerides. [a]Significant difference between $_{lo}$LA/$_{hi}$ALA diet week 0 and $_{hi}$LA/$_{lo}$ALA diet week 0 (*t*-test for paired samples). [b]Significant difference between $_{lo}$LA/$_{hi}$ALA diet week 1 and $_{hi}$LA/$_{lo}$ALA diet week 1 (*t*-test for paired samples). [c]Significant difference between $_{lo}$LA/$_{hi}$ALA diet week 2 and $_{hi}$LA/$_{lo}$ALA diet week 2 (*t*-test for paired samples). [d]Significant difference between week 0 and week 2 of $_{lo}$LA/$_{hi}$ALA diet /$_{hi}$LA/$_{lo}$ALA diet (*t*-test for paired samples). [e]Significant difference within $_{lo}$LA/$_{hi}$ALA diet (one-factorial ANOVA for repeated measures). [f]Significant difference within $_{hi}$LA/$_{lo}$ALA diet (one-factorial ANOVA for repeated measures). [g]Significant difference within $_{lo}$LA/$_{hi}$ALA diet between week 0 and week 1 (*t*-test for paired samples with Holm–Bonferroni correction). [h]Significant difference within $_{lo}$LA/$_{hi}$ALA diet between week 0 and week 2 (*t*-test for paired samples with Holm–Bonferroni correction). [i]Significant difference within $_{hi}$LA/$_{lo}$ALA between week 0 and week 1 (*t*-test for paired samples with Holm–Bonferroni correction). [k]Significant difference within $_{hi}$LA/$_{lo}$ALA between week 0 and week 2 (*t*-test for paired samples with Holm–Bonferroni correction). [l]Significant difference between $_{lo}$LA/$_{hi}$ALA diet und $_{hi}$LA/$_{lo}$ALA (two-factorial ANOVA for repeated measures). $*p \leq 0.05$, $\#p \leq 0.005$, $‡p < 0.001$.

$_{hi}$LA/$_{lo}$ALA diet, LA concentrations non-significantly increased from 99.1 ± 2.89 μg mL^{-1} at baseline to 110 ± 3.51 μg mL^{-1} at day 14 (Table 5 and Fig. 2F).

EPA

EPA concentrations increased during $_{lo}$LA/$_{hi}$ALA diet from 5.49 ± 0.48 μg mL^{-1} at baseline to 6.97 ± 0.55 μg mL^{-1} at day 7 (p = 0.019) and to 8.27 ± 0.82 μg mL^{-1} at day 14 (p = 0.008), corresponding to a mean change of 35.0 ± 13% and 57.6 ± 18% (Table 4 and Fig. 2B). During $_{hi}$LA/$_{lo}$ALA diet EPA concentrations decreased from 5.86 ± 0.41 μg mL^{-1} at baseline to 5.21 ± 0.35 μg mL^{-1} at day 7 (p = 0.002) and 5.11 ± 0.41 μg mL^{-1} at day 14 (p = 0.025), corresponding to a mean change of −11.2 ± 2.1% and −12.9 ± 3.6% (Table 5 and Fig. 2B). Differences in EPA concentrations between both interventions at time point day 7 and day 14 were highly significant ($p < 0.001$) (Table S1†).

DPAn3 and DPAn6

DPAn3 concentrations remained constant during both intervention phases (Tables 4, 5 & S1,† Fig. 2C). However, the relative DPAn3 amount slightly increased (p = 0.004) during $_{lo}$LA/$_{hi}$ALA diet from 2.62 ± 0.09% of total fatty acids at baseline to 2.91 ± 0.10% at day 14 (Table 4 and Fig. 2C) and slightly decreased (p = 0.021) during $_{hi}$LA/$_{lo}$ALA diet from 2.82 ± 0.09% at baseline to 2.67 ± 0.10% at day 14 (Table 5 and Fig. 2C). However, it has to be noted that baseline relative amounts of DPAn3 were significantly different (p = 0.005) between $_{lo}$LA/$_{hi}$ALA and $_{hi}$LA/$_{lo}$ALA diet (Table S1†).

DPAn6 concentrations remained unchanged during $_{lo}$LA/$_{hi}$ALA and $_{hi}$LA/$_{lo}$ALA diet (Tables 4, 5 & S1†). Again, significantly (p = 0.026) different baseline DPAn6 concentrations were observed between $_{lo}$LA/$_{hi}$ALA and $_{hi}$LA/$_{lo}$ALA diet (Table S1†).

DHA

Also DHA concentrations remained unchanged in both intervention phases (Tables 4, 5 & S1,† Fig. 2D), even though the two-factorial ANOVA detected a significant difference (p = 0.025) between the $_{lo}$LA/$_{hi}$ALA and the $_{hi}$LA/$_{lo}$ALA diet (Table S1†) and DHA concentrations were significantly different (p = 0.006) at day 7 of $_{lo}$LA/$_{hi}$ALA diet (35.8 ± 1.75 μg mL^{-1}) and $_{hi}$LA/$_{lo}$ALA diet (32.4 ± 1.75 μg mL^{-1}) (Table S1†).

AA

AA concentrations did not change significantly in both intervention periods (Tables 4, 5 & S1†). However, in the course of the $_{lo}$LA/$_{hi}$ALA diet AA concentrations slightly decreased (n.s.) from 152 ± 4.08 μg mL^{-1} (baseline) to 147 ± 3.61 μg mL^{-1} (day 7) and to 139 ± 3.16 μg mL^{-1} (day 14) (Table 4), whereas during the $_{hi}$LA/$_{lo}$ALA diet AA concentrations increased (n.s.) marginally from 145 ± 4.10 μg mL^{-1} (baseline) to 151 ± 3.86 μg mL^{-1} (day 14) (Table 5). These opposite trends are supported by significantly lower (p = 0.007) AA concentrations at day 14 of $_{lo}$LA/$_{hi}$ALA diet (139 ± 3.16 μg mL^{-1}) compared to day 14 of $_{hi}$LA/$_{lo}$ALA diet (151 ± 3.86 μg mL^{-1}) (Table S1†).

∑EPA + DHA

The concentration of ∑EPA + DHA in RBCs increased slightly (n.s.) in response to the 14-day $_{lo}$LA/$_{hi}$ALA diet from 41.6 ± 2.04 μg mL^{-1} (baseline) to 42.8 ± 2.04 μg mL^{-1} (day 7) and to 44.1 ± 1.96 μg mL^{-1} (day 14) (Table 4 and Fig. 2E). The relative amount of ∑EPA + DHA in RBCs increased marginally but sig-

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Table 4 Concentration of fatty acids in red blood cells during $_{lo}$LA/$_{hi}$ALA diet at baseline (day 0), after seven days (day 7) and after 14 days (day 14)

	Day 0 Mean ± SE	Day 7 Mean ± SE	*t*-Test[a] *p* (day 7–day 0)	Day 14 Mean ± SE	*t*-Test[a] *p* (day 14–day 0)	1-fact. An reM[b] *p*
C10:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C11:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C12:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C13:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C14:0 (μg mL^{-1})	3.31 ± 0.16	3.15 ± 0.17	—	2.78 ± 0.21	—	n.s.
% of total FA	0.32 ± 0.02	0.31 ± 0.01	—	0.30 ± 0.02	—	n.s.
C14:1n5 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C15:0 (μg mL^{-1})	1.59 ± 0.08	1.61 ± 0.06	—	1.37 ± 0.07	—	n.s.
% of total FA	0.15 ± 0.01	0.16 ± 0.00	—	0.15 ± 0.01	—	n.s.
C15:1n5 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C16:0 (μg mL^{-1})	212 ± 7.22	209 ± 5.89	n.s.	184 ± 6.24	n.s.	0.017
% of total FA	20.4 ± 0.18	20.7 ± 0.13	n.s.	19.8 ± 0.18	n.s.	0.005
C16:1n7 (μg mL^{-1})	2.88 ± 0.18	3.11 ± 0.32	—	2.89 ± 0.37	—	n.s.
% of total FA	0.28 ± 0.02	0.30 ± 0.02	—	0.31 ± 0.03	—	n.s.
C17:0 (μg mL^{-1})	3.10 ± 0.12	3.07 ± 0.08	<0.001	2.76 ± 0.08	n.s.	<0.001
% of total FA	0.30 ± 0.01	0.31 ± 0.01	<0.001	0.30 ± 0.01	n.s.	<0.001
C17:1n8 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C18:0 (μg mL^{-1})	157 ± 4.53	154 ± 3.82	—	141 ± 3.85	—	n.s.
% of total FA	15.2 ± 0.18	15.3 ± 0.13	—	15.2 ± 0.15	—	n.s.
C18:1n9 (μg mL^{-1})	139 ± 4.78	130 ± 4.69	n.s.	117 ± 5.25	n.s.	0.016
% of total FA	13.4 ± 0.18	12.8 ± 0.21	0.001	12.6 ± 0.25	<0.001	<0.001
C18:1n7 (μg mL^{-1})	14.5 ± 0.59	14.3 ± 0.42	—	13.5 ± 0.33	—	n.s.
% of total FA	1.40 ± 0.02	1.42 ± 0.02	n.s.	1.45 ± 0.02	n.s.	0.019
C18:2n6 (μg mL^{-1})	101 ± 3.78	91.6 ± 2.59	n.s.	82.8 ± 2.75	n.s.	0.029
% of total FA	9.72 ± 0.20	9.12 ± 0.19	n.s.	8.90 ± 0.15	n.s.	0.012
C18:3n6 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C19:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C18:3n3 (μg mL^{-1})	1.44 ± 0.17	5.63 ± 0.45	<0.001	6.34 ± 0.63	<0.001	<0.001
% of total FA	0.14 ± 0.02	0.55 ± 0.03	<0.001	0.67 ± 0.05	<0.001	<0.001
C18:4n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:0 (μg mL^{-1})	3.87 ± 0.14	3.92 ± 0.12	—	3.71 ± 0.14	—	n.s.
% of total FA	0.37 ± 0.01	0.39 ± 0.01	—	0.40 ± 0.01	—	n.s.
C20:1n9 (μg mL^{-1})	2.86 ± 0.21	2.79 ± 0.13	—	2.52 ± 0.10	—	n.s.
% of total FA	0.27 ± 0.01	0.28 ± 0.01	—	0.27 ± 0.01	—	n.s.
C20:2n6 (μg mL^{-1})	2.00 ± 0.12	1.87 ± 0.10	—	1.62 ± 0.07	—	n.s.
% of total FA	0.19 ± 0.01	0.19 ± 0.01	—	0.18 ± 0.01	—	n.s.
C20:3n9 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:3n6 (μg mL^{-1})	15.9 ± 0.93	14.1 ± 0.96	—	12.9 ± 0.94	—	n.s.
% of total FA	1.53 ± 0.07	1.39 ± 0.08	—	1.38 ± 0.10	—	n.s.
C21:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:4n6 (μg mL^{-1})	152 ± 4.08	147 ± 3.61	—	139 ± 3.16	—	n.s.
% of total FA	14.7 ± 0.18	14.7 ± 0.20	—	15.0 ± 0.27	—	n.s.
C20:3n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:4n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:5n3 (μg mL^{-1})	5.49 ± 0.48	6.97 ± 0.55	0.019	8.27 ± 0.82	0.008	0.002
% of total FA	0.53 ± 0.04	0.69 ± 0.05	0.009	0.88 ± 0.08	<0.001	<0.001
C22:0 (μg mL^{-1})	16.6 ± 0.36	16.5 ± 0.54	—	15.5 ± 0.49	—	n.s.
% of total FA	1.61 ± 0.04	1.64 ± 0.03	—	1.66 ± 0.04	—	n.s.
C22:1n9 (μg mL^{-1})	1.56 ± 0.20	2.08 ± 0.28	—	1.94 ± 0.34	—	n.s.
% of total FA	0.15 ± 0.02	0.20 ± 0.03	—	0.21 ± 0.04	—	n.s.
C22:2n6 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C22:4n6 (μg mL^{-1})	32.5 ± 1.58	30.7 ± 1.21	—	27.9 ± 1.11	—	n.s.
% of total FA	3.13 ± 0.11	3.06 ± 0.11	—	3.01 ± 0.11	—	n.s.
C22:5n6 (μg mL^{-1})	5.84 ± 0.35	5.49 ± 0.34	—	5.11 ± 0.32	—	n.s.

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Table 4 (Contd.)

	Day 0 Mean ± SE	Day 7 Mean ± SE	*t*-Test[a] *p* (day 7–day 0)	Day 14 Mean ± SE	*t*-Test[a] *p* (day 14–day 0)	1-fact. An reM[b] *p*
% of total FA	0.56 ± 0.03	0.54 ± 0.03	—	0.55 ± 0.03	—	n.s.
C22:5n3 (μg mL^{-1})	27.3 ± 1.42	27.1 ± 1.05	—	27.1 ± 1.34	—	n.s.
% of total FA	2.62 ± 0.09	2.70 ± 0.09	n.s.	2.91 ± 0.10	0.004	0.002
C24:0 (μg mL^{-1})	47.1 ± 1.14	45.9 ± 1.21	—	44.1 ± 1.29	—	n.s.
% of total FA	4.56 ± 0.06	4.56 ± 0.05	n.s.	4.75 ± 0.06	n.s.	0.031
C22:6n3 (μg mL^{-1})	36.1 ± 1.75	35.8 ± 1.75	—	35.8 ± 1.44	—	n.s.
% of total FA	3.50 ± 0.15	3.56 ± 0.16	—	3.87 ± 0.16	—	n.s.
C24:1n9 (μg mL^{-1})	51.9 ± 2.42	50.7 ± 1.73	—	49.1 ± 1.34	—	n.s.
% of total FA	4.99 ± 0.13	5.04 ± 0.11	—	5.30 ± 0.14	—	n.s.
∑TFA (μg mL^{-1})	1040 ± 30.9	1010 ± 25.9	—	933 ± 25.8	—	n.s.
∑SFA (μg mL^{-1})	446 ± 13.1	438 ± 11.1	n.s.	396 ± 11.5	n.s.	0.031
% of total FA	42.9 ± 0.30	43.4 ± 0.13	n.s.	42.5 ± 0.18	n.s.	0.025
∑MUFA (μg mL^{-1})	213 ± 7.87	203 ± 6.87	n.s.	188 ± 6.85	n.s.	0.039
% of total FA	20.5 ± 0.31	20.1 ± 0.27	n.s.	20.1 ± 0.32	n.s.	0.036
∑PUFA (μg mL^{-1})	381 ± 11.0	369 ± 8.72	—	349 ± 8.62	—	n.s.
% of total FA	36.7 ± 0.19	36.5 ± 0.20	n.s.	37.5 ± 0.28	n.s.	0.002
∑EPA + DHA (μg mL^{-1})	41.6 ± 2.04	42.8 ± 2.04	—	44.1 ± 1.96	—	n.s.
% of total FA	4.03 ± 0.17	4.25 ± 0.18	n.s.	4.76 ± 0.20	0.019	0.001

Levels are shown as concentration [μg mL^{-1}] in blood and as relative amount [%] of total fatty acids. An reM: ANOVA for repeated measures; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; MUFA: monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; n.s.: not significant; SFA: saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; PUFA: polyunsaturated fatty acids: C18:2n6, C18:3n6, C18:3n3, C:18:4n3, C20:2n6, C20:3n3, C20:3n6, C20:3n9, C20:4n3, C20:4n6, C20:5n3, C22:2n6, C22:4n6, C22:5n3, C22:5n6, C22:6n3; TFA: total fatty acids. [a] *t*-Test for paired samples with Holm–Bonferroni correction (within intervention); significance level $p \leq 0.05$ [b] One-factorial ANOVA for repeated measures (An reM); significance level $p \leq 0.05$.

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nificantly (p = 0.019) in response to the $_{lo}$LA/$_{hi}$ALA diet from initially 4.03 ± 0.17% of total fatty acids to 4.76 ± 0.20% of total fatty acids at day 14 (Table 4 and Fig. 2E). In the course of the $_{hi}$LA/$_{lo}$ALA diet the concentration of ∑EPA + DHA in RBCs remained constant, though, the relative amount of ∑EPA + DHA in RBCs decreased slightly (p = 0.031) from 4.03 ± 0.18% of total fatty acids to 3.93 ± 0.17% at day 14 (Table 5 and Fig. 2E).

Discussion

Study design

The design of clinical trials to investigate the effect of different dietary LA/ALA ratios on PUFA concentrations in blood is challenging. Although ALA is only present in a few plant oils and its intake can easily be controlled, LA is nowadays ubiquitous in our daily diet due to the widespread use of LA-rich vegetable oils especially in ready meals and take-away foods. This makes attempts to reduce the LA intake of free-living individuals difficult.[26]

Numerous human studies have attempted to establish a defined dietary ratio of the precursor fatty acids LA and ALA to investigate the effect on the n3 PUFA status.[20,27–44]

According to a current review from Wood *et al.*,[26] the majority of these studies, exhibited some or several methodological weaknesses such as studying males and females together,[28–36] inappropriate or missing run-in and wash-out periods,[28,39,43] inconsistent composition of the background diet, *e.g.* changes of EPA and DHA intake during interventions,[31,37] inaccurate dietary records.[29,31,33–35,37] These methodological weaknesses question the control and documentation of the PUFA intake and limit the ability to draw robust conclusions.[26] Besides controlled PUFA intake and study conditions, several other factors have been proposed to influence ALA conversion in humans including age,[21,22] BMI,[22] smoking status,[45] sex[46–49] and genotype.[13,50] For example, males and females are known to have different capacities for ALA conversion.[13] Therefore, it is suggested that males and females are either stratified or studied separately.[26]

The aim of our study was to compare the effects of two different dietary LA/ALA ratios on fatty acid concentrations in RBC with special emphasis on LC n3 PUFAs. A homogenous study collective of healthy, non-smoking men within a narrow range regarding age (mean age 26.1 ± 4.53 years) and BMI (24.0 ± 1.65 kg m^{-2}) was chosen to prevent/minimize the influence of gender, smoking, age, and BMI on PUFA metabolism. Likewise, our study approach includes further methodological considerations with the aim to overcome methodological weaknesses listed above. This involves in particular constant background diet and defined low-variable PUFA intake as well as subjects acting as their own controls (cross-over design), and run-in and wash-out phase. Other studies with the aim to modify LA and ALA intake mostly supplemented margarine and/or plant oils additionally to the normal background nutrition of the participants to achieve the desired ratio of LA to ALA.[20,28,29,32,33,38–41,43] Studies, where subjects acted as their own controls are rare.[20,39]

With the two experimental diets a low-LA (2.78 en%) and a high-ALA (4.98 en%) diet and a high-LA (6.95 en%) and low-ALA (0.27 en%) diet were achieved, which correspond to a LA/ALA ratio of 0.56 ± 0.27 : 1 and 25.6 ± 2.41 : 1, respectively. These ratios can be classified as extreme examples of a desirable presumably health-promotive LA/ALA ratio ($_{lo}$LA/$_{hi}$ALA

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Table 5 Concentration of fatty acids in red blood cells during $_{hi}$LA/$_{lo}$ALA diet at baseline (day 0), after seven days (day 7) and after 14 days (day 14)

	Day 0 Mean ± SE	Day 7 Mean ± SE	*t*-Test[a] *p* (day 7–day 0)	Day 14 Mean ± SE	*t*-Test[a] *p* (day 14–day 0)	1-fact. An reM[b] *p*
C10:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C11:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C12:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C13:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C14:0 (μg mL^{-1})	3.07 ± 0.17	3.06 ± 0.12	n.s.	3.53 ± 0.21	0.029	0.006
% of total FA	0.31 ± 0.01	0.32 ± 0.01	—	0.34 ± 0.01	—	n.s.
C14:1n5 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C15:0 (μg mL^{-1})	1.51 ± 0.05	1.51 ± 0.05	n.s.	1.70 ± 0.07	0.045	0.008
% of total FA	0.15 ± 0.00	0.16 ± 0.00	n.s.	0.16 ± 0.01	n.s.	0.041
C15:1n5 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C16:0 (μg mL^{-1})	200 ± 4.48	194 ± 4.23	n.s.	218 ± 6.47	0.043	0.001
% of total FA	20.1 ± 0.13	20.5 ± 0.11	<0.001	21.0 ± 0.13	<0.001	<0.001
C16:1n7 (μg mL^{-1})	2.87 ± 0.13	2.98 ± 0.16	n.s.	3.67 ± 0.47	n.s.	0.041
% of total FA	0.29 ± 0.01	0.32 ± 0.02	—	0.35 ± 0.04	—	n.s.
C17:0 (μg mL^{-1})	2.96 ± 0.09	2.87 ± 0.06	<0.001	3.10 ± 0.08	n.s.	<0.001
% of total FA	0.30 ± 0.01	0.30 ± 0.01	<0.001	0.30 ± 0.01	n.s.	<0.001
C17:1n8 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C18:0 (μg mL^{-1})	151 ± 3.50	142 ± 3.20	0.018	154 ± 4.25	n.s.	0.017
% of total FA	15.3 ± 0.10	15.1 ± 0.12	n.s.	14.8 ± 0.15	0.029	0.009
C18:1n9 (μg mL^{-1})	130 ± 2.65	121 ± 2.56	0.005	133 ± 5.29	n.s.	0.011
% of total FA	13.1 ± 0.20	12.8 ± 0.18	0.011	12.7 ± 0.25	n.s.	0.036
C18:1n7 (μg mL^{-1})	13.8 ± 0.34	13.5 ± 0.30	n.s.	15.2 ± 0.53	n.s.	0.008
% of total FA	1.39 ± 0.02	1.44 ± 0.02	n.s.	1.46 ± 0.03	n.s.	0.022
C18:2n6 (μg mL^{-1})	99.1 ± 2.89	94.5 ± 2.92	n.s.	110 ± 3.51	n.s.	0.001
% of total FA	9.99 ± 0.24	10.0 ± 0.23	n.s.	10.6 ± 0.22	n.s.	0.011
C18:3n6 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C19:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C18:3n3 (μg mL^{-1})	1.47 ± 0.13	1.09 ± 0.09	0.011	1.41 ± 0.17	n.s.	0.008
% of total FA	0.15 ± 0.01	0.12 ± 0.01	0.023	0.13 ± 0.01	n.s.	0.016
C18:4n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:0 (μg mL^{-1})	4.06 ± 0.11	3.70 ± 0.13	—	3.95 ± 0.15	—	n.s.
% of total FA	0.41 ± 0.01	0.39 ± 0.01	—	0.38 ± 0.01	—	n.s.
C20:1n9 (μg mL^{-1})	2.73 ± 0.13	2.57 ± 0.13	0.038	2.87 ± 0.12	n.s.	0.010
% of total FA	0.28 ± 0.01	0.27 ± 0.01	—	0.28 ± 0.01	—	n.s.
C20:2n6 (μg mL^{-1})	1.89 ± 0.08	1.85 ± 0.09	—	2.21 ± 0.11	—	n.s.
% of total FA	0.19 ± 0.01	0.20 ± 0.01	—	0.21 ± 0.01	—	n.s.
C20:3n9 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:3n6 (μg mL^{-1})	14.5 ± 0.82	14.1 ± 0.82	n.s.	15.9 ± 0.89	n.s.	0.010
% of total FA	1.47 ± 0.08	1.50 ± —	n.s.	1.54 ± 0.09	—	n.s.
C21:0 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:4n6 (μg mL^{-1})	145 ± 4.10	138 ± 3.33	n.s.	151 ± 3.86	n.s.	0.011
% of total FA	14.6 ± 0.19	14.7 ± 0.14	—	14.6 ± 0.20	—	n.s.
C20:3n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:4n3 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C20:5n3 (μg mL^{-1})	5.86 ± 0.41	5.21 ± 0.35	0.002	5.11 ± 0.41	0.025	0.001
% of total FA	0.59 ± 0.04	0.55 ± 0.04	0.001	0.49 ± 0.03	<0.001	<0.001
C22:0 (μg mL^{-1})	16.5 ± 0.55	15.28 ± 0.54	0.025	16.6 ± 0.51	n.s.	0.017
% of total FA	1.66 ± 0.04	1.62 ± 0.03	—	1.60 ± 0.04	—	n.s.
C22:1n9 (μg mL^{-1})	2.03 ± 0.20	1.83 ± 0.21	n.s.	1.30 ± 0.12	n.s.	0.033
% of total FA	0.21 ± 0.02	0.19 ± 0.02	n.s.	0.13 ± 0.01	n.s.	0.023
C22:2n6 (μg mL^{-1})	<0.25	<0.25	—	<0.25	—	—
% of total FA	—	—	—	—	—	—
C22:4n6 (μg mL^{-1})	29.5 ± 0.92	28.58 ± 1.25	n.s.	30.4 ± 1.13	n.s.	0.014
% of total FA	2.98 ± 0.11	3.03 ± 0.11	—	2.93 ± 0.09	—	n.s.
C22:5n6 (μg mL^{-1})	5.12 ± 0.24	5.04 ± 0.29	n.s.	5.42 ± 0.25	n.s.	0.015

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Table 5 *(Contd.)*

	Day 0 Mean ± SE	Day 7 Mean ± SE	*t*-Test[a] *p* (day 7–day 0)	Day 14 Mean ± SE	*t*-Test[a] *p* (day 14–day 0)	1-fact. An reM[b] *p*
% of total FA	0.52 ± 0.02	0.53 ± 0.03	—	0.52 ± 0.02	—	n.s.
C22:5n3 (μg mL^{-1})	28.0 ± 0.98	27.03 ± 0.91	—	27.9 ± 1.51	—	n.s.
% of total FA	2.82 ± 0.09	2.87 ± 0.08	n.s.	2.67 ± 0.10	0.021	0.001
C24:0 (μg mL^{-1})	46.7 ± 1.11	44.00 ± 1.36	0.032	46.6 ± 1.48	n.s.	n.s.
% of total FA	4.70 ± 0.06	4.65 ± 0.07	n.s.	4.48 ± 0.08	0.020	0.001
C22:6n3 (μg mL^{-1})	34.3 ± 2.04	32.4 ± 1.75	n.s.	35.68 ± 1.74	n.s.	0.024
% of total FA	3.44 ± 0.16	3.42 ± 0.15	—	3.44 ± 0.16	—	n.s.
C24:1n9 (μg mL^{-1})	49.9 ± 1.45	47.7 ± 1.66	n.s.	49.9 ± 1.64	n.s.	n.s.
% of total FA	5.03 ± 0.11	5.04 ± 0.11	n.s.	4.81 ± 0.12	0.031	0.001
∑TFA (μg mL^{-1})	996 ± 20.6	947 ± 20.6	0.027	1043 ± 28.2	n.s.	0.003
∑SFA (μg mL^{-1})	428 ± 9.33	408 ± 9.05	0.045	449 ± 12.3	n.s.	0.008
% of total FA	42.9 ± 0.15	43.0 ± 0.14	—	43.1 ± 0.19	—	n.s.
∑MUFA (μg mL^{-1})	202 ± 4.06	190 ± 4.23	0.009	206 ± 7.00	n.s.	0.013
% of total FA	20.3 ± 0.25	20.1 ± 0.21	0.028	19.7 ± 0.26	0.012	0.003
∑PUFA (μg mL^{-1})	367 ± 8.40	350 ± 7.94	0.039	387 ± 9.65	n.s.	0.002
% of total FA	36.8 ± 0.23	36.9 ± 0.16	n.s.	37.2 ± 0.19	0.015	0.021
∑EPA + DHA (μg mL^{-1})	40.2 ± 2.27	37.6 ± 1.95	—	40.8 ± 1.91	—	n.s.
% of total FA	4.03 ± 0.18	3.97 ± 0.17	n.s.	3.93 ± 0.17	0.031	0.005

Levels are shown as concentration [μg mL^{-1}] in blood and as relative amount [%] of total fatty acids. An reM: ANOVA for repeated measures; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; MUFA: monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; n.s.: not significant; SFA: saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; PUFA: polyunsaturated fatty acids: C18:2n6, C18:3n6, C18:3n3, C:18:4n3, C20:2n6, C20:3n3, C20:3n6, C20:3n9, C20:4n3, C20:4n6, C20:5n3, C22:2n6, C22:4n6, C22:5n3, C22:5n6, C22:6n3; TFA: total fatty acids. [a] *t*-Test for paired samples with Holm–Bonferroni correction (within intervention); significance level $p \leq 0.05$ [b] One-factorial ANOVA for repeated measures (An reM); significance level $p \leq 0.05$.

diet, <5 : 1) and an unfavorable LA/ALA ratio ($_{hi}$LA/$_{lo}$ALA diet, ≫5 : 1) as it is typical for Western diets. The variations in LA and ALA intake between the probands were minimal in both intervention periods. Moreover, no differences in the (extremely low) background intake of the LC PUFAs AA, EPA, DPAn3 and DHA were observed between the two intervention periods. Total PUFA intake was significantly higher in the $_{lo}$LA/$_{hi}$ALA diet compared to the $_{hi}$LA/$_{lo}$ALA diet due to the higher PUFA content of linseed oil (69.8% of total fatty acids) compared to sunflower oil (62.5% of total fatty acids). The higher MUFA intake in the $_{hi}$LA/$_{lo}$ALA diet was possibly the result of a higher MUFA content of sunflower oil (28.2% of total fatty acids) as compared to linseed oil (19.3% of total fatty acids). The intake of main nutrients (protein, carbohydrates, fat) and energy remained constant between the two intervention periods. These results suggest the applicability of our experimental design and the good compliance of the probands.

RBC fatty acid concentrations

With a few minor exceptions, no significant differences regarding the RBC fatty acid concentrations were observed between the baseline time points of both intervention phases revealing that the run-in and wash-out phase was sufficiently long.

The **$_{lo}$LA/$_{hi}$ALA diet** was effective in increasing ALA and EPA concentrations in RBC membranes. This is in line with the observations of the systematic review of Wood *et al.*[26] who state that a combination of a decrease of LA and a simultaneous increase of ALA intake is most effective in improving the n3 PUFA status.

As expected, the $_{lo}$LA/$_{hi}$ALA diet resulted in a strong increase of ALA concentrations of 332 ± 40% (day 7) and 354 ± 47% (day 14) in RBCs. This comparably large increase of ALA concentrations is greater compared to our previous study, where only ALA was given (12.9 g d^{-1}) *via* the same linseed oil but without LA restriction.[51] In this study the ALA increase in RBCs was 238 ± 24% after 7 days and 294 ± 23% after 3 weeks.[51] The study collective was almost identical in both studies. Obviously, in this study the low LA content of the diet (all other dietary factors remained constant) contributed to the greater increase of ALA and n3 PUFAs. The reason is probably a competition between LA and ALA for the incorporation into cell membranes.[14]

Our finding that EPA concentrations in RBCs significantly increased by 35.0 ± 13% after 7 days and by 57.6 ± 18% after 14 days following the $_{lo}$LA/$_{hi}$ALA diet is a likely result of an increasing conversion of ALA to EPA, since no EPA was ingested *via* the background diet. Also other studies that increased the total ALA intake to 1.1–6.3 en% observed significantly higher EPA amounts, albeit with great variability[27–29,36,38,40–44] reviewed by Wood *et al.*[26] The efficiency of ALA conversion is, besides other factors, mainly dependent on the ALA dose, which essentially explains the fluctuations of the studies cited above.[26] In a similar study, a 6-week intervention with ALA from linseed oil (LA/ALA ratio of 1 : 1) resulted in a 47.2% increase of relative EPA amounts in RBCs.[33] The reason for this comparatively small increase after 6 weeks of intervention may be the lower ALA dose of 8.7 ± 2.2 g d^{-1}.

Furthermore, it is discussed whether the ALA conversion also depends on the LA intake.[12,13] Our data support this assumption as the ALA conversion to EPA can be further

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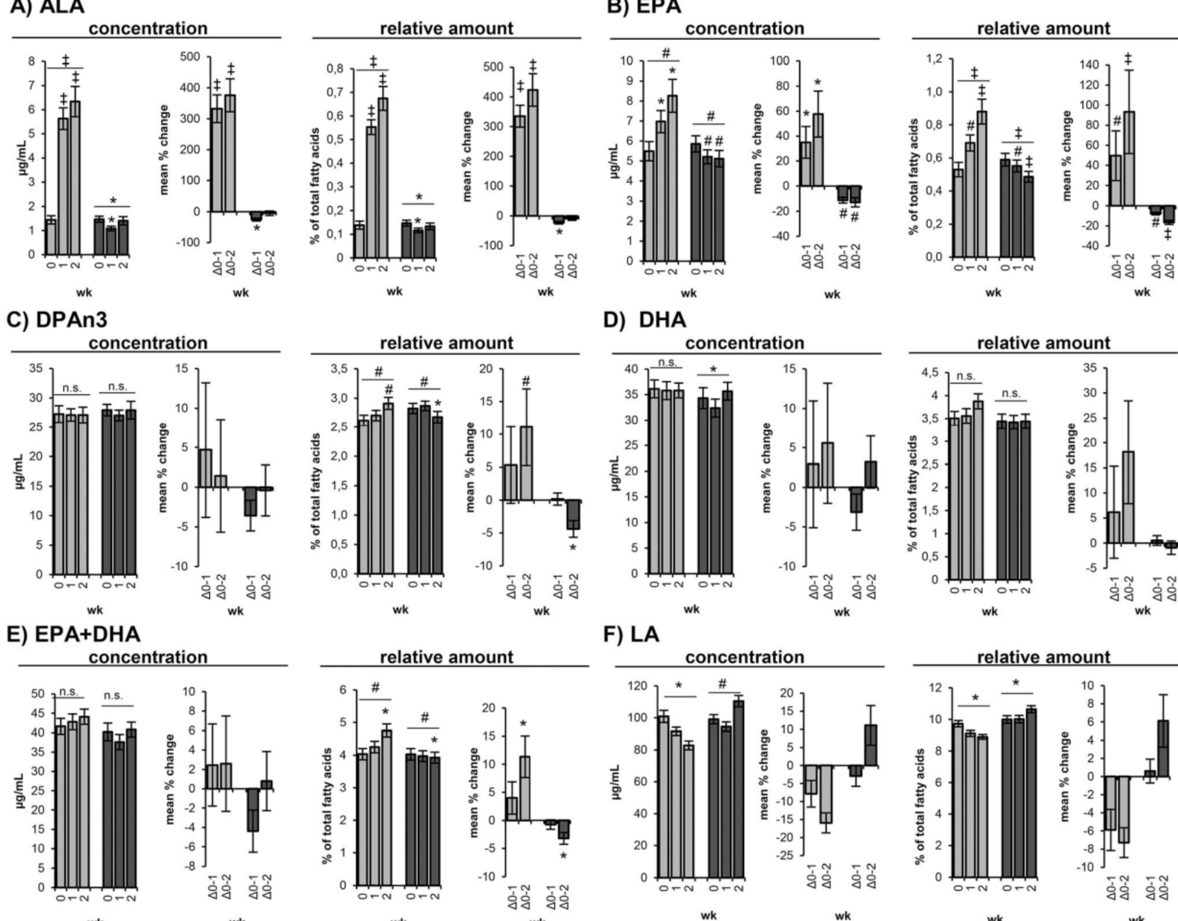

Fig. 2 Levels of (A) α-linolenic acid (ALA, C18:3n3), (B) eicosapentaenoic acid (EPA, C20:5n3), (C) n3 docosapentaenoic acid (DPAn3, C22:5n3), (D) docosahexaenoic acid (DHA, C22:6n3), (E) (EPA + DHA), (F) linoleic acid (LA, C18:2n6) in red blood cells during $_{lo}$LA/$_{hi}$ALA diet (light grey)and $_{hi}$LA/$_{lo}$ALA diet (dark grey) at baseline (week 0), after one (week 1) and two weeks (week 2). Levels are shown as concentrations [µg mL^{-1}] and as relative amounts [%] of total fatty acids in red blood cells. All data are shown as mean ± SE. Levels of significance of one-factorial ANOVAs with repeated measurements and *t*-tests for paired samples with Holm–Bonferroni correction are indicated as follows: *$p \le 0.05$, #$p \le 0.005$, ‡$p < 0.001$.

enhanced by reducing the LA content compared to our previous study with a higher LA intake.[51] In our previous study with a similar ALA dose, but uncontrolled and highly variable LA intake (the LA intake was 9.32 ± 5.93 g d^{-1}; 3.2 en%), a smaller increase of EPA concentrations in RBCs was observed (28.5 ± 10% after one week and 49.2 ± 14% after three weeks).[51] However, due to the daily contact with the subjects in this study compared to the previous study, a higher level of compliance can be assumed. An influence of the EPA status can be excluded as the relative EPA amount in RBCs was almost the same in the study collective of both studies (0.53 ± 0.04% *vs.* 0.60 ± 0.04% of total fatty acids of RBC in the previous study). If larger studies confirm this phenomenon, the meaningfulness of recommending a LA/ALA ratio of 1 : 1 (ref. 19 and 52) without considering absolute LA and ALA intake amounts is questionable. At least the effect of the $_{lo}$LA/$_{hi}$ALA diet on the relative amount of ∑EPA + DHA in RBCs – which is an established marker for protective effects of n3 PUFA with regard to cardiac, cerebral and general health – is stronger compared to a simple ALA supplementation. The relative amount of ∑EPA + DHA in RBCs significantly (p = 0.019) increased by 11.3 ± 3.7% (4.03 ± 0.17% to 4.76 ± 0.20% of total fatty acids) in only 14 days after the $_{lo}$LA/$_{hi}$ALA diet, whereas it remained constant over 12 weeks of daily ALA supplementation without LA restriction.[51]

During the **$_{hi}$LA/$_{lo}$ALA diet** EPA concentrations slightly decreased by −11.2 ± 2% after 7 days and −12.9 ± 3.6% after 14 days. A study with a similar LA/ALA ratio of 26 : 1 including 7.8 g d^{-1} LA (1.4 en%) and 0.30 g d^{-1} ALA (0.09 en%) also observed decreasing EPA levels in cholesterol ester fatty acids.[43] The reason for this EPA decrease may be the fish restricted diet which is lacking EPA and DHA[43] and the small

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amount of ALA available for conversion to EPA. It is likely that the high amount of LA may competitively displace ALA for conversion enzymes.

Regarding the effects of ALA supplementation and different LA/ALA ratios on DHA, the results of current studies are heterogeneous. In some studies, DHA in blood remained constant,[40] while others found increasing[13,53–57] or decreasing[51] levels. In the present study, we observed constant DHA concentrations in both intervention phases. Since DHA intake *via* the background diet was practically unchanged at a very low rate (30 mg *vs.* 20 mg per day), it is likely that increasing ALA and EPA levels after the **$_{lo}$LA/$_{hi}$ALA diet** are not converted to DHA. However, the timeframe of 14 days may be too short to observe significant changes in DHA concentrations. Wood *et al.*[26] concluded that ALA supplementation studies with decreased LA intake – as our study – were able to increase DHA concentrations. Our results cannot confirm this observation, on the one hand possibly due to the short intervention time and on the other hand also due to the high ALA dose in this study. The high ALA intake may lead to a competitive saturation of the delta-6 desaturase, and thus the conversion of EPA to DHA (precisely of 24:5n3 to 24:6n3) may be inhibited by the conversion of ALA to EPA (precisely of 18:3n3 to 18:4n3).[11] The shift in the ALA/EPA ratio in RBCs from 0.28 ± 0.03 (baseline) to 0.87 ± 0.10 (day 7) and 0.83 ± 0.10 (day 14) indicates that there is indeed a change in the substrate availability for the delta-6 desaturase. Hence, no clear conclusions can be drawn on whether it is possible to improve DHA status without eating fish or other marine products. However, the relative amount of ∑EPA + DHA in RBCs – which is an established marker for health protective effects of n3 PUFA – increased significantly ($p = 0.019$) in response to the $_{lo}$LA/$_{hi}$ALA from $4.03 \pm 0.17\%$ (day 0) to $4.76 \pm 0.20\%$ of total fatty acids (day 14). Although the increase is relatively small (and mainly due to the increase in EPA), it would be interesting to investigate the (health) effects on this parameter in an intervention period of more than two weeks.

Only minor changes of the RBC fatty acid patterns were observed in consequence of the **$_{hi}$LA/$_{lo}$ALA diet**. Concentrations of LA, ALA, AA and DHA were unchanged. Considering that the investigated LA/ALA ratio of 25.6 : 1 is close to that of the Western diet of 10–20 : 1,[18,19] the almost constant fatty acid pattern is plausible and therefore in line with the expectations.

Limitations

Despite extensive efforts to create a methodical set-up that allows an adequate examination of the effect of different LA/ALA ratios on the n3 PUFA pattern in blood, this study is also subject to methodical limitations. **First**, our study is limited by a small sample size and a short duration time, which owes primarily to the extremely elaborate methodology. Additionally, it is questionable if the compliance of the subjects to follow the manifold dietary restrictions to consume a low-fat and low-PUFA diet would have declined with longer study duration. **Second**, changes in the fatty acid pattern were only measured in RBCs. The changes of PUFA concentrations in RBCs are determined by the blood cell turnover (mean life span of red blood cell is approximately 120 days in circulation) and thus PUFA changes do not fully reach the RBCs. Nevertheless, strong changes in PUFA concentration in RBCs were observed already after seven days suggesting that the PUFA incorporation into newly formed RBCs is sufficient to reflect changes in the PUFA status. Likewise, the fatty acid patterns in RBCs showed the lowest intra-individual variability compared to plasma and plasma phospholipids levels, and thus, appearing as the most suitable biomarker.[58]

Conclusion

We observed a greater increase in RBC EPA concentrations when a high ALA intake was combined with a reduced LA intake ($_{lo}$LA/$_{hi}$ALA diet) compared to a previous study, where we simply increased the ALA intake without LA restriction. Our data support that a high LA intake might impede the ALA conversion to EPA. Further studies are needed to investigate the influence of high LA doses on the n3 PUFA status, especially in view of the high LA and low ALA intake and low ∑EPA + DHA status in many Western countries. Minor changes in the fatty acid profile in consequence of the $_{hi}$LA/$_{lo}$ALA diet suggest that the LA/ALA ratio of 25.6 : 1 is similar to that of the Western diet.

Abbreviations

AA	Arachidonic acid
ALA	Alpha linolenic acid
BMI	Body mass index
DHA	Docosahexaenoic acid
DPAn3	n3 docosapentaenoic acid
DPAn6	n6 docosapentaenoic acid
en%	Percent of total energy
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
HDL	High density lipoprotein
IS	Internal standard
LA	Linoleic acid
LC	Long chain
LDL	Low density lipoprotein
LLOQ	Lower limit of quantification
MUFA(s)	Monounsaturated fatty acid(s)
n.s.	Not significant
n3	Omega-3
n6	Omega-6
PUFA(s)	Polyunsaturated fatty acid(s)
RBCs	Red blood cells
SD	Standard deviation
SE	Standard error
SFA(s)	Saturated fatty acid(s)
TC	Total cholesterol
TG	Triglycerides

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Ethics approval and consent to participate

This investigator initiated study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethic committee at the medical chamber of Lower Saxony (Hannover, Germany). Written informed consent was obtained from all subjects.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Funding

This study was supported by grants from the Deutsche Forschungsgemeinschaft (Grant SCHE 1801 and SCHU 2516) to NHS and JPS.

Author contributions

The manuscript was written through contributions of all authors. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

We would like to thank the participants who contributed their time to this project. We thank Thade Konrad for the help with the analysis of the fat content and the fatty acid profile of food samples.

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4. Paper III

Effects of docosahexaenoic acid supplementation on PUFA levels in red blood cells and plasma

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Published in: *Prostaglandins Leukotrienes and Essential Fatty Acids*, 115 (2016), 12-23

Link: *https://www.plefa.com/article/S0952-3278(16)30130-2/abstract*

Prostaglandins, Leukotrienes and Essential Fatty Acids 115 (2016) 12–23

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Effects of docosahexaenoic acid supplementation on PUFA levels in red blood cells and plasma

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ARTICLE INFO

Keywords:
LC n3 PUFA
Purified DHA
EPA
Retroconversion
Metabolism

ABSTRACT

Introduction: Polyunsaturated fatty acids (PUFA) are metabolized in a complex network of elongation, desaturation and beta oxidation.

Material and methods: The short (1 and 3 wk), and long term (6 and 12 wk) effect of 1076 mg/d docosahexaenoic acid (DHA, free of eicosapentaenoic acid (EPA)) on (absolute) PUFA concentrations in plasma and red blood cells (RBC) of 12 healthy men (mean age 25.1 ± 1.5 years) was investigated.

Results: RBC DHA concentrations significantly ($p < 0.001$) increased from 28 ± 1.6 µg/mL to 38 ± 2.0 µg/mL (wk 1), 52 ± 3.3 µg/mL (wk 3), 68 ± 2.6 µg/mL (wk 6), and 79 ± 3.5 µg/mL (wk 12). Arachidonic acid (AA) concentrations declined in response to DHA treatment, while the effect was more pronounced in plasma (wk 0: 183 ± 9.9 µg/mL, wk 12: 139 ± 8.0 µg/mL, −24%, $p < 0.001$) compared to RBC (wk 0: 130 ± 3.7 µg/mL, wk 12: 108 ± 4.0 µg/mL, −16%, $p=0.001$). Furthermore, an increase of EPA concentrations in plasma (wk 0: 15 ± 1.5 µg/mL, wk 1:19 ± 1.6 µg/mL, wk 3: 27 ± 2.3 µg/mL, wk 6: 23 ± 1.2 µg/mL, wk 12: 25 ± 1.7 µg/mL, $p < 0.001$) and RBC (wk 0: 4.7 ± 0.33 µg/mL, wk 1: 6.7 ± 1.3 µg/mL, wk 3: 8.0 ± 0.66 µg/mL, wk 6: 6.9 ± 0.44 µg/mL, wk 12: 6.7 ± 0.45 µg/mL, n.s.) was observed suggesting a retroconversion of DHA to EPA.

Conclusion: Based on PUFA concentrations we showed that DHA supplementation results in increased EPA levels, whereas it is not known if this impacts the formation of EPA-derived lipid mediators. Furthermore, shifts in the entire PUFA pattern after supplementation of EPA or DHA should be taken into account when discussing differential physiological effects of EPA and DHA.

1. Introduction

Polyunsaturated fatty acids (PUFA) are metabolized in a complex network of conversion, retroconversion and oxidation. The essential omega-3 (n3) PUFA α-linolenic acid (ALA, C18:3n3) occurring in plant based foods can be converted in humans to the long chain (LC) PUFA eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3) in a multistage chain elongation and desaturation process [1,2]. ALA levels in human blood are generally low, accounting for less than 0.30% of total fatty acids in red blood cells (RBC) [3,4]. Dependent on the dietary supply, DHA levels in RBC are considerably higher, ranging between 1.87% and 8.30% of total fatty acids [4]. Several tissues such as brain, retina, or testes contain substantial higher DHA amounts. For example, DHA accounts for approximately 40% of all PUFA in brain [5].

In most diets the intake of preformed EPA and DHA is low due to low fish consumption. Hence, the endogenous status of EPA and DHA relies largely on the dietary intake of ALA. However, several studies suggest that the conversion rate from ALA to EPA (~5%) and especially to DHA is low (~1%) [6,7]. One reason is the high intake of n6 PUFA. Particularly linoleic acid (LA, C18:2n6) competes for the same conversion enzymes [8] in the transformation into the LC n6 PUFA arachidonic acid (AA, C20:4n6).

Taken the beneficial health effect of increased endogenous levels of LC n3 PUFA into account, most n3 PUFA supplements contain a mixture of EPA and DHA from aquatic organisms. It is well described that intake of these fatty acids directly increases the endogenous LC n3 PUFA status [3,9,10], e.g. expressed as relative amount of EPA+DHA in RBC (omega-3 index), which is negatively correlated with the risk for cardiovascular diseases [11]. However, the molecular mechanisms of how LC n3 PUFA elicit their physiological effects remain controversial. It is even unclear whether EPA or DHA is the biologically (more) active

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http://dx.doi.org/10.1016/j.plefa.2016.10.005
Received 26 August 2016; Received in revised form 10 October 2016; Accepted 11 October 2016
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n3 PUFA. The human body possesses all enzymes to convert EPA to DHA via elongation and desaturation as well as for retroconversion of DHA to EPA. Combined supplementation of EPA and DHA consistently resulted in a strong increase in endogenous EPA levels [3,10,12] and metabolites thereof [10,12]. Yet, since both EPA and DHA were supplemented, it remains unclear whether this increase is only caused by EPA absorption or also by intake and retroconversion of DHA.

Retroconversion of DHA via docosapentaenoic acid (DPA, C22:5n3) to EPA has been shown in a few human studies [13–22] and in vitro [23]. In most of the human studies only the relative amount of fatty acids was determined [13–16,18–20,22] which hampers the calculation of the actual conversion rate since a stronger increase in the relative amount of DHA may lead to a decrease in the relative amount of EPA, although its absolute concentration is increased. Nevertheless, previous authors suggested the in vivo retroconversion rate of dietary DHA to EPA to be between 7% and 14% based on the net mol% rise in EPA in serum phospholipids as the percentage of the net corresponding mol% rise of DHA plus EPA, [13–15].

The aim of the present study was to compare the effects of DHA supplementation on absolute PUFA concentrations and relative fatty acid amounts in plasma (acute effects) and RBC (long-term-effects) over a short (1 and 3 wk) and long term (6 and 12 wk) period. Since changes of each PUFA can be evaluated independent from one another, absolute concentrations are more applicable to calculate a DHA-EPA retroconversion rate. A homogenous collective of healthy men in a narrow age class as well as RBC as sample types were chosen to minimize variability. The LC n-3 PUFA content in RBC had the lowest biological variability compared to plasma and plasma phospholipids and thus may be the preferred sample type for assessing LC n-3 PUFA status [24].

2. Materials and methods

This investigator initiated study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethic committee at the medical chamber of Lower Saxony (Hannover, Germany). Written informed consent was obtained from all subjects. The study is registered in the German clinical trial register (ID DRKS00006765; http://apps.who.int/trialsearch/Trial2.aspx?TrialID=DRKS00006765).

The study was conducted in the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany. It consisted of a screening phase and a twelve wk lasting intervention period with a total of 5 examinations: at the beginning (wk 0), after 1 (wk 1), 3 (wk 3), 6 (wk 6) and 12 (wk 12) weeks. During the intervention period subjects daily ingested two 1000 mg-weight capsules with 538 mg DHA per capsule. Hence, total DHA intake was 1076 mg DHA. According to the manufacturer's specification the concentration of C18:2n6, C18:3n3, C20:5n3, C22:5n3 and C22:5n6 is < 0.1% of total fatty acids (Table 1). The fatty acid pattern corresponds with our own GC analysis of the capsules (supplementary Table S1). The free fatty acid content in the used oil was 0.1%; the peroxide value was 1.8 meq/kg. The oil contained fatty acids as reesterified triacylglycerides. In addition the oil contained 250 ppm tocopherol as an antioxidant. The study supplement DHASCO® oil (DHA Single-Cell Oil) produced by marine microalgae, Crypthecodinium cohnii, was kindly provided by DSM Nutritional Products (Columbia, MD, USA). During each visit, fasting blood was collected, blood pressure was measured and subjects completed a questionnaire to obtain information about changes in medication, diet and lifestyle habits (e.g. physical activity), as well as the tolerability of the capsules. Prior to visit wk 0, wk 6, and wk 12 subjects completed a 3-d dietary questionnaire including two working days and one weekend day. Moreover, participants were requested to abstain from fish, seafood, and alpha-linolenic acid (ALA, 18:3n3)-rich vegetable oils such as linseed oil or chia seeds during the intervention period to minimize nutritional effects on variability in LC-PUFA status and blood levels. The compliance was assessed by a count of left-over capsules at the end of the intervention period.

Table 1
Selected fatty acids of the study supplement according to the manufacturer specifications.

Fatty acid	Common name	% of total fatty acids
C10:0	Capric acid	1.7
12:0	Lauric acid	4.6
C14:0	Myristic acid	10.8
C16:0	Palmitic acid	7.8
C16:1n7	Palmitoleic acid	3.3
C18:0	Stearic acid	0.2
C18:1n9	Oleic acid	7.7
C18:1n7	Vaccenic acid	< 0.1
C18:2n6	Linoleic acid	< 0.1
C18:3n3	α-Linolenic acid	< 0.1
C20:0	Arachidic acid	< 0.1
C20:3n6	Dihomo-γ-Linolenic acid	< 0.1
C20:4n6	Arachidonic acid	< 0.1
C20:5n3	Eicosapentaenoic acid	< 0.1
C22:0	Behenic acid	< 0.1
C22:5n3	Docosapentaenoic acid (Clupanodonic acid)	< 0.1
C22:5n6	Docosapentaenoic acid (Osbond acid)	< 0.1
C22:6n3	Docosahexaenoic acid	60.1

Participants were recruited from the general population in Hannover, Germany by advertisements. Several selection criteria were defined to assemble a homogenous study collective. In particular, only men of a limited age class from 20 to 40 years were included to minimize potential fluctuations in lipid profiles due to age [21] or hormonal influence [20,25,26]. Subjects were preselected via telephone interviews according to the following inclusion criteria: Male sex, age between 20 and 40 years, and a body mass index (BMI) between 20 and 27 kg/m^2. Exclusion criteria were defined as followed: Smoking, serum triglyceride levels ≥150 mg/dl (≥1.7 mmol/l); serum total cholesterol levels ≥200 mg/dl (≥5.2 mmol/l); a relative amount of EPA/DHA in RBC ≥8%, intake of fish (> 2 times per wk) as well as addiction to alcohol, drugs and/or medications and diseases: chronic diseases (e.g. malignant tumors, manifest cardiovascular disease, insulin-dependent type 1 and 2 diabetes, severe renal or liver diseases); chronic gastrointestinal disorders (especially small intestine, pancreas, liver) as well as prior gastrointestinal surgical procedures (e.g. gastrectomy); hormonal disorders (e.g. Cushing's syndrome and untreated hyperthyroidism); uncontrolled hypertension; blood coagulation disorders and intake of coagulation-inhibiting drugs; periodic intake of laxatives; intake of anti-inflammatory drugs (incl. acetylsalicylic acid); intake of lipid lowering drugs (incl. statins, fibrates, bile acid binders, nicotinic acid, Ezetimibe) or supplements (incl. omega-3 and −6 fatty acids, beta-glucans, betaine, ketosan, glucomannan, guar resins, hydropropyl methylcellulose, red yeast rice products, oleic acid, pectins, plant sterols/ stanols and their esters) during the last 3 month before baseline examination. Inclusion and exclusion criteria were assessed via questionnaires. The pre-selected subjects were invited for a screening examination to collect fasting blood for the analysis of serum lipid levels and fatty acid profiles in RBC.

Blood samples were collected in the morning between 6:45 and 10:00 a.m. after overnight fasting. The examinations were scheduled at the same time for each subject. The samples were obtained by venipuncture of an arm vein using Multiflyneedles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt). For analysis of fatty acids in plasma, EDTA blood monovettes were centrifuged for 10 min at 1500×*g* and 4 °C and plasma was transferred into 1.5 mL plastic tubes (Sarstedt) and immediately frozen and stored at −80 °C until extraction and analysis. For analysis of fatty acids in RBC, the cell sediment in EDTA blood monovettes was washed twice with PBS (containing 1.5 mg/mL EDTA) after removal of plasma. Finally, the

Table 2
Clinical, biochemical and anthropometric parameters of the study population.

	wk 0 mean	±	SD	wk 1 mean	±	SD	*t*-test* p (wk 1-0)	wk 3 mean	±	SD	*t*-test* p (wk 3-0)	wk 6 Mean	±	SD	*t*-test* p (wk 6-0)	wk 12 mean	±	SD	*t*-test* p (wk 12-0)	An reM** p
Age (years)	25.1	±	5.2																	
Weight (kg)	74.3	±	8.7					74.7	±	8.4	n.s.	74.8	±	8.5	n.s.	74.5	±	9.2	n.s.	n.s.
BMI (kg/m²)	22.1	±	3.0					22.2	±	2.9	n.s.	22.3	±	3.0	n.s.	22.2	±	3.1	n.s.	n.s.
Sys BP (mmHg)	121.7	±	9.4	115.8	±	9.0	n.s.	115.8	±	7.9	n.s.	117.5	±	10.6	n.s.	120.0	±	11.3	n.s.	n.s.
Dias BP (mmHg)	60.8	±	5.1	61.7	±	5.8	n.s.	60.8	±	7.9	n.s.	60.0	±	6.0	n.s.	64.2	±	10.0	n.s.	n.s.
TC (mg/dl)	172.7	±	16.2	179.4	±	29.3	n.s.	190.7	±	19.9	n.s.	179.4	±	25.3	n.s.	179.3	±	19.9	n.s.	0.032
LDL (mg/dl)	109.1	±	17.7	111.9	±	25.5	n.s.	117.7	±	15.0	n.s.	116.8	±	20.0	n.s.	114.0	±	16.7	n.s.	n.s.
HDL (mg/dl)	52.9	±	13.3	54.7	±	13.0	n.s.	57.2	±	13.6	n.s.	55.4	±	14.2	n.s.	59.6	±	14.4	n.s.	0.002
TG (mg/dl)	101.3	±	33.4	108.8	±	51.1	n.s.	106.3	±	48.5	n.s.	84.8	±	20.5	n.s.	77.2	±	19.5	n.s.	0.030
hsCRP (mg/l)	1.5	±	2.2	1.4	±	1.7	n.s.	1.0	±	1.3	n.s.	0.9	±	1.0	n.s.	1.3	±	2.3	n.s.	n.s.

* *t*-tests for paired samples with Holm-Bonferroni correction.
** Anova for repeated measures (An reM).

RBC were reconstituted in PBS to the initial blood volume, transferred into 1.5 mL Eppendorf tubes and immediately frozen and stored at −80 °C until extraction and analysis. All transfer steps were carried out on ice. Other sets of blood samples (serum and EDTA monovettes) were sent to external laboratories for the measurement of clinical parameters. Serum lipid levels, liver enzymes, small blood picture as well as high sensitive C-reactive protein (hsCRP) were determined in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany.

Concentrations of fatty acids were determined by means of gas chromatography (GC) with flame ionization detection as described [27]. In brief, lipids were extracted with MTBE/MeOH and derivatized with methanolic hydrogen chloride, and the resulting FAME were quantified using C23:0 FAME as internal standard. In addition to the determined absolute concentration, expressed as µg fatty acid per mL blood, the relative amount (% of total fatty acids) of each fatty acid was calculated directly based on peak areas as described [27].

Results of anthropometrical measures and serum lipid levels are presented as mean ± SD, while PUFA levels in plasma and red blood cell membranes and its relative change (%) are presented as mean ± SE. If the concentration of an analyte was below the limit of quantification (LOQ) in more than 50% of the samples at one time point, the LOQ is given for this analyte. Relative changes in the variables (v) were calculated individually for each subject at each time point (x) as Δ%, calculated by: Δ%=100*(v wk x − v wk 0)/v wk 0. The percentage retroconversion of DHA to EPA was calculated as (net EPA concentration rise/net EPA+DHA concentration rise) ×100 according to Conquer and Holub [14]. The calculation is based on molar EPA and DHA concentrations [µmol/l] and provides an estimated percentage retroconversion of DHA to EPA. The distribution of the sample sets were analyzed by means of the Kolmogorov-Smirnov test. Differences between baseline (wk 0) levels and different time points after capsule ingestion (wk 1, wk 3, wk 6, wk 12) were analyzed by ANOVA for repeated measurements. To determine sphericity Mauchly's test was used. In term, that sphericity cannot be assumed the Greenhouse-Geisser correction was used. To determine statistical significance between baseline levels and each time point, *t*-tests for paired samples with Holm-Bonferroni correction were carried out. Statistical significance was accepted at p≤0.05. All statistical analyses were carried out with SPSS software (Version 22, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Study population

Twelve subjects of 30 screened men fulfilled the inclusion and showed no exclusion criteria and thus were included in the study. The participants (mean age 25.1 ± 1.5 years) were healthy and showed a normal BMI (22.1 ± 0.9 kg/m²) and serum lipid profile (Table 2). In view of the nutrition behavior the study collective consumed a normal mixed diet (including meat) with low fish consumption (≤1 serving fish per wk). Eating habits (especially fish consumption) and physical activities did not change during the intervention period as investigated by dietary questionnaire. All probands completed the twelve wk intervention period and attended at all five examination time points (no drop-outs). The capsules were well tolerated and no adverse events were observed during the twelve wk intervention period. Count of left-over capsules at the end of the intervention period revealed a compliance rate of 99%. Weight, blood pressure, LDL, and hsCRP levels were unchanged during the intervention. Total cholesterol (TC) and HDL levels were slightly elevated after three wk. As expected, TG levels were decreased after twelve wk of DHA supplementation due to the TG lowering effect of LC n3 PUFA [3,27].

3.2. DHA, EPA and AA levels in RBC and plasma

At baseline, AA was present in highest concentrations in RBC (130 ± 3.7 µg/mL) and plasma (183 ± 10 µg/mL) among all LC PUFA, followed by DHA (28 ± 1.6 µg/mL, 32 ± 2.4 µg/mL) and EPA (4.7 ± 0.33 µg/mL, 15 ± 1.5 µg/mL; Tables 3 and 4; Figs. 1–3).

Supplementation of DHA did not change total fatty acid concentrations in RBC (1006 ± 131 µg/mL at wk 0 vs. 1099 ± 89.3 µg/mL at wk 12; Table 3), whereas a slight reduction in total fatty acid concentrations (2534 ± 292 µg/mL at wk 0 vs. 2177 ± 264 µg/mL) was observed in plasma (Table 4).

In the course of the DHA supplementation, absolute and relative DHA levels in RBC (Table 3) and plasma (Table 4) significantly increased (Fig. 1). Regarding RBC, the absolute and relative increase was time-dependent. During the twelve-wk supplementation, DHA concentrations in RBC rose from 28 ± 1.6 µg/mL (2.81 ± 0.14% of total fatty acids) at wk 0 to 79 ± 3.5 µg/mL (7.20 ± 0.22% of total fatty acids) at wk 12 (~3-fold increase).

Plasma fatty acid levels changed rapidly after starting the intervention. After one wk the DHA levels were 3-fold increased from 32 ± 2.4 µg/mL (1.26 ± 0.08% of total fatty acids) at wk 0 to 102 ± 7.3 µg/mL (3.86 ± 0.20% of total fatty acids) at wk 1, corresponding to a mean change of 234 ± 33% in one wk. The DHA concentration in plasma almost remained at the same level during the whole intervention period with a steady state of about 120 µg/mL.

In contrast, the relative amount of DHA in RBC increased more slowly and mean%-change from baseline was slightly lower, e.g. after twelve wk 190 ± 18% in absolute DHA concentrations vs. 163 ± 14%

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Table 3
Concentration of fatty acids in red blood cells at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/d).

	wk 0 mean	±	SE	wk 1 mean	±	SE	*t*-test* p (wk 1-0)	wk 3 mean	±	SE	*t*-test* p (wk 3-0)	wk 6 Mean	±	SE	*t*-test* p (wk 6-0)	wk 12 Mean	±	SE	*t*-test* p (wk 12-0)	An reM** p
C10:0 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C11:0 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C12:0 µg/mL	< 0.25			< 0.25				2.61	±	0.17		< 0.25				< 0.25				
% of total FA	–			–				0.23	±	0.01		–				–				
C13:0 µg/mL	< 0.25			< 0.25				2.01	±	0.13		< 0.25				< 0.25				
% of total FA				0.21	±	0.01		0.18	±	0.01		0.12	±	0.00		–				
C14:0 µg/mL	4.18	±	0.63	5.93	±	0.88	n.s.	7.52	±	1.06	**0.021**	3.79	±	0.82	n.s.	3.46	±	0.36	n.s.	**0.015**
% of total FA	0.48	±	0.03	0.54	±	0.07	n.s.	0.78	±	0.03	**0.001**	0.42	±	0.05	n.s.	0.34	±	0.02	n.s.	**< 0.001**
C14:1n5 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C15:0 µg/mL	1.16	±	0.19	1.90	±	0.45	n.s.	3.63	±	0.16	**< 0.001**	< 0.25			n.s.	< 0.25				**< 0.001**
% of total FA	0.14	±	0.01	0.22	±	0.04	n.s.	0.32	±	0.01	**0.018**	0.27	±	0.01	**0.049**	–				**0.001**
C15:1n5 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C16:0 µg/mL	230	±	11.8	243	±	13.9	n.s.	268	±	10.4	n.s.	278	±	5.73	**0.009**	267	±	5.70	n.s.	**0.030**
% of total FA	22.7	±	0.47	22.8	±	0.41	n.s.	23.6	±	0.13	n.s.	23.9	±	0.11	n.s.	24.3	±	0.20	**0.012**	**0.011**
C16:1n7 µg/mL	3.31	±	0.32	3.46	±	0.44		4.43	±	0.46		3.74	±	0.32		3.79	±	0.31		n.s.
% of total FA	0.33	±	0.03	0.35	±	0.02		0.38	±	0.03		0.32	±	0.02		0.35	±	0.03		n.s.
C17:0 µg/mL	3.76	±	0.20	3.92	±	0.26	n.s.	4.39	±	0.12	n.s.	4.55	±	0.11	**0.008**	4.43	±	0.10	**0.010**	**0.034**
% of total FA	0.37	±	0.01	0.37	±	0.01	n.s.	0.39	±	0.01	n.s.	0.39	±	0.01	n.s.	0.40	±	0.01	n.s.	**0.007**
C17:1n7 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C18:0 µg/mL	219	±	16.4	232	±	20.2	n.s.	268	±	8.63	n.s.	279	±	4.99	**0.023**	271	±	3.88	**0.011**	**0.038**
% of total FA	21.5	±	1.12	21.5	±	1.14	n.s.	23.7	±	0.39	n.s.	24.0	±	0.27	n.s.	24.8	±	0.43	n.s.	**0.048**
C18:1n9 µg/mL	119	±	4.12	124	±	5.77		126	±	6.92		129	±	3.71		119	±	5.09		n.s.
% of total FA	11.9	±	0.34	11.7	±	0.32	n.s.	11.1	±	0.23	n.s.	11.0	±	0.21	n.s.	10.8	±	0.26	**0.022**	**0.015**
C18:1n7 µg/mL	13.0	±	0.57	13.0	±	0.58		13.4	±	0.77		13.9	±	0.39		12.9	±	0.57		n.s.
% of total FA	1.30	±	0.03	1.23	±	0.05	n.s.	1.17	±	0.03	**0.014**	1.20	±	0.03	**0.001**	1.17	±	0.03	**< 0.001**	**0.032**
C18:2n6 µg/mL	85.8	±	4.65	89.8	±	7.2		87.0	±	3.99		89.2	±	3.65		80.9	±	2.54		n.s.
% of total FA	8.55	±	0.38	8.38	±	0.38	n.s.	7.68	±	0.24	n.s.	7.65	±	0.21	n.s.	7.36	±	0.17	**0.036**	**0.010**
C18:3n6 µg/mL	0.45	±	0.05	0.32	±	0.03	n.s.	0.33	±	0.02	n.s.	0.33	±	0.03	n.s.	< 0.25			**0.009**	**0.003**
% of total FA	0.05	±	0.00	0.03	±	0.00		0.03	±	0.00		0.03	±	0.00		–				n.s.
C18:3n3 µg/mL	1.37	±	0.13	1.61	±	0.32	n.s.	1.36	±	0.13	n.s.	1.29	±	0.13	n.s.	1.20	±	0.17	**< 0.001**	**0.002**
% of total FA	0.14	±	0.02	0.15	±	0.02		0.12	±	0.01		0.11	±	0.01		0.11	±	0.01		n.s.
C20:0 µg/mL	4.91	±	0.29	5.26	±	0.39		5.44	±	0.22		5.68	±	0.16		5.53	±	0.13		n.s.
% of total FA	0.48	±	0.01	0.49	±	0.02		0.48	±	0.01		0.49	±	0.01		0.50	±	0.01		n.s.
C20:1n9 µg/mL	2.48	±	0.15	2.62	±	0.17		2.57	±	0.17		2.61	±	0.14		2.41	±	0.19		n.s.
% of total FA	0.25	±	0.01	0.25	±	0.01	n.s.	0.23	±	0.01	n.s.	0.22	±	0.01	n.s.	0.22	±	0.01	**0.045**	**0.050**
C20:2n6 µg/mL	2.02	±	0.09	2.04	±	0.13		2.01	±	0.13		2.12	±	0.09		1.82	±	0.11		n.s.
% of total FA	0.20	±	0.01	0.19	±	0.01	n.s.	0.18	±	0.01	**0.001**	0.18	±	0.01	**< 0.001**	0.16	±	0.01	**< 0.001**	**< 0.001**
C20:3n6 µg/mL	13.6	±	0.95	13.2	±	0.87	n.s.	12.1	±	0.64	n.s.	12.1	±	0.61	n.s.	10.5	±	0.60	**0.016**	**0.006**
% of total FA	1.35	±	0.07	1.26	±	0.08	n.s.	1.07	±	0.05	**0.001**	1.03	±	0.04	**< 0.001**	0.96	±	0.05	**< 0.001**	**< 0.001**
C21:0 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25			–	–
% of total FA	–			–				–				–				–			–	–
C20:4n6 µg/mL	130	±	3.65	133.4	±	6.18	n.s.	127	±	6.38	n.s.	125	±	2.85	n.s.	108	±	4.04	**0.010**	**0.001**
% of total FA	13.0	±	0.52	12.7	±	0.58	n.s.	11.2	±	0.14	**0.030**	10.8	±	0.18	**0.002**	9.78	±	0.18	**< 0.001**	**< 0.001**
C20:3n3 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25			–	–
% of total FA	–			–				–				–				–			–	–
C20:5n3 µg/mL	4.74	±	0.33	6.72	±	1.28		8.00	±	0.66		6.92	±	0.44		6.72	±	0.45		n.s.
% of total FA	0.48	±	0.05	0.61	±	0.08	n.s.	0.70	±	0.04	**0.003**	0.59	±	0.03	n.s.	0.61	±	0.04	n.s.	**0.035**
C22:0 µg/mL	13.1	±	0.66	13.4	±	1.01		13.2	±	0.84		13.3	±	0.73		12.4	±	0.74		n.s.
% of total FA	1.32	±	0.08	1.28	±	0.10	**< 0.001**	1.15	±	0.04	**< 0.001**	1.14	±	0.06	**< 0.001**	1.13	±	0.07	**< 0.001**	**< 0.001**
C22:1n9 µg/mL	1.19	±	0.17	1.28	±	0.15	n.s.	2.57	±	0.19	**0.008**	2.01	±	0.37	n.s.	1.15	±	0.38	n.s.	**0.002**
% of total FA	0.12	±	0.02	0.12	±	0.01		0.23	±	0.03		0.20	±	0.03		0.19	±	0.04		n.s.

(continued on next page)

Table 3 *(continued)*

	wk 0 mean	±	SE	wk 1 mean	±	SE	*t*-test* p (wk 1-0)	wk 3 mean	±	SE	*t*-test* p (wk 3-0)	wk 6 Mean	±	SE	*t*-test* p (wk 6-0)	wk 12 Mean	±	SE	*t*-test* p (wk 12-0)	An reM** p
C22:2n6 µg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C22:4n6 µg/mL	25.6	±	1.05	26.1	±	1.28	n.s.	24.2	±	1.59	n.s.	21.9	±	0.86	**0.008**	16.1	±	0.81	**< 0.001**	**< 0.001**
% of total FA	2.56	±	0.10	2.49	±	0.14	n.s.	2.11	±	0.07	n.s.	1.88	±	0.06	n.s.	1.47	±	0.06	**0.003**	**< 0.001**
C22:5n3 µg/mL	21.1	±	0.74	21.2	±	0.89	n.s.	19.2	±	1.21	n.s.	17.3	±	0.79	**0.003**	12.6	±	0.59	**< 0.001**	**< 0.001**
% of total FA	2.12	±	0.09	2.02	±	0.10	n.s.	1.68	±	0.06	n.s.	1.48	±	0.05	**0.013**	1.14	±	0.04	**0.006**	**0.003**
C24:0 µg/mL	36.8	±	1.64	37.6	±	1.75		36.8	±	1.66		38.8	±	1.00		37.0	±	1.34		n.s.
% of total FA	3.71	±	0.21	3.59	±	0.19		3.24	±	0.06		3.33	±	0.07		3.37	±	0.09		n.s.
C22:6n3 µg/mL	28.2	±	1.6	38.3	±	2.04	**0.003**	52.4	±	3.3	**< 0.001**	67.6	±	2.61	**< 0.001**	79.3	±	3.46	**< 0.001**	**< 0.001**
% of total FA	2.81	±	0.14	3.62	±	0.13	n.s.	4.59	±	0.17	n.s.	5.80	±	0.16	**0.023**	7.20	±	0.22	**0.026**	**< 0.001**
C24:1n9 µg/mL	41.3	±	1.75	42.6	±	2.13		41.9	±	2.58		43.6	±	1.75		41.4	±	2.17		n.s.
% of total FA	4.15	±	0.20	4.05	±	0.21		3.67	±	0.12		3.74	±	0.12		3.75	±	0.14		n.s.
Total FA µg/mL	1006	±	131	1065	±	184		1136	±	163		1164	±	84.0		1099	±	89.3		n.s.
SFA µg/mL	513	±	99.7	545	±	124	n.s.	612	±	76.3	n.s.	626	±	42.7	**0.012**	601	±	35.6	n.s.	**0.040**
% of total FA	50.6	±	4.8	50.9	±	4.80	n.s.	54.0	±	1.60	n.s.	53.8	±	1.40	n.s.	54.8	±	2.10	n.s.	**0.041**
MUFA µg/mL	181	±	21.8	187	±	29.2		191	±	36.0		194	±	21.1		181	±	26.7		n.s.
% of total FA	13.9	±	1.30	13.7	±	1.30	n.s.	13.1	±	0.80	n.s.	13.0	±	0.90	n.s.	12.6	±	1.0	**0.018**	**0.020**
PUFA µg/mL	313	±	33.5	333	±	52.6		334	±	53.5		344	±	31.0		317	±	34.4		n.s.
% of total FA	28.5	±	3.1	27.8	±	3.10	n.s.	24.7	±	0.90	**0.020**	23.7	±	0.80	**0.001**	21.6	±	0.9	**< 0.001**	**< 0.001**
Σn3 PUFA µg/mL	55.4	±	7.3	67.8	±	12.3	n.s.	81.0	±	16.1	**0.001**	93.1	±	10.8	**< 0.001**	99.8	±	13.0	**< 0.001**	**< 0.001**
% of total FA	2.7	±	0.5	2.80	±	0.50	n.s.	2.50	±	0.30	n.s.	2.20	±	0.20	**0.002**	1.90	±	0.20	**< 0.001**	**< 0.001**
Σn6 PUFA µg/mL	257	±	29.4	265	±	42.1	n.s.	253	±	39.5	n.s.	251	±	22.3	n.s.	217	±	23.1	n.s.	**0.005**
% of total FA	25.7	±	2.8	25.0	±	2.80	n.s.	22.2	±	1.0	**0.014**	21.5	±	0.80	**0.002**	19.7	±	0.80	**< 0.001**	**< 0.001**
n3/n6	0.22	±	0.03	0.26	±	0.03	**0.008**	0.32	±	0.04	**< 0.001**	0.37	±	0.03	**< 0.001**	0.46	±	0.04	**< 0.001**	**< 0.001**
AA/EPA	28.6	±	6.28	24.0	±	7.86	n.s.	16.6	±	3.55	**< 0.001**	18.9	±	4.46	**0.006**	16.7	±	3.5	**0.002**	**< 0.001**
AA/DHA	4.74	±	0.91	3.55	±	0.66	**< 0.001**	2.47	±	0.35	**< 0.001**	1.88	±	0.24	**< 0.001**	1.37	±	0.17	**< 0.001**	**< 0.001**
DHA/EPA	6.26	±	1.51	6.85	±	2.55	n.s.	6.77	±	1.39	n.s.	10.09	±	2.06	**0.001**	12.2	±	2.57	**< 0.001**	**< 0.001**

Levels are shown as concentration [µg/mL] in blood and as relative amount [%] of total fatty acids.

AA, Arachidonic acid; DGLA, Dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETE, Eicosatrienoic acid; GLA, Gamma-linolenic acid; LA, Linoleic acid; MUFA, monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; SFA, saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; Σ n-3 PUFA, polyunsaturated fatty acids: C18:3n3, C20:3n3, C20:5n3, C22:5n3, C22:6n3; Σ n-6 PUFA: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6.

* *t*-tests for paired samples with Holm-Bonferroni correction.

** Anova for repeated measures (An reM).

calculated based on relative amounts.

DHA supplementation led to an increase of EPA levels in RBC and plasma (Tables 3 and 4; Fig. 2). Highest EPA concentrations in RBC were reached after three wk (8.0 ± 0.66 µg/mL). After six and twelve wk the concentration slightly declined to 6.9 ± 0.44 µg/mL and 6.7 ± 0.45 µg/mL. These changes correspond to a relative increase of 40 ± 21% (wk 1), 72 ± 13% (wk 3), 52 ± 13% (wk 6), and 50 ± 16% (wk 12).

The increase of EPA was more pronounced in plasma compared to RBC. In plasma the mean%-change of EPA levels was generally stronger. Following a rapid increase, the plasma concentration almost doubled from 15 ± 1.5 µg/mL to 27 ± 2.3 µg/mL between baseline and wk three. After wk six and twelve, the EPA plasma concentrations, likewise, slightly decline to 23 ± 1.2 µg/mL and 25 ± 1.7 µg/mL. In RBC, the calculated percentage retroconversion rate of DHA to EPA based on molar concentrations is 13.2 ± 4.3% (wk 1), 12.0 ± 1.3% (wk 3), 5.3 ± 1.1% (wk 6), and 3.3 ± 1.2% (wk 12) while it is 3.9 ± 2.1% (wk 1), 11.6 ± 1.7% (wk 3), 8.7 ± 1.3% (wk 6), and 10.6 ± 1.8% (wk 12) based on absolute concentrations in plasma (Table 5).

In plasma, DPAn3 was detected in low concentrations at baseline (wk 0: 11.8 ± 0.78 µg/mL; Table 4). Substantial DPAn3 levels were found in RBC (wk 0: 21.1 ± 0.74 µg/mL; Table 3). In response to DHA supplementation DPAn3 levels decreased. In RBC changes in DPAn3 levels occurred after six wk (wk 6: 17.3 ± 0.79 µg/mL); and were reduced almost by half after twelve wk (wk 12: 12.6 ± 0.59 µg/mL). In plasma a significant decline of baseline DPAn3 concentrations were already observed after one and three wk (wk 1: 7.77 ± 0.59 and wk 3: 7.57 ± 0.56 µg/mL), thereafter the concentration dropped under the limit of quantification (< 0.50 µg/mL) at wk six and twelve.

During the intervention the concentration and relative amount of saturated fatty acids (SFA) monounsaturated fatty acids (MUFA) and total PUFA remained constant in RBC (Table 3; Fig. S1). Thus, increase in EPA and DHA in RBC directly led to decreased total n6 PUFA levels (25.7 ± 2.8% of all fatty acids at wk 0 vs. 19.7 ± 0.8% of all fatty acids at wk 12), as also shown for AA in Fig. 3. In consequence the n3/n6 PUFA ratio in RBC increased from 0.22 ± 0.03 at wk 0 to 0.46 ± 0.04 at wk 12. In plasma the increase in the n3 PUFA concentration and relative amount (2.9 ± 0.6% of all fatty acids at wk 0 vs. 7.3 ± 0.9% of all fatty acids at wk 12) was not accompanied by a decrease in the relative amount of n6 PUFA (31.8 ± 2.8% of all fatty acids at wk 0 vs. 31.2 ± 2.2% at wk 12; Table 4; Fig. S1). Rather, concentration and relative amount of both SFA and MUFA fractions also slightly decreased. The n3/n6 PUFA ratio in plasma increased from 0.09 ± 0.02 at wk 12 to 0.23 ± 0.04 at wk 12.

In RBC the AA concentration remained almost unchanged in the first six wk and then dropped to 108 ± 4.0 µg/mL at wk 12 (Table 3; Fig. 3). The relative amount of AA in RBC decreased in a time dependent manner with a mean%-change of −13 ± 2.8%, −17 ± 2.3%, and −24 ± 2.0% at wk three, six, and twelve.

Table 4
Concentration of fatty acids in plasma at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/d).

	wk 0 mean	±	SE	wk 1 mean	±	SE	*t*-test* p (wk 1-0)	wk 3 mean	±	SE	*t*-test* p (wk 3-0)	wk 6 Mean	±	SE	*t*-test* p (wk 6-0)	wk 12 mean	±	SE	*t*-test* p (wk 12-0)	An reM** p
C10:0 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C11:0 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C12:0 μg/mL	1.70	±	0.29	1.65	±	0.33		1.62	±	0.32		1.39	±	0.39		1.57	±	0.38		n.s.
% of total FA	0.07	±	0.01	0.06	±	0.01		0.06	±	0.01		0.07	±	0.02		0.09	±	0.02		n.s.
C13:0 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C14:0 μg/mL	22.4	±	3.06	21.8	±	4.05		23.1	±	2.93		14.9	±	1.57		18.3	±	2.54		n.s.
% of total FA	0.88	±	0.01	0.78	±	0.10		0.93	±	0.11		0.64	±	0.06		0.84	±	0.11		n.s.
C14:1n5 μg/mL	1.98	±	0.27	1.71	±	0.29	n.s.	2.02	±	0.27	n.s.	1.39	±	0.13	n.s.	1.46	±	0.18	n.s.	n.s.
% of total FA	0.08	±	0.01	0.06	±	0.01		0.08	±	0.01		0.06	±	0.00		0.07	±	0.01		n.s.
C15:0 μg/mL	< 0.25			< 0.25				3.71	±	0.60		< 0.25				< 0.25				
% of total FA	–			–				0.19	±	0.01		–				–				
C15:1n5 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C16:0 μg/mL	610	±	27.8	623	±	39.4	n.s.	573	±	31.7	n.s.	532	±	16.8	**0.044**	509	±	21.2	**0.013**	**0.005**
% of total FA	24.0	±	0.47	23.6	±	0.37		23.1	±	0.45		23.1	±	0.27		23.4	±	0.42		n.s.
C16:1n7 μg/mL	50.5	±	5.11	44.8	±	4.20	n.s.	39.3	±	3.71	n.s.	35.4	±	2.51	n.s.	39.1	±	2.77	n.s.	**0.018**
% of total FA	1.96	±	0.15	1.68	±	0.08	n.s.	1.56	±	0.10	n.s.	1.54	±	0.10	n.s.	1.79	±	0.10	n.s.	**0.005**
C17:0 μg/mL	7.53	±	0.41	7.33	±	0.49	n.s.	7.08	±	0.34	n.s.	6.10	±	0.35	**0.031**	5.25	±	0.25	**0.003**	**< 0.001**
% of total FA	0.30	±	0.01	0.28	±	0.01	n.s.	0.29	±	0.01	n.s.	0.26	±	0.01	n.s.	0.24	±	0.01	**0.021**	**< 0.001**
C17:1n7 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C18:0 μg/mL	311	±	20.2	297	±	17.7	n.s.	254	±	10.9	n.s.	258	±	9.10	n.s.	234	±	14.3	**0.013**	**0.001**
% of total FA	12.3	±	0.68	11.4	±	0.53	n.s.	10.3	±	0.35	n.s.	11.2	±	0.27	n.s.	10.7	±	0.50	n.s.	**0.039**
C18:1n9 μg/mL	537	±	26.2	573	±	50.6	n.s.	517	±	41.6	n.s.	464	±	26.9	n.s.	427	±	20.1	**0.042**	**0.004**
% of total FA	21.1	±	0.58	21.4	±	0.84		20.6	±	0.76		20.0	±	0.73		19.6	±	0.51		n.s.
C18:1n7 μg/mL	48.5	±	2.28	45.1	±	2.38	n.s.	42.3	±	3.01	n.s.	39.9	±	1.82	**0.011**	38.1	±	1.77	**0.005**	**< 0.001**
% of total FA	1.91	±	0.06	1.72	±	0.04	**0.011**	1.69	±	0.06	**< 0.001**	1.73	±	0.06	**0.037**	1.75	±	0.07	n.s.	**0.002**
C18:2n6 μg/mL	563	±	17.9	573	±	26.9	n.s.	571	±	22.2	n.s.	541	±	18.2	n.s.	506	±	19.4	n.s.	**0.017**
% of total FA	22.4	±	0.84	22.0	±	0.79		23.3	±	0.73		23.6	±	0.69		23.4	±	0.68		n.s.
C18:3n6 μg/mL	8.42	±	0.78	6.78	±	0.71	n.s.	5.68	±	0.56	n.s.	4.25	±	0.42	**0.001**	4.19	±	0.81	**0.036**	**< 0.001**
% of total FA	0.33	±	0.02	0.25	±	0.02	n.s.	0.25	±	0.01	n.s.	0.18	±	0.02	**< 0.001**	0.22	±	0.03	n.s.	**< 0.001**
C18:3n3 μg/mL	14.0	±	1.30	14.9	±	1.98		15.4	±	1.70		13.1	±	1.28		13.5	±	2.02		n.s.
% of total FA	0.56	±	0.06	0.56	±	0.07		0.61	±	0.06		0.57	±	0.06		0.62	±	0.09		n.s.
C20:0 μg/mL	7.28	±	0.47	7.30	±	0.44	n.s.	5.95	±	0.28	**0.024**	6.32	±	0.24	n.s.	6.08	±	0.31	n.s.	**0.006**
% of total FA	0.29	±	0.02	0.29	±	0.02		0.25	±	0.02		0.28	±	0.01		0.28	±	0.01		n.s.
C20:1n9 μg/mL	4.11	±	0.24	4.10	±	0.43	n.s.	3.54	±	0.32	n.s.	3.06	±	0.27	n.s.	2.89	±	0.32	n.s.	**0.003**
% of total FA	0.16	±	0.01	0.15	±	0.01	n.s.	0.14	±	0.01	n.s.	0.13	±	0.01	n.s.	0.13	±	0.02	n.s.	**0.042**
C20:2n6 μg/mL	4.37	±	0.31	4.08	±	0.31	n.s.	4.36	±	0.25	n.s.	3.59	±	0.17	n.s.	3.41	±	0.20	n.s.	**0.01**
% of total FA	0.17	±	0.01	0.15	±	0.01		0.18	±	0.01		0.16	±	0.00		0.16	±	0.01		n.s.
C20:3n6 μg/mL	35.5	±	1.79	30.2	±	1.62	**0.023**	26.7	±	1.00	**< 0.001**	23.1	±	1.39	**< 0.001**	21.5	±	1.46	**< 0.001**	**< 0.001**
% of total FA	1.40	±	0.06	1.16	±	0.05	**0.045**	1.09	±	0.03	**0.002**	1.00	±	0.05	**0.001**	0.98	±	0.05	**0.003**	**< 0.001**
C21:0 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C20:4n6 μg/mL	183	±	9.86	179	±	8.31	n.s.	157	±	6.85	**0.006**	149	±	7.08	**0.001**	139	±	7.99	**< 0.001**	**< 0.001**
% of total FA	7.21	±	0.26	6.98	±	0.42	n.s.	6.40	±	0.29	n.s.	6.49	±	0.28	n.s.	6.36	±	0.23	n.s.	**0.026**
C20:3n3 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C20:5n3 μg/mL	15.4	±	1.53	19.2	±	1.62	n.s.	27.0	±	2.29	**0.001**	23.2	±	1.16	**0.002**	25.1	±	1.70	**0.001**	**< 0.001**
% of total FA	0.60	±	0.06	0.74	±	0.07	n.s.	1.10	±	0.09	**< 0.001**	1.01	±	0.05	**0.001**	1.16	±	0.07	**< 0.001**	**< 0.001**
C22:0 μg/mL	14.3	±	0.45	15.5	±	0.42	n.s.	15.1	±	0.71	n.s.	15.2	±	0.55	n.s.	15.4	±	0.58	n.s.	n.s.
% of total FA	0.57	±	0.02	0.61	±	0.03	n.s.	0.62	±	0.03	n.s.	0.67	±	0.03	**< 0.001**	0.72	±	0.03	**< 0.001**	**< 0.001**
C22:1n9 μg/mL	< 0.25			< 0.25				3.47	±	0.80		< 0.25				< 0.25				
% of total FA	–			–				0.16	±	0.04		–				–				

(continued on next page)

Table 4 *(continued)*

	wk 0 mean	±	SE	wk 1 mean	±	SE	*t*-test* p (wk 1-0)	wk 3 mean	±	SE	*t*-test* p (wk 3-0)	wk 6 Mean	±	SE	*t*-test* p (wk 6-0)	wk 12 mean	±	SE	*t*-test* p (wk 12-0)	An reM** p
C22:2n6 μg/mL	< 0.25			< 0.25				< 0.25				< 0.25				< 0.25				
% of total FA	–			–				–				–				–				
C22:4n6 μg/mL	6.11	±	0.34	4.89	±	0.21	**0.002**	3.54	±	0.16	**< 0.001**	3.18	±	0.19	**< 0.001**	2.97	±	0.22	**< 0.001**	**< 0.001**
% of total FA	0.24	±	0.01	0.19	±	0.01	**< 0.001**	0.14	±	0.01	**< 0.001**	0.14	±	0.01	**< 0.001**	0.14	±	0.01	**< 0.001**	**< 0.001**
C22:5n3 μg/mL	11.8	±	0.78	7.77	±	0.59	**< 0.001**	7.57	±	0.56	**0.003**	< 0.25				< 0.25				**< 0.001**
% of total FA	0.46	±	0.02	0.30	±	0.02	**< 0.001**	0.31	±	0.02	**< 0.001**	–				–				**< 0.001**
C24:0 μg/mL	12.7	±	0.40	13.7	±	0.42	n.s.	14.2	±	0.61	**0.012**	13.9	±	0.49	n.s.	13.2	±	0.48	n.s.	**0.05**
% of total FA	0.51	±	0.02	0.54	±	0.03	n.s.	0.58	±	0.03	n.s.	0.61	±	0.02	**< 0.001**	0.61	±	0.02	**0.016**	**< 0.001**
C22:6n3 μg/mL	31.9	±	2.37	102	±	7.32	**< 0.001**	124	±	8.22	**< 0.001**	115	±	6.01	**< 0.001**	118	±	7.27	**< 0.001**	**< 0.001**
% of total FA	1.26	±	0.08	3.86	±	0.20	**< 0.001**	4.99	±	0.23	**< 0.001**	5.00	±	0.21	**< 0.001**	5.43	±	0.29	**< 0.001**	**< 0.001**
C24:1n9 μg/mL	28.1	±	1.30	29.5	±	1.57		28.9	±	1.39		29.8	±	1.39		29.6	±	1.21		n.s.
% of total FA	1.12	±	0.06	1.16	±	0.08	n.s.	1.19	±	0.07	n.s.	1.31	±	0.07	**0.017**	1.37	±	0.07	**0.029**	**< 0.001**
Total FA μg/mL	2534	±	292	2628	±	482	n.s.	2477	±	394	n.s.	2301	±	233	n.s.	2177	±	264	**0.019**	**0.002**
SFA μg/mL	989	±	154	988	±	202	n.s.	898	±	148	n.s.	849	±	93.6	**0.023**	804	±	121	**0.007**	**0.002**
% of total FA	39.0	±	3.20	37.6	±	2.60	n.s.	36.3	±	2.30	n.s.	36.9	±	1.90	n.s.	36.9	±	2.80	n.s.	**0.039**
MUFA μg/mL	671	±	112	698	±	197	n.s.	637	±	164	n.s.	574	±	105	n.s.	539	±	83.5	**0.045**	**0.005**
% of total FA	26.4	±	2.30	26.2	±	2.90		25.4	±	2.70		24.8	±	2.80		24.7	±	2.20		n.s.
PUFA μg/mL	874	±	86.3	942	±	132	n.s.	942	±	116	n.s.	878	±	82.5	n.s.	835	±	101	n.s.	**0.005**
% of total FA	34.7	±	2.9	36.2	±	3.90	n.s.	38.3	±	3.10	n.s.	38.3	±	2.30	**0.001**	38.4	±	2.30	**0.001**	**< 0.001**
Σn3 PUFA μg/mL	73.1	±	15.1	144	±	31.8	**< 0.001**	174	±	37.3	**< 0.001**	154	±	22.0	**< 0.001**	158	±	25.1	**< 0.001**	**< 0.001**
% of total FA	2.90	±	0.60	5.50	±	0.90	**< 0.001**	7.0	±	1.0	**< 0.001**	6.70	±	0.70	**< 0.001**	7.30	±	0.90	**< 0.001**	**< 0.001**
Σn6PUFA μg/mL	801	±	80.1	798	±	106	n.s.	768	±	93.3	n.s.	725	±	73.8	n.s.	677	±	86.5	**0.001**	**< 0.001**
% of total FA	31.8	±	2.80	30.8	±	3.50		31.3	±	3.20		31.6	±	2.50		31.2	±	2.20		n.s.
n3/n6	0.09	±	0.02	0.18	±	0.03	**< 0.001**	0.23	±	0.04	**< 0.001**	0.21	±	0.03	**< 0.001**	0.23	±	0.04	**< 0.001**	**< 0.001**
AA/EPA	12.8	±	3.10	10.1	±	3.16	**0.026**	6.19	±	1.80	**< 0.001**	6.55	±	1.23	**0.001**	5.73	±	1.37	**< 0.001**	**< 0.001**
AA/DHA	5.92	±	1.44	1.88	±	0.60	**< 0.001**	1.32	±	0.36	**< 0.001**	1.34	±	0.38	**< 0.001**	1.22	±	0.30	**< 0.001**	**< 0.001**
DHA/EPA	2.19	±	0.48	5.70	±	2.13	**< 0.001**	4.93	±	1.75	**0.001**	5.07	±	1.10	**< 0.001**	4.92	±	1.54	**0.001**	**< 0.001**

Levels are shown as concentration [μg/mL] in blood and as relative amount [%] of total fatty acids.

AA, Arachidonic acid; DGLA, Dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETE, Eicosatrienoic acid; GLA, Gamma-linolenic acid; LA, Linoleic acid; MUFA, monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; SFA, saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; Σ n-3 PUFA, polyunsaturated fatty acids: C18:3n3, C20:3n3, C20:5n3, C22:5n3, C22:6n3; Σ n-6 PUFA: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6.

* *t*-tests for paired samples with Holm-Bonferroni correction.

** Anova for repeated measures (An reM).

The AA plasma concentration declined in a time dependent manner following DHA supplementation. Mean%-change was −14 ± 2.9% after three wk, −18 ± 2.3% after six wk and −24 ± 2.6% after twelve wk. The relative amount of AA of all fatty acids insufficiently reflects these changes, yielding a mean%-decrease of ~10% between wk three and twelve. Similarly to AA, concentrations of several other n6 PUFA (e.g. C18:3n6, C20:2n6, C20:3n6, and C22:4n6) declined in a time dependent fashion (Tables 3 and 4) with some differences between RBC and plasma. Changes in concentrations of the major food n6 and n3 PUFA LA and ALA were small during DHA intervention. ALA concentrations in plasma and RBC were not influenced. However, for LA discrepancies between concentrations and relative amounts were observed. LA concentrations in plasma were significantly reduced at wk 12, whereas no changes were observed regarding relative amounts (Table 4). In contrast, LA concentrations in RBC were unchanged, but relative amounts were significantly reduced at wk 3 and wk 12 (Table 3).

After twelve wk of DHA supplementation, AA was still the most abundant PUFA in RBC (wk 12: 108 ± 4.0 μg/mL, −16%) and plasma (wk 12: 139 ± 8.0 μg/mL, −24%); however, the difference to DHA was significantly diminished (Tables 3 and 4). At wk 0 the AA/DHA ratio in RBC was 4.7 ± 0.9 and in plasma 5.9 ± 1.4. After DHA supplementation the ratio AA/DHA was only 1.4 ± 0.2 for RBC and 1.2 ± 0.3 for plasma, respectively. Due to the strong increase of DHA concentration in RBC (wk 12: 79 ± 3.5 μg/mL, +190%) and plasma (wk 12: 118 ± 7.3 μg/mL, +294%), the DHA/EPA ratio in RBC increased from 6.3 ± 1.5 at wk 0 to 12.2 ± 2.6 at wk 12 and from 2.2 ± 0.5 to 4.9 ± 1.5 in plasma, respectively. As a consequence of the EPA increase in RBC (wk 12: 6.7 ± 0.45 μg/mL, +50%) and plasma (wk 12: 25 ± 1.7 μg/mL, +78%) – as well as the AA decrease – the AA/EPA ratio in RBC was diminished from 28.6 ± 6.3 at wk 0 to 16.7 ± 3.5 at wk 12 and from 12.8 ± 3.1 to 5.7 ± 1.4 in plasma, respectively.

4. Discussion

The present study evaluated the effects of DHA supplementation (free of EPA) on fatty acid patterns in RBC and plasma as well as on serum lipids and blood pressure over time. A homogenous collective of healthy men in a narrow age class was chosen to analyze whether relevant retroconversion of DHA to EPA occurs. Previous studies found that the retroconversion of DHA to EPA was significantly lower in postmenopausal women receiving hormone replacement therapy vs. women receiving no hormones [20], possibly due to an influence of female hormones on hepatic lipid and fatty acid metabolism [25]. Consequently, sex differences in the proportions of n3 PUFA blood levels were observed [26]. Furthermore, a difference in the DHA to EPA retroconversion rate was detected between healthy elderly (mean age 77 years) vs. young adults (mean age 27 years) [21]. To minimize variability, these findings suggest sex-specific studies on LC PUFA

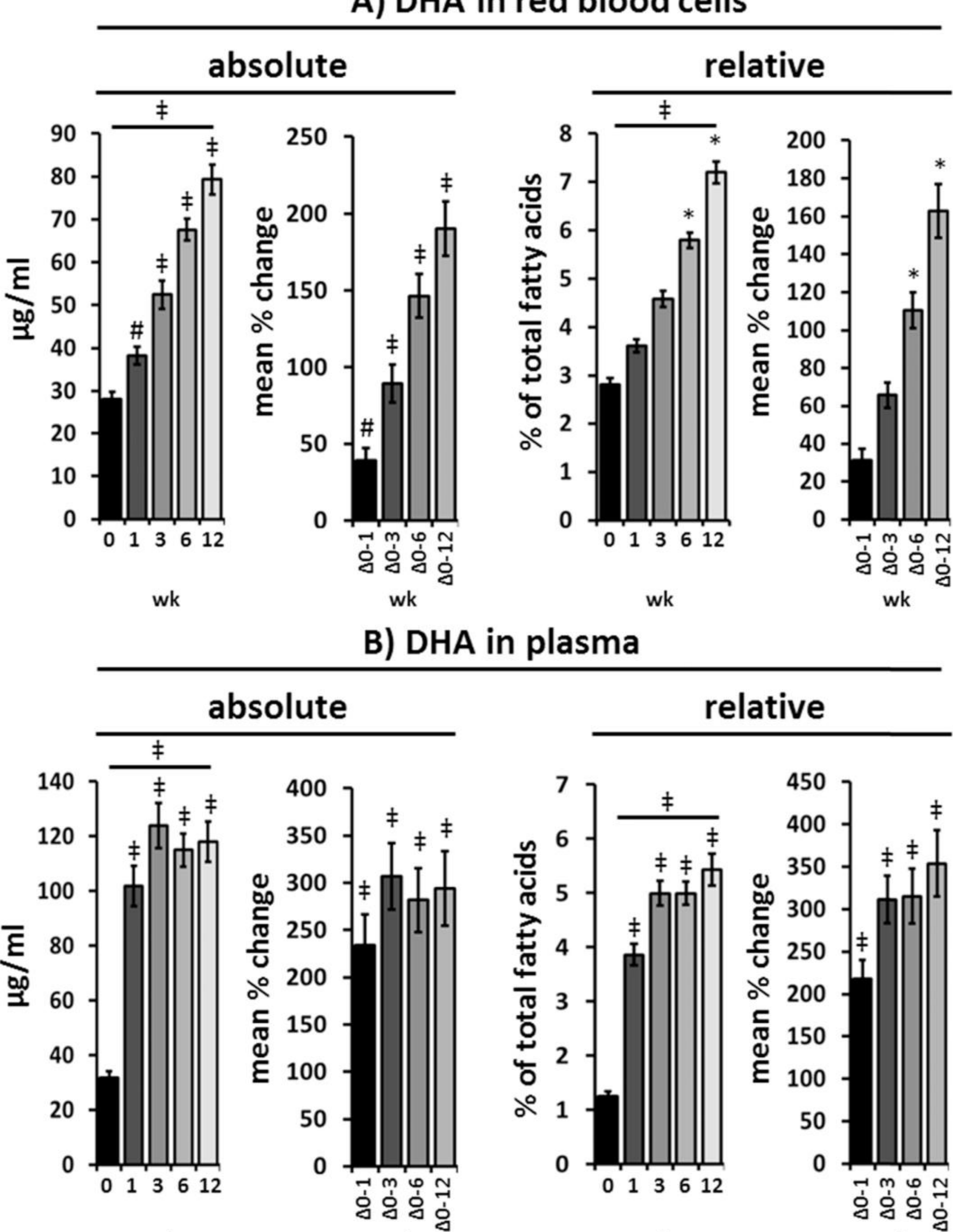

Fig. 1. Levels of docosahexaenoic acid (DHA, C22:6n3) in red blood cells and plasma at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/day). DHA levels are shown as absolute concentrations [µg/mL] and as relative amounts [%] of total fatty acids in (A) plasma and (B) red blood cells. All data are shown as mean ± SE (n=12). $^{*}p \le 0.05$, $^{\#}p \le 0.005$, $^{\ddagger}p < 0.001$.

metabolism with subjects in a comparable age class. In our study, at baseline all subjects showed a similar pattern of PUFA in blood and interperson-variability was low (Figs. 1–3).

The DHA supplementation resulted – as expected for fish oils or LC n3 PUFA – in a significant (p=0.030) TG reduction [3,28]. Even in healthy subjects with low baseline TG levels (101.3 ± 33.4 mg/dl), a time dependent TG reduction after twelve wk (77.2 ± 19.5 mg/dl) was observed. As a result of the TG reduction, total fatty acid concentrations in plasma were also reduced.

As expected, DHA supplementation resulted in a strong increase in DHA levels in both, RBC and plasma. Overall, changes in PUFA plasma concentrations occurred faster than in RBC. The incorporation of DHA

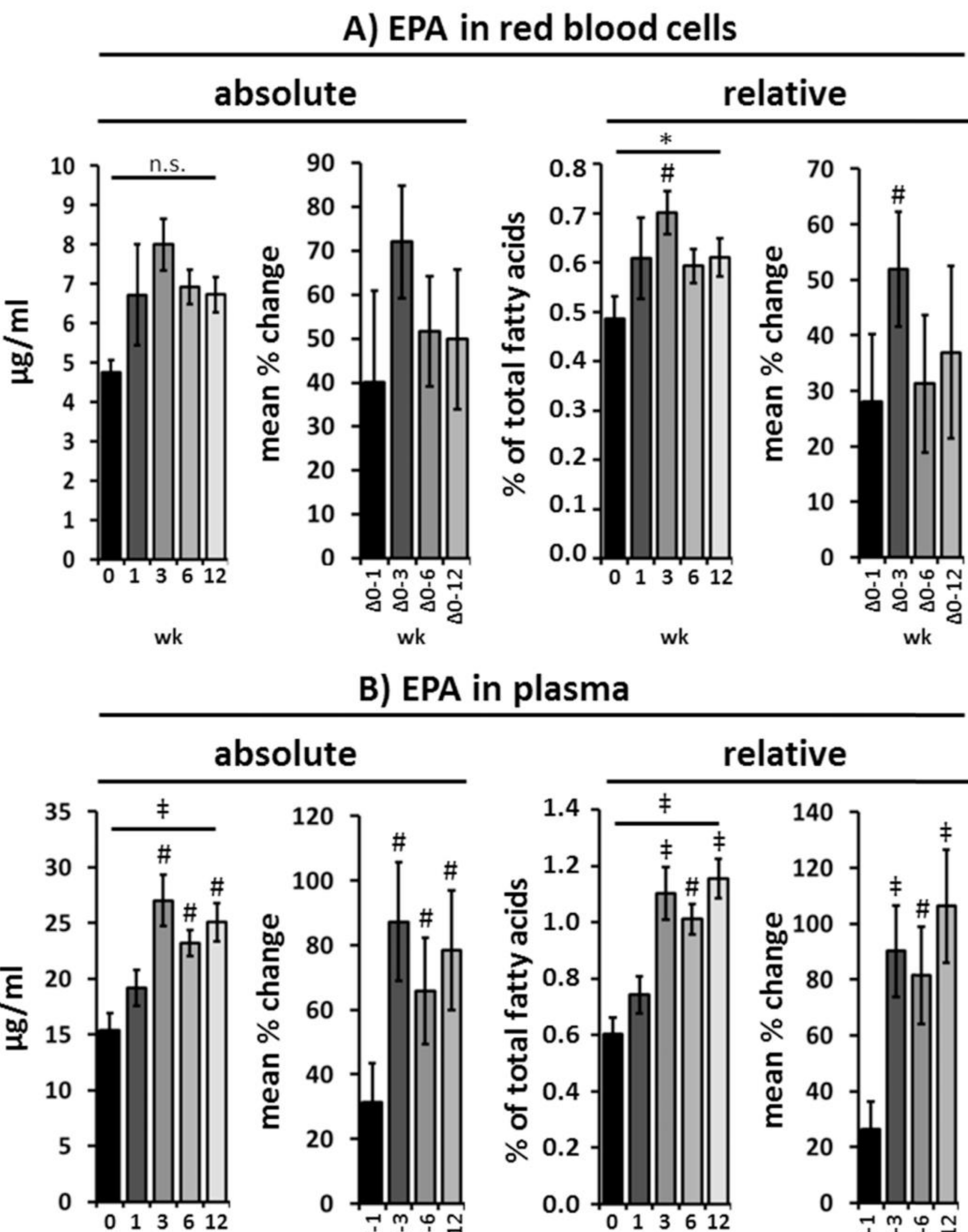

Fig. 2. Levels of eicosapentaenoic acid (EPA, C20:5n3) in red blood cells and plasma at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/day). EPA levels are shown as absolute concentrations [µg/mL] and as relative amounts [%] of total fatty acids in (A) plasma and (B) red blood cells. All data are shown as mean ± SE (n=12). * p≤0.05, #p≤0.005, ‡p < 0.001.

(and other PUFA) into blood cells – mainly RBC – is determined by red blood cell turnover (mean life span of a red blood cell is approx. 120 days in circulation) and thus requires several wk, whereas plasma fatty acids are directly affected by nutrition.

In RBC the increase of DHA concentrations was linear over the intervention period and saturation was not reached after twelve wk. In contrast, DHA concentrations in plasma reached a maximum steady state almost after one wk which remained constant after three, six and twelve wk. Similarly, EPA plasma concentrations were elevated during the whole intervention period. Interestingly, AA plasma concentrations slightly dropped over time indicating a decrease in the endogenous AA pool as also reflected by the AA red blood cell concentration. The decrease in relative AA amounts in RBC (1.2-fold after six wk) is consistent with similar studies, observing a 1.2-fold [15] or 1.5-fold [13] decrease of relative AA amounts after six wk of DHA supplementation (1.50 and 1.62 g DHA/d, respectively). The decline in AA

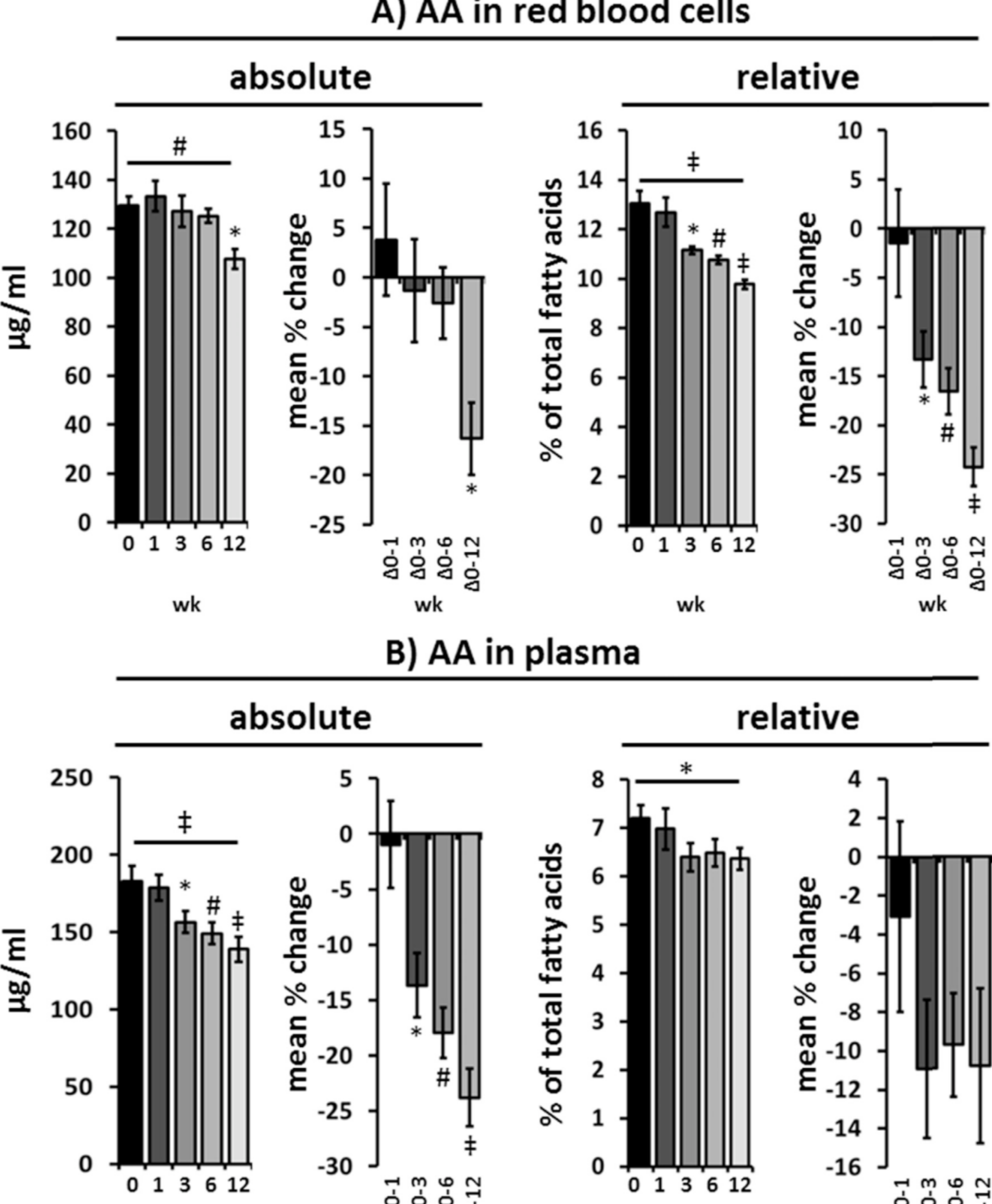

Fig. 3. Levels of arachidonic acid (AA, C20:4n6) in red blood cells and plasma at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/day). AA levels are shown as absolute concentrations [μg/mL] and as relative amounts [%] of total fatty acids in (A) plasma and (B) red blood cells. All data are shown as mean ± SE (n=12). * p≤0.05, #p≤0.005, ‡p < 0.001.

concentrations is likely the consequence of a displacement by DHA as well as a decreased activity of Δ5 and Δ6 desaturases leading to lower production of AA from LA [23].

In numerous LC n3 PUFA supplementation studies the increase in EPA and DHA blood levels is accompanied by a decrease in AA levels [3,9,10]. Quantifying fatty acid concentrations is advantageous in comparison to the commonly used relative fatty acid pattern since absolute changes of each PUFA can be evaluated independent from one another. This becomes clear from the following two examples: *First,* in plasma we showed that AA concentrations decreased in a time dependent manner (14%, −18%, and −24% at wk 3, wk 6, and wk 12), while relative AA amounts indicate a reduction of just ~10%. Similarly, only little changes in the relative amounts of Σ n6 PUFA in plasma (32% at wk 0 vs. 31% at wk 12) result, while Σ n6 PUFA plasma concentrations clearly decrease (802 μg/mL at wk 0 vs. 678 μg/mL at wk 12). *Second,* apparent changes in the (relative) fatty acid amounts are induced by a change in the fatty acid pattern, for example in RBC after three and six wk of DHA treatment, AA concentrations were unchanged, while relative AA amounts showed a significant reduction (−13% and −16.6% at wk 3 and wk 6) due to a DHA increase. The

Table 5
Net increase in eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3) concentrations in red blood cells and plasma after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation (1076 mg/d). Molar EPA and DHA concentrations [µmol/l] and net increase of (EPA/EPA+DHA)×100 ratio are used as a measure to estimate the percentage retroconversion of DHA to EPA [14].

		Δ wk 1-wk 0			Δ wk 3-wk 0			Δ wk 6-wk 0			Δ wk 12-wk 0		
Red blood cells		**mean**	**±**	**SE**	**mean**	**±**	**SE**	**mean**	**±**	**SE**	**mean**	**±**	**SE**
ΔEPA	µmol/l	6.5	±	3.9	10.8	±	1.9	7.2	±	1.6	6.6	±	1.9
ΔDHA	µmol/l	30.7	±	6.0	73.8	±	9.1	120	±	7.6	156	±	10.9
ΔEPA+ΔDHA	µmol/l	37.3	±	8.8	84.6	±	10.7	127	±	8.5	162	±	12.1
ΔEPA/ΔEPA+ΔDHA	relative%	13.2	±	4.3	12.0	±	1.3	5.3	±	1.1	3.3	±	1.2
Plasma													
ΔEPA	µmol/l	12.5	±	3.9	38.5	±	6.1	25.9	±	4.8	32.1	±	5.5
ΔDHA	µmol/l	213	±	21.3	280	±	24.4	253	±	19.3	262	±	23.8
ΔEPA+ΔDHA	µmol/l	225	±	23.9	319	±	27.9	279	±	22.6	294	±	26.6
ΔEPA/ΔEPA+ΔDHA	relative%	3.9	±	2.1	11.6	±	1.7	8.7	±	1.3	10.6	±	1.8

discrepancy between PUFA concentrations and relative fatty acid amounts in blood is consistent with earlier studies, e.g. longitudinal studies with pregnant woman showed that relative AA and DHA amounts progressively decrease during pregnancy, while absolute levels increase [29,30].

We could demonstrate that supplementing DHA in humans led to an increase in EPA concentration in plasma and RBC over time. This indicates that, indeed, a modest but significant part of DHA is converted to EPA. In RBC, the estimated percentage retroconversion of DHA to EPA [14] is initially high (13.2% and 12.0% at wk 1 and wk 3) and gradually declines thereafter (5.3% and 3.3% at wk 6 and wk 12). The retroconversion rate is comparable to those of other studies [13–15], where a rate between 7% and 14% based on relative amounts (mol%) was calculated. The slight decline in EPA concentrations in RBC and plasma after six and twelve wk and the resulting gradual decline in the retroconversion rate in blood indicates that the retroconversion is probably down-regulated and may reach a saturation point consistent with earlier findings [17]. This assumption is also supported by the finding that concentrations of DPAn3, which is an intermediate product in the (retro-)conversion of DHA to EPA, are strongly reduced with the supplementation in a time dependent manner. This decline in blood DPAn3 levels after DHA supplementation is also consistent with earlier studies [13,15,20]. However, the assumption of a down-regulated retro-conversion is speculative has to be confirmed in, for example, isolated liver cells considering the activity of enzymes involved in the metabolism of DHA to EPA. Further, mechanistic explanations for the decline in DPAn3 are a replacement of DPAn3 with dietary DHA in circulating and cellular phospholipids [13]. Moreover, decreased DPAn3 concentrations after DHA supplementation alongside with increased EPA concentrations could also result in lower conversion of EPA to DHA [18], which may be – similarly to decreasing AA concentrations – explained by an inhibitory role of DHA on Δ5 and Δ6 desaturase enzymes [23]. A limitation of the present study is that the genetic status of the subjects in view of the fatty acid desaturase (FADS) and elongation of very LC fatty acids is unknown which potentially impact the PUFA pathways [31].

The increase in EPA concentrations in RBC (50% or 2.0 µg/mL) and plasma (80% or 9.7 µg/mL) after twelve wk is considerably smaller compared to changes of DHA and AA. Considering the low baseline level of EPA, the increase and the final concentrations are marginal. In contrast, the decrease of AA concentrations (−16% or −22 µg/mL in RBC and −24% or −44 µg/mL in plasma) and the increase of DHA concentrations (190% or 51 µg/mL in RBC and 294% or 86 µg/mL in plasma) are more pronounced.

5. Conclusions

After DHA supplementation, we observed discrepancies between resulting absolute PUFA concentrations and relative fatty acid amounts in RBC as well as in plasma. Absolute concentrations are more reliable to evaluate changes in PUFA patterns since changes of each PUFA can be evaluated independent from one another. Based on PUFA concentrations it could be shown that DHA supplementation results in slightly, but significantly increased EPA levels. The elevations of EPA levels are small and the physiological significance is not known. Moreover, an initial increase and subsequent drop of EPA levels suggest a downregulation in the EPA formation over the time. Further mechanistic studies including tracer techniques as well as genomic and proteomic analyzes are required to confirm our findings.

Author contributions

JPS and NHS conceived and designed the study. The study was performed by JPS and HK. Analytical experiments were performed by AIO, LS and AH. Data analysis was performed by JPS and TG. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Funding

This study was supported by grants from the German Research Foundation (Grant SCHE 1801 and SCHU 2516) to NHS and JPS.

Acknowledgments

This study was supported by grants from the German Research Foundation (Grant SCHE 1801 and SCHU 2516) to NHS and JPS. The provision of the DHASCO® oil capsules by DSM Nutritional Products (Columbia, MD, USA) is kindly acknowledged. We would like to thank the participants who contributed their time to this project.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plefa.2016.10.005.

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5. Paper IV

Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood

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Published in: *Prostaglandins Leukotrienes and Essential Fatty Acids*, 121 (2017), 76-87

Link: *https://www.plefa.com/article/S0952-3278(17)30119-9/abstract*

Prostaglandins, Leukotrienes and Essential Fatty Acids 121 (2017) 76–87

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood

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ARTICLE INFO

Keywords:
LC n-3 PUFA
DHA
EPA
Free oxylipins
Immune cell stimulation
Lipid mediators

ABSTRACT

Introduction: EPA and DHA cause different physiological effects, which are in many cases mediated via their oxidative metabolites (oxylipins). However, metabolism studies investigating the effect of either EPA or DHA on comprehensive oxylipin patterns are lacking.

Material and methods: The short and long term (1, 3, 6, and 12 week) effect of 1076 mg/d DHA (free of EPA) on free (unesterified) oxylipin concentrations in plasma and lipopolysacharid (LPS) stimulated blood of 12 healthy men (mean age 25.1 ± 1.5 years) was investigated.

Results: After DHA supplementation, plasma levels of all DHA-oxylipins (HDHAs, EpDPEs, DiHDPEs) significantly increased (up to 600%) in a time-dependent fashion. Oxylipins of EPA and arachidonic acid (AA) were also affected. Whereas a slight increase in several EPA-derived hydroxy-FAs (including the RvE1 precursor 18-HEPE) and dihydroxy-FAs was observed after DHA supplementation, a trend to a slight decline in AA-derived oxylipin levels was found. In LPS stimulated blood, it is shown that DHA supplementation significantly reduces the ability of immune cells to form AA-derived COX (TXB2 and PGB2) and 12-LOX (12-HETE) eicosanoids. While no increase in EPA COX metabolites was found, n-3 PUFA 12-LOX metabolites of EPA (12-HEPE) and DHA (14-HDHA) were highly induced.

Conclusion: We demonstrated that DHA supplementation causes a time-dependent shift in the entire oxylipin profile suggesting a cross-linked metabolism of PUFAs and subsequent formation of oxygenated lipid mediators.

1. Introduction

There is a debate whether eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) possess differential physiological effects [1]. For example, in a recent carefully controlled comparative human study it was shown that a similar dose of DHA (3 g/d for 10 wk) was more potent than EPA in modulating specific markers of inflammation as well as blood lipids [2]. Current research therefore attempts to discover tailored EPA/DHA-ratios for treatment needs of specific indications with the aim to produce evidence based recommendations for the intake of omega-3 (n-3) polyunsaturated fatty acids (PUFA) [1].

Thereby, it should not be forgotten that PUFAs are metabolized in a complex network of conversion, retro-conversion and oxidation. **First**, the parent essential PUFA of the n-3 family α-linolenic acid (ALA, 18:3n3) can be converted to the longer chain (LC) physiologically more active PUFAs EPA and DHA in a multistage enzymatic chain elongation and desaturation process [3,4], which is greatly influenced by genetic heritability [5]. Indeed, the status of EPA and DHA relies largely on the dietary intake of EPA and DHA since the conversion rate from ALA to EPA and especially to DHA is low (~ 1%) [3,6,7]. **Second**, a metabolic retro-conversion of DHA via docosapentaenoic acid (DPAn3, C22:5n3) to EPA has been shown in vitro [8] and in vivo [9–12]. Vice versa the administration of EPA caused decreasing DHA levels in blood [11].

Third, it becomes increasingly evident that a significant portion of the physiological effects of EPA and DHA are mediated via their oxidative metabolites, which are involved in the regulation of numerous processes such as inflammation, pain and blood coagulation [13,14]. Via enzymatic catalysis and autoxidation AA, EPA and DHA among other PUFAs are converted to oxidized metabolites (eicosanoids and other oxylipins). In humans, the enzymatic conversion of oxylipins is catalyzed by cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-LOX, 12-LOX and 15-LOX), cytochrome P450s (CYPs, e.g., CYP4 and CYP2 family) [14,15], soluble epoxide hydrolase (sEH) [16], and

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http://dx.doi.org/10.1016/j.plefa.2017.06.007
Received 5 May 2017; Received in revised form 12 June 2017; Accepted 13 June 2017
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further enzymes. In addition, oxylipins can be also formed by non-enzymatic autoxidation of LC PUFAs giving rise to hydroperoxy-FA as well as prostanoids like molecules such as isoprostanes. While the class of COX-mediated-metabolites (e.g., 1- and 2-series prostaglandins) has been intensively studied during the last decades, the most recently recognized lipid mediators include the "specialized pro-resolving metabolites" (SPMs: resolvins, protectins, and maresins) [17] and the EPA- and DHA-derived epoxides generated by CYP enzymes [15]. Recent studies showed that CYP-dependent n-3 epoxides are involved in biological processes like heart control [18], vascular tone [19], platelet aggregation [20] as well as angiogenesis, tumor growth and metastasis [21]. Based on these findings it is assumed that EPA- and DHA-epoxides play an important role in mediating cardioprotective and anti-inflammatory effects of EPA and DHA [22]. CYP epoxygenase products are rapidly metabolized to less active dihydroxy-FAs by sEH [16], which limits their biological activity [1]. Comprehensive profiling of classical and novel LC PUFA-derived lipid mediators using targeted metabolomics is important to identify biomarkers that are closely linked to the cardiovascular actions of LC n-3 and n-6 PUFAs [1,14].

The number of human trials that studied the effects of LC n-3 PUFA supplementation on comprehensive patterns of hydroxy-, epoxy- and dihydroxy-FAs is limited [22–31]. In the majority of human studies total oxylipins were measured after conjugate cleavage [22–24,26,27] and only a few studies focused on free oxylipins [28,29]. Another study compared total with free oxylipin concentrations [30]. Free oxylipins represents their bioactive form [32], whereas the biological role of the esterified oxylipins is more unclear. It is suggested that they represent a pool for a rapid release upon stimulation [33]. The concentrations of total oxylipins are considerably higher for several oxylipins classes compared to free oxylipins since the majority of hydroxy-, epoxy-, and dihydroxy-FAs in circulating blood are esterified [30].

In a recent study we showed that DHA supplementation affects not only DHA concentrations in plasma and blood cells of healthy male adults but also changes the entire PUFA pattern [34]. In particular, we observed declined AA levels and slightly, but significantly increased EPA concentrations. In the present study, we investigated the effects of DHA supplementation on levels of free plasma oxylipins in the same study cohort. To measure the effect of DHA supplementation on oxylipin formation in response to an inflammatory scenario like acute sepsis, an ex vivo assay with immune stimulated blood cells was used. Similar to common whole blood assays [35] for the assessment of COX-2 activity, fresh blood was incubated with lipopolysaccharide (LPS) from E.Coli cell walls triggering an (initiate) immune response via toll-like receptors. This leads to an enhanced release of PUFAs from lipids by phospholipase activation as well as induction of oxylipin generating enzymes such as COX. Based on this ex vivo assay, a modulation of the oxylipins formation during a phase of acute inflammation could be investigated.

2. Materials and methods

This investigator initiated study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethic committee at the medical chamber of Lower Saxony (Hannover, Germany). All included subjects gave their written informed consent to take part in the study. The study is registered in the German clinical trial register (no. DRKS00006765).

2.1. Study design

The study was conducted in the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany. The study protocol is described in detail in [34]. In brief, the study consisted a screening phase and a 12 week lasting intervention period with 5 examinations: at the beginning (wk 0), after 1 (wk 1), 3 (wk 3), 6 (wk 6) and 12 (wk 12) weeks. The study supplement DHASCO® oil, produced by the marine microalgae *Crypthecodinium cohnii*, was kindly provided by DSM Nutritional Products (Columbia, MD, USA). The fatty acid pattern of the DHA oil was analyzed by means of solid phase extraction and gas chromatography as described in [36]. The concentration of other LC PUFAs such as C18:2n6, C18:3n3, C20:5n3, C22:5n3 and C22:5n6 is < 0.1% of total fatty acids (Table 1). The free fatty acid content was 0.1%. During the intervention period subjects daily ingested two 1000 mg-weight capsules with 538 mg DHA (as reesterified triacylglycerides) per capsule. Hence, total DHA intake was 1076 mg DHA per day.

Table 1
Fatty acid pattern of the study supplement. The presented results are mean values ± SD of three analyses.

Fatty acid	Common name	g/100 g Mean ± SD	% of total fatty acids Mean ± SD
C10:0	Capric acid	0.06 ± 0.02	0.07 ± 0.02
C12:0	Lauric acid	3.19 ± 0.23	3.72 ± 0.16
C14:0	Myristic acid	9.98 ± 0.32	11.66 ± 0.03
C14:1n5	Myristoleic acid	0.38 ± 0.03	0.44 ± 0.02
C16:0	Palmitic acid	7.55 ± 0.21	8.82 ± 0.04
C16:1n7	Palmitoleic acid	3.70 ± 0.12	4.32 ± 0.02
C18:0	Stearic acid	0.16 ± 0.01	0.19 ± 0.01
C18:1n9	Oleic acid	7.18 ± 0.20	8.38 ± 0.03
C18:1n7	Vaccenic acid	0.10 ± 0.03	0.11 ± 0.03
C18:2n6	Linoleic acid	0.03 ± 0.0	0.03 ± 0.0
C20:0	Arachidic acid	0.04 ± 0.0	0.05 ± 0.0
C20:1n9	Gadoleic acid	0.06 ± 0.02	0.07 ± 0.02
C20:4n6	Arachidonic acid	0.05 ± 0.0	0.06 ± 0.0
C20:5n3	Eicosapentaenoic acid	0.06 ± 0.0	0.07 ± 0.0
C22:0	Behenic acid	0.02 ± 0.0	0.03 ± 0.0
C22:1n9	Cetoleic acid	0.04 ± 0.0	0.05 ± 0.0
C22:5n3	Docosapentaenoic acid (Clupanodonic acid)	0.77 ± 0.03	0.90 ± 0.01
C22:6n3	Docosahexaenoic acid	52.05 ± 1.50	60.78 ± 0.18
C24:1n9	Docosapentaenoic acid (Osbond acid)	0.22 ± 0.02	0.26 ± 0.01
ALL FA		85.63 ± 2.71	100
SFA	Saturated fatty acids	21.01 ± 0.81	24.53 ± 0.18
MUFA	Monounsaturated fatty acids	11.67 ± 0.38	13.63 ± 0.03
PUFA	Polyunsaturated fatty acids	52.95 ± 1.53	61.84 ± 0.18
n3	Omega-3 fatty acids	52.87 ± 1.53	61.75 ± 0.18
n6	Omega-6 fatty acids	0.08 ± 0.0	0.09 ± 0.0
n9	Omega-9 fatty acids	7.50 ± 0.22	8.71 ± 0.02

During each visit, fasting blood was collected from each proband. Likewise, blood pressure was measured and subjects completed a questionnaire to obtain information about changes in medication, diet and lifestyle habits (e.g., physical activity), as well as the tolerability of the capsules. Prior to visit at wk 0, wk 6, and wk 12, subjects completed a 3-day dietary questionnaire including two working days and one weekend day. The dietary questionnaires were analyzed using PRODI (Nutri-Science GmbH, Freiburg, Germany) to obtain data on energy and nutrient intake. Prior to the intervention phase, comprehensive instructions on food intake or sport activities were given to the probands. Usual exercise and dietary habits (especially fish and seafood consumption) should be maintained throughout the intervention time. Moreover, subjects were requested to abstain from alpha-linolenic acid (ALA, 18:3n-3)-rich vegetable oils such as linseed oil or chia seeds during the intervention period to minimize variability in LC-PUFA status and blood levels. The subject's compliance was assessed by a count of left-over capsules at the end of the intervention period.

2.2. Subjects

Several selection criteria were defined to assemble a homogenous study collective. In particular, only men of a limited age class from 20 to 40 years were included to minimize potential fluctuations in lipid

profiles due to age [37] or an influence of (female) hormones [38–40]. Healthy probands had similar eating habits (e.g., mixed diet including meat, ≤ two times fish intake per week). Likewise, only subjects with a relative amount of EPA/DHA in blood cells ≥ 8% were selected for the study to ensure a similar LC n-3 PUFA status. Subjects were recruited from the general population by an advertisement. Subject selection including inclusion and exclusion criteria is described in detail in [34].

2.3. Sample collection and pre-analytics

Blood samples were collected in the morning between 6:45 and 10:00 a.m. after overnight fasting (last meal and caloric beverage before 8:00 p.m.). The examinations were scheduled at the same time for each subject. The samples were obtained by venipuncture of an arm vein using Multifly needles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt, Nümbrecht, Germany) and TruCulture blood collection tubes (Myriad RBM Austin, TX, USA). All blood samples for oxylipin analysis were immediately processed after blood draw. For analysis of free oxylipins in plasma, EDTA blood monovettes were centrifuged for 10 min at 1500 × *g* and 4 °C and plasma was transferred into 1.5 mL micro-centrifuge tubes (Rotilabo, Roth, Karlsruhe, Germany) and immediately frozen and stored at −80 °C until extraction and analysis. For analysis of free oxylipins in ex vivo immune cell stimulated (whole) blood, TruCulture blood collection tubes were used. The TruCulture System is an integrated blood collection device including a whole blood culture system with proprietary culture medium and LPS as stimulant. The frozen TruCulture blood collection tubes were thawed 30 min before blood collection at room temperature. 1 mL of blood was drawn directly into the TruCulture tube, which were instantly incubated in the dark at 37 °C for 24 h. After incubation, the supernatant was transferred into 1.5 mL micro-centrifuge tubes, immediately frozen and stored at −80 °C until extraction and analysis. Other sets of blood samples (serum and EDTA monovettes) were sent to external laboratories for the measurement of clinical parameters. Serum lipid levels, liver enzymes, small blood count (red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, leucocyte count, platelet count) as well as high sensitive C-reactive protein (hsCRP) were determined in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany.

2.4. Analysis of free oxylipins in plasma

Oxylipins were analyzed by LC-MS following solid phase extraction as described in [41,42]. Briefly, after addition of internal standards and antioxidant solution 500 µL plasma was diluted with 1200 µL water and 300 µL methanol. Samples were acidified with concentrated acetic acid to a pH of 3 directly before extraction on C18 cartridges (500 mg, Macherey-Nagel, Düren, Germany) using methyl formate for elution [41,42].

In addition to DHA-oxylipins, we also evaluated plasma concentrations of AA- and EPA-derived oxylipins because of their physiological importance. While several AA-derived prostanoids such as prostaglandins (e.g., PGF2a, PGE2, and PGD2) and thromboxanes (e.g., TXB2) were analyzed, we focus especially on hydroxy-, epoxy- and dihydroxy-FA formed by LOXs, CYPs, sEH, and (aut-)oxidation. SPMs (resolvins, protectins, and maresins) were not detected since these analytes were below the limit of quantification (e.g., Resolvin E1) or not included in the utilized method because of limited access to authentic reference compounds at the time of analysis (Table S2).

Only oxylipins which exceeded the limit of quantification in > 50% of the plasma samples were used for data analysis. Based on this criterion THF diol; PGD1; PGE3; 6-keto-PGF1α; PGJ2; 15-deoxy-PGJ2; PGB2; LTB3; LTB4; LTB5; 6-trans-LTB4; 20-COOH-LTB4; 20-OH-LTB4; 9-HETE; 11,12,15-TriHETrE; Resolvin E1; PGD3; 8(9)-EpETE; 11(12)-EpETE; 14(15)-EpETE; 5,6-DiHETE; 8,9-DiHETE; 8,15-DiHETE; and 5,15-DiHETE were excluded.

2.5. Data analysis and statistics

Results for body mass index (BMI) are presented as mean ± SD, while plasma oxylipin levels and its relative change (%) are presented as mean ± SE. Changes in the variables (v) were calculated for each individual at each time point (x) as Δ%, calculated by: Δ% = 100 * (v tx - v wk 0)/v wk 0. The Kolmogorov-Smirnov test indicated that the levels of numerous oxylipins were not normally distributed. Therefore, statistical analyzes were carried out with log transformed data.

Differences between baseline (wk 0) levels and different time points (wk 1, wk 3, wk 6, wk 12) were analyzed by ANOVA for repeated measurements. To determine sphericity Mauchly's test was used. In case sphericity cannot be assumed, the Greenhouse-Geisser correction was used. To determine statistical significance between baseline levels and each time point, *t*-tests for paired samples after Bonferroni correction were carried out. Values of $p \le 0.05$ were considered to be statistically significant.

Correlation analyzes (Spearman) were conducted to determine associations between oxylipin concentrations in plasma and related precursor FA (AA, EPA and DHA) concentrations in plasma (for FA concentrations in plasma see [34]). The analyzes were conducted at the respective time point (wk 0, wk 1, wk 3, wk 6 or wk 12) as well as for Δ% between the different time points. Correlation between the variables was accepted with a correlation coefficient (r) of ≥ 0.5 or ≤ −0.5 and $p \le 0.05$.

All statistical analyses were carried out with SPSS software (Version 23, SPSS Inc., Chicago, IL, USA).

3. Results

12 healthy male subjects fulfilling the inclusion and exclusion criteria were included in the study. Questionnaires on food frequency and physical activity revealed that eating habits (especially fish consumption) and physical activities were not changed during the intervention period. No statistical differences in saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and PUFA intake from background diet were observed in the course of the study (Table S1).

All probands completed the 12 week intervention period and attended at all 5 examination time points (no drop-outs). Thus, the data of the total study population was involved in the analysis. None of the probands had a cold or other disease with inflammatory activity during the examination time points. DHA capsules were well tolerated and no adverse events were observed during the 12 week intervention period. Count of left-over capsules at the end of the intervention period revealed a compliance rate of 99%.

The clinical parameters of the probands are described in detail in [34]. In brief, the probands (mean age 25.1 ± 1.5y) were healthy and showed a normal BMI (22.1 ± 0.9 kg/m^2) and serum lipid profile. Weight, blood pressure, or LDL levels were unchanged during the intervention [34]. Total cholesterol (TC) and HDL levels were slightly elevated after three weeks, while TG levels were slightly decreased after 12 weeks of DHA supplementation. hsCRP levels were in the range of ~ 1 at all time points showing that probands did not show any signs of acute or chronic inflammatory disease during the intervention period.

3.1. Effect of DHA supplementation on free oxylipins in plasma

95 oxylipins were covered by the applied method (Table S2) and 43 free (i.e., non-esterified) DHA-, EPA-, and AA-derived oxylipins could be quantified in plasma before DHA supplementation while 46 exceeded the limit of quantification after supplementation (Table 2). Relative changes in free plasma oxylipin levels in response to DHA supplementation are shown in Fig. 1.

Table 2
Concentration of free DHA-, EPA- and AA-oxylipins (pM) in plasma at baseline (wk 0) and after one, three, six and twelve weeks of DHA supplementation.

	wk 0 Mean ± SE	wk 1 Mean ± SE	t-test[a] p (wk1-0)	wk 3 Mean SE	t-test[a] p (wk3-0)	wk 6 Mean SE	t-test[a] p (wk6-0)	wk 12 Mean ± SE	t-test[a] p (wk12-0)	An reM[b] p
DHA-oxylipins										
Hydroxy fatty acids										
4-HDHA	240 ± 46	510 ± 36	0.020	630 ± 32	0.004	770 ± 71	0.002	840 ± 58	0.001	< 0.001
7-HDHA	50 ± 10	140 ± 10	0.003	170 ± 13	< 0.001	220 ± 18	0.001	230 ± 19	< 0.001	< 0.001
8-HDHA	410 ± 69	1400 ± 110	< 0.001	1700 ± 120	< 0.001	1900 ± 130	< 0.001	2100 ± 130	< 0.001	< 0.001
10-HDHA	88 ± 13	290 ± 21	0.001	380 ± 34	< 0.001	420 ± 19	< 0.001	430 ± 20	< 0.001	< 0.001
11-HDHA	130 ± 19	280 ± 24	0.005	360 ± 21	0.001	400 ± 24	0.001	420 ± 21	< 0.001	< 0.001
13-HDHA	130 ± 19	310 ± 33	0.008	350 ± 34	< 0.001	370 ± 27	0.001	370 ± 24	< 0.001	< 0.001
14-HDHA	580 ± 93	2300 ± 220	< 0.001	2800 ± 330	< 0.001	2700 ± 420	0.001	2400 ± 550	0.028	< 0.001
16-HDHA	160 ± 21	420 ± 31	0.002	500 ± 66	< 0.001	530 ± 39	< 0.001	530 ± 24	< 0.001	< 0.001
17-HDHA	520 ± 81	1200 ± 78	0.001	1600 ± 110	< 0.001	1700 ± 97	< 0.001	2100 ± 193	< 0.001	< 0.001
20-HDHA	380 ± 55	830 ± 49	0.005	1000 ± 110	0.001	1200 ± 72	0.001	1300 ± 58	< 0.001	< 0.001
Epoxy fatty acids										
10(11)-EpDPE	120 ± 25	260 ± 24	0.002	330 ± 23	0.019	340 ± 43	0.024	460 ± 48	0.004	< 0.001
13(14)-EpDPE	< LLOQ	86 ± 8	n.s.	100 ± 7	n.s.	99 ± 10	n.s.	130 ± 13	n.s.	0.021
16(17)-EpDPE	40 ± 5	120 ± 12	< 0.001	150 ± 10	< 0.001	140 ± 8	0.001	180 ± 20	< 0.001	< 0.001
19(20)-EpDPE	160 ± 30	400 ± 33	0.025	480 ± 26	0.005	480 ± 49	0.013	680 ± 65	0.003	< 0.001
Dihydroxy fatty acids										
4,5-DiHDPE	770 ± 120	2100 ± 170	0.014	2300 ± 110	0.006	2600 ± 210	0.004	3400 ± 190	0.001	< 0.001
7,8-DiHDPE	< LLOQ	160 ± 6	n.s.	200 ± 11	n.s.	220 ± 15	n.s.	250 ± 15	0.012	0.001
10,11-DiHDPE	190 ± 36	380 ± 30	n.s.	440 ± 31	0.019	460 ± 44	0.014	600 ± 40	0.004	< 0.001
13,14-DiHDPE	250 ± 36	460 ± 28	0.027	570 ± 37	0.003	630 ± 58	0.001	770 ± 48	< 0.001	< 0.001
16,17-DiHDPE	270 ± 40	530 ± 28	0.029	620 ± 35	0.003	690 ± 54	0.002	800 ± 45	0.002	< 0.001
19,20-DiHDPE	2600 ± 470	5100 ± 320	n.s.	5600 ± 230	0.015	6500 ± 680	0.004	7200 ± 530	0.007	< 0.001
EPA-oxylipins										
Hydroxy fatty acids										
5-HEPE	180 ± 30	170 ± 20	n.s.	180 ± 22	n.s.	250 ± 51	n.s.	300 ± 54	0.025	< 0.001
8-HEPE	< LLOQ	89 ± 6		110 ± 8	0.047	120 ± 10	< 0.001	140 ± 10	0.001	< 0.001
12-HEPE	130 ± 22	180 ± 21	n.s.	210 ± 21	0.005	220 ± 45	n.s.	180 ± 25	n.s.	0.009
15-HEPE	< LLOQ	150 ± 13		180 ± 15	0.038	200 ± 23	0.036	230 ± 31	0.009	< 0.001
18-HEPE	270 ± 49	270 ± 27	n.s.	350 ± 32	n.s.	340 ± 51	n.s.	410 ± 48	n.s.	< 0.001
Epoxy fatty acids										
17(18)-EpETE	140 ± 33	< LLOQ		< LLOQ		< LLOQ		72 ± 11	n.s.	
Dihydroxy fatty acids										
8,9-DiHETE	71 ± 9	88 ± 9	n.s.	110 ± 8	n.s.	110 ± 11	n.s.	140 ± 9	0.006	< 0.001
11,12-DiHETE	36 ± 6	29 ± 2	n.s.	35 ± 3	n.s.	39 ± 5	n.s.	50 ± 7	n.s.	0.002
14,15-DiHETE	110 ± 16	130 ± 7	n.s.	150 ± 11	n.s.	150 ± 14	n.s.	190 ± 16	0.034	0.002
17,18-DiHETE	660 ± 130	820 ± 58	n.s.	940 ± 70	n.s.	970 ± 120	n.s.	1300 ± 130	n.s.	< 0.001
AA-oxylipins										
Thromboxanes, prostaglandins										
TXB2	880 ± 190	480 ± 55	n.s.	380 ± 42	n.s.	390 ± 100	n.s.	270 ± 49	n.s.	0.004
PGF2a	120 ± 17	90 ± 8		87 ± 7		< LLOQ		76 ± 4		n.s.
PGE2	210 ± 36	120 ± 27	n.s.	110 ± 29	n.s.	100 ± 25	n.s.	210 ± 32	n.s.	0.029
PGD2	230 ± 70	160 ± 15	n.s.	< LLOQ		< LLOQ		< LLOQ		
Hydroxy fatty acids										
5-HETE	840 ± 110	580 ± 71		480 ± 48		630 ± 150		710 ± 160		n.s.
8-HETE	370 ± 30	310 ± 23		280 ± 14		320 ± 32		300 ± 29		n.s.
11-HETE	460 ± 50	330 ± 32	n.s.	290 ± 18	n.s.	290 ± 34	n.s.	280 ± 29	n.s.	0.007
12-HETE	1400 ± 170	1500 ± 120		1300 ± 100		1300 ± 210		1100 ± 170		n.s.
15-HETE	1000 ± 100	900 ± 55		790 ± 46		860 ± 82		890 ± 99		n.s.
20-HETE	940 ± 170	510 ± 54	n.s.	440 ± 51	n.s.	500 ± 89	n.s.	520 ± 99	n.s.	0.003
Epoxy fatty acids										
5(6)-EpETrE	410 ± 35	340 ± 19		310 ± 22		340 ± 29	n.s.	290 ± 33		n.s.
11(12)-EpETrE	110 ± 11	110 ± 6	n.s.	110 ± 6	n.s.	88 ± 6	n.s.	78 ± 7	n.s.	0.035
14(15)-EpETrE	140 ± 19	120 ± 7		110 ± 9		110 ± 13		100 ± 11		n.s.
Dihydroxy fatty acids										
5,6-DiHETrE	290 ± 34	210 ± 14	n.s.	190 ± 11	n.s.	200 ± 16	n.s.	200 ± 19	n.s.	0.015
8,9-DiHETrE	310 ± 34	240 ± 14	n.s.	190 ± 11	n.s.	210 ± 20	n.s.	210 ± 16	n.s.	0.010
11,12-DiHETrE	790 ± 86	550 ± 24	n.s.	480 ± 24	n.s.	510 ± 50	n.s.	550 ± 50	n.s.	0.012
14,15-DiHETrE	950 ± 94	740 ± 34		660 ± 32		690 ± 69		720 ± 61		n.s.

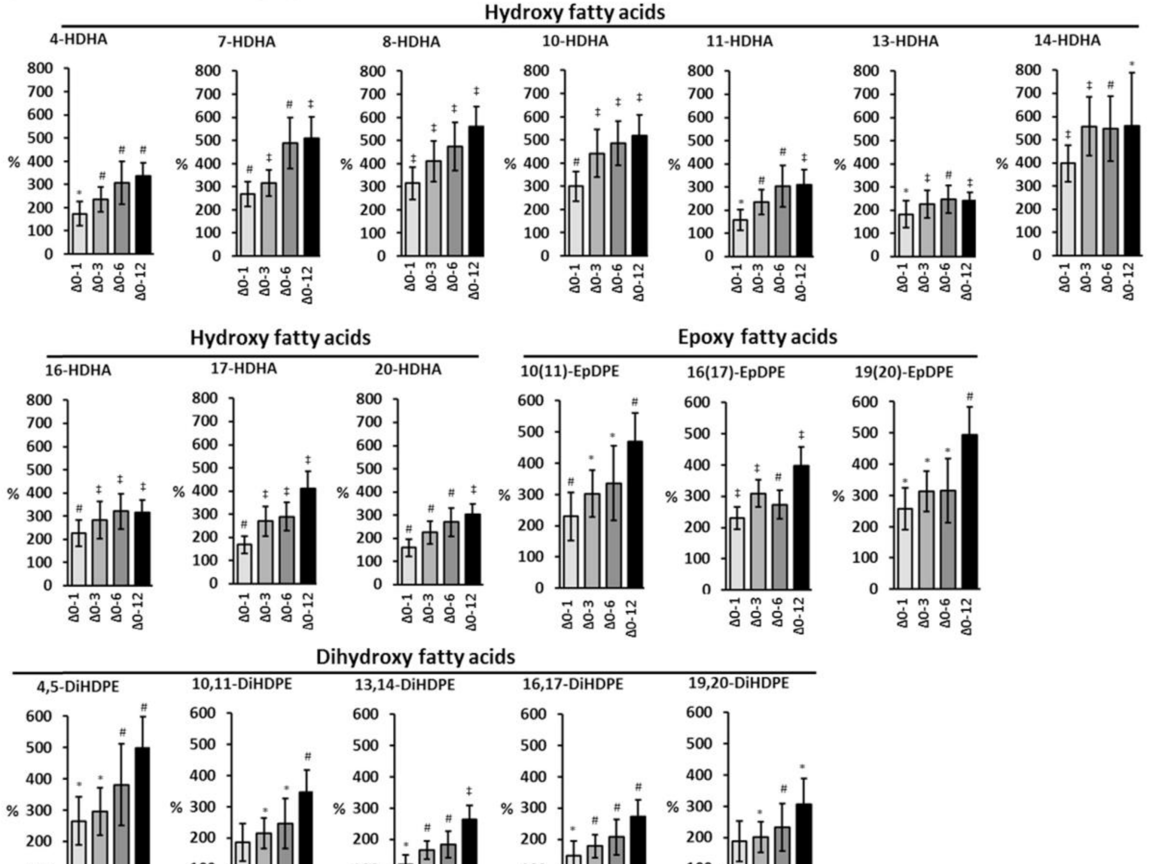
[a] *t*-test for paired samples with Holm-Bonferroni correction.
[b] Anova for repeated measures (An reM).

3.1.1. *Effect on DHA-oxylipins*

20 free DHA-derived oxylipins were detected and quantified in plasma (Table 2) including 10 hydroxy-FAs (4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, 20-HDHA), 4 epoxy-FAs (10(11)-, 13(14)-, 16(17)-, 19(20)-EpDPE) and 6 dihydroxy-FAs (4,5-, 7,8-, 10,11-, 13,14-, 16,17- and 19,20-DiHDPE).

Plasma levels of all DHA-oxylipins were significantly increased post DHA supplementation (Table 2, Fig. 1a). Already after one week of DHA intake, the plasma levels of free DHA-oxylipins increased by 150–400%.

The highest relative increase with 500–600% compared to wk 0 was observed for 7-, 8-, 10-, and 14-HDHA, 10(11)-EpDPE, 19(20)-EpDPE and 4,5-DiHDPE after 12 weeks of DHA supplementation. While concentrations of several analytes were in the range of 100–300 pM (7-HDHA, 13(14)-EpDPE, 16(17)-EpDPE and 7,8-DiHDPE) after 12 weeks



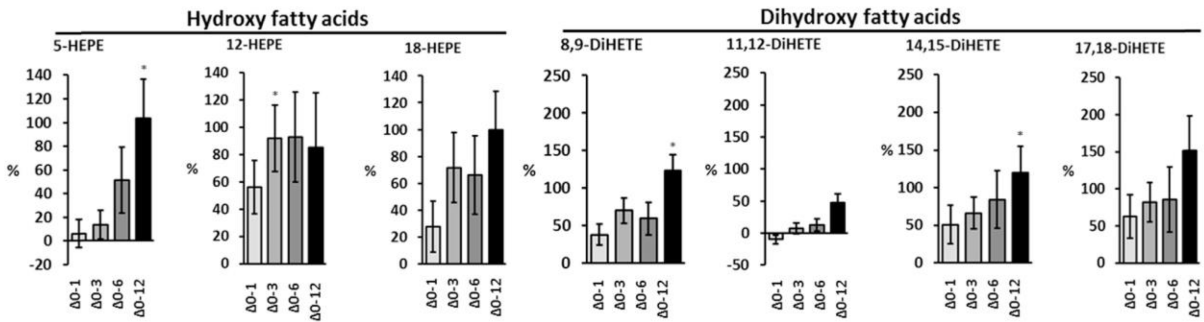


Fig. 1. Relative change (%) of free A) DHA-, B) EPA- and D) AA-derived oxylipin levels in plasma between baseline (wk 0) and one, three, six and twelve weeks of DHA supplementation. Bars represent mean ± SE. * $p \leq 0.05$, # $p \leq 0.005$, ‡ $p < 0.001$.

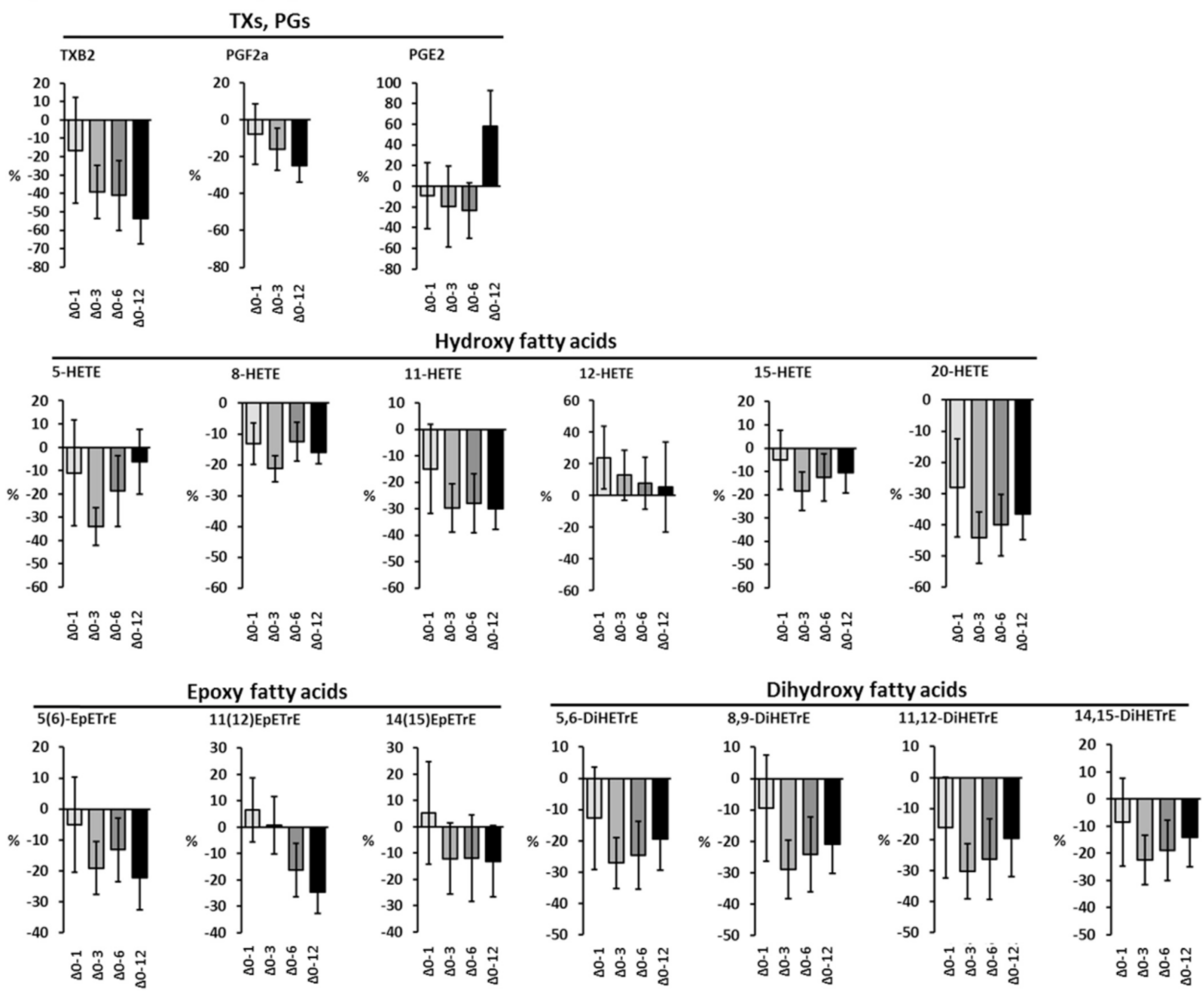


Fig. 1. (*continued*)

of DHA supplementation, concentrations of 14-HDHA, 4,5-DiHDPE and 19,20-DiHDPE extends to 2400 ± 550 pM, 3400 ± 190 pM and 7200 ± 530 pM, respectively.

The sum of all covered DHA-oxylipins significantly ($p < 0.001$) increased from 7000 ± 1100 pM (wk 0) to 22,400 ± 1500 pM (wk 6) and 25,200 ± 1000 pM (wk 12). The sum of all detected DHA-, EPA-, and AA-derived oxylipins was also highly ($p < 0.001$) increased from 18,100 ± 2200 pM (wk 0) to 31,300 ± 2400 pM (wk 6) and 34,700 ± 1600 pM (wk 12) which was mainly due to the shift in DHA-oxylipins.

The levels of most DHA-derived oxylipins correlated at baseline (wk 0) with their precursor FA DHA in plasma (Table 3a). After DHA supplementation only few correlations (mainly at wk 1) were observed between DHA levels in plasma and levels of its related oxylipins in plasma at respective time points. This may be explained by the high level of plasma DHA in response to the supplementation which is not reflected by the oxylipin levels. Nevertheless, for nearly all DHA-oxylipins strong correlations among changes in precursor DHA and related oxylipins between the different time points were observed, in particular in the first week (Δwk 0–1) of DHA supplementation and between the 3rd and 6th week (Δwk 3–6, Table 3b). Surprisingly, no correlations between DHA-oxylipins and DHA in blood cells were found.

3.1.2. *Effect on EPA-oxylipins*

DHA supplementation led to a slight increase in EPA-derived hydroxy- and dihydroxy-FA plasma levels, reaching level of statistical significance for a few oxylipins (Table 2 and Fig. 1b). 8- and 15-HEPE were below the limit of quantification at baseline but quantified after one week, while levels linearly increase with time of DHA supplementation. EPA-derived epoxides were below the limit of quantification (25–100 pM, Table S2) at all time points, except 17(18)-EpETE at wk 0 and 12 (Table 2). On a total level, EPA-oxylipins significantly ($p < 0.001$) increased from 1600 ± 260 pM (wk 0) to 2400 ± 300 pM (wk 6) and 3000 ± 290 pM (wk 12).

At baseline, EPA levels in plasma correlate with some EPA-oxylipins in plasma (Table S3), while EPA plasma levels were rarely correlated with its corresponding oxylipins after DHA supplementation (Table S3). Similarly, only few correlations between changes in plasma EPA levels and related plasma oxylipins were found (Table S3).

3.1.3. *Effect on AA-oxylipins*

In general, a trend to a slight decline in AA-derived oxylipin levels is visible in the course of DHA supplementation, although not statistically significant (Table 2 and Fig. 1c). In total, the sum value of AA-oxylipins, however, significantly ($p = 0.015$) decreased from 9500 ± 900 pM (wk

Table 3
Analysis of correlations between the precursor fatty acid DHA and its oxylipins in plasma. A) Correlations between plasma levels of DHA and oxylipins are shown at baseline (wk 0) and after one, three, six and twelve weeks of DHA supplementation. B) Correlations among changes in precursor DHA and related oxylipins between the different time points (Δwk). Only significant correlation (correlation coefficient r ≥ +0.5 or r ≤ −0.5 and p ≤ 0.05) are presented.

Correlations between DHA and DHA-oxylipin levels

A)

DHA-oxylipins	wk	Correlation coefficient, p-value
7-HDHA	0	r = 0.658 (p = 0.020)
	1	r = 0.581 (p = 0.048)
8-HDHA	0	r = 0.663 (p = 0.019)
	1	r = 0.613 (p = 0.034)
10-HDHA	0	r = 0.740 (p = 0.006)
	1	r = 0.669 (p = 0.017)
11-HDHA	0	r = 0.635 (p = 0.027)
13-HDHA	0	r = 0.800 (p = 0.002)
14-HDHA	0	r = 0.808 (p = 0.001)
	3	r = 0.662 (p = 0.019)
	12	r = 0.609 (p = 0.047)
16-HDHA	0	r = 0.767 (p = 0.004)
	1	r = 0.678 (p = 0.015)
17-HDHA	0	r = 0.654 (p = 0.021)
20-HDHA	0	r = 0.653 (p = 0.021)
	1	r = 0.727 (p = 0.007)
10(11)-EpDPE	0	r = 0.811 (p = 0.001)
	1	r = 0.772 (p = 0.003)
16(17)-EpDPE	0	r = 0.934 (p < 0.001)
	1	r = 0.732 (p = 0.007)
19(20)-EpDPE	0	r = 0.694 (p = 0.012)
	1	r = 0.749 (p = 0.005)
4,5-DiHDPE	0	r = 0.662 (p = 0.019)
	1	r = 0.612 (p = 0.034)
7,8-DiHDPE	3	r = 0.621 (p = 0.031)
10,11 DiHDPE	0	r = 0.649 (p = 0.022)
13,14-DiHDPE	0	r = 0.575 (p = 0.050)
19,20-DiHDPE	0	r = 0.630 (p = 0.028)

B)

DHA-oxylipins	Δwk	Correlation coefficient, p-value
4-HDHA	0–1	r = 0.587 (p = 0.045)
	3–6	r = 0.806 (p = 0.005)
7-HDHA	0–1	r = 0.818 (p = 0.001)
	3–6	r = 0.818 (p = 0.004)
8-HDHA	0–1	r = 0.867 (p < 0.001)
	3–6	r = 0.794 (p = 0.006)
10-HDHA	0–1	r = 0.888 (p < 0.001)
	1–3	r = 0.601 (p = 0.039)
	3–6	r = 0.782 (p = 0.008)
11-HDHA	3–6	r = 0.855 (p = 0.002)
13-HDHA	0–1	r = 0.713 (p = 0.009)
	3–6	r = 0.794 (p = 0.006)
16-HDHA	0–1	r = 0.832 (p = 0.001)
	3–6	r = 0.855 (p = 0.002)
17-HDHA	0–1	r = 0.699 (p = 0.011)
	3–6	r = 0.733 (p = 0.016)
20-HDHA	0–1	r = 0.818 (p = 0.001)
	3–6	r = 0.855 (p = 0.002)
10(11)-EpDPE	0–1	r = 0.573 (p = 0.051)
	3–6	r = 0.673 (p = 0.033)
16(17)-EpDPE	0–1	r = 0.699 (p = 0.011)
19(20)-EpDPE	0–1	r = 0.720 (p = 0.008)
	3–6	r = 0.891 (p = 0.001)
4,5-DiHDPE	0–1	r = 0.727 (p = 0.007)
	1–3	r = 0.706 (p = 0.010)
	3–6	r = 0.891 (p = 0.001)
7,8-DiHDPE	3–6	r = 0.697 (p = 0.025)
10,11-DiHDPE	0–1	r = 0.818 (p = 0.001)
	3–6	r = 0.746 (p = 0.005)
13,14-DiHDPE	0–1	r = 0.755 (p = 0.005)
	3–6	r = 0.806 (p = 0.005)
16,17-DiHDPE	0–1	r = 0.776 (p = 0.003)
	3–6	r = 0.879 (p = 0.001)
19,20-DiHDPE	0–1	r = 0.783 (p = 0.003)
	3–6	r = 0.758 (p = 0.011)

0) to 6500 ± 800 pM (wk 6) and 6500 ± 600 pM (wk 12). Several AA-oxylipins such as TXB2, 11-HETE, 20-HETE and all dihydroxy-FAs are reduced by 30–50%. PGD2 – occurring in concentrations of 230 ± 70 pM at wk 0 and 160 ± 15 pM at wk 1 – was below the limit of quantification (100 pM) after 3, 6 and 12 week of DHA supplementation.

With a few exceptions, concentrations of AA in plasma and their related AA-oxylipins in plasma were not correlated at any time point (Table S3). Likewise, only few correlations were found in changes between AA plasma levels and changes in AA-oxylipin levels in plasma between different time points (Table S3).

3.2. *Effect of DHA supplementation on free oxylipins in LPS stimulated blood*

In LPS stimulated blood a total of 45 free DHA-, EPA-, and AA-oxylipin species were quantified (Tables S2, S4, Fig. S1).

3.2.1. *COX metabolites*

PGE2, PGF2a, and TXB2 are significantly induced (6- to 13-fold) compared to plasma (Fig. 2a). Moreover, PGB2 – a degradation product of PGE2 – which was below the limit of quantification in plasma at all time points – was also highly induced in LPS stimulated blood.

In response to DHA supplementation all prostanoids were reduced, reaching statistical significance for TXB2 and PGB2 (Table S4, Fig. S1). In comparison, a significant decrease in prostanoid concentrations in plasma was also observed for PGE2 and TXB2 (Table 2).

3.2.2. *CYP and sEH metabolites*

Levels of CYP metabolites (Fig. 2e) and sEH metabolites (Fig. 2f) were significantly lower in LPS stimulated blood compared to plasma. The differences were marginal for 14(15)-EpETrE, but pronounced for 19(20)-EpDPE and all dihydroxy-FAs of AA, EPA and DHA.

After DHA supplementation a slight but significant reduction in AA-derived 14(15)-EpETrE and 14,15-DiHETrE in LPS stimulated blood was observed, while n-3 epoxides and dihydroxy-FA were increased. The DHA-induced changes in epoxides and dihydroxy-FAs are exemplarily shown for 14(15)-EpETrE, 19(20)-EpDPE, 14,15-DiHETrE, 17,18-DiHETE and 19,20-DiHDPE, but similar trends were observed for other epoxides (i.e., 11(12)-EpETrE, 10(11)-EpDPE, 13(14)-EpDPE, 16(17)-EpDPE) and dihydroxy-FAs (i.e., 5,6-DiHETrE, 8,9- DiHETrE, 11,12- DiHETrE, 14,15-DiHETE, 4,5-DiHDPE, 7,8-DiHDPE, 10,11-DiHDPE, 13,14-DiHDPE, 16–17-DiHDPE).

3.2.3. *5-LOX, 15-LOX metabolites*

Levels of 5-HETE, 15-HETE, and 17-HDHA were slightly higher in LPS stimulated blood compared to plasma, while no differences were observed for 5-HEPE and 15-HEPE as well as 4-HDHA and 7-HDHA (Fig. 2c/d).

After DHA supplementation, levels of 5-HETE and 15-HETE were significantly reduced in LPS stimulated blood, while LC n-3 PUFA-derived 5-HEPE and 15-HEPE as well as 4-HDHA, 7-HDHA, and 17-HDHA were significantly enhanced (Fig. 2c/d). Whereas the increase of LC n-3 PUFA-derived oxylipins was similar, the differences in AA-oxylipins between LPS stimulated blood and plasma were more pronounced. In

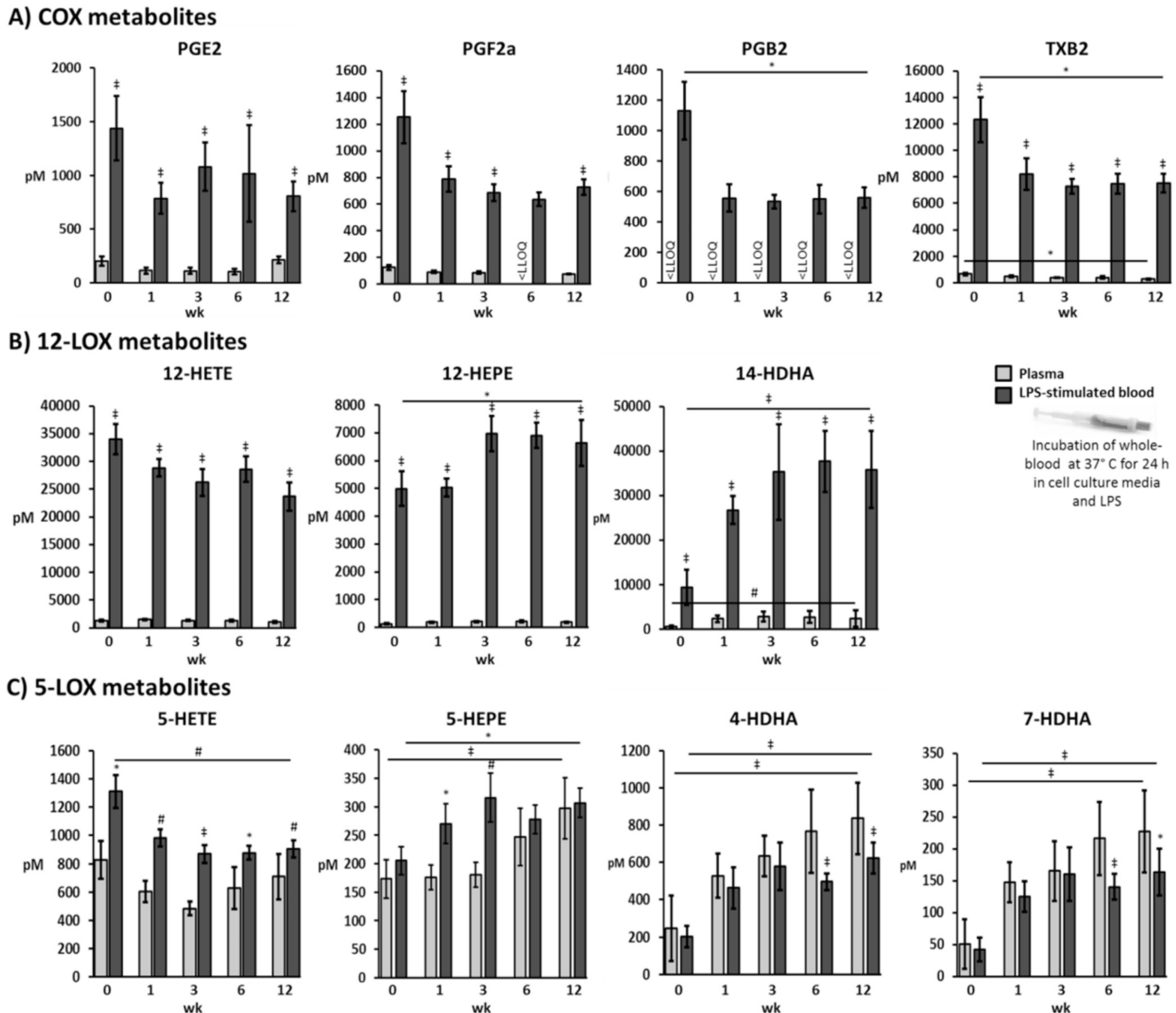


Fig. 2. LPS stimulated oxylipin formation via COX (A), 12-LOX (B), 5-LOX (C), 15-LOX (D), and CYP (E). Shown are the levels (pM) of oxylipins in plasma (light grey bars) and ex vivo LPS stimulated (whole) blood (dark grey bars) at baseline (wk 0) and after one, three, six and twelve weeks of DHA supplementation. Bars represent mean ± SE. Statistic differences between oxylipin levels in plasma and LPS stimulated blood were measured by *t*-test for paired samples (symbol on top of dark grey bars). Differences between different time points were analyzed by ANOVA for repeated measurements (symbol above line). * $p \leq 0.05$, # $p \leq 0.005$, ‡ $p < 0.001$.

comparison, no changes in levels of 5-HETE and 15-HETE were observed in plasma.

3.2.4. 12-LOX metabolites

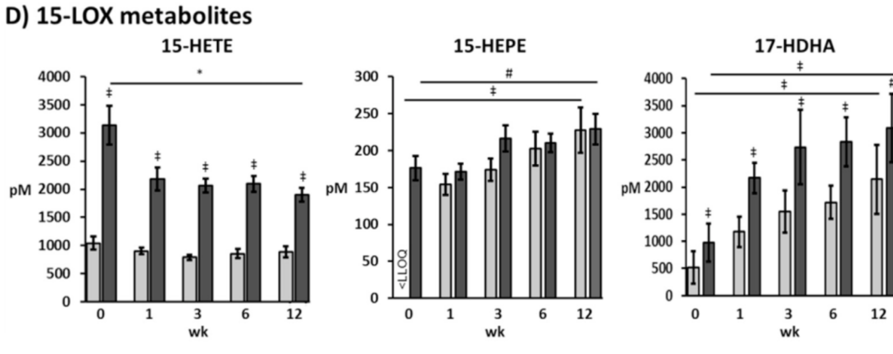
At baseline, concentrations of 12-HETE, 12-HEPE and 14-HDHA in LPS stimulated blood were 24-, 38-, and 16-fold higher compared to plasma (Fig. 2b).

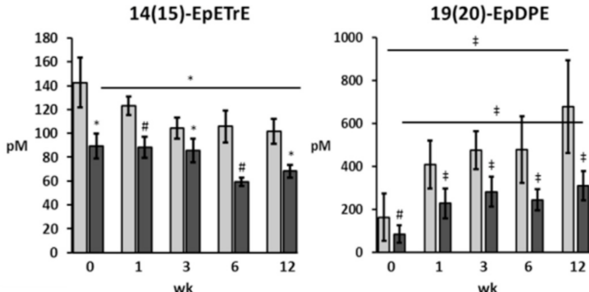
In the course of DHA supplementation, a trend for a time-dependent 12-HETE reduction was observed. As expected, levels of the DHA-derived 12-LOX metabolite 14-HDHA are significantly increased in LPS stimulated blood. Similarly, the EPA-metabolite 12-HEPE is highly induced in LPS stimulated blood in response to DHA supplementation.

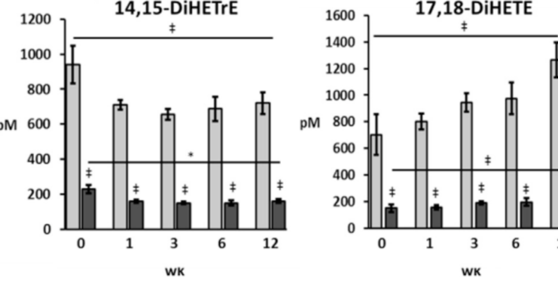
4. Discussion

In our study the variations of oxylipin concentrations and its LC n-3 PUFA induced changes were considerably lower compared to earlier studies [23,28–30]. This is probably caused by the study design used and the carefully selected subjects with respect to sex, age, health status, as well as eating habits and nutrition status. Previous studies observed effects of female hormones on PUFA metabolism [38–40]. Furthermore, a difference in the DHA to EPA retro-conversion rate was detected between healthy elderly (mean age of 77 years) vs. young adults (mean age of 27 years) [37] suggesting an effect of age on PUFA metabolism. One should note that varying precursor PUFA levels directly influence their downstream oxylipin levels [43]. Although a recent study observed that the basal level of some of the investigated oxylipins in elderly people (mean age: 53 years) were higher compared to the younger study participants (mean age: 22 years), e.g. 5-LOX products 5-HETE, 5-HEPE and 7-HDHA or 20-HETE [44], no data exist on age dependent differences in comprehensive oxylipin patterns. Otherwise, factors influencing the oxylipin pattern are largely unknown.

Hence, a homogenous collective of healthy men (e.g., normolipidemic), in a narrow age range (20–40 y), with similar physical constitution and eating habits (primarily fish intake) was chosen to analyze the effect of DHA on concentrations of DHA, AA and EPA and their oxylipins in blood. Furthermore, due to pre-selection of suitable







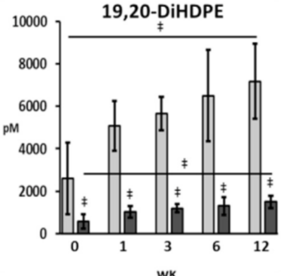


Fig. 2. (*continued*)

probands based on LC n-3 PUFA levels in blood in order to confirm the reported fish consumption, the subjects showed similar concentrations of EPA and DHA in blood cells (e.g., mean ΣEPA/DHA in % of total fatty acid: 3.3 ± 0.2) and a low interperson-variability [34]. Also, careful pre-analytic procedures, such as short time between blood collection and plasma preparation – a factor known to influence oxylipin levels [45] – led to low variations in oxylipin concentrations. Moreover, blood was collected from each proband at the same time point. The latter was a precautionary measure since it is unclear whether concentrations of free oxylipins in plasma underlie circadian variations; a question which we investigate in a present study.

4.1. Effect of DHA supplementation on free oxylipins in plasma

In previous human studies, that investigated the effects of LC n-3 PUFAs on comprehensive hydroxy-, epoxy- and dihydroxy-FA patterns in blood, mixtures of EPA and DHA (mainly of fish origin) were supplemented [[22–31], reviewed in [43]]. In these studies relative changes in EPA-oxylipins (particularly CYP-derived epoxides) were considerably higher compared to DHA-oxylipins [22–24,26,29,30]. A possible explanation is the low abundance of EPA in human tissues. In addition, a retro-conversion of DHA to EPA or a preferred oxidation of EPA by metabolizing enzymes (or both) may contribute. Comparing the CYP-product/substrate ratios, EPA is more efficiently converted to epoxy-FAs compared to DHA in vitro [15] and in vivo [29].

However, the effect of long-term DHA supplementation on comprehensive blood oxylipin patterns and possible effects on EPA- and AA-oxylipins has not been studied in humans so far [43]. Only one study [46] investigated the effect of EPA and DHA on oxylipin concentrations. However, in this “acute test meal study” only seven analytes were quantified (15S-HEPE, 18S-HEPE, 14,15-EpETE, 17,18-EpETE, 14,15-DiHETE, 17,18-DiHETE, 19,20-DiHDPA).

It is remarkable, that – with a few exceptions – all quantified DHA-, EPA-, and AA-oxylipins were affected by DHA supplementation, even though not statistically significantly for each analyte. As expected, all DHA-oxylipins linearly increased in a time-dependent fashion similarly as their precursor DHA in blood cells [34], which is in line with earlier studies [29]. DHA-derived CYP epoxides – which showed potent anti-arrhythmic [18], vasodilatory [19], anti-thrombotic [20] and anti-angiogenic effects [21] – were increased by 400–500% after 12 weeks. In contrast, DHA levels in plasma rapidly increase already after one week and remain high over 12 weeks [34]. This is consistent with the intracellular formation of oxylipins and the reason why the PUFA pattern is for most tissues well reflected by the red blood cell composition [43].

A slight but significant increase of several EPA-oxylipins in plasma was observed after DHA supplementation. For example, 18-HEPE, a

precursor for SPM resolvin E1 (18-RvE1) [47], is significantly elevated. Whether this potent anti-atherogenic lipid mediator [48] was affected cannot be clarified since 18-RvE1 was not covered by our analytical method. An increase of anti-inflammatory and cardioprotective EPA-epoxides could not be detected because their levels were below the limit of quantification of the method even after supplementation. However, their degradation products, the dihydroxy FAs, increased time-dependently during the supplementation period. Hence, it can be concluded, that EPA-epoxides equally increased but are rapidly metabolized to less active dihydroxy-FAs by sEH [16].

The observed correlation of EPA levels in plasma and EPA-oxylipin levels in plasma is in line with our previous findings [29,30], where we found similar associations. The slight increase in EPA levels in plasma and blood cells prior DHA supplementation is a possible result of a retro-conversion from DHA [34]. The concurrent increase in EPA levels in plasma and EPA-oxylipin levels in plasma is consistent with the finding of previous intervention studies that the oxylipin pattern reflects changes of the parent PUFA [22,29,43]. As we previously showed, the changes in plasma oxylipins occur rapidly even after a single dose of EPA/DHA [49]. Interestingly, a single dose of DHA also leads to a rapid (4 h) increase of EPA-oxylipins (17,18-EpETE, 14,15-DiHETE, and 17,18-DiHETE) suggesting a release of EPA from the membrane pool by DHA, which evokes the increase of oxylipins [46].

In contrast to AA-oxylipins, the decline of AA levels in plasma was linear and time dependent, although less pronounced [34]. The finding that the decline in AA-derived oxylipin levels was not statistically significant is possibly due to (inter-subject) variations. However, in total, the sum value of AA-oxylipins was drastically and significantly decreased already after 6 weeks of DHA supplementation.

4.2. *Effect of DHA supplementation on free oxylipins in LPS stimulated blood*

The majority of hydroxy-, epoxy-, and dihydroxy-FAs in circulating blood are esterified [30,33,45], and thus not detected as free oxylipins. However, upon an inflammatory stimulus, these oxylipins can be directly released by e.g. phospholipase hydrolysis of lipids. In order to monitor the effect of DHA supplementation on oxylipin release in response to an inflammatory scenario like acute sepsis, an ex vivo whole-blood culture systems with LPS as stimulator of leukocytes (LPS stimulated blood) was used.

LPS is a potent stimulant of the innate immune response through the activation of circulating monocytes including the induction of COX-2 [50]. As a result the production of PGs such as PGE2 is triggered. Consequently, in LPS stimulated blood, COX metabolites of AA PGE2, PGF2a, and TXB2 are significantly induced (6- to 13-fold) compared to plasma. The finding that DHA supplementation reduces the formation of prostanoids in immune stimulated blood cells can be explained by two factors. First, DHA supplementation cause reduced AA levels. However, a significant reduction of AA levels in blood cell membranes was observed only at the end of the intervention period after 12 weeks of DHA supplementation [34]. The second factor are the increased LC n-3 PUFA levels (occurring already after the first week of supplementation [34]), which compete with AA for the active site of the COXs and reduce the formation of AA-derived prostanoids accordingly. Thus, the results from the ex vivo inflammation assay support the most common mode of action how LC n-3 PUFA from fatty fish or fish-oil reduce the formation of pro-inflammatory PGs [51]. However, it is somewhat remarkable, that DHA alone causes this massive reduction of PGs.

In contrast to COX and 12-LOX metabolites, levels of CYP and sEH metabolites were significantly lower in LPS stimulated blood compared to plasma. Possibly, epoxides and dihydroxy-FAs are degraded in LPS stimulated blood during the 24 h incubation or absorbed, esterified and incorporated in blood cell membranes. After DHA supplementation a slight but significant reduction in AA-derived epoxides in LPS stimulated blood was observed, while n-3 epoxides and dihydroxy-FA were increased.

Slight (or absent) differences in 5-LOX and 15-LOX metabolite concentrations between LPS stimulated blood and plasma can be explained by a lower activation of these AA cascade pathways compared to the COX-2 stimulation in monocytes. In order to investigate the activation of 5-LOX and 15-LOX, a stimulation using calcium ionophore – as carried out in other studies [52] – would have been more efficient.

Nevertheless, the time-dependent trends in 5-LOX and 15-LOX metabolites after DHA supplementation were similar to 12-LOX metabolites: Significant reduced AA-derived hydroxy-FAs (5-HETE and 15-HETE) and significant enhanced EPA-derived hydroxy-FAs (5-HEPE and 15-HEPE) and DHA-derived hydroxy-FAs (4-HDHA, 7-HDHA, 17-HDHA) in LPS stimulated blood. Notable, 17-HDHA – a precursor for SPM Rv D1 (17-RvD1 or protectin D1) [17] – is significantly elevated in both LPS stimulated blood and plasma. In LPS stimulated blood, 12-LOX metabolites of AA, EPA, and DHA were highly induced compared to plasma, which is probably caused by the activation of platelets during the 24 h incubation.

After DHA supplementation, DHA-derived 14-HDHA and EPA-derived 12-HEPE are significantly increased in LPS stimulated blood, which can be explained by a higher substrate availability. The time-dependent decrease in 12-HETE levels in LPS stimulated blood may be the result of an AA displacement by DHA. These results shows the strength of the use of the ex vivo activation assay in order to monitor changes in the AA cascade in healthy subjects.

4.3. *Limitations*

In the present study only the free oxylipins in plasma were quantified. Given the autocrine and paracrine mode of action of the oxylipins acting as lipid mediators, the concentration in plasma is only a proxy for the biologically relevant changes occurring in the tissues, which cannot be sampled from healthy volunteers.

5. Conclusion

We demonstrated that DHA supplementation causes a time-dependent shift in the entire oxylipin profile suggesting a cross-linked metabolism of PUFAs. We show that DHA supplementation not only impacts the concentrations of DHA-oxylipins but also provokes increasing EPA-oxylipins and decreasing AA-oxylipins, which certainly affects resulting biological effects. This finding complicates the disentanglement of differential physiological effects of EPA and DHA since effects of DHA may be also conducted by EPA and its oxylipins. Utilizing a commercially available ex vivo inflammation assay, we could show that the changes in the oxylipins pattern upon LPS stimulus were particularly pronounced in the COX pathway. This indicated that the 12 week DHA supplementation would change the acute inflammatory response in the subjects since the ability of the monocytes/macrophages to form pro-inflammatory PGs is significantly reduced.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The provision of the DHA capsules by DSM Nutritional Products (Columbia, MD, USA) is kindly acknowledged. We would like to thank the participants who contributed their time to this project. This study was supported by grants from the German Research Foundation (Grant

SCHE 1801 and SCHU 2516) to NHS and JPS.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2017.06.007.

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6. Paper V

Intra-individual variance of human plasma oxylipin pattern: Low inter-day variability in fasting blood samples versus high variability during the day

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Published in: *Analytical Methods*

Link: *https://pubs.rsc.org/en/content/articlelanding/2018/ay/c8ay01753k*

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Intra-individual variance of the human plasma oxylipin pattern: low inter-day variability in fasting blood samples *versus* high variability during the day†

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Received 6th August 2018
Accepted 22nd September 2018

DOI: 10.1039/c8ay01753k

rsc.li/methods

Introduction: Several eicosanoids and other oxylipins are important lipid mediators. Reliable quantification in plasma is important to assess the state of disease, action of drugs and the biology of oxylipins. In order to monitor biological changes, low background variability of oxylipin concentrations in biological samples is essential for proper interpretation of oxylipin biology. However, only little is known about the variation in the oxylipin profile in healthy human subjects. *Experimental*: Inter-day variation in circulating oxylipins after overnight fasting was investigated in healthy young men on either a standardized or non-standardized diet during a (24 to) 48 h time interval. Intra-day variance was investigated during an 8 h time interval (covering breakfast and lunch meals) in men on a standardized diet with blood sampling at 0, 2, 4, 6 and 8 hours. Free oxylipins in plasma were analyzed using a targeted metabolomics platform for the quantification of 160 oxylipins from different precursors. Analytical variation was evaluated based on quality control plasma samples. *Results*: Free oxylipins in quality control plasma samples showed low variations (<20% for most analytes). Inter-day variations in fasting blood were in the same range, while significant differences were observed within the day (intra-day variance). *Conclusion*: Based on the low intra-individual inter-day variance in concentrations of free oxylipins, our results demonstrate the suitability of fasting plasma for the investigation of oxylipin biology. In non-fasting plasma samples, the variations were high during the day. Thus, non-fasting plasma samples appear to be unsuitable to evaluate biologically relevant changes, for instance, those caused by disease or drugs. However, it remains to be determined if the same standardized meal results in reproducible modulations of the oxylipin profile allowing evaluation of the oxylipin pattern during the postprandial state.

1 Introduction

Eicosanoids and other oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFAs) and many of them have high biological activity in different physiological processes. They are formed endogenously within the arachidonic acid (C20:4n6, ARA) cascade in a variety of enzymatic and non-enzymatic reactions. Conversion of PUFAs by cyclooxygenases (COXs) may lead to the formation of prostanoids like PGE_2, which is involved in the regulation of pain and inflammation or thromboxane A_2, a potent mediator in the regulation of platelet aggregation.[1] Action of lipoxygenases (LOXs) may give rise to hydroperoxy-PUFAs, which can either be reduced to hydroxy-PUFAs or further metabolized, *e.g.* by LOXs, to multiple hydroxylated PUFAs.[2] In particular, multiple hydroxylated metabolites from the omega-3 (n3)-PUFAs eicosapentaenoic acid (C20:5n3, EPA) or docosahexaenoic acid (C22:6n3, DHA) have been shown to be potent mediators in the resolution of inflammation.[2] Cytochrome P450 (CYP) monooxygenase enzymes can act as epoxygenases or ω-hydroxylases resulting dominantly in epoxy-PUFAs or terminally (ω and ω-n) hydroxylated PUFAs.[3,4] Epoxy-PUFAs are highly active, *e.g.* the terminally epoxygenated EPA (17(18)-EpETE) shows strong anti-arrhythmic potency[3,5] and epoxy-PUFAs from ARA, EPA and DHA show anti-inflammatory properties.[6] Moreover, ARA derived epoxy-PUFAs display angiogenic activity,[6] while the terminally epoxygenated DHA (19(20)-EpDPE) shows anti-angiogenic properties.[7] These highly potent epoxy-PUFAs are converted by the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ay01753k

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soluble epoxide hydrolase (sEH) to dihydroxy-PUFAs[8] with so far poorly characterized biological activity. Additionally, autoxidation of PUFAs may give rise, for instance, to hydro(pero)xy-PUFAs or prostaglandin-like structures, such as isoprostanes.[9,10]

Reliable quantification of oxylipins in biological samples is essential for the understanding of their biological role. However, different endogenous and exogenous factors have been shown to have an impact on the oxylipin profile, which hampers the interpretation of biological data. For instance, genetic variants in PUFA metabolizing enzymes[11–13] or enzyme preferences for specific PUFAs might influence oxylipin production.[3] Other factors, including age,[14–16] sex,[17–19] physical exercise[20] or health status[21–23] may also impact oxylipin concentrations, either directly or indirectly *via* an influence on PUFA metabolism. Dietary factors, such as fatty acid intake, also influence the oxylipin profile, for example, as recently shown for the amount of alpha-linolenic acid (C18:3n3, ALA) in the diet[24] or for supplementation with n3-PUFAs.[25] Of note, supplementation with n3-PUFAs resulted in high variations in the individual response to n3-PUFAs[25–29] and only part of the effect could be accounted for by the basal status of n3-PUFAs.[25,27] This means that even when aiming at modulating the profile of oxylipins, interpretation of results might be complicated due to high inter-individual variations. Apart from these biological variations, (pre-)analytical procedures, such as the time between blood collection and plasma preparation, comprise a factor that is known to influence oxylipin concentrations in the sample.[30]

In most studies analyzing oxylipin concentrations, blood from the fasting state is used for analysis.[24,26,27,31] However, only little is known about the modulation of free oxylipin concentrations in plasma following food ingestion or whether the oxylipin profile underlies circadian variations[30,32] and which background variations (inter-day variation of the oxylipin profile in fasting plasma) may have to be expected. Therefore, this study aims to (i) investigate the inter-day variation in the oxylipin profile in samples collected 48 hours apart (with and without standardized nutrition) and (ii) to investigate the intra-day variation of free oxylipins in plasma.

2 Experimental

The data published here are derived from two different studies. The investigator initiated studies were conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of the Medical Chamber of Lower Saxony (Hannover, Germany). Written informed consent was obtained from all subjects. Both studies are registered in the German Clinical Trial Register (DRKS00006765 and DRKS00012257) and were conducted at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany.

Study design

To investigate the inter- and intra-day variation in the oxylipin profile in a population of healthy volunteers, the samples of two separate studies were used:

Study (A) Inter-day variation in a study population on a non-standardized diet. An unpublished subset of samples from the study described in detail in ref. 24 is presented here. In brief, after a screening and a four-week run-in phase with an ALA-poor diet, blood samples of 18 male healthy subjects were taken at baseline (t0) and after 48 hours (t48) during normal (non-standardized) nutrition.

Study (B) Intra- and inter-day variation in a study population on a standardized diet. Following a screening and a four-week run-in phase, blood samples were taken at baseline (t0) and after 2 (t2), 4 (t4), 6 (t6), 8 (t8), 24 (t24), 48 (t48), and 72 (t72) hours during standardized nutrition. The standardized nutrition started with the lunch meal one day prior to baseline blood collection and ended with the last examination. The diet was PUFA-poor and portion sizes were adjusted to the energy demands (small and large portion size) of the participants. Volunteers were allowed to drink water, tea and coffee (without milk/sugar). The macronutrient and fatty acid composition of the standardized nutrition is shown in Table S1.† During the four-week run-in phase, participants were requested to abstain from fish, seafood, and ALA-rich vegetables oil, such as linseed oil or chia seeds to minimize nutritional effects on the variability of oxylipin patterns. Recruitment, inclusion and exclusion criteria matched those of Study A published in ref. 24 and can be found in the ESI† along with a description of the pre-screening procedure.

Proband examination, blood sampling and pre-analytical procedures

At baseline examination (t0), blood pressure, body height, body weight and pulse were measured and the subjects completed a questionnaire to obtain information about changes in medication, diet and lifestyle habits (*e.g.* physical activity) compared to the screening questionnaire. Additionally, fasting blood samples were taken at baseline (t0), after 24 (t24), 48 (t48) and 72 hours (t72). Non-fasting blood samples were taken after 2 (t2), 4 (t4), 6 (t6) and 8 (t8) hours. Blood samples were obtained by venipuncture of an arm vein using Multiflyneedles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt). For analysis of plasma oxylipins and triglycerides (TGs), EDTA blood monovettes were centrifuged for 10 min at 1500 × *g* and 4 °C. Plasma was transferred into 1.5 mL Eppendorf tubes (Sarstedt) and immediately frozen and stored at −80 °C until analysis. All transfer steps were carried out on ice. Serum lipid levels, liver enzymes and small blood picture at baseline (t0) and triglycerides in plasma at t0, t2, t4, t6 and t8 were determined in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany.

Fatty acid analyses in food samples

The total fat content of food samples from Study B was determined by gravimetry after lipid extraction according to Weibull–Stoldt performed as rapid microextraction.[33] Concentrations of fatty acids in the lipid extracts were determined by means of gas chromatography with flame ionization detection (GC-FID)

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following (trans-)esterification to fatty acid methyl esters (FAMEs) as described[34] using methyl pentacosanoate (C25:0 methyl ester) as an internal standard (IS). Fatty acid concentrations in food samples were calculated as mg fatty acid/100 g meal.

Oxylipin analysis

The concentrations of oxylipins in the plasma of Study A were determined by means of liquid chromatography-mass spectrometry (LC-MS) following solid phase extraction as described.[24] Oxylipins in the plasma of Study B were analyzed accordingly with slight modifications described in ref. 35. In brief, after addition of internal standards, antioxidant solution and methanol, plasma samples were frozen at −80 °C overnight. Oxylipins were extracted from the supernatant using Bond Elut Certify II cartridges (200 mg, Agilent, Waldbronn, Germany) and ethyl acetate/*n*-hexane (75 : 25, v/v) with 1% acetic acid as the eluent. Before elution, the samples were dried under vacuum (−200 mbar, 30 sec). LC-MS analysis was carried out using a 1290 Infinity LC System (Agilent, Waldbronn, Germany) with a 6500 QTrap (Sciex, Darmstadt, Germany) operated in scheduled selected reaction monitoring mode following negative electrospray ionization as described.[35]

Within the sample batch of Study B, human quality control (QC) plasma samples ($n = 15$) and randomly assigned duplicate samples of the study population ($n = 12$) were prepared and analyzed to characterize intra-batch variation of the analytical method (including sample preparation, LC-MS analysis and peak integration).

Calculations and statistics

Oxylipin concentrations in plasma and their relative change [%] are stated as mean ± standard error of the mean (SEM). If the concentration in a sample was below the lower limit of quantification (LLOQ) the 1/2 LLOQ was used to calculate the mean and SEM. The concentration was set to LLOQ if the analyte could not be quantified in more than 50% of the samples. Relative changes were calculated individually for each subject at each time point (x) using the following formula: rel. change [%] $= 100 \times (conc_{tx}/conc_{t0})$. In QC samples, only those analytes were evaluated which were >LLOQ in ≥80% of the samples. Means were calculated by filling in the LLOQ for analytes <LLOQ and the 95% interval of the standard deviation (95% SD = SD × 1.96) was calculated.

The distributions of the sample sets (Study A and B) were analyzed by a Kolmogorov–Smirnov test. Statistical differences between the time points were tested for parametric data with a *t*-test (Study A) or ANOVA with repeated measurements followed by *post hoc* *t*-tests for paired samples with Holm–Bonferroni-adjusted levels of significance (Study B) and for non-parametric data with Wilcoxon-tests (Study A) or the Friedman Test followed by the Dunn–Bonferroni *post hoc* test (Study B). Statistical tests were only performed for analytes which were quantified in the study population at all time points of the respective studies. Statistical significance was set at $p \leq 0.05$ for all analyses. All statistical analyses were carried out with SPSS software (Version 24, SPSS Inc., Chicago, IL, USA).

3 Results

Study population

The study population of Study A is described in detail elsewhere.[24] 18 male, healthy subjects were included in the study with a mean age of 26.2 ± 4.5 years and a BMI of 24.9 ± 2.0 kg m^{-2}. In Study B, 13 male subjects met the criteria and were included in the study. All participants (mean age 24.6 ± 2.5 years) were healthy and had a normal BMI (24.6 ± 2.0 kg m^{-2}). Liver enzymes and serum lipid profiles were in the normal range (ESI Table S2A†).

Quality control plasma

The results of selected oxylipins in the QC samples can be found in Fig. 1. Absolute concentrations of all analytes quantified in the QC samples along with the variation are presented in ESI Table S4.† 73 (out of 160) analytes were quantified in QC plasma in the range of 50 ± 5 pM (11,12-DiHETE) to 11 ± 1 nM (15,16-DiHODE). Based on the relative 95% SD, *i.e.* the deviation covering 95% of all values measured, 61 of the quantified analytes showed a variation of <20% and only 12 analytes fluctuated >20%. In general, the relative 95% SD decreased with higher concentrations, or more specifically with increasing ratio of determined concentration to LLOQ. However, the variation did not decrease below 4.8%.

The deviation between the first and second analysis of the randomly assigned duplicate samples from the study population was within the range of the deviation of the analytes in the QC samples.

Inter-day variation of free oxylipins in plasma

The results for the inter-day variation of plasma oxylipin concentrations in a non-standardized diet can be found for representative oxylipins in Fig. 2 (all data in ESI Table S5†). Differences between fasting plasma oxylipin concentrations collected at baseline (t0) and after 48 hours (t48) for a non-standardized diet were not significant. However, for some analytes the inter-day variance exceeded the analytical fluctuation, *i.e.* PGE_2, 9(10)-Ep-stearic acid, 11,12-DiHETE and various linoleic acid (C18:2n6, LA) and ALA metabolites.

The results for the inter-day variation of oxylipins in fasting plasma samples of subjects on a standardized diet can be found for representative oxylipins in Fig. 3 (all data in ESI Table S6†). Variation of most analytes was within the analytical fluctuation and plasma concentrations remained constant during the observation period (ESI Table S6†). However, the concentration of few hydroxy-PUFAs significantly decreased during the observation period for a standardized diet, *i.e.* 5-HETE, 8-HETE, 5-HEPE, 4-HDHA, 8-HDHA, 10-HDHA and 17-HDHA. The concentration of 5(*R*,*S*)-5-F_{2t}-IsoP, 15-HETE, 20-HDHA and 8,9-DiHETrE also decreased significantly; however, the changes were within the analytical fluctuation. Although not statistically

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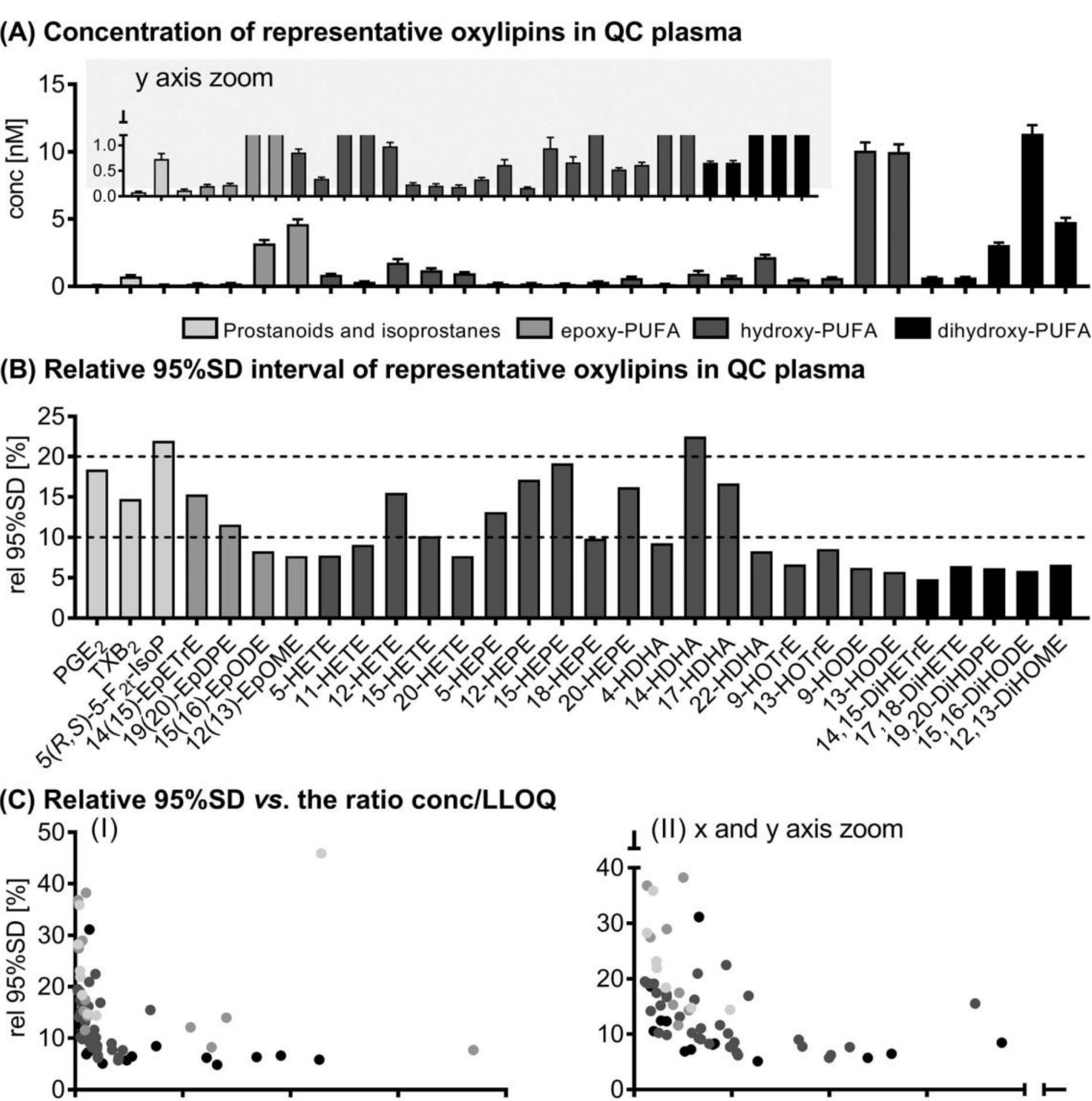

Fig. 1 Variation of the analytical method. Shown are concentrations ± 95% interval of the SD (A) and the relative 95% interval of the SD (B) of selected oxylipins in quality control plasma samples ($n = 15$). In (C) the relative 95% SD is plotted against the ratio of concentration to the lower limit of quantification (conc./LLOQ). Shown are all analytes quantified in quality control plasma (73 analytes, see ESI Table S4†).

significant, differences for some analytes were higher than expected from the analytical fluctuation, *e.g.* 12-HHTrE, 10(11)-EpDPE, 11-HETE, 20-HETE, 18-HEPE and 8,9-DiHETE.

The relative concentrations of representative oxylipins within a 48 hour time interval in both studies (t0 *vs.* t48 for Study A and t24 *vs.* t72 for study B) are shown in ESI Fig. S3.† This direct comparison revealed similar inter-day differences in oxylipin concentration in both studies (non-standardized *vs.* standardized diet). However, the variation in relative concentrations of some analytes was higher in Study A, *e.g.* 12(13)-EpOME, 5-HEPE or PGE_2.

Intra-day variation of free oxylipins in (non-fasting) plasma samples

The results for the intra-day variation can be found for selected oxylipins in Fig. 4 (means, all data in ESI Table S7†) as well as Fig. S4† (individual data values). Plasma oxylipin concentrations were subject to large fluctuations throughout the day (t0 (following overnight fasting) to t8) with significant differences for almost all analytes (ESI Table S7†). Most metabolites from the individual PUFAs showed similar trends during the observation period. Moreover, trends for ARA, EPA and DHA derived metabolites were comparable (except ARA prostanoids

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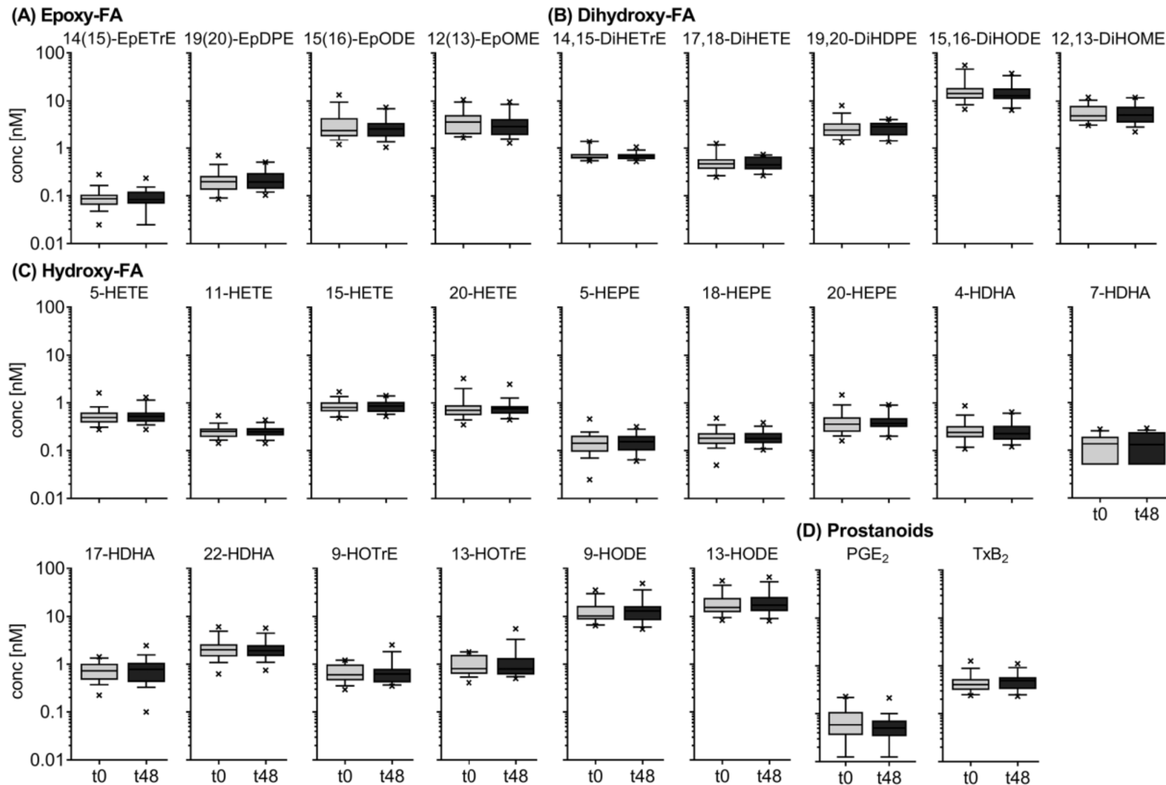

Fig. 2 Inter-day variation of circulating oxylipins in subjects on a non-standardized diet. Shown are concentrations ± SEM of selected epoxy-FA (A), dihydroxy-FA (B), hydroxy-FA (C) and prostanoids (D) ($n = 18$). Plasma was collected at baseline (t0) and after 48 h (t48). No intervention was carried out.

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increasing from t0 to t8): starting from the highest concentration at t0, concentrations were decreased to a minimum at t2 and increased again at t4. At t6 and t8, concentrations were decreased compared to t4, for some analytes to a similar level compared to t2 (*e.g.* dihydroxy-PUFAs, 5-HETE, 5-HEPE and 4-HDHA, Fig. 4). In an analogous manner, trends for LA and ALA derived analytes were similar: an increase in analyte concentrations up to t4 with a following decrease. The lowest concentrations were observed at t8 (*e.g.* epoxy- and hydroxy-PUFAs, Fig. 4). The largest intra-day variations were observed for different LA and ALA metabolites (up to 400% at t4 compared to t0).

4 Discussion

The physiological effects of oxylipins are diverse and range, for example, from important roles in the regulation of fever and inflammation (*e.g.* PGE_2) to anti-arrhythmic (*e.g.* 17(18)-EpETE) and anti-angiogenic effects (*e.g.* 19(20)-EpDPE). However, due to crosstalk between the different pathways of the ARA cascade, physiological effects result more from changes in the whole product pattern rather than from changes in individual mediators.[36] In order to investigate and understand oxylipin biology, exact and reliable quantification of a comprehensive pattern of oxylipins is crucial. The pattern of oxylipins can reflect the pathophysiology of diseases,[21–23] which may allow using oxylipins as biomarkers of the disease state. Moreover, drugs[37] as well as the diet[24,25,38] modulate the oxylipin pattern, which is an important factor for their effect on physiology and health. However, biological, time-dependent variations in the oxylipin pattern in healthy individuals have to be kept in mind when interpreting biological data. Therefore, the aim of the present study was to characterize inter- and intra-day variations of the oxylipin profile as an important basis for the interpretation of biological effects.

The precision of the analytical method was assessed in QC plasma samples (different aliquots of the same sample). Oxylipin concentrations in the QC samples were in the same range as in the plasma samples of both studies. Most oxylipins (about 84% of 73 analytes) showed a fluctuation of less than 20% within the batch; only 12 analytes showed a higher fluctuation. The variations observed here were comparable to or lower than those previously described for other LC-MS based analytical approaches for the quantification of oxylipins.[39,40] It is not surprising that the degree of variation decreased with increasing ratio of concentration to LLOQ, *i.e.* in general with higher levels in plasma. The minimum variation was ~5%, which is consistent with the expected (random) relative error of

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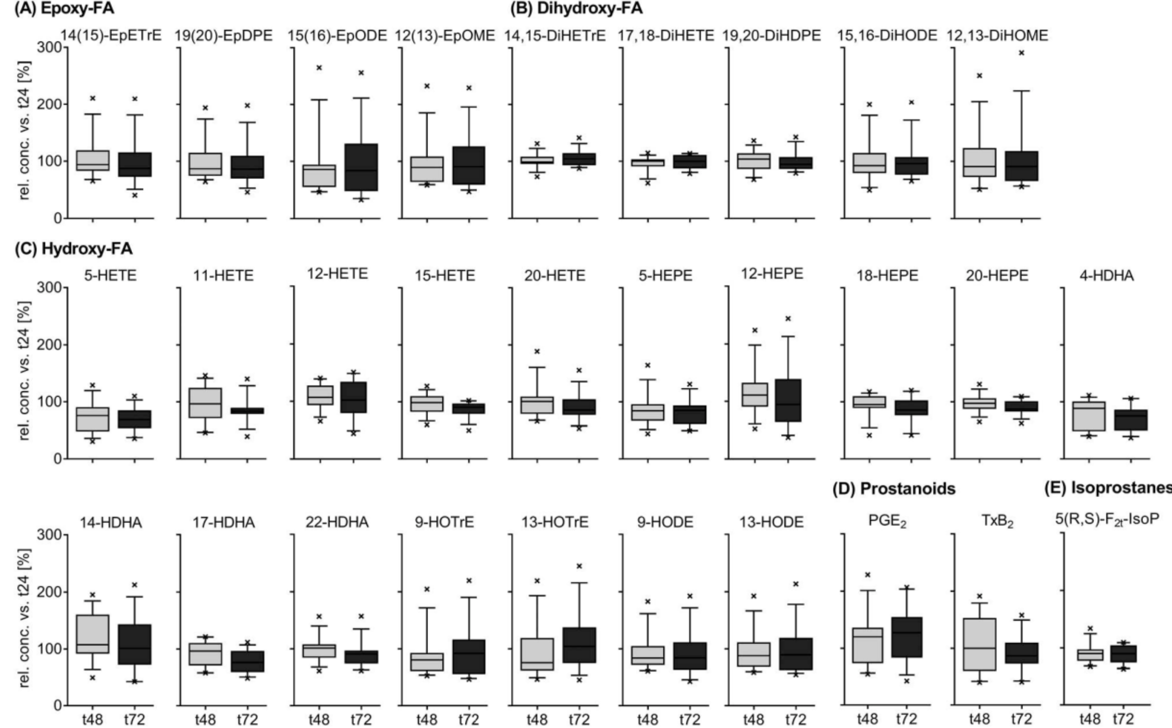

Fig. 3 Inter-day variation of circulating oxylipins in subjects on a standardized diet. Shown are means of the relative inter-day change within each subject ± SEM of selected epoxy-FA (A), dihydroxy-FA (B), hydroxy-FA (C), prostanoids (D) and isoprostanes (E) ($n = 13$). Plasma was collected from study participants on a standardized diet at t24, t48 and t72 and the relative concentrations of oxylipins at t48 and t72 were calculated against t24.

LC(-MS) methods.[41] For the interpretation of biological data it is important to keep this analytical variation in mind since high variations might mask biological effects. Therefore, it is crucial to have a well-characterized quantification method to reliably determine endogenous concentrations.

In most studies investigating the biology of oxylipins, fasting blood is used and is usually collected in the morning following overnight fasting.[24,26,27,32] Therefore, another crucial parameter for the interpretation of biological effects is the inter-day variability (*i.e.* from one morning to the next) in the oxylipin profile of a healthy individual. This background variation should be reduced as much as possible in order to allow a better evaluation of biologically relevant effects, *e.g.* in the course of diseases or during pharmacological intervention. Our results support the suitability of fasting plasma for the investigation of biological effects since inter-day variations were low for oxylipins from all chemical groups in both studies (non-standardized and standardized diet). Except for a few analytes (mainly LA and ALA metabolites), the variation of all oxylipins in the study population on a non-standardized diet was comparable to the analytical variance, indicating stable levels of oxylipins in a healthy person as expected for a homeostasis of lipid mediators. For a standardized diet, the variations observed in the oxylipin profile were slightly more pronounced and often higher than expected from the analytical method. Comparing absolute concentrations between t24, t48 and t72, a slight negative trend could be observed which was more pronounced when baseline concentrations (t0) were taken into account. This decrease might be a result of changes in dietary PUFA intake with the standardized nutrition (which started at lunch the day before baseline blood sampling). For a western diet the consumption of (n6-)PUFA – especially LA – is higher compared to the standardized diet.[42] Moreover, it has been previously shown for increases of LA in plasma lipids of subjects changing from a low- to a high-PUFA diet[43] that – depending on the lipid class – most changes occurred during the first five days for the high-PUFA diet.

Nevertheless, it can be summarized that inter-day variations in the oxylipin profile of fasting plasma in healthy human subjects are small, which is a prerequisite for the investigation of oxylipin biology in intervention studies. However, the study population of male subjects has been well-characterized and further studies have to show to what extent different lifestyles, physical activity, or the menstrual cycle might influence the oxylipin profile.

The postprandial state differs substantially from the fasting state regarding blood – and thus plasma – lipid composition and fatty acid metabolism. Taking into account that the postprandial state is more representative for the plasma composition during the day for individuals of western countries

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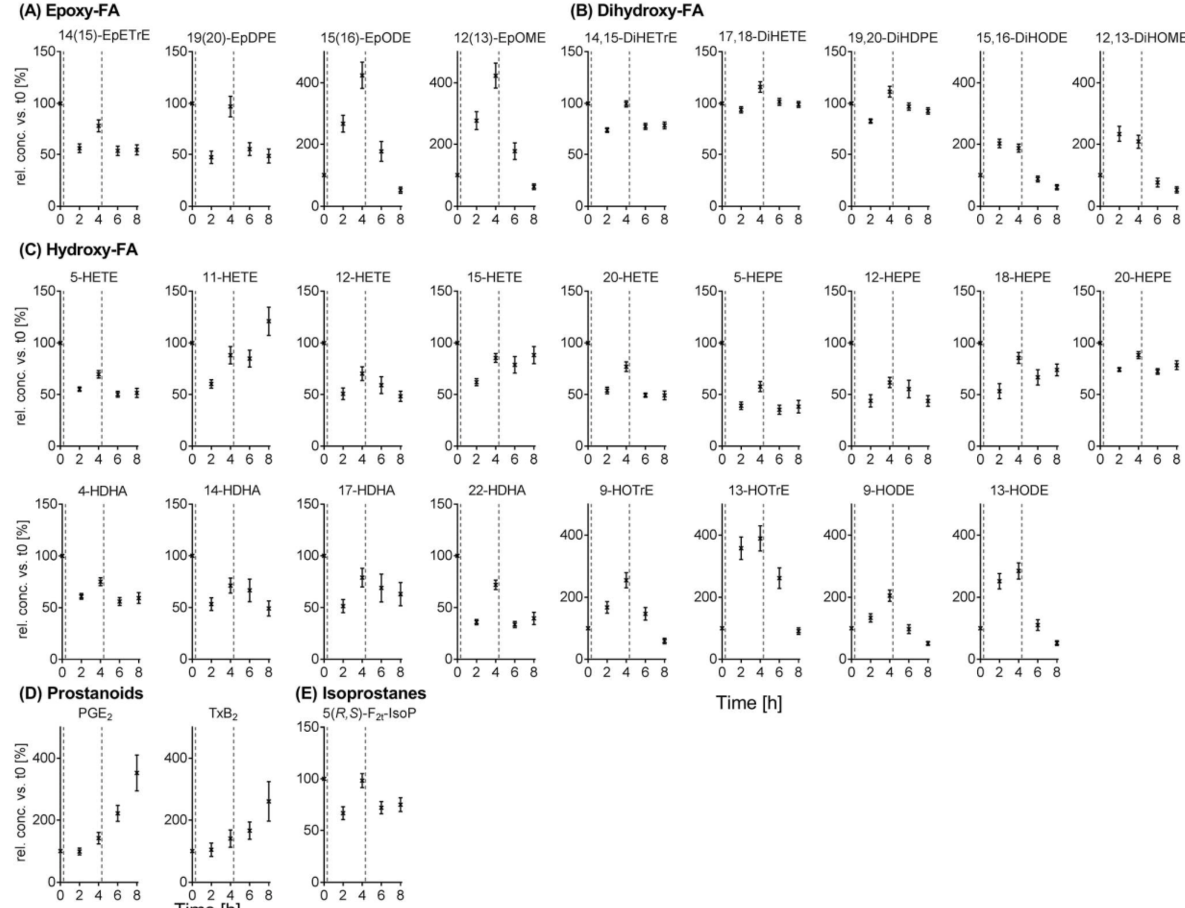

Fig. 4 Intra-day, intra-person variation of circulating oxylipins in subjects on a standardized diet. Shown are means of the relative intra-day change within each subject ± SEM of epoxy-FA (A), dihydroxy-FA (B), hydroxy-FA (C), prostanoids (D) and isoprostanes (E) ($n = 13$). Plasma was collected from study participants on a standardized diet at baseline (t0) and t2, t4, t6 and t8. The relative concentrations of oxylipins were calculated against the baseline. The dotted lines in the diagrams indicate food intake (20 min past sample collection at t0 and t4).

compared to the fasting state, the question arises whether postprandial plasma might also be a suitable matrix for the analysis of the oxylipin profile. Following a meal, fatty acids are absorbed and reach the blood *via* lipoproteins, *i.e.* chylomicrons, resulting in a peak of plasma TGs, which is accompanied by elevated lipolysis.[44] Thus, it is not surprising that the intake of fat (within a meal) resulted in remarkable changes in the oxylipin profile; LA and ALA metabolites showed the highest changes throughout the day with a maximum at 4 h post breakfast, which was also reflected in mean plasma TGs (individual maxima at 2–6 h). In a previous study with a (breakfast) meal containing more fat compared to our study (1 g fat per kg body weight (BW) *vs.* ~0.44 g kg^{-1} BW) plasma TGs reached a maximum after 4 h,[45] while in another study with a similar fat content compared to our breakfast (~0.56 g kg^{-1} BW) most individuals showed two maxima in plasma TGs: the first between 1 and 3 h and the second between 4 and 7 h post ingestion.[46] In contrast to breakfast, the lunch meal did not show a marked effect on the profile of LA and ALA derived oxylipins, which might be explained by the higher fat content of the breakfast (36.6 g total fat, 17.8 g saturated fatty acids (SFAs), and 8.68 g monounsaturated fatty acids (MUFAs)) in comparison to the lunch meal (6.8 g total fat, 2.71 g SFAs, and 1.24 g MUFAs). Moreover, the gastric emptying of the more complex lunch meal may occur later and slower because of the higher content in vegetables (fiber) and proteins.

Kardinaal *et al.* found an increased level of different eicosanoids, *i.e.* 11- and 12-HETE and 19,20-DiHDPE as well as 8,9- and 11,12-DiHETrE following a high fat challenge (milk shake (~500 mL) containing 16 g fat/100 g milk shake).[47] Strassburg *et al.* found that a high fat shake (95 g fat) with high SFA content (51 g; and 6 g PUFA (no ALA, EPA or DHA)) increased LA derived oxylipins involved in the LOX pathway while high MUFA (79 g; and 8 g PUFA (no ALA, EPA or DHA)) content led to increased LA derived oxylipins derived from the CYP450 pathway.[38] Although a similar differentiation was not possible in our study because

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of the fat composition of the breakfast, our observations are still in line with those from Strassburg *et al.*[38] since LA oxylipin concentrations from both the LOX as well as the CYP pathway were elevated. However, although increased in the present study, the concentrations of ALA derived oxylipins were not affected by the high fat/high SFA or high fat/high MUFA shakes, which might be a result of the higher ALA content of the meals in comparison to the shakes.

In contrast to LA and ALA metabolites, the concentration of oxylipins derived from ARA, EPA and DHA – with only a few exceptions – decreased 2 h post ingestion, which was accompanied by an increase at t4. The initial decrease – which is in contrast to the observed increase in LA and ALA metabolites – might be a result of the small amount of these PUFAs in the meals and has been observed previously following a meal challenge with low PUFA content.[38,39] Moreover, this time course in the concentrations may be explained by changing insulin concentrations as previously discussed by Strassburg *et al.*:[38] a postprandial increase of insulin concentrations is associated with reduced lipolysis and therefore decreased oxylipin concentrations at t2. Decreasing insulin concentrations following t2 may lead to a release of fatty acids and corresponding oxylipin formation at t4.[48] A similar decrease – as observed after breakfast – was found following the lunch meal; however, oxylipin concentrations did not increase 4 h post lunch.

Interestingly, the prostanoids PGE_2 and TxB_2 showed a different course compared to other oxylipins since both increased (almost linearly) over all non-fasting blood samplings from the baseline to t8. PGE_2 and TxA_2 (the biologically active precursor of TxB_2) are important metabolites derived from ARA and are involved in inflammatory responses by regulation of pain and fever (PGE_2) or platelet aggregation (TxA_2).[1] It has been discussed before that a high fat meal might induce postprandial inflammation.[49] Regarding changes in pro-inflammatory oxylipins, previous results have been ambiguous[38,39,50] while our results clearly show increased concentrations of PGE_2 and TxB_2 during the day. This increase, however, could not be associated with the time of meal ingestion (as observed for other oxylipin classes). Moreover, at the fasting time points following t8, *i.e.* t24, t48 and t72, both analytes were found in plasma with similar concentrations as compared to t8. Thus, instead of being associated with the postprandial state, the observed increase in prostanoids PGE_2 and TxB_2 could be a result of increasing stress levels in the participants due to the number of blood samplings during the study.

In line with previous results,[38] our results indicate that intake of dietary fat and its fatty acid composition influence the oxylipin profile. However, it has to be determined if they act as direct precursors for the synthesis of oxylipins or if they indirectly activate intermediate pathways that may lead to oxylipin formation or their release.[51] Moreover, further research is needed to investigate whether postprandial plasma might be useful for the investigation of oxylipin patterns, for example, for the identification of biomarkers of diseases or the efficacy of pharmaceutical drugs. Although the pattern of oxylipins is subject to changes induced by ingestion of fat, this diet induced modulation of the oxylipin profile might be reproducible as previously shown for the individual intra-day response of different plasma fatty acids in lipid classes to the same standardized meal.[52]

5 Conclusion

Overall, our data demonstrate the suitability of fasting plasma for the investigation of the biological role of the oxylipin pattern. Background variations are low, for most analytes within the variation of the analytical method and independent of a standardization of the diet. This makes fasting plasma an ideal matrix for the investigation of oxylipins in pathophysiological states and may allow the identification of biomarkers as well as evaluating the modulation of oxylipin formation by pharmaceuticals, food ingredients and diet.

In non-fasting plasma, oxylipin concentrations fluctuated strongly over the day and ingestion of food was followed by changes in the oxylipin profile. Since in western countries individuals are in a postprandial state during most of the day it would be highly interesting to investigate whether a standardized meal might result in similar modifications of the oxylipin profile and if postprandial plasma might be suitable for the investigation of oxylipin biology.

Funding

This study was supported by grants from the German Research Foundation (Grant SCHE 1801 and SCHU 2516) to NHS and JPS.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Abbreviations

ALA	Alpha-linolenic acid (C18:3n3)
ARA	Arachidonic acid (C20:4n6)
BW	Body weight
COX	Cyclooxygenase
CYP	Cytochrome P450
DHA	Docosahexaenoic acid (C22:6n3)
DiHETE	Dihydroxy eicosatetraenoic acid
DiHETrE	Dihydroxy eicosatrienoic acid
DiHODE	Dihydroxy octadecadienoic acid
EPA	Eicosapentaenoic acid (C20:5n3)
EpDPE	Epoxy docosapentaenoic acid
EpETE	Epoxy eicosatetraenoic acid
EpETrE	Epoxy eicosatrienoic acid
FAMEs	Fatty acid methyl esters
GC-FID	Gas chromatography with flame ionization detection
HDHA	Hydroxy docosahexaenoic acid
HEPE	Hydroxy eicosapentaenoic acid
HETE	Hydroxy eicosatetraenoic acid
HODE	Hydroxy octadecadienoic acid
IS	Internal standard
IsoP	Isoprostanes
LA	Linoleic acid (C18:2n6)

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LC-MS	Liquid chromatography-mass spectrometry
LLOQ	Lower limit of quantification
LOX	Lipoxygenase
MUFA	Monounsaturated fatty acid
n3/6	Omega 3/6
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
QC	Quality control
SD	Standard deviation
sEH	Soluble epoxide hydrolase
SFA	Saturated fatty acid
TG	Triglyceride
Tx	Thromboxane

Acknowledgements

We would like to thank Vanessa Christa for helping with the fatty acid analyses in food samples and the participants who contributed their time to this project.

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7. General discussion

PUFA data of the **ALA** and **LA/ALA study** are collectively discussed in chapter 7.1 while PUFA data of the **DHA study** is discussed in chapter 7.2. In chapter 7.3 oxylipin data of the **ALA** and **DHA study** are compared. Chapter 7.4 deals with analytical-, inter- and intra-day variations of oxylipin patterns investigated in the **Oxylipin study**. Lastly, strengths and limitations of all conducted studies are subject of discussion in chapter 7.5.

7.1. Conversion of ALA to EPA, DPAn3 and DHA

In both the **ALA** (Paper I) and the $_{lo}$LA/$_{hi}$ALA diet of the **LA/ALA study** (Paper II) the effects of high-ALA diets were investigated. Both study collectives consisted of healthy, non-smoking, male subjects and were with a mean age of 26.2±4.53 and 26.1±4.53 years and a mean BMI of 24.9±2.0 kg/m^2 and 24.0±1.65 kg/m^2 comparable to each other. In the LA/ALA study, the high-ALA diet was accompanied by a low dietary LA intake ($_{lo}$LA/$_{hi}$ALA). Both high-ALA diets were successful at increasing ALA as well as EPA concentrations in RBCs. Highly significant increases of ALA concentrations in RBCs have been observed in both studies already after one week of high-ALA diet although changes in RBCs are determined by their turnover and the mean life span of an RBC is about 120 days. The daily ALA doses from linseed oil were with 12.9 g in the ALA study and 12.5 g in the LA/ALA study nearly identical and resulted in a total daily ALA intake of 13.9±0.34 (4.44 en%) and 13.1±0.22 g (4.98 en%), respectively. However, when comparing the percentage increase of ALA and EPA between the ALA and LA/ALA study, the increase was higher after 2 weeks of $_{lo}$LA/$_{hi}$ALA diet compared to 3 weeks of high-ALA diet without LA restriction: in the ALA study, ALA and EPA increase after 3 weeks was 294±23 % and 49.2±14 %, respectively and in the LA/ALA study, ALA and EPA increase after 2 weeks was 354±47 % and 57.6±18, respectively. LA intake was uncontrolled and highly variable in the ALA study (9.32±5.93 g/day; 3.2 en%) compared to a lower and less variable LA intake of the participants in the LA/ALA study (7.30±0.37 g/day; 2.8 en%). LA and ALA not only compete for the same conversion enzymes, especially for the rate-limiting enzyme D6D [27,30,47,53], but also for incorporation into cell membrane phospholipids [241,242]. Therefore, the higher and more variable LA intake in the ALA study may be responsible for both, the smaller increase of ALA due to competition for incorporation into cell membranes, as well as the smaller increase of EPA due to lower conversion efficiency. Moreover, the daily personnel contact with the participants in the LA/ALA study compared to the ALA study, might contribute to a higher level of compliance, which may also be (partly) responsible for the higher and faster increase of ALA and EPA concentrations in RBCs in the LA/ALA study.

The baseline ratios of LA to ALA in RBCs (ALA study: 74.8±3.54; LA/ALA study: 78.0±6.38) deviated from their respective dietary supply (ALA study: 7.70±3.75; not measured in the LA/ALA study, but probably similar to that from the ALA study). After a 12-week dietary LA/ALA ratio of 0.67±0.21 in the ALA study the ratio of LA to ALA concentrations in RBCs remained high (17.5±0.97), compared to the low dietary ratio. The $_{lo}$LA/$_{hi}$ALA diet with a dietary LA/ALA ratio of 0.56±0.27 led to a similarly high ratio of LA to ALA concentrations in RBCs (13.9±0.95) in only 2 weeks. The phenomenon of deviating LA/ALA ratios in RBCs compared to dietary ratios demonstrates that LA and ALA differ substantially regarding absorption, tissue distribution, membrane incorporation, and/or degradation. A high percentage of 60-85 % of ALA is rapidly degraded by beta-oxidation [3] which has been shown to increase with increasing ALA concentrations [47].

DPAn3 concentrations increased slowly in consequence of the high-ALA diet of the ALA study and reached statistical significance only after 6 (18±5 %; p=0.033) and 12 weeks (27±7 %; p=0.014), which may be the result of ALA conversion. Conversely, in the LA/ALA study, DPAn3 concentrations remained constant, which is probably due to the short duration of the intervention (2 weeks). In contrast, expressed as percent of total FAs, DPAn3 increased significantly after 2 weeks of $_{lo}$LA/$_{hi}$ALA diet (11±6 % p=0.004), which demonstrates the abovementioned discrepancies between the two parameters.

In the ALA study, DHA concentrations in RBCs dropped linearly after one (-7±3 %; n.s.), 3 (-9±4 %; n.s.), 6 (-11±3 %; p=0.010) and 12 weeks (-23±3 %; p<0.001) of ALA supplementation. Likewise, the relative weight percent of total FAs decreased slightly from 4.03±0.16 µg/mL at baseline to 3.90±0.15 % after one week and 3.74±0.13 % after 3 weeks. In combination with increasing EPA concentrations this resulted in a constant low omega-3 index of 4.63±0.14 % over the whole intervention period. The observation of decreasing DHA concentrations stands in contrast to the literature, where a conversion rate of ALA to DHA of 0.5-5 % [29,62,63,243–245] has been observed. The daily ALA intake was with 13.9±0.34 g and 4.44 en% much higher compared to other studies [110,112,115,244,246]. One explanation might be that excessive amounts of LA and ALA have been shown to further inhibit D6D to catalyze the formation of tetracosahexaenoic acid (24:6n3) from tetracosapentaenoic acid (24:5n3), which is the last step before peroxisomal beta-oxidation to DHA occurs [247]. The reduction of DHA in RBCs, however, does not necessarily go hand in hand with a reduction of DHA in the tissues. For example, in a rat study a high-ALA diet led to accumulation of DHA in heart and liver, whereas plasma levels remained constant. An accumulation of DHA in the brain would also be conceivable [248], however, human studies are limited to blood as medium of investigation. Another possible explanation for decreasing concentrations of DHA in RBCs might be that EPA- and DHA-rich oily fish should be avoided throughout the duration of the

intervention. The intake of EPA, DPAn3 and DHA was slightly, albeit insignificantly reduced during the intervention compared to the wash-out phase. These minor changes are, however, unlikely to have caused the decrease of DHA concentrations in RBCs. The selection of participants with generally low fish intake potentially further minimized this effect.

Interestingly, the $_{lo}$LA/$_{hi}$ALA diet in the LA/ALA study led to a slight increase of the relative weight percent of DHA of total FAs from 3.50±0.15 % at baseline to 3.87±0.16 % after 2 weeks, while DHA concentrations remained constant. The omega-3 index increased significantly (p=0.019) from 4.03±0.17 % to 4.76±0.20 %, which is in line with observations of a systematic review of Wood et al. [27] who state that a combination of a decrease of LA and a simultaneous increase of ALA intake is most effective in improving the n3 PUFA status. Even though this is only a minor change, it would still be interesting to investigate if the trend of a rising omega-3 index continues over a longer intervention period. However, due to the ubiquitous occurrence of LA in the diet and the widespread use of LA-rich vegetable oils the longer-term maintenance of a defined dietary LA/ALA ratio is challenging. Attempts to reduce the LA intake of free-living individuals are especially difficult [27]. Moreover, the basal status affects FA responses to n3 PUFA supplementation in healthy volunteers with higher improvements with low basal status [72,249]. In both, ALA study and 3, the basal omega-3 index was low – slightly lower in the LA/ALA study (4.03±0.17 %) compared to the ALA study (4.63±0.19 %). Consequently, a simple ALA supplementation in subjects on a Western diet (with high LA intake) does not improve the omega-3 index. A simultaneous reduction of LA may have the potential to slightly increase the omega-3 index. It remains questionable whether this marginal increase has any biological significance, also from the point of view that a reduction of LA intake in the Western diet is extremely difficult to implement. The intake of preformed EPA and DHA is an effective strategy in improving the LC n3 PUFA status/omega-3 index [72]. Against this background, every nutrition society should give recommendations for the intake of EPA and DHA.

As a result of the $_{hi}$LA/$_{lo}$ALA diet, only minor changes in the RBC FA pattern were observed and, in particular, the concentrations of LA, ALA, AA and DHA remained unchanged. The LA/ALA ratio in the typical Western diet is about 10-20:1 [35,250] and hence close to the investigated ratio of 25.6:1. The almost constant FA pattern is reasonable and therefore in line with expectations. The absence of changes in the PUFA pattern suggests that the high LA/ALA ratio of the Western diet already leads to a saturation and a further increase of the ratio does not affect the PUFA pattern.

7.2. Effects of DHA supplementation: Retroconversion of DHA to EPA

In the **DHA study**, the effects of a 12-week supplementation of 1076 mg of DHA per day on the PUFA pattern in RBCs and plasma were investigated. As expected, DHA concentrations increased in both sample types, RBCs (190 % or 51 µg/mL) and plasma (294% or 86 µg/mL), but plasma concentrations increased faster and reached a steady state quickly. In RBCs, the increase was time-dependent and no saturation was reached during the 12 weeks of DHA supplementation. Although the supplement did not contain EPA, EPA concentrations increased in plasma (80 % or 9.7 µg/mL) and RBCs (50 % or 2.0 µg/mL) after 12 weeks of DHA supplementation possibly due to retroconversion from DHA. Hence, ingested DHA may serve as a reservoir for EPA, which is in line with earlier findings [251]. The estimated retroconversion percentage of DHA to EPA in RBCs calculated according to [60] was initially high (13.2 % and 12.0 % after one and 3 weeks) and gradually declined thereafter (5.3 % and 3.3 % after 6 and 12 weeks). It should be noted that these retroconversion rates were calculated based on concentrations, which might be more reliable compared to calculation based on relative weight percent of total FAs. Nevertheless, the estimated retroconversion rates are comparable to those of other studies [51,60,61], where a rate between 7 and 14 % based on relative weight percent of total FAs has been calculated. Also in line with earlier findings is the decline of EPA concentrations in RBCs and plasma after 6 and 12 weeks with accompanying lower retroconversion rates, which is possibly caused by a saturation [251].

Decreasing AA concentrations (-16 % or -22 µg/mL in RBC and -24 % or -44 µg/mL in plasma) in the course of the DHA supplementation may be explained by an inhibitory effect of DHA on D5D and D6D enzymes, which consequently results in a lower conversion of LA to AA [56].

DPAn3 concentrations declined in response to the DHA supplementation, although DPAn3 is an intermediate product of the (retro)conversion of DHA to EPA or vice versa. Though, this finding is consistent with those of other studies [51,52,61]. A possible mechanistic explanation might be that DHA replaces DPAn3 in phospholipids likely by competition from DHA-containing precursors [51]. The inhibitory effect of DHA on D6D may also inhibit the conversion of EPA to DHA [252]. This could be another driver for increasing EPA and decreasing DPAn3 concentrations.

The fast and comparatively stronger increase of ALA concentrations in RBCs (after 6 weeks: 352±27 %) following ALA supplementation stands in contrast to a slower and less pronounced rise of DHA concentrations in RBCs (190±18 %). A possible explanation may be the lower baseline concentration of ALA (1.44±0.10 µg/mL) compared to DHA (31.9±2.37 µg/mL), which in combination with the higher supplemented daily ALA dose of 12.9 g (vs. 1076 mg of DHA) might have led to a more rapid increase of ALA in RBCs compared to DHA.

Overall, changes of DHA and AA concentrations were much more pronounced compared to the increase of EPA resulting in a low retroconversion rate. Concentrations in RBCs have been shown to be more suitable to evaluate long-term changes compared to plasma.

7.3. Modulation of oxylipins by ALA and DHA supplementation

The effects of ALA and DHA supplementation on the profile of free oxylipins in plasma were investigated in the **ALA** and **DHA study**. In order to investigate the oxylipin patterns in response to a treatment, it would be most appropriate to analyze oxylipin concentrations in the tissue of interest. However, in humans, this is not feasible due to ethical reasons. Therefore, mainly plasma is used as a proxy for tissue concentrations to quantitatively evaluate changes in oxylipin patterns [4].

A correlation between concentrations of precursor PUFAs in blood and their oxylipins in plasma was demonstrated in different intervention studies [155,161,253]. Accordingly, in the ALA and DHA study, changes of free oxylipin concentrations in plasma – apart from some exceptions – reflect changes of their respective precursor PUFAs. Thus, concentrations of ALA-derived oxylipins increased in response to ALA supplementation and concentrations of DHA-derived oxylipins increased in response to DHA supplementation. However, in contrast to ALA concentrations in RBCs, which increased more than 3-fold after only one week of ALA supplementation, ALA-derived oxylipins were only slightly but not significantly elevated after one week. Moreover, whilst ALA concentration in RBCs seemed to reach a steady state after 6 weeks of ALA supplementation, ALA-derived oxylipins, such as the hydroxy-PUFAs 9- and 13-HOTrE increased steadily until week 12 up to 1.9-fold and 2.6-fold, respectively. The biological role of ALA-derived oxylipins is only poorly understood. Some studies demonstrated positive biological effects of ALA-derived oxylipins [254,255]. Further investigations of ALA-derived oxylipins are needed as these lipid mediators are present in relevant concentrations in humans on a Western diet [7].

Following DHA supplementation, DHA-derived oxylipins increased linearly in a time-dependent manner similar to their precursor FA DHA in RBCs. DHA-derived CYP epoxides – which are claimed to act anti-arrhythmic [256], vasodilatory [257], anti-thrombotic [258] and anti-angiogenic [208] – were increased by 400-500 % after 12 weeks of DHA supplementation. Plasma concentrations of DHA, however, reached a steady state after almost one week while plasma oxylipins kept linearly increasing. This is consistent with the intracellular formation of oxylipins and the reason why the PUFA pattern is for most tissues well reflected by the RBC composition [4].

Consistent with elevated levels of EPA in RBCs (1.8-fold increase after 6 weeks) in the ALA study, the high-ALA diet led to increasing concentrations of EPA-derived hydroxy- and dihydroxy-PUFA in plasma (~1.3-1.8-fold after 6 weeks). Moreover, a slight but significant increase of several EPA-oxylipins in plasma was observed after DHA supplementation in the DHA study. Higher levels of EPA-derived oxylipins may have beneficial health effects, e.g. 18-HEPE concentrations – a precursor of pro-resolving and anti-inflammatory E-series resolvins [259] – increased significantly both in the ALA study and the DHA study. An increase of anti-inflammatory and cardioprotective EPA-epoxides could not be detected (below lower limit of quantification (LLOQ) of the method). However, their degradation products, the dihydroxy FAs (e.g. 17,18-DiHETE), increased time-dependently during the supplementation period in the ALA study and DHA study. Hence, it can be hypothesized that EPA-epoxides may be equally increased, but rapidly metabolized to less active dihydroxy-FAs by sEH [135]. Finally, it has to be mentioned that direct supplementation with EPA more efficiently raises EPA-derived oxylipin levels compared to ALA and DHA supplementation [145].

Although DHA concentrations in RBCs were significantly lowered in response to the high-ALA diet in the ALA study, DHA-derived oxylipin concentrations showed no consistent shift towards lower levels. A similar observation was made in a rat study in which ALA supplementation resulted even in an elevation of the DHA oxylipins in serum, but not of the precursor FA DHA [260]. Thus, oxylipin data may provide information on PUFA conversion that is not apparent from PUFA data in RBCs.

Conversion rates of ALA to EPA and DHA as well as formation of oxylipins from n3 PUFAs are influenced by the presence of n6 PUFAs competing for the same enzymes [48,261]. Several studies demonstrated that n3 PUFA supplementation leads to declining AA and AA-derived oxylipin concentrations, but the results were heterogeneous between different intervention studies [145]. Despite the significant decrease of AA concentrations in RBCs both in the ALA and DHA study, only slight but not significant declines of AA-derived oxylipin concentrations have been observed in both studies. In the ALA study, e.g., the concentrations of hydroxy-PUFAs 5- and 20-HETE decreased in the course of the supplementation while no effect was observed for e.g. the 15-LOX product 15-HETE. The reason for lacking statistical significance in the decline of AA-derived oxylipin concentrations may be the high inter-individual variations especially in the DHA study.

Similarly, for LA-derived oxylipins no significant reduction in response to ALA supplementation was observed in the ALA study. Most likely the excess amount of LA in the diet and the preponderance of LA in RBCs was too high to be modified by ALA supplementation. A reduction of LA and its oxylipins is assumed to be beneficial, as negative health effects were observed for LA metabolites such as sEH products of the CYP-derived epoxy-PUFA [262–

264]. A reduction of LA metabolite 9,10-DiHOME by supplementation with a lower (6 g/day) dose of ALA compared to our study has been demonstrated by Caligiuri et al. in young individuals (19-28 years) [147], after only 4 weeks of intervention. However, participants had to abstain from dietary oils, which might have altered their normal eating habits; thus, leading to shifts in FA and oxylipin pattern.

Overall, the influence of ALA and DHA on the profile of oxylipins is diverse and seems to result more from a shift in the whole oxylipin pattern rather than from changes of selected metabolites [265]. For the interpretation of intervention-resulting effects it is therefore important to analyze a comprehensive set of oxylipins from all branches of the AA cascade [156,265]. Many studies in this field, however, only analyze and/or report selected groups of oxylipins, e.g. epoxy-FA [266], SPM and precursors thereof [267,268], SPM and epoxy-FA [269], or mainly AA-derived mediators [148].

Ex vivo induction of inflammation led to changes in the oxylipins pattern compared to baseline and untreated plasma particularly pronounced in the metabolites that derive from the COX pathway. This indicates that the DHA supplementation led to changes in the acute inflammatory response of the subjects since the ability of the monocytes and macrophages to form pro-inflammatory PGs has shown to be significantly reduced.

7.4. Variability of oxylipin patterns

The oxylipin profile has been shown to be subject to high (biological) variability. The reliable quantification of a comprehensive pattern of oxylipins is thus crucial in order to investigate oxylipin biology [126]. Moreover, biological, time-dependent variations in the oxylipin pattern in healthy individuals have to be kept in mind when interpreting biological data. Hence, precision of the analytical method and inter- and intra-day variations of the oxylipin profile have been investigated in the **Oxylipin study**.

The variations of oxylipin quantification have been shown to be comparable to previously described variations for other liquid chromatography-mass spectrometry (LC-MS) based analytical approaches for the quantification of oxylipins [158,270]. Most oxylipins (about 84% of analytes) fluctuated less than 20 % within the batch; only 12 analytes showed a higher fluctuation. The minimum variation was with ~5 % consistent with the expected (random) relative error of LC(-MS)-based methods [271]. For the interpretation of biological data, it is important to keep this analytical variation in mind since high variations might mask biological effects.

In most studies – including those of the ALA and DHA study – fasting blood is used for the quantification of oxylipins which is usually collected in the morning following overnight fasting

[144,160–162]. Therefore, another crucial parameter for the interpretation of biological effects is the inter-day variability of fasting oxylipins of a healthy individual. This parameter should be small in order to be a suitable timepoint for blood collection. The results support the suitability of fasting plasma for the investigation of biological effects since inter-day variations were comparable to the analytical variance for nearly all oxylipins on a standardized and non-standardized diet. Only for a few LA- and ALA-derived oxylipins on a standardized diet, higher variations than expected from the analytical method could be observed. This might be a result of changes in dietary PUFA intake as part of the standardized diet and therefore questions the method of standardized nutrition for the investigation of short-term changes of the oxylipin profiles. Thus, when using standardized diets for the investigation of PUFA-related changes in the oxylipin profile, it is indispensable to include a null-control group in the cross-over study design.

On the other hand, inter-day variations were shown to be high and the results indicate that intake of dietary fat and its FA composition has an influence on the oxylipin profile. This observation is in line with previous results [146].

7.5. Strengths and Limitations

The human studies conducted for this thesis have several limitations, but also certain strengths. First, the studies are all limited by small sample sizes (of 12 to 19 participants), which owes primarily to the high methodological expense.

Second, the direct quantification of conversion and retroconversion rates of PUFAs is only feasible via tracer studies with labeled FAs. However, while these types of studies have been done several times and constant background factors prevail, the studies are nonetheless meaningful.

Third, genetic factors which are known to have an influence on the PUFA and oxylipin status and the response to supplementation induced changes have not been considered.

Fourth, the LA/ALA study has a short intervention duration (of 2 weeks) which owes primarily to the elaborate methodology. It is questionable whether the compliance of the subjects to follow the manifold dietary restrictions to consume a low-fat and low-PUFA diet would have declined with longer study duration. Also changes in the FA pattern were only measured in RBCs and thus PUFA changes do not fully reach the RBCs. Nevertheless, strong changes in PUFA concentration in RBCs were observed already after 7 days suggesting that the PUFA incorporation into newly formed RBCs is sufficient to reflect changes in the PUFA status.

On the other side, strengths are the homogenous study collectives of all conducted studies regarding sex, age, BMI, smoking status, diet and baseline EPA and DHA levels. This leads to a reduction of interindividual variations especially of the oxylipin profile compared to earlier investigations [141,155–157] and facilitates the interpretation of observed effects. The cross-over design of the LA/ALA study also led to a reduction in interindividual variability of RBC PUFA concentrations.

Overall, the RBC and plasma FA data are presented as both concentrations in µg/mL and as relative weight percent of total FAs. In the majority of previous studies, FA data are only quantified as relative weight percent of total FAs. However, the (additional) quantification of concentrations may have several advantages: First, incoherent development between the two parameters can be uncovered and intervention-related changes of selective PUFAs can be evaluated independent from one another. This allows for the calculation of actual conversion (ALA and LA/ALA study) and retroconversion rates (DHA study). Second, the sum of total FAs as the basis of relative weight percent can differ depending on e.g. sample preparation or expertise of the chromatographer. Inclusion of an internal standard (IS) and subsequent calculation of FA concentrations allow for literature comparisons of FA data [2]. Third, biological effects are dependent on the concentrations, hence their interpretation should be based on this parameter as well [4].

Moreover, the ALA and DHA study had several intermediate timepoints (1, 3, and 6 weeks) apart from the baseline and final measurement timepoint after 12 weeks. Also, the LA/ALA study had one intermediate measurement timepoint after one week. This greatly enhances the possible depth of interpretation since it allows for analyzing concentrations over the course of time.

Finally, our own analyses have confirmed the suitability of fasting plasma for the investigation of intervention-induced effects of oxylipin concentrations as well as the accuracy of the oxylipin analysis method in the Oxylipin study.

8. General conclusion and perspectives

This thesis contributes to a better understanding of PUFA metabolism in humans by providing detailed data on the modulation of PUFAs and their oxylipins induced by different dietary interventions.

It has been demonstrated that a 12-week high-ALA diet results in an increase of ALA, EPA and DPAn3 concentrations in RBCs and a decline in DHA concentrations suggesting a closely connected PUFA metabolism. The omega-3 index – which is mainly associated with CVD and inflammatory conditions – is nevertheless not affected in response to the high-ALA diet. This demonstrates that even a 10-fold increase in ALA intake, compared to the background intake of this FA, does not significantly improve the status of the physiological important PUFAs EPA and DHA. When combining the high-ALA diet with a reduced and less fluctuating LA intake ($_{lo}$LA/$_{hi}$ALA diet), a larger increase in RBC ALA and EPA concentrations and no decline in DHA concentrations has been observed. This supports the notion that LA might impede the conversion to EPA and DHA. These results indicate that on a Western diet (with high LA intake) ALA is no significant source for endogenous EPA and DHA. A simultaneous reduction of LA may have the potential to slightly increase the omega-3 index. Still, the biological significance of this slight increase remains questionable, because a reduction of LA intake in the Western diet is very difficult to implement. Further studies are needed to investigate the influence of LA on the n3 PUFA status, especially regarding a combination of high LA and low ALA intake as well as the low omega-3 index in many Western countries.

DHA supplementation leads to a strong increase in DHA and a slight but significant increase in EPA concentrations in both RBCs and plasma, suggesting likely retroconversion of DHA. The physiological significance of this small increase remains unknown. In contrast to ALA, the intake of preformed DHA is an effective strategy for improving the omega-3 index. Therefore, it is suggested that every nutrition society might give recommendations for the intake of (EPA and) DHA.

As expected, plasma DHA concentrations, in contrast to RBCs, reach a steady state quickly and are therefore not suitable to evaluate long-term changes of FAs in response to an intervention. Observed incoherent developments between concentrations in µg/mL and relative weight percent of total FAs in the implemented studies indicate a need for additional quantification of concentrations in all future studies.

Changes in plasma oxylipin concentrations generally reflect their precursor FAs in RBCs but not in plasma. DHA supplementation leads to an increase of EPA-derived oxylipins which makes the distinction of physiological effects stemming from EPA vs. DHA complicated, since effects of DHA may also be mediated by EPA and its oxylipins. Oxylipin data may provide more

information on PUFA conversion not directly deducible from PUFA data in RBCs. Ex vivo stimulation of an inflammatory response reveals that DHA supplementation leads to changes in the acute inflammatory response of the subjects. Evaluation of the oxylipin profiles is paradigm to reveal the underlying mechanisms of this process.

Fasting plasma seems to be suitable for the investigation of the biological role of the oxylipin pattern. Background variations are low and for most analytes within the variation of the analytical method. Variations are independent from a standardization of the diet as well. This makes fasting plasma an ideal matrix for the investigation of oxylipin patterns in response to interventions or in pathophysiological states. During the day, oxylipin concentrations fluctuate strongly and ingestion of food is directly followed by changes in the oxylipin profile. Since in Western countries individuals are in a postprandial state during most of the day, it would be highly interesting to investigate whether a standardized meal results in similar intra-day modifications of the oxylipin profile and if postprandial plasma might be suitable for the investigation of oxylipin biology.

9. References

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Appendix Paper I

Table S1: Fatty acid profile of the linseed oil used in the study (own analysis).

Fatty acid	Common name	% of total fatty acids	rel. dev.[a] [%]
C14:0	Myristic acid	0.06	25.4
C15:0	Pentadecylic acid	0.03	31.4
C16:0	Palmitic acid	5.14	0.03
C16:1n7	Palmitoleic acid	0.05	4.71
C17:0	Margaric acid	0.06	1.45
C18:0	Stearic acid	4.23	0.09
C18:1n9	Oleic acid	15.2	0.03
C18:1n7	Vaccenic acid	0.65	0.33
C18:2n6	Linoleic acid	15.7	0.01
C19:0	Nonadecylic acid	0.12	0.46
C18:3n3	α-Linolenic acid	58.0	0.04
C20:0	Arachidic acid	0.17	0.76
C20:1n9	Icosenoic acid	0.11	1.69
C20:2n6	Eicosadienoic acid	0.03	18.6
C20:3n3	Eicosatrienoic acid	0.05	7.99
C22:0	Behenic acid	0.12	0.69
C24:0	Lignoceric acid	0.09	4.36
C22:6n3	Docosahexaenoic acid	0.19	1.07

[a] relative deviation from the mean

Table S2: LC-ESI-MS/MS analysis of free oxylipins in plasma.

Analyte	Mass transition		Internal standard	LLOQ[a]	LLOQ[a]	Included in data analysis
	m/z			Vial	500 µL Plasma	
	MS1	MS3	IS	(nM)	(nM)	Plasma
20-OH-PGE$_2$	367.2	189.1	$^2H_4$-PGE$_2$	0.25	0.025	no, <LLOQ in 50% of samples
Δ^{17}-6-keto-PGF$_{1\alpha}$	367.2	163.2	$^2H_4$-6-keto-PGF$_{1\alpha}$	1.0	0.1	no, <LLOQ in 50% of samples
2,3-dinor-TxB$_1$	343.0	142.9	$^2H_4$-TxB$_2$	5.0	0.5	no, <LLOQ in 50% of samples
2,3-dinor-TxB$_2$	341.2	167.0	$^2H_4$-TxB$_2$	1.0	0.1	no, <LLOQ in 50% of samples
6-keto-PGF$_{1a}$	369.3	163.2	$^2H_4$-6-keto-PGF$_{1\alpha}$	1.8	0.1805	no, <LLOQ in 50% of samples
RvE1	349.3	195.0	$^2H_4$-TxB$_2$	1.2	0.12	no, <LLOQ in 50% of samples
20-COOH-LTB$_4$	365.2	347.2	$^2H_4$-TxB$_2$	1.0	0.1	no, <LLOQ in 50% of samples
TxB$_3$	367.3	169.3	$^2H_4$-TxB$_2$	0.25	0.025	yes
20-OH-LTB$_4$	351.2	195.2	$^2H_4$-PGD$_2$	0.25	0.025	no, <LLOQ in 50% of samples
13,14-dihydro-15-keto-tetranor-PGE$_2$	296.9	109.0	$^2H_4$-PGE$_2$	0.25	0.025	no, <LLOQ in 50% of samples
TxB$_1$	371.3	171.2	$^2H_4$-TxB$_2$	0.5	0.05	no, <LLOQ in 50% of samples
15-F$_{2t}$-IsoP (8-*iso*-PGF$_{2\alpha}$)	353.1	193.1	$^2H_4$-15-F$_{2t}$-IsoP	0.5	0.05	no, <LLOQ in 50% of samples
TXB$_2$	369.2	169.1	$^2H_4$-TxB$_2$	1.3	0.125	yes
11-dehydro-TxB$_3$	365.3	161.2	$^2H_4$-TxB$_2$	1.0	0.1	no, <LLOQ in 50% of samples
PGE$_3$	349.3	269.2	$^2H_4$-PGE$_2$	0.3	0.03	no, <LLOQ in 50% of samples
11β-PGF$_{2\alpha}$	353.3	193.1	$^2H_4$-PGE$_2$	0.5	0.05	no, <LLOQ in 50% of samples
5(*R,S*)-5-F$_{2t}$-IsoP (5-iPF$_{2\alpha}$-VI)	353.2	114.8	$^2H_{11}$-5(*R,S*)-5-F$_{2t}$-IsoP	0.5	0.05	no, <LLOQ in 50% of samples
PGD$_3$	349.3	269.2	$^2H_4$-PGD$_2$	1.0	0.1	no, <LLOQ in 50% of samples
PGF$_{1\alpha}$	355.4	293.2	$^2H_4$-PGE$_2$	0.25	0.025	no, <LLOQ in 50% of samples
PGE$_2$	351.2	271.3	$^2H_4$-PGE$_2$	0.25	0.025	yes
11-dehydro-TxB$_2$	367.0	161.1	$^2H_4$-TxB$_2$	0.50	0.05	no, <LLOQ in 50% of samples
PGE$_1$	353.3	317.2	$^2H_4$-PGE$_2$	0.33	0.0325	no, <LLOQ in 50% of samples
PGD$_1$	353.3	317.2	$^2H_4$-PGD$_2$	0.50	0.05	no, <LLOQ in 50% of samples
PGD$_2$	351.2	271.3	$^2H_4$-PGD$_2$	1.0	0.1	yes
15-keto-PGF$_{1\alpha}$	353.3	193.1	$^2H_4$-PGE$_2$	0.25	0.025	no, <LLOQ in 50% of samples
11,12,15-TriHETrE	353.2	167.1	$^2H_4$-PGE$_2$	0.50	0.05	no, <LLOQ in 50% of samples
LXA$_4$	351.2	115.2	$^2H_4$-PGE$_2$	0.18	0.0175	no, <LLOQ in 50% of samples
RvD1	375.3	141.0	$^2H_4$-PGE$_2$	0.25	0.025	no, <LLOQ in 50% of samples
13,14-dihydro-15-keto-PGF$_{2\alpha}$	353.3	183.3	$^2H_4$-PGE$_2$	0.50	0.05	yes
13,14-dihydro-15-keto-PGE$_1$	353.3	221.2	$^2H_4$-PGE$_2$	0.50	0.05	yes
dihomo-PGF$_{2\alpha}$	381.4	221.1	$^2H_4$-PGE$_2$	0.10	0.01	no, <LLOQ in 50% of samples
RvE2	333.2	253.3	$^2H_4$-PGE$_2$	2.0	0.2	no, <LLOQ in 50% of samples
PGJ$_2$	333.3	189.2	$^2H_4$-PGE$_2$	1.6	0.16	no, <LLOQ in 50% of samples
LTB$_5$	333.3	195.2	$^2H_4$-LTB$_4$	0.10	0.01	no, <LLOQ in 50% of samples
PGB$_2$	333.3	175.1	$^2H_4$-PGE$_2$	0.40	0.04	no, <LLOQ in 50% of samples
THF diol	353.2	127.1	$^2H_4$-LTB$_4$	0.25	0.025	no, <LLOQ in 50% of samples
18(*S*)-RvE3	333.2	201.3	$^2H_4$-PGE$_2$	1.0	0.1	no, <LLOQ in 50% of samples
12-OH-17(18)-EpETE	333.1	179.3	$^2H_4$-9,10-DiHOME	0.50	0.05	no, <LLOQ in 50% of samples
15,16-DiHODE	311.2	223.2	$^2H_4$-9,10-DiHOME	1.0	0.1	yes
9,10-DiHODE	311.2	201.2	$^2H_4$-9,10-DiHOME	0.20	0.02	yes
12,13-DiHODE	311.2	183.1	$^2H_4$-9,10-DiHOME	1.0	0.1	yes
8,15-DiHETE	335.2	235.2	$^2H_{11}$-14,15-DiHETrE	0.80	0.08	no, <LLOQ in 50% of samples
18(*R*)-RvE3	333.2	201.3	$^2H_4$-PGE$_2$	0.50	0.05	no, <LLOQ in 50% of samples
6-*trans*-LTB$_4$	335.2	195.1	$^2H_4$-LTB$_4$	0.50	0.05	no, <LLOQ in 50% of samples
5,15-DiHETE	335.3	173.2	$^2H_{11}$-14,15-DiHETrE	0.25	0.025	no, <LLOQ in 50% of samples
17,18-DiHETE	335.3	247.2	$^2H_{11}$-14,15-DiHETrE	0.25	0.025	yes
LTB$_4$	335.2	195.1	$^2H_4$-LTB$_4$	0.25	0.025	no, <LLOQ in 50% of samples
14,15-DiHETE	335.3	207.2	$^2H_{11}$-14,15-DiHETrE	0.25	0.025	yes
11,12-DiHETE	335.2	167.1	$^2H_{11}$-14,15-DiHETrE	0.25	0.025	yes
12,13-DiHOME	313.2	183.2	$^2H_4$-9,10-DiHOME	0.50	0.05	yes
8,9-DiHETE	335.2	127.1	$^2H_{11}$-14,15-DiHETrE	0.50	0.05	yes
9,10-DiHOME	313.2	201.2	$^2H_4$-9,10-DiHOME	0.50	0.05	yes
14,15-DiHETrE	337.2	207.1	$^2H_{11}$-14,15-DiHETrE	0.10	0.01	yes

19,20-DiHDPE	361.2	273.2	$^{2}H_{11}$-14,15-DiHETrE	0.50	0.05	yes
LTB$_3$	337.2	195.2	$^{2}H_{4}$-LTB$_4$	0.50	0.05	no, <LLOQ in 50% of samples
9,10-diH-stearic acid	315.0	170.8	$^{2}H_{4}$-9,10-DiHOME	2.0	0.2	yes
16,17-DiHDPE	361.2	233.2	$^{2}H_{11}$-14,15-DiHETrE	0.50	0.05	yes
11,12-DiHETrE	337.2	167.1	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes
19-HEPE	317.2	229.3	$^{2}H_{8}$-12-HETE	0.71	0.071	yes
13,14-DiHDPE	361.2	193.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes
20-HEPE	317.2	287.3	$^{2}H_{8}$-12-HETE	1.0	0.1	yes
9-HOTrE	293.2	171.2	$^{2}H_{4}$-9-HODE	0.50	0.05	yes
10,11-DiHDPE	361.2	153.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes
8,9-DiHETrE	337.2	127.1	$^{2}H_{11}$-14,15-DiHETrE	0.50	0.05	yes
13-HOTrE	293.2	195.1	$^{2}H_{4}$-9-HODE	0.60	0.06	yes
18-HEPE	317.2	259.2	$^{2}H_{4}$-9-HODE	1.0	0.1	yes
15-deoxy-PGJ$_2$	315.2	271.2	$^{2}H_{11}$-14,15-DiHETrE	0.50	0.05	no, <LLOQ in 50% of samples
7,8-DiHDPE	361.2	113.1	$^{2}H_{11}$-14,15-DiHETrE	1.0	0.1	yes
20-HETE	319.2	289.1	$^{2}H_{6}$-20-HETE	1.0	0.1	yes
15-HEPE	317.2	219.2	$^{2}H_{8}$-12-HETE	1.3	0.125	yes
5,6-DiHETrE	337.2	145.1	$^{2}H_{11}$-14,15-DiHETrE	0.50	0.05	yes
11-HEPE	317.0	167.0	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
8-HEPE	317.2	155.2	$^{2}H_{8}$-12-HETE	0.63	0.0625	yes
12-HEPE	317.2	179.2	$^{2}H_{8}$-12-HETE	0.63	0.0625	yes
9-HEPE	317.2	166.9	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
21-HDHA	343.0	255.0	$^{2}H_{8}$-12-HETE	1.65	0.165	yes
5-HEPE	317.2	115.1	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
22-HDHA	343.2	313.2	$^{2}H_{8}$-12-HETE	2.80	0.28	yes
4,5-DiHDPE	361.2	229.3	$^{2}H_{11}$-14,15-DiHETrE	2.0	0.2	yes
13-HODE	295.2	195.2	$^{2}H_{4}$-9-HODE	5.0	0.5	yes
9-HODE	295.2	171.1	$^{2}H_{4}$-9-HODE	5.0	0.5	yes
20-HDHA	343.2	241.201	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
15(16)-EpODE	293.3	235.2	$^{2}H_{4}$-9(10)-EpOME	0.50	0.05	no[b]
15-HETE	319.2	219.2	$^{2}H_{8}$-12-HETE	1.3	0.125	yes
9(10)-EpODE	293.3	171.2	$^{2}H_{4}$-9(10)-EpOME	0.40	0.04	no[b]
17(18)-EpETE	317.2	215.2	$^{2}H_{11}$-14(15)-EpETrE	1.0	0.1	no[b]
16-HDHA	343.2	233.201	$^{2}H_{8}$-12-HETE	0.25	0.025	yes
17-HDHA	343.2	201.2	$^{2}H_{8}$-12-HETE	2.0	0.2	yes
13-HDHA	343.2	193.1	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
12(13)-EpODE	293.2	183.1	$^{2}H_{4}$-9(10)-EpOME	0.50	0.05	no[b]
11-HETE	319.2	167.2	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
10-HDHA	343.2	153.201	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
14-HDHA	343.2	205.2	$^{2}H_{8}$-12-HETE	1.0	0.1	yes
14(15)-EpETE	317.2	207.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
8-HETE	319.2	155.2	$^{2}H_{8}$-12-HETE	1.3	0.125	yes
12-HETE	319.2	179.2	$^{2}H_{8}$-12-HETE	0.50	0.05	yes
11(12)-EpETE	317.2	167.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
11-HDHA	343.2	121.1	$^{2}H_{8}$-5-HETE	0.25	0.025	yes
7-HDHA	343.2	141.2	$^{2}H_{8}$-5-HETE	1.0	0.1	yes
8(9)-EpETE	317.2	127.2	$^{2}H_{11}$-14(15)-EpETrE	1.0	0.1	no[b]
9-HETE	319.2	167.2	$^{2}H_{8}$-5-HETE	2.5	0.25	yes
15(S)-HETrE	321.2	221.2	$^{2}H_{8}$-5-HETE	0.50	0.05	yes
8-HDHA	343.2	189.2	$^{2}H_{8}$-5-HETE	0.50	0.05	yes
5-HETE	319.2	115.2	$^{2}H_{8}$-5-HETE	0.50	0.05	yes
4-HDHA	343.2	101.1	$^{2}H_{8}$-5-HETE	0.25	0.025	yes
19(20)-EpDPE	343.2	241.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
12(13)-EpOME	295.3	195.2	$^{2}H_{4}$-9(10)-EpOME	0.25	0.025	no[b]
14(15)-EpETrE	319.2	219.3	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
9(10)-EpOME	295.3	171.1	$^{2}H_{4}$-9(10)-EpOME	0.25	0.025	no[b]
16(17)-EpDPE	343.2	233.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
13(14)-EpDPE	343.2	193.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]
5-oxo-ETE	317.2	273.2	$^{2}H_{4}$-9(10)-EpOME	2.0	0.2	no, <LLOQ in 50% of samples
10(11)-EpDPE	343.2	153.2	$^{2}H_{11}$-14(15)-EpETrE	0.25	0.025	no[b]
11(12)-EpETrE	319.3	167.2	$^{2}H_{11}$-14(15)-EpETrE	0.50	0.05	no[b]

8(9)-EpETrE	319.2	155.2	$^{2}H_{11}$-14(15)-EpETrE	1.0	0.1	no[b]
5(6)-EpETrE	319.2	191.1	$^{2}H_{11}$-14(15)-EpETrE	2.0	0.2	no[b]
9(10)-ep-stearic acid	297.0	170.8	$^{2}H_{4}$-9(10)-EpOME	2.0	0.2	no[b]

Shown are the covered analytes, the mass transition used for quantification in scheduled selected reaction monitoring mode, the internal standard (IS) and the lower limit of quantification (LLOQ).
[a] LLOQ was set to the lowest calibration standard injected within the sample set yielding a signal to noise ratio ≤ 5 and accuracy in the calibration within ±20%.
[b] Epoxy-FA not included in data analysis due to high variation in quality control samples.

Table S3: Concentration and relative amount of fatty acids in red blood cells in the follow-up period.

		wk 14			t-test[a]	wk 20			t-test[a]	An reM[b]
		mean	±	SE	p (wk 14 - wk 12)	mean	±	SE	p (wk 20 - wk 12)	p
C12:0	µg/mL	<0.25			-	<0.25			-	-
	% of total FA	-			-	-			-	-
C14:0	µg/mL	3.08	±	0.18	-	3.14	±	0.18	-	n.s.
	% of total FA	0.31	±	0.02	-	0.32	±	0.01	-	n.s.
C14:1n5	µg/mL	<0.25			-	<0.25			-	-
	% of total FA	-			-	-			-	-
C15:0	µg/mL	1.65	±	0.08	n.s.	1.67	±	0.07	n.s.	**0.047**
	% of total FA	0.17	±	0.01	-	0.17	±	0.01	-	n.s.
C16:0	µg/mL	191	±	6.09	-	194	±	5.01	-	n.s.
	% of total FA	19.7	±	0.11	-	19.7	±	0.19	-	n.s.
C16:1n7	µg/mL	2.64	±	0.20	-	2.79	±	0.28	-	n.s.
	% of total FA	0.27	±	0.02	-	0.28	±	0.02	-	n.s.
C17:0	µg/mL	3.08	±	0.09	n.s.	3.19	±	0.12	n.s.	**0.043**
	% of total FA	0.32	±	0.01	**0.020**	0.32	±	0.01	n.s.	**0.028**
C18:0	µg/mL	149	±	3.67	-	150	±	2.88	-	n.s.
	% of total FA	15.4	±	0.12	n.s.	15.3	±	0.11	n.s.	**0.039**
C18:1n9	µg/mL	123	±	4.99	-	127	±	4.78	-	n.s.
	% of total FA	12.6	±	0.20	-	12.9	±	0.23	-	n.s.
C18:1n7	µg/mL	13.1	±	0.49	-	13.4	±	0.46	-	n.s.
	% of total FA	1.35	±	0.02	-	1.36	±	0.02	-	**0.032**
C18:2n6	µg/mL	97.8	±	3.73	-	98.4	±	3.76	-	n.s.
	% of total FA	10.1	±	0.33	n.s.	10.1	±	0.37	**0.004**	**0.001**
C18:3n6	µg/mL	<0.25			-	<0.25			-	-
	% of total FA	-			-	-			-	-
C19:0	µg/mL	<0.25			-	<0.25			-	-
	% of total FA	-			-	-			-	-
C18:3n3	µg/mL	2.62	±	0.16	**<0.001**	2.27	±	0.21	**<0.001**	**<0.001**
	% of total FA	0.27	±	0.01	**<0.001**	0.23	±	0.02	**<0.001**	**<0.001**
C20:0	µg/mL	4.60	±	0.21	**0.006**	4.57	±	0.19	**0.032**	**0.011**
	% of total FA	0.48	±	0.02	n.s.	0.46	±	0.01	**0.039**	**0.045**
C20:1n9	µg/mL	2.95	±	0.12	n.s.	3.03	±	0.15	n.s.	**0.047**
	% of total FA	0.30	±	0.01	-	0.31	±	0.01	-	n.s.
C20:2n6	µg/mL	1.98	±	0.12	-	2.13	±	0.15	-	n.s.
	% of total FA	0.21	±	0.01	-	0.22	±	0.01	-	n.s.
C20:3n6	µg/mL	14.3	±	1.05	**0.43**	16.4	±	0.93	**0.001**	**<0.001**
	% of total FA	1.47	±	0.09	**0.043**	1.67	±	0.09	**<0.001**	**<0.001**
C20:4n6	µg/mL	136	±	4.17	n.s.	139	±	3.45	n.s.	**0.023**
	% of total FA	14.0	±	0.15	**0.012**	14.2	±	0.15	**0.001**	**<0.001**
C20:5n3	µg/mL	10.0	±	0.52	**0.009**	8.53	±	0.69	**<0.001**	**<0.001**
	% of total FA	1.03	±	0.04	**0.002**	0.87	±	0.06	**<0.001**	**<0.001**
C22:0	µg/mL	17.0	±	0.53	n.s.	16.5	±	0.46	n.s.	**0.046**

	% of total FA	1.76 ± 0.04	**0.006**	1.68 ± 0.03	n.s.	**0.004**
C22:1n9	µg/mL	2.18 ± 0.17	-	1.72 ± 0.20	-	n.s.
	% of total FA	0.23 ± 0.02	-	0.17 ± 0.02	-	n.s.
C22:4n6	µg/mL	26.5 ± 0.99	n.s.	28.3 ± 1.00	n.s.	**0.035**
	% of total FA	2.74 ± 0.06	n.s.	2.87 ± 0.08	n.s.	**<0.001**
C22:5n3	µg/mL	35.3 ± 1.63	-	32.6 ± 1.49	-	n.s.
	% of total FA	3.64 ± 0.12	n.s.	3.31 ± 0.11	n.s.	**0.001**
C24:0	µg/mL	48.2 ± 1.24	n.s.	47.8 ± 1.15	n.s.	**0.077**
	% of total FA	5.00 ± 0.10	-	4.87 ± 0.08	-	n.s.
C22:6n3	µg/mL	32.8 ± 1.15	-	33.8 ± 1.57	-	n.s.
	% of total FA	3.41 ± 0.13	-	3.46 ± 0.17	-	n.s.
C24:1n9	µg/mL	50.5 ± 1.84	n.s.	52.0 ± 1.69	-	**0.008**
	% of total FA	5.21 ± 0.09	**0.012**	5.28 ± 0.09	**0.003**	**0.001**
TFA	µg/mL	969 ± 27.4	-	984 ± 22.3	-	n.s.
SFA	µg/mL	417 ± 11.2	-	421 ± 9.18	-	n.s.
	% of total FA	43.1 ± 0.17	-	42.9 ± 0.14	-	n.s.
MUFA	µg/mL	194 ± 7.27	-	200 ± 7.05	-	n.s.
	% of total FA	20.0 ± 0.28	**0.002**	20.3 ± 0.31	n.s.	**0.001**
PUFA	µg/mL	357 ± 9.63	-	362 ± 7.16	-	n.s.
	% of total FA	37.0 ± 0.23	n.s.	36.9 ± 0.32	**0.023**	**0.012**
Σn3 PUFA	µg/mL	80.7 ± 2.47	-	77.2 ± 2.60	-	n.s.
	% of total FA	8.35 ± 0.15	**0.009**	7.86 ± 0.22	**0.001**	**<0.001**
Σn6 PUFA	µg/mL	277 ± 7.82	-	285 ± 5.98	-	n.s.
	% of total FA	28.6 ± 0.28	n.s.	29.0 ± 0.33	**0.050**	**0.018**
ΣEPADHA	µg/mL	42.9 ± 1.44	-	42.3 ± 1.88	-	n.s.
	% of total FA	4.45 ± 0.14	-	4.32 ± 0.20	-	n.s.
Σ n6/Σ n3 PUFA		3.44 ± 0.08	**0.014**	3.73 ± 0.13	**0.002**	**<0.001**
AA/EPA		13.8 ± 0.47	**<0.001**	17.3 ± 1.06	**<0.001**	**<0.001**
D5D index		10.1 ± 0.60	n.s.	8.75 ± 0.45	**0.001**	**0.002**
D6D index		0.15 ± 0.01	**0.006**	0.17 ± 0.01	**<0.001**	**<0.001**
% n3 in HUFA		30.7 ± 0.39	n.s.	28.9 ± 0.63	**<0.001**	**<0.001**
% n6 in HUFA		69.3 ± 0.39	n.s.	71.1 ± 0.63	**<0.001**	**<0.001**

Levels are shown as concentration [µg/mL] in blood and as relative amount [%] of total fatty acids at wk 14 and wk 20 (2 and 8 weeks after completion of the 12-week intervention).

AA: arachidonic acid; D5D/D6D index, delta-5/6 desaturase index: calculated according to [272]: D5D=C20:4n6/C20:3n6 and D6D=C20:3n6/C18:2n6; EPA: eicosapentaenoic acid; HUFA: highly unsaturated fatty acids; indices of HUFA calculated as follows, modified from Lands (2008): % n3 in HUFA = 100*(C20:5n3 + C22:5n3 + C22:6n3)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3); % n6 in HUFA = 100*(C20:3n6 + C20:4n6 + C22:4n6)/(C20:3n6 + C20:4n6 + C22:4n6 + C20:5n3 + C22:5n3 + C22:6n3); MUFA: monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; n.s.: not significant; SFA: saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; PUFA: polyunsaturated fatty acids: C18:2n6, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:4n6, C20:5n3, C22:4n6, C22:5n3, C22:6n3; SE: standard error; TFA: total fatty acids; Σ n3 PUFA: C18:3n3, C20:3n3, C20:5n3, C22:5n3, C22:6n3; Σ n6 PUFA: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6; wk: week.

[a] t-test for paired samples with Holm-Bonferroni correction; significance level p≤0.05

[b] ANOVA for repeated measures (An reM); significance level p≤0.05

Table S4: Concentration of free oxylipins (pM) in plasma.

	wk 0			wk 1			t-test[a]	wk 3			t-test[a]	wk 6			t-test[a]	wk 12			t-test[a]	An reM[b]
	mean	**±**	**SE**	**mean**	**±**	**SE**	**p (wk 1-0)**	**mean**	**±**	**SE**	**p (wk 3-0)**	**mean**	**±**	**SE**	**p (wk 6-0)**	**mean**	**±**	**SE**	**p (wk 12-0)**	**p**
ALA-oxylipins																				
Hydroxy fatty acids																				
9-HOTrE	693	±	71.7	814	±	58.5	n.s.	897	±	88.0	n.s.	1141	±	127	**0.021**	1285	±	106	**0.001**	**<0.001**
13-HOTrE	993	±	113	1384	±	109	n.s.	1888	±	165	**0.001**	1990	±	256	**0.008**	2569	±	234	**<0.001**	**<0.001**
Dihydroxy fatty acids																				
9,10-DiHODE	403	±	142	280	±	23.0	-	291	±	27.9	-	405	±	81.3	-	579	±	253	-	n.s.
12,13-DiHODE	284	±	33.7	282	±	18.9	n.s.	295	±	17.5	n.s.	414	±	48.8	n.s.	380	±	31.9	**0.017**	**0.001**
15,16-DiHODE	18243	±	2970	19895	±	1804	n.s.	21065	±	2057	n.s.	28086	±	3631	n.s.	24795	±	1802	**0.033**	**0.007**
EPA-oxylipins																				
Hydroxy fatty acids																				
5-HEPE	160	±	22.2	134	±	8.42	-	161	±	11.6	-	238	±	52.4	-	200	±	13.5	-	n.s.
8-HEPE	<LLOQ			<LLOQ				<LLOQ			-	144	±	61.6	-	94.70	±	5.59	-	-
12-HEPE[c]	6032	±	1108	3590	±	471	n.s.	11064	±	1081	**0.030**	5790	±	814	n.s.	16534	±	1701	**<0.001**	**<0.001**
15-HEPE	156	±	10.3	175	±	12.5	n.s.	199	±	13.4	**0.030**	211	±	18.0	**0.040**	230	±	22.3	**0.033**	**<0.001**
18-HEPE	203	±	21.5	231	±	12.1	n.s.	247	±	13.8	n.s.	348	±	30.9	**0.002**	317	±	22.0	**0.001**	**<0.001**
19-HEPE	747	±	113	929	±	63.2	n.s.	1057	±	127	n.s.	1272	±	199	n.s.	1082	±	104	n.s.	**0.005**
20-HEPE	429	±	72.1	463	±	31.4	-	457	±	33.0	-	557	±	64.8	-	492	±	49.5	-	n.s.
Dihydroxy fatty acids																				
8,9-DiHETE	<LLOQ			67.4	±	3.23	-	71.1	±	5.01	-	92.1	±	12.6	-	80.0	±	5.97	-	**-**
11,12-DiHETE	42.8	±	5.71	47.4	±	2.52	n.s.	53.7	±	3.35	n.s.	68.9	±	7.41	n.s.	64.7	±	4.21	**0.008**	**<0.001**
14,15-DiHETE	88.3	±	9.94	100	±	5.01	n.s.	111	±	6.44	n.s.	142	±	15.7	**0.032**	125	±	7.23	**0.006**	**<0.001**
17,18-DiHETE	537	±	65.4	690	±	41.3	n.s.	760	±	56.1	**0.045**	946	±	95.7	**0.006**	854	±	53.7	**0.001**	**<0.001**
DHA-oxylipins																				
Hydroxy fatty acids																				
4-HDHA	284	±	42.0	230	±	22.8	n.s.	197	±	18.9	n.s.	375	±	46.3	n.s.	305	±	24.3	n.s.	**0.002**
7-HDHA	151	±	13.3	133	±	10.6	n.s.	158	±	16.2	n.s.	243	±	54.3	n.s.	227	±	16.1	**0.001**	**0.007**
8-HDHA	439	±	55.2	380	±	39.7	n.s.	416	±	40.4	n.s.	787	±	148	n.s.	627	±	44.4	**0.024**	**<0.001**

10-HDHA	273 ± 30.1	184 ± 18.0	n.s.	318 ± 30.5	n.s.	323 ± 80.4	n.s.	380 ± 31.5	n.s.	**0.014**
11-HDHA	8962 ± 1111	4265 ± 550	**0.004**	10903 ± 1313	n.s.	5677 ± 819	n.s.	12049 ± 1064	n.s.	**<0.001**
13-HDHA	167 ± 20.1	122 ± 12.2	n.s.	151 ± 13.2	n.s.	200 ± 33.9	n.s.	206 ± 15.9	n.s.	**0.010**
14-HDHA[c]	9910 ± 1114	5058 ± 621	**0.004**	13189 ± 1349	n.s.	6846 ± 1026	n.s.	15098 ± 1405	**0.016**	**<0.001**
16-HDHA	173 ± 13.9	156 ± 13.9	n.s.	147 ± 9.21	n.s.	227 ± 39.1	n.s.	172 ± 9.35	n.s.	**0.038**
17-HDHA	774 ± 82.9	658 ± 80.9	-	756 ± 91.6	-	868 ± 136	-	882 ± 84.6	-	n.s.
20-HDHA	423 ± 39.2	367 ± 28.0	-	372 ± 24.3	-	455 ± 64.6	-	351 ± 20.3	-	n.s.
21-HDHA	2098 ± 218	1692 ± 148	-	1661 ± 89.4	-	1806 ± 255	-	1438 ± 103	-	n.s.
22-HDHA	2283 ± 308.3	1731 ± 160	n.s.	1757 ± 116	n.s.	1909 ± 254	n.s.	1480 ± 114	n.s.	**0.039**
Dihydroxy fatty acids										
4,5-DiHDPE	696 ± 76.6	540 ± 50.6	-	502 ± 24.2	-	717 ± 169	-	492 ± 32.5	-	n.s.
7,8-DiHDPE	<LLOQ	<LLOQ	-	<LLOQ	-	<LLOQ	-	<LLOQ	-	-
10,11-DiHDPE	188 ± 29.3	128 ± 14.7	n.s.	124 ± 8.52	n.s.	150 ± 23.9	n.s.	111 ± 7.76	n.s.	**0.029**
13,14-DiHDPE	232 ± 23.3	184 ± 13.5	n.s.	182 ± 10.6	n.s.	189 ± 15.2	n.s.	162 ± 8.84	n.s.	**0.008**
16,17-DiHDPE	321 ± 32.2	254 ± 15.3	n.s.	256 ± 16.6	n.s.	284 ± 32.0	n.s.	227 ± 14.7	n.s.	**0.047**
19,20-DiHDPE	2958 ± 383	2318 ± 199	-	2310 ± 165	-	2337 ± 219	-	2006 ± 138	-	n.s.
LA-Oxylipins										
Hydroxy fatty acids										
9-HODE	13621 ± 1868	11311 ± 856	-	11169 ± 757	-	12594 ± 1021	-	13625 ± 1746	-	n.s.
13-HODE	20227 ± 2952	14821 ± 1201	-	15538 ± 1103	-	15725 ± 1209	-	19240 ± 2855	-	n.s.
Dihydroxy fatty acids										
9,10-DiHOME	5276 ± 764	4019 ± 584	-	4002 ± 551	-	4625 ± 668	-	6091 ± 2161	-	n.s.
12,13-DiHOME	5998 ± 654	4355 ± 384	n.s.	4253 ± 313	n.s.	5343 ± 444	n.s.	5038 ± 465	n.s.	**0.010**
AA-oxylipins										
Thromboxanes, prostaglandins										
PGD2	<LLOQ	<LLOQ		<LLOQ		<LLOQ		<LLOQ		
PGE2	81.7 ± 14.8	35.3 ± 2.58	n.s.	57.9 ± 7.52	n.s.	47.9 ± 6.51	n.s.	90.0 ± 9.40	n.s.	**<0.001**
13,14-dihydro-15-keto-PGF2a	136 ± 7.46	130 ± 7.01	-	136 ± 8.35	-	156 ± 9.12	-	147 ± 8.81	-	n.s.
TXB2	477 ± 58.7	293 ± 16.2	n.s.	418 ± 45.3	n.s.	387 ± 45.6	n.s.	660 ± 57.3	n.s.	**<0.001**
TXB3	<LLOQ	<LLOQ	-	44.7 ± 4.74	-	56.3 ± 9.34	-	<LLOQ	-	**-**

13,14-dihydro-15-keto-PGE1	125 ± 24.7	<LLOQ	-	110 ± 21.8	-	<LLOQ	-	115 ± 27.1	-	n.s.
Hydroxy fatty acids										
5-HETE	550 ± 70.4	413 ± 21.9	-	408 ± 28.4	-	476 ± 25.0	-	474 ± 32.5	-	n.s.
8-HETE	317 ± 23.1	256 ± 16.4	n.s.	326 ± 17.5	n.s.	325 ± 16.4	n.s.	390 ± 24.9	n.s.	**0.001**
9-HETE	<LLOQ	<LLOQ	-	<LLOQ	-	<LLOQ	-	<LLOQ	-	-
11-HETE	261 ± 21.2	213 ± 12.5	-	233 ± 11.2	-	266 ± 12.3	-	310 ± 18.8	-	n.s.
12-HETE[c]	18479 ± 2315	9146 ± 873	**0.015**	24454 ± 2148	n.s.	10471 ± 1135	n.s.	29175 ± 2434	**0.005**	**<0.001**
15-HETE	864 ± 72.8	751 ± 40.4	-	838 ± 45.3	-	795 ± 35.8	-	872 ± 58.2	-	n.s.
20-HETE	896 ± 160	654 ± 57.3	-	693 ± 61.7	-	652 ± 41.3	-	672 ± 40.4	-	n.s.
Dihydroxy fatty acids										
5,6-DiHETrE	220 ± 24.0	182 ± 10.0	n.s.	181 ± 11.2	n.s.	177 ± 8.44	n.s.	173 ± 10.2	n.s.	**0.044**
8,9-DiHETrE	247 ± 22.0	201 ± 12.4	n.s.	201 ± 10.5	n.s.	200 ± 8.89	n.s.	194 ± 8.83	n.s.	**0.024**
11,12-DiHETrE	611 ± 52.5	492 ± 29.0	n.s.	516 ± 25.7	n.s.	494 ± 17.9	n.s.	490 ± 21.1	n.s.	**0.016**
14,15-DiHETrE	727 ± 59.0	611 ± 25.7	n.s.	626 ± 26.8	n.s.	608 ± 16.4	n.s.	600 ± 21.8	n.s.	**0.044**
DGLA-Oxylipins										
Hydroxy fatty acids										
15(S)-HETrE	268 ± 17.7	245 ± 14.3	-	258 ± 16.5	-	282 ± 11.2	-	292 ± 14.5	-	n.s.

Levels are shown at wk 0, 1, 3, 6, and 12 of high ALA diet (14.0±0.45 g/d).

DiHDPE: dihydroxy docosapentaenoic acid; DiHETE: dihydroxy eicosatetraenoic acid; DiHETrE: dihydroxy eicosatrienoic acid; DiHODE: dihydroxy octadecadienoic acid; DiHOME: dihydroxy octadecenoic acid; HDHA: hydroxy docosahexaenoic acid; HETrE: hydroxy eicosatrienoic acid; HEPE: hydroxy eicosapentaenoic acid; HETE: hydroxy eicosatetraenoic acid; HODE: hydroxy octadecadienoic acid; HOTrE: hydroxy octadecatrienoic acid; LLOQ: lower limit of quantification; n.s.: not significant; PG: prostaglandin; SE: standard error; TX: Thromboxane; wk: week.

[a] t-test for paired samples with Holm-Bonferroni correction; significance level p≤0.05

[b] ANOVA for repeated measures (An reM); significance level p≤0.05

[c] 12-LOX metabolites: highly variable concentration in quality control samples, most likely due to residual enzyme activity

Table S5: Concentration of free oxylipins (pM) in the follow-up period.

	wk 14			t-test[a]	wk 20			t-test[a]	An reM[b]
	mean	±	SE	p (wk 14 - wk 12)	mean	±	SE	p (wk 20 - wk 12)	p
ALA-Oxylipins									
Hydroxy fatty acids									
9-HOTrE	834	±	78.6	**0.015**	914	±	102	n.s.	**0.011**
13-HOTrE	1195	±	109	**0.002**	980	±	116	**0.002**	**<0.001**
Dihydroxy fatty acids									
9,10-DiHODE	248	±	32.8	-	268	±	31.7	-	n.s.
12,13-DiHODE	277	±	30.7	**0.014**	299	±	34.7	n.s.	**0.009**
15,16-DiHODE	18892	±	2417	n.s.	18880	±	2449	n.s.	**0.043**
EPA-oxylipins									
Hydroxy fatty acids									
5-HEPE	187	±	13.5	-	179	±	26.2	-	n.s.
8-HEPE	81.8	±	5.89	-	78.5	±	7.67	-	n.s.
12-HEPE[c]	5921	±	861	**<0.001**	1896	±	387	**<0.001**	**<0.001**
15-HEPE	159	±	7.81	**0.012**	<LLOQ				**0.012**
18-HEPE	265	±	10.7	-	265	±	44.0	-	n.s.
19-HEPE	949	±	77.8	-	825	±	145	-	n.s.
20-HEPE	449	±	36.5	-	444	±	63.9	-	n.s.
Dihydroxy fatty acids									
8,9-DiHETE	64.7	±	3.65	-	64.6	±	8.52	-	n.s.
11,12-DiHETE	48.2	±	2.46	**0.006**	46.9	±	7.02	n.s.	**0.031**
14,15-DiHETE	97.1	±	5.20	**0.025**	88.5	±	9.26	**0.011**	**0.002**
17,18-DiHETE	635	±	41.4	**0.013**	594	±	76.0	**0.005**	**0.001**
DHA-oxylipins									
Hydroxy fatty acids									
4-HDHA	364	±	28.0	-	320	±	60.7	-	n.s.
7-HDHA	175	±	15.6	**0.042**	142	±	23.6	**0.037**	**0.013**
8-HDHA	575	±	42.6	-	508	±	104	-	n.s.
10-HDHA	264	±	24.4	**0.006**	172	±	22.8	**0.001**	**<0.001**
11-HDHA	5844	±	859	**0.001**	2309	±	452	**<0.001**	**<0.001**
13-HDHA	180	±	13.6	-	134	±	24.0	-	n.s.
14-HDHA[c]	7582	±	1085	**0.001**	3049	±	566	**<0.001**	**<0.001**
16-HDHA	200	±	10.6	-	182	±	23.9	-	n.s.
17-HDHA	718	±	83.2	n.s.	544	±	78.0	n.s.	**0.018**
20-HDHA	448	±	30.0	-	408	±	49.9	-	n.s.
21-HDHA	1942	±	149	-	1972	±	245	-	n.s.
22-HDHA	2027	±	164	-	1995	±	258	-	n.s.
Dihydroxy fatty acids									
4,5-DiHDPE	632	±	56.9	-	653	±	105	-	n.s.
7,8-DiHDPE	<LLOQ			-	<LLOQ			-	-
10,11-DiHDPE	139	±	11.3	-	145	±	22.2	-	n.s.
13,14-DiHDPE	195	±	11.3	-	191	±	19.9	-	n.s.
16,17-DiHDPE	268	±	12.9	-	257	±	21.8	-	n.s.
19,20-DiHDPE	2502	±	147	-	2391	±	255	-	n.s.
LA-Oxylipins									

Hydroxy fatty acids									
9-HODE	11374	±	964	-	12350	±	1728	-	n.s.
13-HODE	14322	±	1271	-	14998	±	1906	-	n.s.
Dihydroxy fatty acids									
9,10-DiHOME	4114	±	771	-	4792	±	1035	-	n.s.
12,13-DiHOME	4628	±	556	-	5309	±	641	-	n.s.
AA-oxylipins									
Thromboxanes, prostaglandins									
PGD2	<LLOQ			-	<LLOQ			-	-
PGE2	74.7	±	6.97	-	<LLOQ			-	n.s.
13,14-dihydro-15-keto-PGF2a	147	±	11.8	-	159	±	14.0	-	n.s.
TXB2	539	±	44.4	-	2689	±	2447	-	n.s.
TXB3	67.6	±	6.83	-	64.6	±	30.7	-	n.s.
13,14-dihydro-15-keto-PGE1	<LLOQ				93.8	±	16.6	-	n.s.
Hydroxy fatty acids									
5-HETE	561	±	42.0	-	532	±	75.4	-	n.s.
8-HETE	332	±	16.4	-	289	±	29.9	-	n.s.
9-HETE	<LLOQ			-	<LLOQ			-	-
11-HETE	288	±	12.0	-	329	±	96.3	-	n.s.
12-HETE[c]	13790	±	1645	**<0.001**	6266	±	925	**<0.001**	**<0.001**
15-HETE	890	±	54.3	-	861	±	115	-	n.s.
20-HETE	839	±	62.0	-	762	±	86.0	-	n.s.
Dihydroxy fatty acids									
5,6-DiHETrE	204	±	11.5	-	210	±	19.2	-	n.s.
8,9-DiHETrE	228	±	10.1	-	240	±	22.0	-	n.s.
11,12-DiHETrE	556	±	26.8	-	556	±	40.3	-	n.s.
14,15-DiHETrE	662	±	22.7	-	660	±	37.6	-	n.s.
DGLA-Oxylipins									
Hydroxy fatty acids									
15(S)-HETrE	271	±	16.7	-	291	±	24.5	-	n.s.

Levels are shown at wk 14 and wk 20 (2 and 8 weeks after completion of the 12-week intervention).

DiHDPE: dihydroxy docosapentaenoic acid; DiHETE: dihydroxy eicosatetraenoic acid; DiHETrE: dihydroxy eicosatrienoic acid; DiHODE: dihydroxy octadecadienoic acid; DiHOME: dihydroxy octadecenoic acid; HDHA: hydroxy docosahexaenoic acid; HETrE: hydroxy eicosatrienoic acid; HEPE: hydroxy eicosapentaenoic acid; HETE: hydroxy eicosatetraenoic acid; HODE: hydroxy octadecadienoic acid; HOTrE: hydroxy octadecatrienoic acid; n.s.: not significant; PG: prostaglandin; SE: standard error; TX: Thromboxane; wk: week.

[a] t-test for paired samples with Holm-Bonferroni correction

[b] ANOVA for repeated measures (An reM) wk 12, wk 14 and wk 20; significance level $p \leq 0.05$

[c] 12-LOX metabolites: highly variable concentration in quality control samples, most likely due to residual enzyme activity

Appendix Paper II

Table S1: Concentration of fatty acids in red blood cells during the $_{lo}$LA/$_{hi}$ALA diet and $_{hi}$LA/$_{lo}$ALA diet at baseline (day 0), after seven days (day 7) and after 14 days (day 14).

		Day 0			Day 7			Day 14			
		$_{lo}$LA/$_{hi}$ALA	**$_{hi}$LA/$_{lo}$ALA**	**t-test[a]**	**$_{lo}$LA/$_{hi}$ALA**	**$_{hi}$LA/$_{lo}$ALA**	**t-test[a]**	**$_{lo}$LA/$_{hi}$ALA**	**$_{hi}$LA/$_{lo}$ALA**	**t-test[a]**	**2-fact. An reM[b]**
		mean ± SE	**mean ± SE**	**p (day 0-day 0)**	**mean ± SE**	**mean ± SE**	**p (day 1-day 1)**	**mean ± SE**	**mean ± SE**	**p (day 2-day 2)**	**p**
C10:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C11:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C12:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C13:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C14:0	µg/mL	3.31 ± 0.16	3.07 ± 0.17	n.s.	3.15 ± 0.17	3.06 ± 0.12	n.s.	2.78 ± 0.21	3.53 ± 0.21	**0.001**	n.s.
	% of total FA	0.32 ± 0.02	0.31 ± 0.01	n.s.	0.31 ± 0.01	0.32 ± 0.01	n.s.	0.30 ± 0.02	0.34 ± 0.01	**0.001**	n.s.
C14:1n5	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C15:0	µg/mL	1.59 ± 0.08	1.51 ± 0.05	n.s.	1.61 ± 0.06	1.51 ± 0.05	n.s.	1.37 ± 0.07	1.70 ± 0.07	**0.002**	n.s.
	% of total FA	0.15 ± 0.01	0.15 ± 0.00	n.s.	0.16 ± 0.00	0.16 ± 0.00	n.s.	0.15 ± 0.01	0.16 ± 0.01	**0.008**	n.s.
C15:1n5	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C16:0	µg/mL	212 ± 7.22	200 ± 4.48	n.s.	209 ± 5.89	194 ± 4.23	**0.024**	184 ± 6.24	218 ± 6.47	**<0.001**	n.s.
	% of total FA	20.4 ± 0.18	20.1 ± 0.13	n.s.	20.7 ± 0.13	20.5 ± 0.11	**0.002**	19.8 ± 0.18	21.0 ± 0.13	**<0.001**	**0.006**

C16:1n7	µg/mL	2.88 ± 0.18	2.87 ± 0.13	n.s.	3.11 ± 0.32	2.98 ± 0.16	n.s.	2.89 ± 0.37	3.67 ± 0.47	**0.025**	n.s.
	% of total FA	0.28 ± 0.02	0.29 ± 0.01	n.s.	0.30 ± 0.02	0.32 ± 0.02	n.s.	0.31 ± 0.03	0.35 ± 0.04	n.s.	n.s.
C17:0	µg/mL	3.10 ± 0.12	2.96 ± 0.09	n.s.	3.07 ± 0.08	2.87 ± 0.06	**0.031**	2.76 ± 0.08	3.10 ± 0.08	**0.004**	n.s.
	% of total FA	0.30 ± 0.01	0.30 ± 0.01	n.s.	0.31 ± 0.01	0.30 ± 0.01	n.s.	0.30 ± 0.01	0.30 ± 0.01	n.s.	n.s.
C17:1n8	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C18:0	µg/mL	157 ± 4.53	151 ± 3.50	n.s.	154 ± 3.82	142 ± 3.20	**0.030**	141 ± 3.85	154 ± 4.25	**0.01**	n.s.
	% of total FA	15.2 ± 0.18	15.3 ± 0.10	n.s.	15.3 ± 0.13	15.1 ± 0.12	**0.026**	15.2 ± 0.15	14.8 ± 0.15	n.s.	n.s.
C18:1n9	µg/mL	139 ± 4.78	130 ± 2.65	n.s.	130 ± 4.69	121 ± 2.56	**0.046**	117 ± 5.25	133 ± 5.29	**0.01**	n.s.
	% of total FA	13.4 ± 0.18	13.1 ± 0.20	n.s.	12.8 ± 0.21	12.8 ± 0.18	n.s.	12.6 ± 0.25	12.7 ± 0.25	n.s.	n.s.
C18:1n7	µg/mL	14.5 ± 0.59	13.8 ± 0.34	n.s.	14.3 ± 0.42	13.5 ± 0.30	n.s.	13.5 ± 0.33	15.2 ± 0.53	**0.003**	n.s.
	% of total FA	1.40 ± 0.02	1.39 ± 0.02	n.s.	1.42 ± 0.02	1.44 ± 0.02	n.s.	1.45 ± 0.02	1.46 ± 0.03	n.s.	n.s.
C18:2n6	µg/mL	101 ± 3.78	99.1 ± 2.89	n.s.	91.6 ± 2.59	94.5 ± 2.92	n.s.	82.8 ± 2.75	110 ± 3.51	**<0.001**	**<0.001**
	% of total FA	9.72 ± 0.20	9.99 ± 0.24	n.s.	9.12 ± 0.19	10.0 ± 0.23	**<0.001**	8.90 ± 0.15	10.6 ± 0.22	**<0.001**	**<0.001**
C18:3n6	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C19:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C18:3n3	µg/mL	1.44 ± 0.17	1.47 ± 0.13	n.s.	5.63 ± 0.45	1.09 ± 0.09	**<0.001**	6.34 ± 0.63	1.41 ± 0.17	**<0.001**	**<0.001**
	% of total FA	0.14 ± 0.02	0.15 ± 0.01	n.s.	0.55 ± 0.03	0.12 ± 0.01	**<0.001**	0.67 ± 0.05	0.13 ± 0.01	**<0.001**	**<0.001**
C18:4n3	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C20:0	µg/mL	3.87 ± 0.14	4.06 ± 0.11	n.s.	3.92 ± 0.12	3.70 ± 0.13	n.s.	3.71 ± 0.14	3.95 ± 0.15	n.s.	n.s.
	% of total FA	0.37 ± 0.01	0.41 ± 0.01	**0.012**	0.39 ± 0.01	0.39 ± 0.01	n.s.	0.40 ± 0.01	0.38 ± 0.01	n.s.	n.s.
C20:1n9	µg/mL	2.86 ± 0.21	2.73 ± 0.13	n.s.	2.79 ± 0.13	2.57 ± 0.13	**0.004**	2.52 ± 0.10	2.87 ± 0.12	**0.002**	n.s.
	% of total FA	0.27 ± 0.01	0.28 ± 0.01	n.s.	0.28 ± 0.01	0.27 ± 0.01	n.s.	0.27 ± 0.01	0.28 ± 0.01	n.s.	n.s.
C20:2n6	µg/mL	2.00 ± 0.12	1.89 ± 0.08	n.s.	1.87 ± 0.10	1.85 ± 0.09	n.s.	1.62 ± 0.07	2.21 ± 0.11	**<0.001**	**0.001**
	% of total FA	0.19 ± 0.01	0.19 ± 0.01	n.s.	0.19 ± 0.01	0.20 ± 0.01	**0.049**	0.18 ± 0.01	0.21 ± 0.01	**0.004**	**0.001**
C20:3n9	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-

C20:3n6	µg/mL	15.9 ± 0.93	14.5 ± 0.82	n.s.	14.1 ± 0.96	14.1 ± 0.82	n.s.	12.9 ± 0.94	15.9 ± 0.89	**<0.001**	n.s.
	% of total FA	1.53 ± 0.07	1.47 ± 0.08	n.s.	1.39 ± 0.08	1.50 ± -	**0.003**	1.38 ± 0.10	1.54 ± 0.09	**<0.001**	n.s.
C21:0	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C20:4n6	µg/mL	152 ± 4.08	145 ± 4.10	n.s.	147 ± 3.61	138 ± 3.33	**0.047**	139 ± 3.16	151 ± 3.86	**0.007**	n.s.
	% of total FA	14.7 ± 0.18	14.6 ± 0.19	n.s.	14.7 ± 0.20	14.7 ± 0.14	n.s.	15.0 ± 0.27	14.6 ± 0.20	**0.043**	n.s.
C20:3n3	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C20:4n3	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C20:5n3	µg/mL	5.49 ± 0.48	5.86 ± 0.41	n.s.	6.97 ± 0.55	5.21 ± 0.35	**<0.001**	8.27 ± 0.82	5.11 ± 0.41	**<0.001**	**0.001**
	% of total FA	0.53 ± 0.04	0.59 ± 0.04	n.s.	0.69 ± 0.05	0.55 ± 0.04	**<0.001**	0.88 ± 0.08	0.49 ± 0.03	**<0.001**	**<0.001**
C22:0	µg/mL	16.6 ± 0.36	16.5 ± 0.55	n.s.	16.5 ± 0.54	15.28 ± 0.54	n.s.	15.5 ± 0.49	16.6 ± 0.51	**0.007**	n.s.
	% of total FA	1.61 ± 0.04	1.66 ± 0.04	n.s.	1.64 ± 0.03	1.62 ± 0.03	n.s.	1.66 ± 0.04	1.60 ± 0.04	n.s.	n.s.
C22:1n9	µg/mL	1.56 ± 0.20	2.03 ± 0.20	**0.022**	2.08 ± 0.28	1.83 ± 0.21	n.s.	1.94 ± 0.34	1.30 ± 0.12	n.s.	n.s.
	% of total FA	0.15 ± 0.02	0.21 ± 0.02	**0.003**	0.20 ± 0.03	0.19 ± 0.02	n.s.	0.21 ± 0.04	0.13 ± 0.01	n.s.	n.s.
C22:2n6	µg/mL	<0.25	<0.25	-	<0.25	<0.25	-	<0.25	<0.25	-	-
	% of total FA	-	-	-	-	-	-	-	-	-	-
C22:4n6	µg/mL	32.5 ± 1.58	29.5 ± 0.92	**0.048**	30.7 ± 1.21	28.58 ± 1.25	**0.020**	27.9 ± 1.11	30.4 ± 1.13	**0.024**	**0.017**
	% of total FA	3.13 ± 0.11	2.98 ± 0.11	**0.003**	3.06 ± 0.11	3.03 ± 0.11	n.s.	3.01 ± 0.11	2.93 ± 0.09	n.s.	**0.013**
C22:5n6	µg/mL	5.84 ± 0.35	5.12 0.24	**0.026**	5.49 ± 0.34	5.04 ± 0.29	n.s.	5.11 ± 0.32	5.42 ± 0.25	n.s.	**0.014**
	% of total FA	0.56 ± 0.03	0.52 0.02	**0.010**	0.54 ± 0.03	0.53 ± 0.03	n.s.	0.55 ± 0.03	0.52 ± 0.02	n.s.	**0.008**
C22:5n3	µg/mL	27.3 ± 1.42	28.0 ± 0.98	n.s.	27.1 ± 1.05	27.0 ± 0.91	n.s.	27.1 ± 1.34	27.9 ± 1.51	n.s.	n.s.
	% of total FA	2.62 ± 0.09	2.82 ± 0.09	**0.005**	2.70 ± 0.09	2.87 ± 0.08	**0.015**	2.91 ± 0.10	2.67 ± 0.10	**<0.001**	n.s.
C24:0	µg/mL	47.1 ± 1.14	46.7 ± 1.11	n.s.	45.9 ± 1.21	44.0 ± 1.36	n.s.	44.1 ± 1.29	46.6 ± 1.48	n.s.	n.s.
	% of total FA	4.56 ± 0.06	4.70 ± 0.06	**0.050**	4.56 ± 0.05	4.65 ± 0.07	**0.022**	4.75 ± 0.06	4.48 ± 0.08	**0.002**	n.s.
C22:6n3	µg/mL	36.1 ± 1.75	34.3 ± 2.04	n.s.	35.8 ± 1.75	32.4 ± 1.75	**0.006**	35.8 ± 1.44	35.7 ± 1.74	n.s.	**0.025**
	% of total FA	3.50 ± 0.15	3.44 ± 0.16	n.s.	3.56 ± 0.16	3.42 ± 0.15	**0.050**	3.87 ± 0.16	3.44 ± 0.16	**<0.001**	**0.012**
C24:1n9	µg/mL	51.9 ± 2.42	49.9 ± 1.45	n.s.	50.7 ± 1.73	47.7 ± 1.66	**0.027**	49.1 ± 1.34	49.9 ± 1.64	n.s.	**0.050**
	% of total FA	4.99 ± 0.13	5.03 ± 0.11	n.s.	5.04 ± 0.11	5.04 ± 0.11	n.s.	5.30 ± 0.14	4.81 ± 0.12	**<0.001**	n.s.

ΣTFA	µg/mL	1040 ± 30.9	996 ± 20.6	n.s.	1010 ± 25.9	947 ± 20.6	**0.050**	933 ± 25.8	1043 ± 28.2	**0.004**	n.s.
ΣSFA	µg/mL	446 ± 13.1	428 ± 9.33	n.s.	438 ± 11.1	408 ± 9.05	**0.033**	396 ± 11.5	449 ± 12.3	**0.002**	n.s.
	% of total FA	42.9 ± 0.30	42.9 ± 0.15	n.s.	43.4 ± 0.13	43.0 ± 0.14	**<0.001**	42.5 ± 0.18	43.1 ± 0.19	**0.008**	n.s.
ΣMUFA	µg/mL	213 ± 7.87	202 ± 4.06	n.s.	203 ± 6.87	190 ± 4.23	**0.040**	188 ± 6.85	206 ± 7.00	**0.016**	n.s.
	% of total FA	20.5 ± 0.31	20.3 ± 0.25	n.s.	20.1 ± 0.27	20.1 ± 0.21	n.s.	20.1 ± 0.32	19.7 ± 0.26	n.s.	n.s.
ΣPUFA	µg/mL	381 ± 11.0	367 ± 8.40	n.s.	369 ± 8.72	350 ± 7.94	n.s.	349 ± 8.62	387 ± 9.65	**0.006**	n.s.
	% of total FA	36.7 ± 0.19	36.8 ± 0.23	n.s.	36.5 ± 0.20	36.9 ± 0.16	**0.006**	37.5 ± 0.28	37.2 ± 0.19	**0.037**	n.s.
Σn3 PUFA	µg/mL	71.1 ± 3.00	70.4 ± 2.83	n.s.	76.7 ± 2.96	66.5 ± 2.46	**0.001**	78.9 ± 3.34	70.9 ± 3.05	n.s.	**0.001**
	of total FA	6.79 ± 0.20	7.00 ± 0.20	n.s.	7.55 ± 0.20	6.96 ± 0.19	**<0.001**	8.42 ± 0.26	6.73 ± 0.20	**<0.001**	**<0.001**
Σn6 PUFA	µg/mL	310 ± 9.07	296 ± 6.60	n.s.	292 ± 6.85	283 ± 6.40	n.s.	270 ± 6.58	316 ± 7.45	**<0.001**	n.s.
	% of total FA	29.9 ± 0.27	29.8 ± 0.27	n.s.	29.0 ± 0.27	29.9 ± 0.22	**<0.001**	29.0 ± 0.35	30.4 ± 0.24	**<0.001**	**<0.001**
Σn6/Σn3 PUFA		4.42 ± 0.16	4.27 ± 0.15	n.s.	3.86 ± 0.14	4.31 ± 0.15	**<0.001**	3.48 ± 0.15	4.54 ± 0.16	**<0.001**	**<0.001**
ΣEPA+DHA	µg/mL	41.6 ± 2.04	40.2 ± 2.27	n.s.	42.8 ± 2.04	37.6 ± 1.95	**0.001**	44.1 ± 1.96	40.8 ± 1.91	n.s.	**0.005**
	% of total FA	4.03 ± 0.17	4.03 ± 0.18	n.s.	4.25 ± 0.18	3.97 ± 0.17	**<0.001**	4.76 ± 0.20	3.93 ± 0.17	**<0.001**	**0.001**

Levels are shown as concentration [µg/mL] in blood and as relative amount [%] of total fatty acids.
DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; MUFA, monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; SFA, saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; PUFA, Polyunsaturated fatty acids: C18:2n6, C18:3n6, C18:3n3, C:18:4n3, C20:2n6, C20:3n3, C20:3n6, C20:3n9, C20:4n3, C20:4n6, C20:5n3, C22:2n6, C22:4n6, C22:5n3, C22:5n6, C22:6n3; TFA, Total fatty acids; Σn3 PUFA, polyunsaturated fatty acids: C18:3n3, C:18:4n3, C20:3n3, C20:4n3, C20:5n3, C22:5n3, C22:6n3; Σn6 PUFA: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6, C22:5n6.

[a] t-test for paired samples (between interventions), significance level $p \leq 0.05$

[b] two-factorial ANOVA for repeated measures (An reM); significance level $p \leq 0.05$

Appendix Paper III

Tab. S1: Fatty acid pattern of the study supplement.

Fatty acid	Common name	g/100 g			% of total fatty acids		
		mean		SD	mean		SD
C10:0	Capric acid	0.06	±	0.02	0.07	±	0.02
12:0	Lauric acid	3.19	±	0.23	3.72	±	0.16
C14:0	Myristic acid	9.98	±	0.32	11.66	±	0.03
C14:1n5	Myristoleic acid	0.38	±	0.03	0.44	±	0.02
C16:0	Palmitic acid	7.55	±	0.21	8.82	±	0.04
C16:1n7	Palmitoleic acid	3.70	±	0.12	4.32	±	0.02
C18:0	Stearic acid	0.16	±	0.01	0.19	±	0.01
C18:1n9	Oleic acid	7.18	±	0.20	8.38	±	0.03
C18:1n7	Vaccenic acid	0.10	±	0.03	0.11	±	0.03
C18:2n6	Linoleic acid	0.03	±	0.0	0.03	±	0.0
C20:0	Arachidic acid	0.04	±	0.0	0.05	±	0.0
C20:1n9	Gadoleic acid	0.06	±	0.02	0.07	±	0.02
C20:4n6	Arachidonic acid	0.05	±	0.0	0.06	±	0.0
C20:5n3	Eicosapentaenoic acid	0.06	±	0.0	0.07	±	0.0
C22:0	Behenic acid	0.02	±	0.0	0.03	±	0.0
C22:1n9	Cetoleic acid	0.04	±	0.0	0.05	±	0.0
C22:5n3	Docosapentaenoic acid (Clupanodonic acid)	0.77	±	0.03	0.90	±	0.01
C22:6n3	Docosahexaenoic acid	52.05	±	1.50	60.78	±	0.18
C24:1n9	Docosapentaenoic acid (Osbond acid)	0.22	±	0.02	0.26	±	0.01
ALL FA		85.63	±	2.71	100		
SFA	Saturated fatty acids	21.01	±	0.81	24.53	±	0.18
MUFA	Monounsaturated fatty acids	11.67	±	0.38	13.63	±	0.03
PUFA	Polyunsaturated fatty acids	52.95	±	1.53	61.84	±	0.18
n3	Omega-3 fatty acids	52.87	±	1.53	61.75	±	0.18
n6	Omega-6 fatty acids	0.08	±	0.0	0.09	±	0.0
n9	Omega-9 fatty acids	7.50	±	0.22	8.71	±	0.02

The presented results are mean values±SD of three analyses.

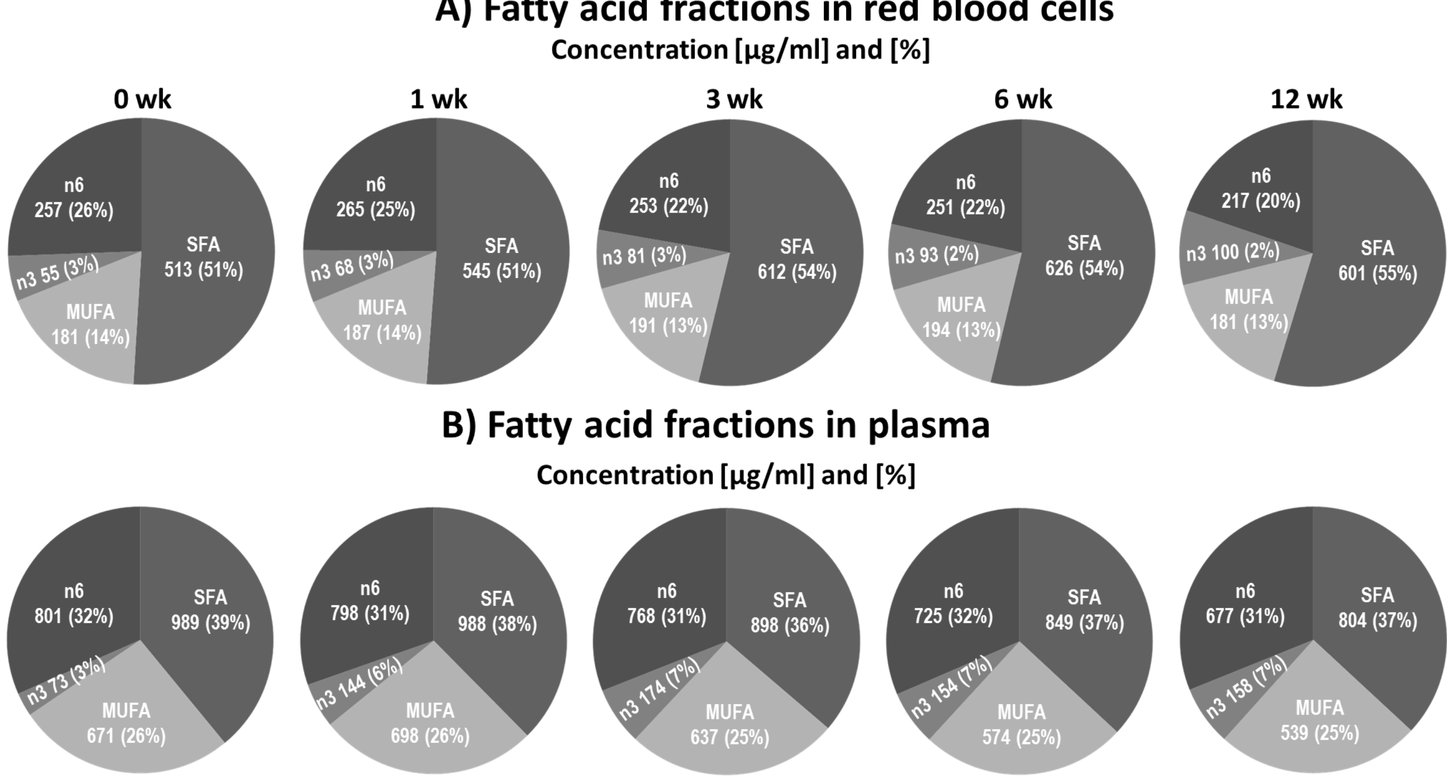


Fig. S1: Levels of Σ saturated fatty acids (SFA), Σ monounsaturated fatty acids (MUFA), Σ omega-3 polyunsaturated fatty acids (n3 PUFA, n3) and Σ n6 PUFA (n6) in (A) red blood cells and (B) plasma at baseline (wk 0) and after one, three, six and twelve weeks (wk 1, 3, 6, and 12) of DHA supplementation.

Levels are shown as concentration [µg/mL] in blood and as relative amount [%] of total fatty acids. MUFA, monounsaturated fatty acids: C14:1n5, C15:1n5, C16:1n7, C17:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, 24:1n9; SFA, saturated fatty acids: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0; n3, omega-3 polyunsaturated fatty acids: C18:3n3, C20:3n3, C20:5n3, C22:5n3, C22:6n3; n6, omega-6 polyunsaturated fatty acids: C18:2n6, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6, C22:4n6. For statistic evaluation please see Tab 3 and 4.

Appendix Paper IV

Tab. S1: No differences in daily SFA, MUFA, and PUFA intake [g] from background diet in the course of the study. PUFA content was calculated from 3-day dietary questionnaires.

	wk	mean	±	SD
SFA	0	54.84	±	36.10
	6	45.98	±	24.73
	12	42.93	±	15.23
MUFA	0	39.94	±	23.67
	6	34.45	±	15.38
	12	35.89	±	12.86
PUFA	0	17.12	±	7.84
	6	14.68	±	5.14
	12	15.56	±	5.34
LA	0	14.57	±	6.93
	6	12.62	±	4.45
	12	13.64	±	4.81
ALA	0	1.57	±	0.99
	6	1.23	±	0.48
	12	1.30	±	0.54
AA	0	0.28	±	0.22
	6	0.31	±	0.22
	12	0.18	±	0.10
EPA	0	0.09	±	0.09
	6	0.07	±	0.14
	12	0.05	±	0.06
DPAn3	0	0.12	±	0.11
	6	0.07	±	0.08
	12	0.07	±	0.11

DHA	0	0.23 ± 0.16
	6	0.19 ± 0.29
	12	0.13 ± 0.15

Tab S2: LC-ESI-MS/MS analysis of free oxylipins in plasma and LPS stimulated blood.

	Transition					Included in data analysis	Included in data analysis
Analyte	m/z (MS1)	m/z (MS3)	IS	LLOQ vial / 500 µL plasma (nM)[1]		Plasma	LPS stimulated blood
THF diol	353.2	127.1	$^{2}H_4$-LTB_4	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
EKODE	309.2	291.1	$^{2}H_4$-9-HODE	0.5	0.05	yes	yes
9-oxo-ODE	293.2	185.1	$^{2}H_4$-9-HODE	1.0	0.1	yes	yes
13-oxo-ODE	293.2	195.1	$^{2}H_4$-9-HODE	1.0	0.1	yes	yes
9-HODE	295.2	171.1	$^{2}H_4$-9-HODE	1.0	0.1	yes	yes
13-HODE	295.2	195.2	$^{2}H_4$-9-HODE	1.0	0.1	yes	yes
9(10)-EpOME	295.3	171.1	$^{2}H_4$-9(10)-EpOME	0.25	0.025	yes	yes
12(13)-EpOME	295.3	195.2	$^{2}H_4$-9(10)-EpOME	0.25	0.025	yes	yes
9,10-DiHOME	313.2	201.2	$^{2}H_4$-9,10-DiHOME	0.5	0.05	yes	yes
12,13-DiHOME	313.2	183.2	$^{2}H_4$-9,10-DiHOME	1.25	0.125	yes	yes
9,10,13-TriHOME	329.2	171.1	$^{2}H_4$-PGE_2	0.5	0.05	yes	yes
9,12,13-TriHOME	329.2	211.1	$^{2}H_4$-PGE_2	1.25	0.125	yes	yes
15(S)-HETrE	321.2	221.2	$^{2}H_8$-5-HETE	0.5	0.05	yes	yes
PGD_1	353.3	317.2	$^{2}H_4$-PGD_2	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGE_1	353.3	317.2	$^{2}H_4$-PGE_2	0.33	0.033	no, <LLOQ in >50% of samples	yes
9-HOTrE	293.2	171.2	$^{2}H_4$-9-HODE	0.5	0.05	yes	yes
13-HOTrE	293.2	195.1	$^{2}H_4$-9-HODE	0.6	0.06	yes	yes
9(10)-EpODE	293.3	171.2	$^{2}H_4$-9(10)-EpOME	0.2	0.02	yes	yes

12(13)-EpODE	293.2	183.1	$^2H_4$-9(10)-EpOME	0.25	0.025	yes	yes
15(16)-EpODE	293.3	235.2	$^2H_4$-9(10)-EpOME	0.25	0.025	yes	yes
9,10-DiHODE	311.2	201.2	$^2H_4$-9,10-DiHOME	0.2	0.02	yes	yes
12,13-DiHODE	311.2	183.1	$^2H_4$-9,10-DiHOME	2.0	0.2	yes	no, <LLOQ in >50% of samples
15,16-DiHODE	311.2	223.2	$^2H_4$-9,10-DiHOME	0.5	0.05	yes	yes
TxB_2	369.2	169.1	$^2H_4$-TxB_2	0.63	0.063	yes	yes
LXA_4	351.2	115.2	$^2H_4$-PGE_2	0.18	0.018	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
$PGF_{2\alpha}$	353.2	309.2	$^2H_4$-PGE_2	0.7	0.07	yes	yes
6-keto-$PGF_{1\alpha}$	369.3	163.2	$^2H_4$-6-keto-$PGF_{1\alpha}$	0.9	0.09	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGE_2	351.2	271.3	$^2H_4$-PGE_2	0.1	0.01	yes	yes
PGJ_2	333.3	189.2	$^2H_4$-PGE_2	1.6	0.16	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGD_2	351.2	271.3	$^2H_4$-PGD_2	1.0	0.1	yes	no, <LLOQ in >50% of samples
15-deoxy-PGJ_2	315.2	271.2	$^2H_{11}$-14,15-DiHETrE	1.0	0.1	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGB_2	333.3	175.1	$^2H_4$-PGE_2	0.4	0.04	no, <LLOQ in >50% of samples	yes
LTB_3	337.2	195.2	$^2H_4$-LTB_4	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
LTB_4	335.2	195.1	$^2H_4$-LTB_4	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
LTB_5	333.3	195.2	$^2H_4$-LTB_4	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
6-trans-LTB_4	335.2	195.1	$^2H_4$-LTB_4	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
20-COOH-LTB_4	365.2	347.2	$^2H_4$-TxB_2	1.0	0.1	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
20-OH-LTB_4	351.2	195.2	$^2H_4$-TxB_2	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
5-oxo-ETE	317.2	273.2	$^2H_4$-9(10)-EpOME	2.0	0.2	no, <LLOQ in >50% of samples	yes
15-oxo-ETE	317.2	113.1	$^2H_8$-5-HETE	0.5	0.05	yes	no, <LLOQ in >50% of samples

5-HETE	319.2	115.2	$^{2}H_{8}$-5-HETE	1.25	0.125	yes	yes
8-HETE	319.2	155.2	$^{2}H_{8}$-12-HETE	2.5	0.25	yes	yes
9-HETE	319.2	167.2	$^{2}H_{8}$-5-HETE	2.5	0.25	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
11-HETE	319.2	167.2	$^{2}H_{8}$-12-HETE	0.5	0.05	yes	yes
12-HETE	319.2	179.2	$^{2}H_{8}$-12-HETE	0.5	0.05	yes	yes
15-HETE	319.2	219.2	$^{2}H_{8}$-12-HETE	1.25	0.125	yes	yes
20-HETE	319.2	275.1	$^{2}H_{6}$-20-HETE	2.6	0.26	yes	no, <LLOQ in >50% of samples
5(6)-EpETrE	319.2	191.1	$^{2}H_{11}$-14(15)-EpETrE	1.0	0.1	yes	yes
8(9)-EpETrE	319.2	155.2	$^{2}H_{11}$-14(15)-EpETrE	2.0	0.2	no, <LLOQ in >50% of samples	yes
11(12)-EpETrE	319.3	167.2	$^{2}H_{11}$-14(15)-EpETrE	0.5	0.05	yes	yes
14(15)-EpETrE	319.2	219.3	$^{2}H_{11}$-14(15)-EpETrE	0.5	0.05	yes	yes
5,6-DiHETrE	337.2	145.1	$^{2}H_{11}$-14,15-DiHETrE	0.5	0.05	yes	yes
8,9-DiHETrE	337.2	127.1	$^{2}H_{11}$-14,15-DiHETrE	0.5	0.05	yes	yes
11,12-DiHETrE	337.2	167.1	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	yes
14,15-DiHETrE	337.2	207.1	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	yes
11,12,15-TriHETrE	353.2	167.1	$^{2}H_{4}$-PGE_{2}	1.0	0.1	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
Resolvin E1	349.3	195.0	$^{2}H_{4}$-TxB_{2}	1.2	0.12	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGE_{3}	349.3	269.2	$^{2}H_{4}$-PGE_{2}	0.3	0.03	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
PGD_{3}	349.3	269.2	$^{2}H_{4}$-PGD_{2}	1.0	0.1	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
5-HEPE	317.2	115.1	$^{2}H_{8}$-12-HETE	0.5	0.05	yes	yes
8-HEPE	317.2	155.2	$^{2}H_{8}$-12-HETE	0.63	0.063	yes	no, <LLOQ in >50% of samples
12-HEPE	317.2	179.2	$^{2}H_{8}$-12-HETE	0.63	0.063	yes	yes

15-HEPE	317.2	219.2	$^{2}H_{8}$-12-HETE	1.25	0.125	yes	yes
18-HEPE	317.2	259.2	$^{2}H_{4}$-9-HODE	0.1	0.01	yes	yes
8(9)-EpETE	317.2	127.2	$^{2}H_{11}$-14(15)-EpETrE	1.0	0.1	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
11(12)-EpETE	317.2	167.2	$^{2}H_{11}$-14(15)-EpETrE	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
14(15)-EpETE	317.2	207.2	$^{2}H_{11}$-14(15)-EpETrE	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
17(18)-EpETE	317.2	215.2	$^{2}H_{11}$-14(15)-EpETrE	0.5	0.05	yes	no, <LLOQ in >50% of samples
5,6-DiHETE	335.2	115.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
8,9-DiHETE	335.2	127.1	$^{2}H_{4}$-9,10-DiHOME	0.5	0.05	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
8,15-DiHETE	335.2	235.2	$^{2}H_{11}$-14,15-DiHETrE	0.8	0.08	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
5,15-DiHETE	335.3	173.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	no, <LLOQ in >50% of samples	no, <LLOQ in >50% of samples
11,12-DiHETE	335.2	167.1	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	no, <LLOQ in >50% of samples
14,15-DiHETE	335.3	207.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	yes
17,18-DiHETE	335.3	247.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	yes
4-HDHA	343.2	101.1	$^{2}H_{8}$-5-HETE	0.1	0.01	yes	yes
7-HDHA	343.2	141.2	$^{2}H_{8}$-5-HETE	0.25	0.025	yes	yes
8-HDHA	343.2	189.2	$^{2}H_{8}$-5-HETE	0.25	0.025	yes	yes
10-HDHA	343.2	153.2	$^{2}H_{8}$-12-HETE	0.1	0.01	yes	yes
11-HDHA	343.2	121.1	$^{2}H_{8}$-5-HETE	0.1	0.01	yes	yes
13-HDHA	343.2	193.1	$^{2}H_{8}$-12-HETE	0.1	0.01	yes	yes
14-HDHA	343.2	205.2	$^{2}H_{8}$-12-HETE	0.25	0.025	yes	yes
16-HDHA	343.2	233.2	$^{2}H_{8}$-12-HETE	0.1	0.01	yes	yes
17-HDHA	343.2	201.2	$^{2}H_{8}$-12-HETE	0.5	0.05	yes	yes

20-HDHA	343.2	241.2	$^{2}H_{8}$-12-HETE	0.25	0.025	yes	yes
10(11)-EpDPE	343.2	153.2	$^{2}H_{11}$-14(15)-EpETrE	0.25	0.025	yes	yes
13(14)-EpDPE	343.2	193.2	$^{2}H_{11}$-14(15)-EpETrE	0.5	0.05	yes	no, <LLOQ in >50% of samples
16(17)-EpDPE	343.2	233.2	$^{2}H_{11}$-14(15)-EpETrE	0.25	0.025	yes	no, <LLOQ in >50% of samples
19(20)-EpDPE	343.2	241.2	$^{2}H_{11}$-14(15)-EpETrE	0.25	0.025	yes	yes
4,5-DiHDPE	361.2	229.3	$^{2}H_{11}$-14,15-DiHETrE	2.0	0.2	yes	yes
7,8-DiHDPE	361.2	113.1	$^{2}H_{11}$-14,15-DiHETrE	1.0	0.1	yes	no, <LLOQ in >50% of samples
10,11-DiHDPE	361.2	153.2	$^{2}H_{11}$-14,15-DiHETrE	0.5	0.05	yes	yes
13,14-DiHDPE	361.2	193.2	$^{2}H_{11}$-14,15-DiHETrE	0.25	0.025	yes	yes
16,17-DiHDPE	361.2	233.2	$^{2}H_{11}$-14,15-DiHETrE	0.5	0.05	yes	yes
19,20-DiHDPE	361.2	273.2	$^{2}H_{11}$-14,15-DiHETrE	1.0	0.1	yes	yes

Shown are the covered analytes, the mass transition used for quantification in scheduled selected reaction monitoring mode, the internal standard (IS) and the lower limit of quantification (LLOQ).

[1] LLOQ was set to the lowest calibration standard injected within the sample set yielding a signal to noise ratio ≤ 9 and accuracy in the calibration within ±20%.

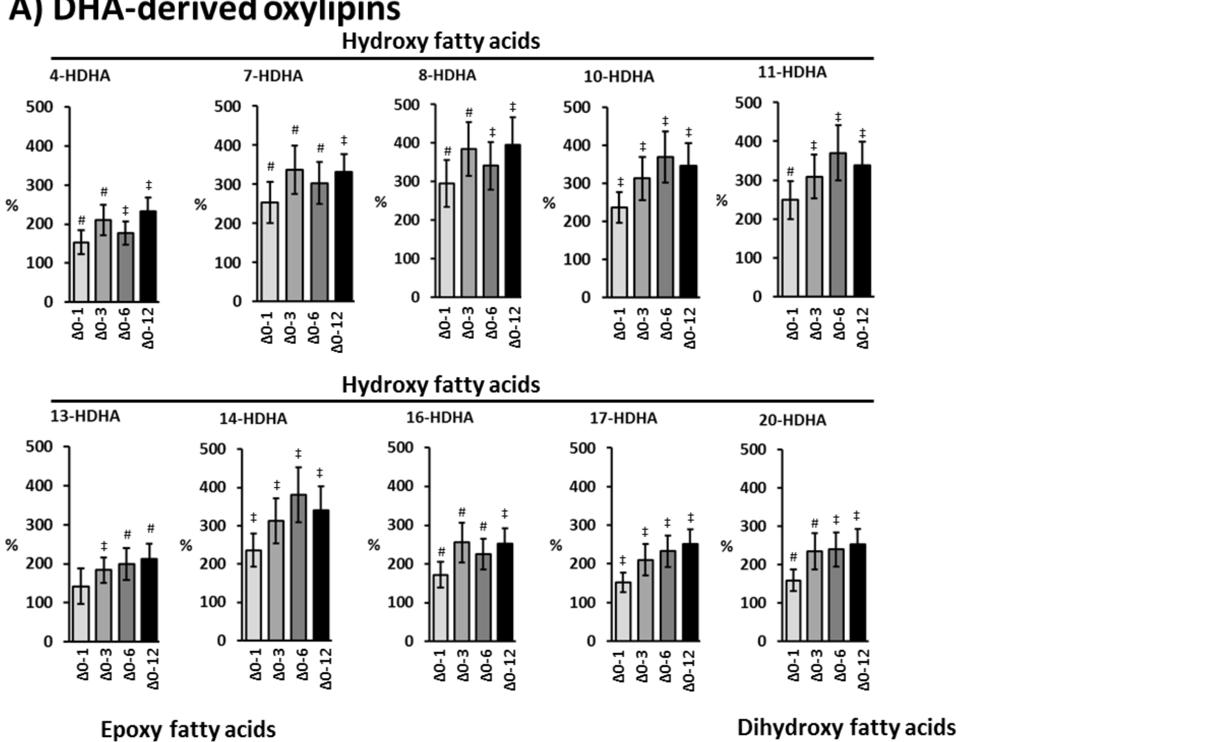
Tab. S3: Concentration of free AA-, EPA- and DHA-oxylipins (pM) in LPS stimulated blood at baseline (wk 0) and after one, three, six and twelve weeks of DHA supplementation.

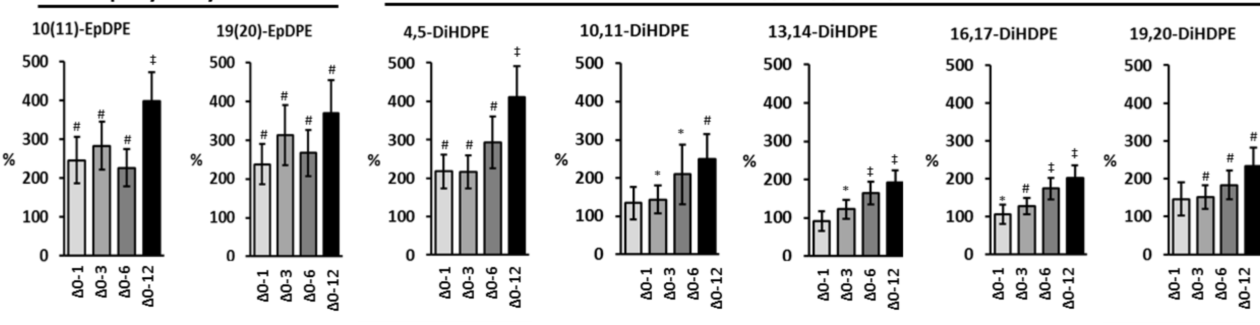
	wk 0			wk 1			t-test*	wk 3			t-test*	wk 6			t-test*	wk 12			t-test*	An reM**
	mean	±	SE	mean	±	SE	p (wk1-0)	mean		SE	p (wk3-0)	mean		SE	p (wk6-0)	mean	±	SE	p (wk12-0)	p
DHA-oxylipins																				
Hydroxy fatty acids																				
4-HDHA	200	±	18	460	±	33	0.001	580	±	37	0.001	500	±	19	<0.001	610	±	27	<0.001	<0.001
7-HDHA	42	±	6	130	±	7	0.002	160	±	12	0.001	140	±	7	0.001	160	±	12	<0.001	<0.001
8-HDHA	250	±	35	790	±	60	0.001	1000	±	66	0.001	870	±	53	<0.001	1000	±	52	<0.001	<0.001
10-HDHA	260	±	34	740	±	31	<0.001	960	±	77	<0.001	1100	±	72	<0.001	980	±	68	<0.001	<0.001
11-HDHA	800	±	120	2300	±	93	0.001	2900	±	250	<0.001	3200	±	200	<0.001	2900	±	220	<0.001	<0.001
13-HDHA	360	±	67	670	±	62	n.s.	860	±	51	<0.001	840	±	39	0.004	900	±	40	0.001	<0.001
14-HDHA	9400	±	1300	26700	±	950	<0.001	35300	±	3100	<0.001	39300	±	2700	<0.001	34800	±	2600	<0.001	<0.001
16-HDHA	100	±	11	240	±	13	0.001	330	±	26	0.001	290	±	18	0.001	320	±	17	<0.001	<0.001
17-HDHA	980	±	110	2200	±	85	<0.001	2700	±	200	<0.001	2900	±	150	<0.001	3000	±	200	<0.001	<0.001
20-HDHA	350	±	35	800	±	33	0.001	1000	±	75	0.001	1000	±	57	<0.001	1100	±	59	<0.001	<0.001
Epoxy fatty acids																				
10(11)-EpDPE	43	±	6	130	±	15	0.003	140	±	13	0.002	110	±	8	0.001	170	±	16	<0.001	<0.001
13(14)-EpDPE	<LLOQ			68	±	7		84	±	7	n.s.	57	±	2	n.s.	92	±	9	0.002	<0.001
16(17)-EpDPE	<LLOQ			79	±	11		95	±	10	n.s.	64	±	4	n.s.	100	±	9	n.s.	0.019
19(20)-EpDPE	85	±	12	230	±	21	0.003	280	±	20	0.002	240	±	16	0.002	300	±	25	0.001	<0.001
Dihydroxy fatty acids																				
4,5-DiHDPE	520	±	62	1400	±	140	0.003	1500	±	110	0.001	1600	±	140	0.002	2200	±	200	<0.001	<0.001
7,8-DiHDPE	<LLOQ			160	±	11		200	±	10	n.s.	200	±	12	n.s.	230	±	13	n.s.	0.008
10,11-DiHDPE	72	±	9	140	±	19	n.s.	160	±	14	0.014	180	±	31	0.022	200	±	23	0.003	<0.001
13,14-DiHDPE	58	±	7	91	±	8	n.s.	120	±	8	0.02	130	±	10	<0.001	140	±	8	<0.001	<0.001
16,17-DiHDPE	73	±	10	130	±	10	0.021	150	±	8	0.001	170	±	12	<0.001	190	±	11	<0.001	<0.001
19,20-DiHDPE	580	±	110	1000	±	80	n.s.	1200	±	55	0.004	1300	±	120	0.002	1500	±	93	0.002	<0.001
EPA-oxylipins																				
Hydroxy fatty acids																				

5-HEPE	210 ± 25	270 ± 35	n.s.	320 ± 43	n.s.	270 ± 22	n.s.	310 ± 23	0.042	0.001
8-HEPE	<LLOQ	<LLOQ		76 ± 4		74 ± 3		77 ± 4		n.s.
12-HEPE	5000 ± 620	5000 ± 320	n.s.	7000 ± 640	n.s.	7100 ± 420	n.s.	6600 ± 760	n.s.	0.003
15-HEPE	180 ± 16	170 ± 11	n.s.	220 ± 18	n.s.	220 ± 12	n.s.	230 ± 19	0.025	<0.001
18-HEPE	130 ± 15	120 ± 11	n.s.	160 ± 13	n.s.	150 ± 9	n.s.	140 ± 9	n.s.	0.003
Dihydroxy fatty acids										
14,15-DiHETE	33 ± 3	31 ± 2	n.s.	37 ± 3	n.s.	39 ± 3	n.s.	44 ± 3	0.046	<0.001
17,18-DiHETE	150 ± 30	160 ± 15	n.s.	190 ± 13	0.047	190 ± 25	0.036	240 ± 21	0.012	<0.001
AA-oxylipins										
Thromboxanes, prostaglandins										
TXB2	12300 ± 1700	8200 ± 1200	n.s.	7300 ± 550	0.033	7200 ± 650	n.s.	7400 ± 670	0.035	0.030
PGF2a	1300 ± 200	790 ± 94		690 ± 62		640 ± 52		710 ± 57		n.s.
PGE2	1400 ± 300	790 ± 150		1100 ± 230		990 ± 370		780 ± 130		n.s.
PGB2	1100 ± 190	560 ± 90	n.s.	530 ± 46	0.007	550 ± 94	n.s.	560 ± 67	n.s.	0.045
Hydroxy fatty acids										
5-HETE	1300 ± 120	980 ± 60	n.s.	870 ± 62	n.s.	870 ± 40	n.s.	910 ± 54	n.s.	0.001
8-HETE	430 ± 24	380 ± 20		360 ± 23		360 ± 15		340 ± 24		n.s.
11-HETE	2200 ± 300	1500 ± 180		1400 ± 88		1380 ± 93		1300 ± 84		n.s.
12-HETE	34000 ± 2300	28900 ± 1600		26200 ± 2400		29100 ± 2000		23100 ± 2400		n.s.
15-HETE	3100 ± 340	2200 ± 210	n.s.	2100 ± 120	0.002	2000 ± 120	n.s.	1900 ± 118	0.011	0.023
20-HETE	570 ± 100	420 ± 56		350 ± 29		<LLOQ		<LLOQ		n.s.
Epoxy fatty acids										
5(6)-EpETrE	490 ± 51	560 ± 51		480 ± 47		430 ± 29		430 ± 25		n.s.
11(12)-EpETrE	91 ± 10	95 ± 9		82 ± 6		63 ± 3		76 ± 5		n.s.
14(15)-EpETrE	89 ± 10	88 ± 9	n.s.	86 ± 10	n.s.	58 ± 3	n.s.	67 ± 5	n.s.	0.017
Dihydroxy fatty acids										
5,6-DiHETrE	1000 ± 100	790 ± 80	n.s.	740 ± 61	n.s.	690 ± 55	n.s.	740 ± 46	n.s.	0.002
8,9-DiHETrE	250 ± 18	190 ± 15	n.s.	170 ± 9	0.010	160 ± 10	0.005	170 ± 10	0.002	0.001
11,12-DiHETrE	210 ± 22	130 ± 9	n.s.	120 ± 6	0.001	130 ± 9	0.006	130 ± 9	0.004	<0.001
14,15-DiHETrE	230 ± 23	160 ± 9	n.s.	150 ± 7	0.003	150 ± 12	0.015	160 ± 11	0.002	0.012

* t-test for paired samples with Holm-Bonferroni correction; ** Anova for repeated measures (An reM).

Fig. S1: Relative change (%) of free A) AA-, B) EPA- and D) DHA-derived oxylipin levels in LPS stimulated blood between baseline (wk 0) and one, three, six and twelve weeks of DHA supplementation. Bars represent mean±SE. * p≤0.05, # p≤0.005, ‡ p<0.001.





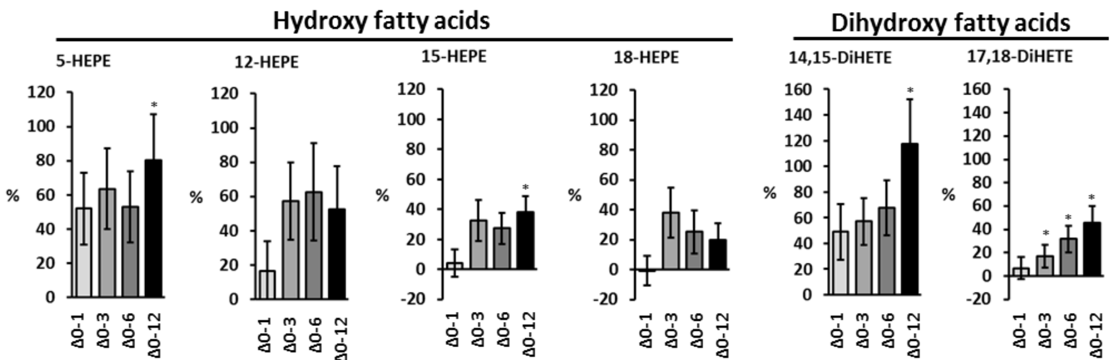
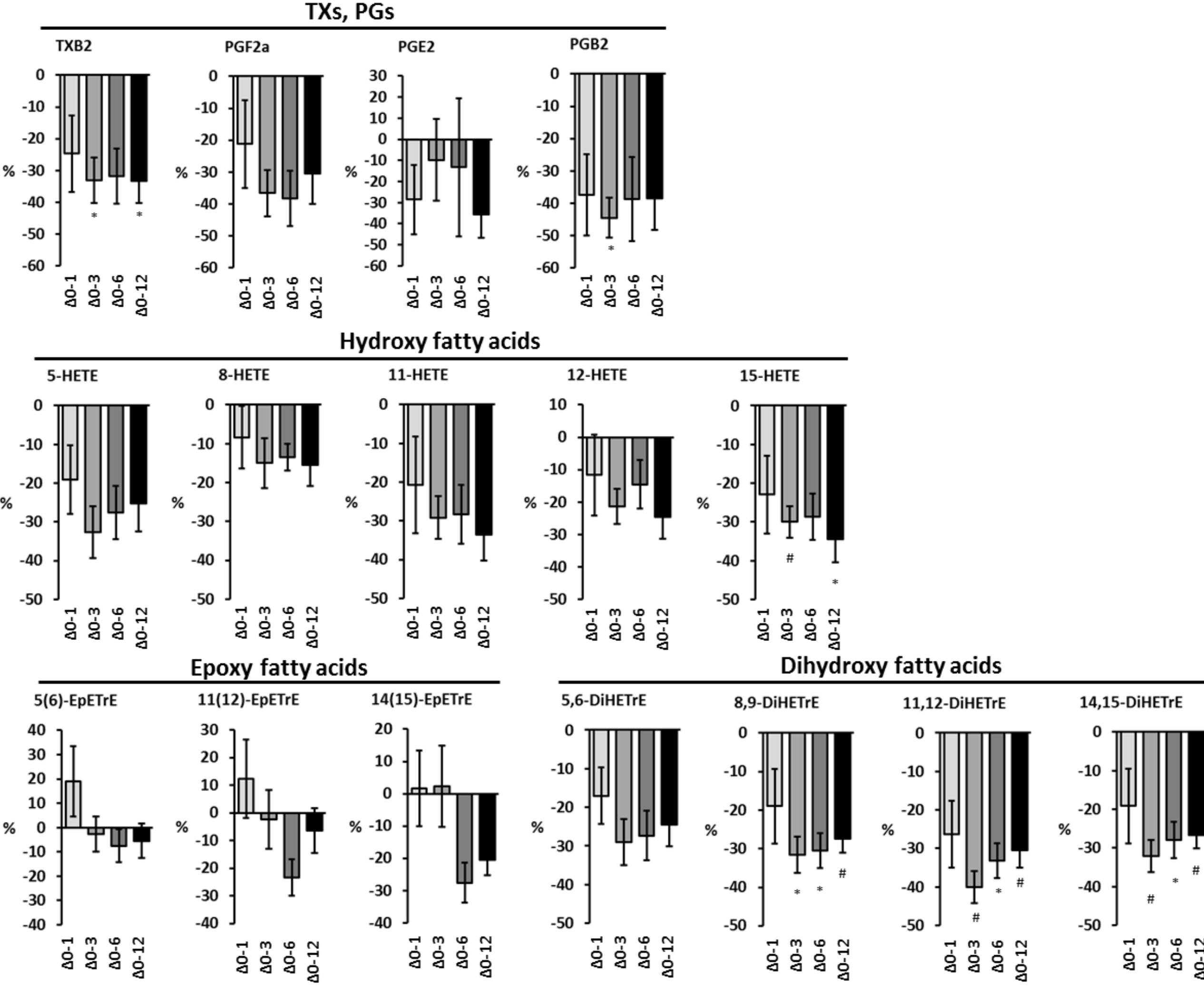


Fig. S1: Continued.



Appendix Paper V

Study B

Details on the study population

Participants were recruited from the general population in Hannover, Germany by advertisements. Subjects were pre-selected via screening questionnaires according to the following inclusion criteria: Male sex, age between 20 and 40 years, body mass index (BMI) between 20 and 27 kg/m², mixed diet with low meat and fish consumption. Exclusion criteria were defined as followed: Smoking, serum triglyceride (TG) levels ≥150 mg/dl (≥1.7 mmol/l); serum total cholesterol levels ≥200 mg/dl (≥5.2 mmol/l); a relative amount of ΣEPA+DHA in red blood cells ≤3 and ≥6%, intake of fish (>2 times per week) as well as addiction to alcohol, drugs and/or medications and diseases: chronic diseases (e.g. malignant tumors, manifest cardiovascular disease, insulin-dependent type 1 and 2 diabetes, severe renal or liver diseases); chronic gastrointestinal disorders (especially small intestine, pancreas, liver) as well as prior gastrointestinal surgical procedures (e.g. gastrectomy); hormonal disorders (e.g. Cushing´s syndrome and untreated hyperthyroidism); uncontrolled hypertension; blood coagulation disorders and intake of coagulation-inhibiting drugs; periodic intake of laxatives; intake of anti-inflammatory drugs (incl. acetylsalicylic acid); intake of lipid lowering drugs or supplements during the last 3 months before baseline examination. Inclusion and exclusion criteria were assessed via questionnaires. The pre-selected subjects were invited to a screening examination to collect fasting blood for the analysis of serum lipid levels, liver enzymes and fatty acid patterns in blood cells.

Pre-screening

Subjects evaluated eligible based on a screening questionnaire (including inclusion and exclusion criteria) were invited to the screening examination 3 weeks before the start of the run-in period. Fasting blood was drawn from the subjects to determine the fatty acid composition in red blood cells, serum triglyceride and serum total cholesterol levels.

Serum triglyceride and total cholesterol were analyzed in the LADR laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V.), Hannover, Germany. For analysis of fatty acids in blood cells, the cell sediment after centrifugation and removal of plasma was reconstituted in PBS to the initial blood volume, transferred into 1.5 mL Eppendorf tubes and immediately frozen and stored at -80 °C until extraction and analysis. Lipids were extracted from 50 µL diluted blood cells using MTBE/MeOH and concentrations of fatty acids were determined by by means of gas chromatography with flame ionization detection (GC-FID) following (trans-)esterification to fatty acid methy esters (FAMEs) as described (1)[using methyl pentacosanoate (C25:0 methyl ester) as internal standard (IS) for quantification.

(1) Ostermann, A.I., Müller, M., Willenberg, I., and Schebb, N.H. (2014). Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography – a comparison of different derivatization and extraction procedures. Prostaglandins Leukot. Essent. Fat. Acids 91, 235–241.

Tab. S1: Daily energy, macronutrient and fatty acid intake of study participants from the study on inter-day and intra-day variation of free oxylipins in plasma on a standardized diet (Study B) during the whole period of the standardized nutrition **(A)** and energy, macronutrient and fatty acid intake of meals from t0 to t24 (Day 1) of the standardized nutrition **(B)**.

A)	**Day 1**		**Day 2**		**Day 3**	
Portion size	**small**	**large**	**small**	**large**	**small**	**large**
Energy intake (kcal)[a]	2924	3152	2687	2946	2907	3179
Carbohydrates (g)[a]	337	378	321	375	335	375
Protein (g)[a]	122	128	103	110	125	136
Total fat intake (g)[a]	82.0	82.3	106	108	85.33	85.32
SFA (g)[a]	37.06	35.31	40.88	38.55	38.50	37.34
MUFA (g)[a]	17.49	16.64	20.87	20.06	18.05	17.67
PUFA (g)[a]	3.98	4.00	10.20	10.16	4.86	5.32
LA (g)[b]	3.25	3.27	9.49	9.45	4.00	4.36
ALA (g)[b]	0.53	0.52	0.51	0.49	0.67	0.74
ARA (g)[b]	0.10	0.11	0.12	0.12	0.11	0.11
EPA (g)[b]	0.03	0.03	0.03	0.03	0.03	0.03
DPAn3 (g)[b]	< 0.01	0.02	< 0.01	0.02	0.01	0.02
DHA (g)[b]	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Levels are shown at day 1, 2 and 3 of standardized nutrition for small and large portion size.

B)	**Breakfast**	**Lunch**	**Snack**		**Dinner**	
Portion size	**small / large**	**small / large**	**small**	**large**	**small**	**large**
Energy intake (kcal)[a]	900	919	223	319	882	1014
Carbohydrates (g)[a]	95.9	125	33.3	48.1	83.3	109
Protein (g)[a]	34.8	37.7	8.00	10.0	41.2	45.3
Total fat intake (g)[a]	36.6	6.80	3.78	4.10	34.9	35.1
SFA (g)[a]	17.82	2.71	2.16		14.37	12.62
MUFA (g)[a]	8.68	1.24	1.07		6.49	5.64
PUFA (g)[a]	1.86	0.33	0.12		1.67	1.69
LA (g)[b]	1.54	0.24	0.09		1.38	1.40
ALA (g)[b]	0.22	0.07	0.02		0.21	0.20
ARA (g)[b]	0.05	0.01	< 0.01		0.04	0.04
EPA (g)[b]	0.02	< 0.01	< 0.01		0.01	0.01
DPAn3 (g)[b]	< 0.01	< 0.01	< 0.01		< 0.01	0.01
DHA (g)[b]	< 0.01	< 0.01	< 0.01		< 0.01	< 0.01

Levels are shown for breakfast, lunch, snack and dinner of standardized nutrition for small and large portion size.

ARA: arachidonic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; DPAn3: docosapentaenoic acid; LA: linoleic acid; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.

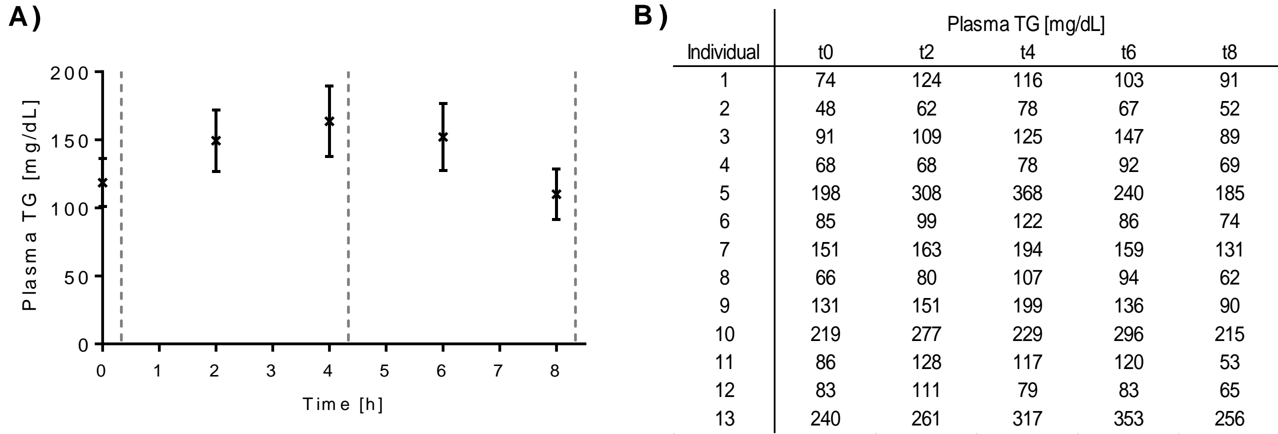
[a] Energy, carbohydrate and protein intake were calculated with PRODI®

[b] Total fat, SFA, MUFA and PUFA LA, ALA, AA, EPA, DPAn3 and DHA intake were calculated from own analyses of meals that were provided by the Institute of Food Science and Human Nutrition

Tab. S2: Baseline clinical, biochemical and anthropometric parameters of study participants from the study on inter-day and intra-day variation of free oxylipins in plasma on a standardized diet (Study B). Shown are mean ± SD (n=13).

	mean	±	SD
Age (years)	24.6	±	2.47
Weight (kg)	83.6	±	9.10
BMI (kg/m^2)	24.6	±	2.02
Sys BP (mmHg)	132	±	19.9
Dias BP (mmHg)	81.5	±	9.13
AST (U/l)	26.7	±	7.35
ALT (U/l)	23.7	±	15.3
GGT (U/l)	21.5	±	8.52
TC (mg/dl)	177	±	43.1
HDL (mg/dl)	50.2	±	8.69
LDL (mg/dl)	111	±	33.5
TG (mg/dl)	136	±	67.5

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; BMI: body mass index; dias BP: diastolic blood pressure; GGT: Gamma-glutamyl transpeptidase; HDL: high density lipoprotein; LDL: low density lipoprotein; SD: standard deviation; sys BP: systolic blood pressure; TC: total cholesterol; TG: triglycerides.



Individual	Plasma TG [mg/dL] t0	t2	t4	t6	t8
1	74	124	116	103	91
2	48	62	78	67	52
3	91	109	125	147	89
4	68	68	78	92	69
5	198	308	368	240	185
6	85	99	122	86	74
7	151	163	194	159	131
8	66	80	107	94	62
9	131	151	199	136	90
10	219	277	229	296	215
11	86	128	117	120	53
12	83	111	79	83	65
13	240	261	317	353	256

Fig. S1: Plasma triglyceride levels of study participants from the study on inter-day and intra-day variation of free oxylipins in plasma on a standardized diet (Study B). Shown are mean ± SEM (n=13) **(A)** and individual values **(B)**. Dotted lines in the diagram indicate food intake (20 min post sample collection at t0, t4 and t8).

Tab. S3: List of analytes included in LC-MS analysis and data evaluation in both studies. Shown are the analyte name and the LLOQ in plasma (2).

Analyte	LLOQ+	Study A	Study B	Analyte	LLOQ+	Study A	Study B	Analyte	LLOQ+	Study A	Study B
20-OH-PGE$_2$	0.025	Y	Y	dihomo-PGF$_{2\alpha}$	0.010	Y	Y	11-HEPE	0.050	Y	Y
ent-16(*R*,*S*)-13-*epi*-ST-Δ^{14}-9-PhytoF 1	0.024	N	Y	4(*R*,*S*)-4-F$_{3t}$-NeuroP$_{n6}$	0.10	N	Y	8-HEPE	0.063	Y	Y
ent-16(*R*,*S*)-13-*epi*-ST-Δ^{14}-9-PhytoF 2	0.026	N	Y	17(*R*,*S*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF 1	0.10	N	Y	12-HEPE	0.063	Y*	Y
ent-16-*epi*-16-F$_{1t}$-PhytoP	0.050	N	Y	17(*R*,*S*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF 2	0.10	N	Y	9-HEPE	0.050	Y	Y
ent-16-F$_{1t}$-PhytoP	0.10	N	Y	RvE2	0.20	Y	Y	21-HDHA	0.17	Y	Y
ent-9-F$_{1t}$-PhytoP	0.025	N	Y	PGJ$_2$	0.16	Y	Y	5-HEPE	0.050	Y	Y
ent-9-*epi*-9-F$_{1t}$-PhytoP	0.050	N	Y	Δ^{12}-PGJ$_2$	0.10	N	Y	22-HDHA	0.28	Y	Y
Δ^{17}-6-keto-PGF$_{1\alpha}$	0.10	Y	Y	LTB$_5$	0.010	Y	Y	4,5-DiHDPE	0.20	Y	Y
2,3-dinor-TxB$_1$	0.50	Y	Y	PGB$_2$	0.040	Y	Y	13-HODE	0.50	Y	Y
15(*R*,*S*)-2,3-dinor-15-F$_{2t}$-IsoP	0.050	N	Y*	THF diol	0.025	Y	Y	9-HODE	0.50	Y	Y
2,3-dinor-TxB$_2$	0.10	Y	Y	18(*S*)-RvE3	0.1	Y	Y	20-HDHA	0.05	Y	Y
6-keto-PGF$_{1\alpha}$	0.18	Y	Y	12-OH-17(18)-EpETE	0.05	Y	Y	15(16)-EpODE	0.05	Y	Y
8-F$_{3t}$-IsoP	0.10	N	Y	15,16-DiHODE	0.1	Y	Y	15-HETE	0.13	Y	Y
8-*epi*-8-F$_{3t}$-IsoP	0.10	N	Y	9,10-DiHODE	0.02	Y	Y	9(10)-EpODE	0.04	Y	Y
RvE1	0.12	Y	Y	12,13-DiHODE	0.1	Y	Y	17(18)-EpETE	0.10	Y	Y
20-COOH-LTB$_4$	0.10	Y	Y	8,15-DiHETE	0.08	Y	Y	16-HDHA	0.03	Y	Y
TxB$_3$	0.025	Y	Y	10(*S*),17(*S*)-diH n3 DPA	0.1	N	Y	17-HDHA	0.20	Y	Y
20-OH-LTB$_4$	0.025	Y	Y	18(*R*)-RvE3	0.05	Y	Y	13-HDHA	0.05	Y	Y
5(*R*,*S*)-5-F$_{3t}$-IsoP	0.20	N	Y	NPD1	0.05	N	Y	12(13)-EpODE	0.05	Y	Y
13,14-dihydro-15-keto-tetranor-PGE$_2$	0.025	Y	Y	6-*trans*-LTB$_4$	0.05	Y	Y	13-oxo-ODE	0.10	Y*	Y
TxB$_1$	0.050	Y	Y	5,15-DiHETE	0.025	Y	Y	11-HETE	0.05	Y	Y
15-F$_{2t}$-IsoP (8-*iso*-PGF$_{2\alpha}$)	0.050	Y	Y	17,18-DiHETE	0.025	Y	Y	10-HDHA	0.05	Y	Y
TxB$_2$	0.13	Y	Y	LTB$_4$	0.025	Y	Y	14-HDHA	0.10	Y*	Y
11-dehydro-TxB$_3$	0.10	Y	Y	7(*S*),17(*S*)-diH n3 DPA	0.075	N	Y	9-oxo-ODE	0.10	Y*	Y
PGE$_3$	0.030	Y	Y	14,15-DiHETE	0.025	Y	Y	15-oxo-ETE	0.05	Y*	Y
11β-PGF$_{2\alpha}$	0.050	Y	Y	11,12-DiHETE	0.025	Y	Y	14(15)-EpETE	0.05	Y	Y
10-F$_{4t}$-NeuroP	0.050	N	Y	12,13-DiHOME	0.05	Y	Y	8-HETE	0.13	Y	Y
10-*epi*-10-F$_{4t}$-NeuroP	0.10	N	Y	8,9-DiHETE	0.05	Y	Y	12-HETE	0.05	Y*	Y
5(*R*,*S*)-5-F$_{2t}$-IsoP (5-iPF$_{2\alpha}$-VI)	0.050	Y	Y	10(*S*),17(*S*)-diH n6 DPA	0.025	N	Y	11(12)-EpETE	0.05	Y	Y*
PGD$_3$	0.10	Y	Y	9,10-DiHOME	0.05	Y	Y	11-HDHA	0.03	Y*	Y
16-B$_1$-PhytoP	0.025	N	Y	10(*S*),17(*S*)-diH AdA	0.1	N	Y	7-HDHA	0.10	Y	Y
9-L$_1$-PhytoP	0.025	N	Y	12(*S*)-HHTrE	0.05	N	Y	8(9)-EpETE	0.10	Y	Y
PGF$_{2\alpha}$	0.070	Y*	Y	14,15-DiHETrE	0.01	Y	Y	9-HETE	0.25	Y	Y
14(*R*,*S*)-14-F$_{4t}$-NeuroP	2.0	N	Y	19,20-DiHDPE	0.05	Y	Y	15-HETrE	0.05	Y	Y
PGF$_{1\alpha}$	0.025	Y	Y	LTB$_3$	0.05	Y	Y	8-HDHA	0.05	Y	Y
PGE$_2$	0.025	Y	Y	9,10-diH-stearic acid	0.2	Y	Y	5-HETE	0.05	Y	Y
11-dehydro-TxB$_2$	0.050	Y	Y	16,17-DiHDPE	0.05	Y	Y	4-HDHA	0.03	Y	Y
PGE$_1$	0.033	Y	Y*	11,12-DiHETrE	0.025	Y	Y	19(20)-EpDPE	0.05	Y	Y
4(*R*,*S*)-4-F$_{4t}$-NeuroP	0.10	N	Y	19-HEPE	0.071	Y	Y	12(13)-EpOME	0.03	Y	Y
PGD$_1$	0.05	Y	Y	13,14-DiHDPE	0.025	Y	Y	14(15)-EpETrE	0.05	Y	Y
PGD$_2$	0.10	Y	Y	20-HEPE	0.1	Y	Y	9(10)-EpOME	0.03	Y	Y
15-keto-PGF$_{1\alpha}$	0.025	Y	Y	9-HOTrE	0.05	Y	Y	16(17)-EpDPE	0.05	Y	Y
4(*R*,*S*)-ST-Δ^5-8-NeuroF	4.0	N	Y	10,11-DiHDPE	0.025	Y	Y	13(14)-EpDPE	0.05	Y	Y
17(*R*,*S*)-17-F$_{2t}$-dihomo-IsoP 1	0.13	N	Y	8,9-DiHETrE	0.05	Y	Y	5-oxo-ETE	0.20	Y	Y
17(*R*,*S*)-17-F$_{2t}$-dihomo-IsoP 2	0.075	N	Y	13-HOTrE	0.06	Y	Y	10(11)-EpDPE	0.03	Y	Y
7(*R*,*S*)-ST-Δ^8-11-dihomo-IsoF	0.20	N	Y	18-HEPE	0.1	Y	Y	11(12)-EpETrE	0.05	Y	Y
ent-7(*R*,*S*)-7-F$_{2t}$-dihomo-IsoP	0.050	N	Y	15-deoxy-PGJ$_2$	0.05	Y	Y	8(9)-EpETrE	0.10	Y	Y
11,12,15-TriHETrE	0.050	Y	Y	19-HETE	1.0	N	Y	5(6)-EpETrE	0.20	Y	Y*
LXA$_4$	0.018	Y	Y	7,8-DiHDPE	0.10	Y	Y	5-HETrE	0.02	Y	Y
RvD1	0.025	Y	Y	20-HETE	0.10	Y	Y	9(10)-ep-stearic acid	0.20	Y	Y
13,14-dihydro-15-keto-PGF$_{2\alpha}$	0.050	Y	Y	15-HEPE	0.13	Y	Y				
13,14-dihydro-15-keto-PGE$_1$	0.050	Y	Y	5,6-DiHETrE	0.050	Y	Y				

N - analyte not included in LC-MS method

Y - Analyte included in LC-MS method and evaluated

Y* - Analyte included in LC-MS method, but no evaluation

+ Lower limit of quantification (LLOQ) in plasma [nM]

(2) Rund, K.M., Ostermann, A.I., Kutzner, L., Galano, J.-M., Oger, C., Vigor, C., Wecklein, S., Seiwert, N., Durand, T., and Schebb, N.H. (2017). Development of an LC-ESI(-)-MS/MS method for the simultaneous quantification of 35 isoprostanes and isofurans derived from the major n3- and n6-PUFAs. Anal. Chim. Acta. 2018; DOI: *doi.org/10.1016/j.aca.2017.11.002.*

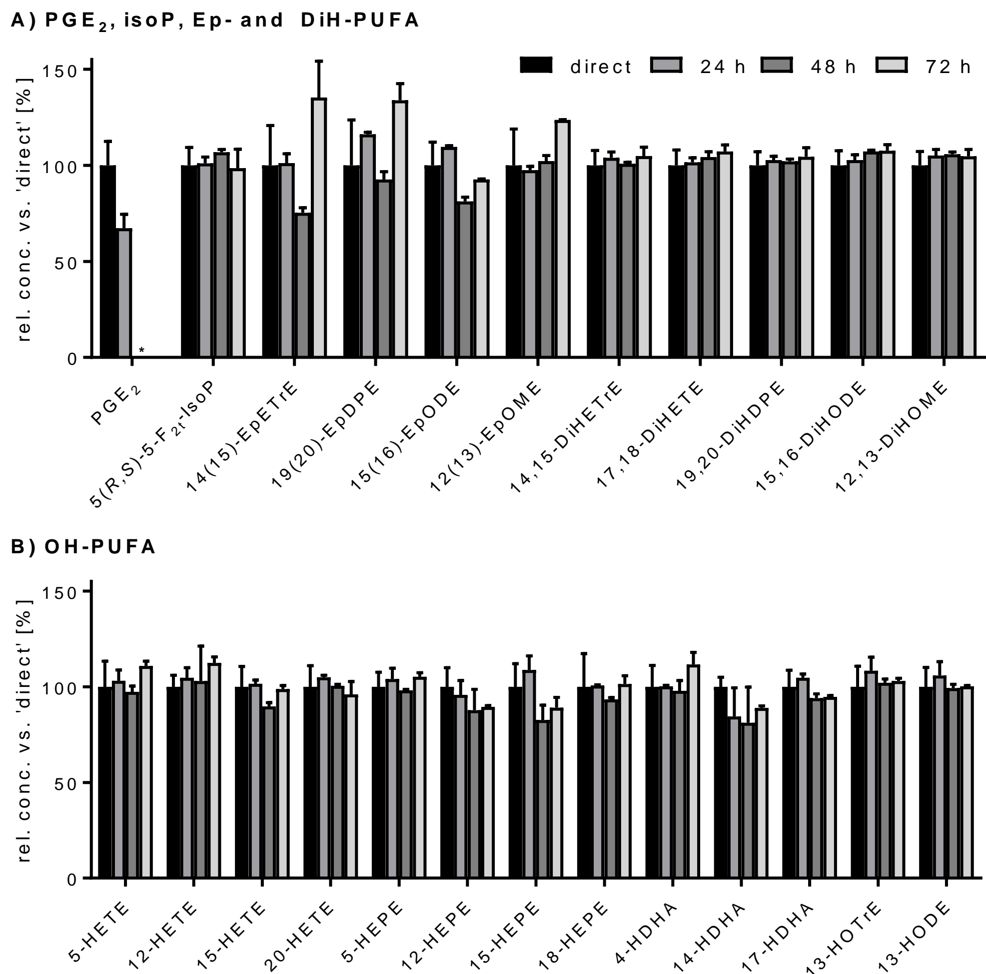


Fig. S2: Changes in selected oxylipins when freezing samples for up to 72 h following protein precipitation, i.e. before solid phase extraction. Relative concentrations of PGE_2 as well as selected isoP, ep-, and diH-PUFA **(A)** and OH-PUFA **(B)** in samples stored for 24-72 h versus direct extraction (30 min) are shown (n=3 for direct analysis; n=2 for 24 h, 48 h and 72 h). For sample preparation, internal standards, antiox-mix and methanol were added to plasma samples. Samples were stored for 30 min, 24 h, 48 h and 72 h at -80°C before extraction of free oxylipins by solid phase extraction (Bond Elut Certify II, Agilent). * PGE_2 was found at very low concentrations in plasma (< 2-fold LLOQ) and was <LLOQ after storage for 48 and 72 h.

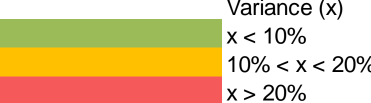
Tab. S4: Concentration of oxylipins found in human quality control plasma. Shown are mean ± 95% interval of the standard deviation (95%SD, n=15) as well as the relative 95%SD. A color code was assigned to each analyte based on the relative 95%SD as a measure for the quality of the analytes' analytical variance.

Analytes			LLOQ [nM]*	Mean±95%SD [nM]	rel 95%SD [%]	color code
LA	epoxy-PUFA	9(10)-EpOME	0.03	1.8 ± 0.25	14	yellow
		12(13)-EpOME	0.03	4.6 ± 0.36	7.7	green
	vic dihydroxy-PUFA	9,10-DiHOME	0.05	4.2 ± 0.27	6.3	green
		12,13-DiHOME	0.05	4.8 ± 0.32	6.6	green
	hydroxy-PUFA	9-HODE	0.50	10 ± 0.62	6.2	green
		13-HODE	0.50	10.0 ± 0.57	5.7	green
	others	13-oxo-ODE	0.10	0.23 ± 0.053	23	red
		9-oxo-ODE	0.10	0.98 ± 0.14	14	yellow
DGLA	hydroxy-PUFA	5-HETrE	0.02	0.20 ± 0.016	7.9	green
		15-HETrE	0.05	0.53 ± 0.033	6.2	green
ARA	prostanoids	PGE_2	0.03	0.081 ± 0.015	18	yellow
		$PGF_{2\alpha}$	0.07	8.0 ± 3.7	46	red
		13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.05	0.097 ± 0.035	36	red
		TxB_2	0.13	0.73 ± 0.11	15	yellow
	isoprostanes	5(*R*,*S*)-5-F_{2t}-IsoP	0.05	0.12 ± 0.025	22	red
	epoxy-PUFA	11(12)-EpETrE	0.02	0.079 ± 0.023	29	red
		14(15)-EpETrE	0.05	0.20 ± 0.030	15	yellow
	vic dihydroxy-PUFA	5,6-DiHETrE	0.05	0.33 ± 0.10	31	red
		8,9-DiHETrE	0.05	0.26 ± 0.018	6.9	green
		11,12-DiHETrE	0.03	0.60 ± 0.034	5.7	green
		14,15-DiHETrE	0.01	0.66 ± 0.032	4.8	green
	hydroxy-PUFA	5-HETE	0.05	0.86 ± 0.067	7.7	green
		8-HETE	0.13	0.34 ± 0.052	15	yellow
		11-HETE	0.05	0.34 ± 0.031	9.1	green
		12-HETE	0.05	1.7 ± 0.27	15	yellow
		15-HETE	0.13	1.2 ± 0.12	10	yellow
		19-HETE	1.00	1.1 ± 0.22	20	yellow
		20-HETE	0.10	0.98 ± 0.075	7.7	green
		12-HHTrE	0.05	1.1 ± 0.084	7.6	green
	others	15-oxo-ETE	0.05	0.067 ± 0.019	28	red
ALA	epoxy-PUFA	9(10)-EpODE	0.04	0.20 ± 0.077	38	red
		12(13)-EpODE	0.05	0.23 ± 0.040	17	yellow
		15(16)-EpODE	0.05	3.2 ± 0.26	8.2	green
	vic dihydroxy-PUFA	9,10-DiHODE	0.02	0.25 ± 0.013	5.1	green
		12,13-DiHODE	0.10	0.27 ± 0.034	12	yellow
		15,16-DiHODE	0.10	11 ± 0.66	5.8	green
	hydroxy-PUFA	9-HOTrE	0.05	0.53 ± 0.035	6.6	green
		13-HOTrE	0.06	0.62 ± 0.053	8.5	green

Tab. S4: Continued.

Analytes			LLOQ [nM]*	Mean±95%SD [nM]	rel 95%SD [%]	color code
EPA	vic dihydroxy-FA	8,9-DiHETE	0.05	0.085 ± 0.016	19	
		11,12-DiHETE	0.03	0.050 ± 0.005	11	
		14,15-DiHETE	0.03	0.083 ± 0.010	12	
		17,18-DiHETE	0.03	0.66 ± 0.043	6.5	
	hydroxy-PUFA	5-HEPE	0.05	0.23 ± 0.031	13	
		8-HEPE	0.06	0.16 ± 0.016	10	
		9-HEPE	0.05	0.10 ± 0.019	19	
		11-HEPE	0.05	0.062 ± 0.012	19	
		12-HEPE	0.06	0.21 ± 0.036	17	
		15-HEPE	0.13	0.18 ± 0.035	19	
		18-HEPE	0.10	0.34 ± 0.033	9.8	
		19-HEPE	0.07	0.83 ± 0.14	17	
		20-HEPE	0.10	0.62 ± 0.100	16	
DHA	epoxy-PUFA	10(11)-EpDPE	0.03	0.14 ± 0.020	14	
		13(14)-EpDPE	0.05	0.067 ± 0.025	37	
		16(17)-EpDPE	0.05	0.083 ± 0.023	27	
		19(20)-EpDPE	0.05	0.23 ± 0.026	12	
	vic dihydroxy-PUFA	4,5-DiHDPE	0.20	0.55 ± 0.068	12	
		10,11-DiHDPE	0.03	0.20 ± 0.016	8.1	
		13,14-DiHDPE	0.03	0.21 ± 0.017	8.3	
		16,17-DiHDPE	0.05	0.29 ± 0.021	7.2	
		19,20-DiHDPE	0.05	3.1 ± 0.19	6.2	
	hydroxy-PUFA	4-HDHA	0.03	0.17 ± 0.015	9.2	
		8-HDHA	0.05	0.44 ± 0.051	12	
		10-HDHA	0.05	0.086 ± 0.012	14	
		11-HDHA	0.03	0.16 ± 0.034	21	
		13-HDHA	0.05	0.12 ± 0.020	17	
		14-HDHA	0.10	0.94 ± 0.21	22	
		16-HDHA	0.03	0.15 ± 0.015	10	
		17-HDHA	0.20	0.66 ± 0.11	17	
		20-HDHA	0.05	0.34 ± 0.038	11	
		21-HDHA	0.17	2.8 ± 0.25	9.0	
		22-HDHA	0.28	2.2 ± 0.18	8.2	
oleic acid	epoxy-PUFA	9(10)-Ep-stearic acid	0.20	11 ± 1.3	12	
	vic dihydroxy-PUFA	9,10-DiH-stearic acid	0.20	7.5 ± 0.64	8.5	

Legend:



Variance (x)
x < 10%
10% < x < 20%
x > 20%

* Lower limit of quantification [nM] in plasma

Tab. S5: Concentration of oxylipins found in plasma of study participants from the study on inter-day variation of free oxylipins in plasma on a non-standardized diet (Study A). Shown are mean ± SEM (n=18). The LLOQ is shown in case the analyte's concentration was <LLOQ in more than 50% of the samples. All analytes are shown which were quantified at least at one time point. The color code refers to the analytes' variation in quality control plasma (relative 95%SD <10%: green, 10-20%: orange, >20% red; see Tab. S4). Statistics were done with paired t-tests and Wilcoxon-tests. No statistical differences between t0 and t48 were found ($p \leq 0.05$; p-values not shown).

Analytes			color code (see Table S4)	t0	t48	factor high *vs.* low*	within analytical variance?[+]
LA	epoxy-PUFA	9(10)-EpOME	orange	1.7 ± 0.23	1.4 ± 0.16	1.26	N
		12(13)-EpOME	green	4.1 ± 0.61	3.3 ± 0.47	1.23	N
	vic dihydroxy-PUFA	9,10-DiHOME	green	5.3 ± 0.76	4.7 ± 0.66	1.12	N
		12,13-DiHOME	green	5.9 ± 0.65	5.9 ± 0.73	1.00	Y
	hydroxy-PUFA	9-HODE	green	14 ± 1.9	15 ± 2.6	1.12	N
		13-HODE	green	20 ± 2.9	23 ± 3.6	1.11	N
DGLA	prostanoids	13,14-dihydro-15-keto-PGE$_1$		0.11 ± 0.026	0.074 ± 0.015	1.56	-
	hydroxy-FA	15-HETrE	green	0.26 ± 0.017	0.27 ± 0.019	1.01	Y
ARA	prostanoids	PGE$_2$	orange	0.079 ± 0.015	0.059 ± 0.010	1.33	N
		13,14-dihydro-15-keto-PGF$_{2\alpha}$	red	0.14 ± 0.007	0.15 ± 0.009	1.08	Y
		TxB$_2$	orange	0.48 ± 0.057	0.53 ± 0.055	1.10	Y
	epoxy-PUFA	5(6)-EpETrE		0.37 ± 0.066	0.34 ± 0.046	1.09	-
		8(9)-EpETrE		0.37 ± 0.059	0.44 ± 0.075	1.21	-
		11(12)-EpETrE	red	0.062 ± 0.011	0.063 ± 0.007	1.02	Y
		14(15)-EpETrE	orange	0.093 ± 0.012	0.093 ± 0.011	1.00	Y
	vic dihydroxy-PUFA	5,6-DiHETrE	red	0.21 ± 0.022	0.22 ± 0.014	1.03	Y
		8,9-DiHETrE	green	0.23 ± 0.014	0.24 ± 0.013	1.06	Y
		11,12-DiHETrE	green	0.58 ± 0.046	0.59 ± 0.037	1.01	Y
		14,15-DiHETrE	green	0.69 ± 0.044	0.69 ± 0.033	1.00	Y
	hydroxy-PUFA	5-HETE	green	0.54 ± 0.069	0.58 ± 0.063	1.08	Y
		8-HETE	orange	0.31 ± 0.023	0.31 ± 0.019	1.01	Y
		11-HETE	green	0.26 ± 0.021	0.25 ± 0.018	1.01	Y
		15-HETE	orange	0.87 ± 0.073	0.89 ± 0.064	1.03	Y
		20-HETE	green	0.84 ± 0.15	0.83 ± 0.11	1.01	Y
ALA	epoxy-PUFA	9(10)-EpODE	red	0.18 ± 0.025	0.16 ± 0.022	1.12	Y
		12(13)-EpODE	orange	0.13 ± 0.019	0.11 ± 0.013	1.17	Y
		15(16)-EpODE	green	3.7 ± 0.73	3.1 ± 0.43	1.21	N
	vic dihydroxy-PUFA	9,10-DiHODE	green	0.41 ± 0.14	0.28 ± 0.045	1.45	N
		12,13-DiHODE	orange	0.28 ± 0.034	0.28 ± 0.032	1.01	Y
		15,16-DiHODE	green	18 ± 3.0	16 ± 2.1	1.13	N
	hydroxy-PUFA	9-HOTrE	green	0.70 ± 0.071	0.78 ± 0.13	1.11	N
		13-HOTrE	green	1.0 ± 0.11	1.3 ± 0.30	1.26	N
EPA	prostanoids	TxB$_3$		< 0.025	0.042 ± 0.007	-	
	vic dihydroxy-PUFA	8,9-DiHETE	orange	< 0.05	< 0.05	-	
		11,12-DiHETE	orange	0.041 ± 0.006	0.033 ± 0.004	1.24	N
		14,15-DiHETE	orange	0.083 ± 0.008	0.076 ± 0.006	1.10	Y
		17,18-DiHETE	green	0.50 ± 0.054	0.47 ± 0.037	1.06	Y
	hydroxy-PUFA	5-HEPE	orange	0.16 ± 0.022	0.16 ± 0.017	1.02	Y
		15-HEPE	orange	0.13 ± 0.016	< 0.13	-	
		18-HEPE	green	0.20 ± 0.022	0.18 ± 0.016	1.07	Y
		19-HEPE	orange	0.68 ± 0.092	0.68 ± 0.067	1.01	Y
		20-HEPE	orange	0.41 ± 0.068	0.43 ± 0.048	1.05	Y
DHA	epoxy-PUFA	13(14)-EpDPE	red	0.13 ± 0.018	0.13 ± 0.015	1.00	Y
		19(20)-EpDPE	orange	0.22 ± 0.032	0.23 ± 0.029	1.06	Y
	vic dihydroxy-PUFA	4,5-DiHDPE	orange	0.66 ± 0.070	0.72 ± 0.078	1.09	Y
		10,11-DiHDPE	green	0.17 ± 0.021	0.17 ± 0.018	1.01	Y
		13,14-DiHDPE	green	0.22 ± 0.019	0.22 ± 0.018	1.02	Y
		16,17-DiHDPE	green	0.30 ± 0.025	0.29 ± 0.022	1.04	Y
		19,20-DiHDPE	green	2.7 ± 0.24	2.7 ± 0.22	1.01	Y
	hydroxy-PUFA	4-HDHA	green	0.28 ± 0.042	0.28 ± 0.039	1.00	Y
		7-HDHA		0.14 ± 0.016	0.15 ± 0.020	1.10	-
		8-HDHA	orange	0.44 ± 0.055	0.44 ± 0.055	1.01	Y
		10-HDHA	orange	0.27 ± 0.030	0.29 ± 0.031	1.06	Y
		13-HDHA	orange	0.17 ± 0.020	0.16 ± 0.018	1.00	Y
		16-HDHA	orange	0.17 ± 0.013	0.17 ± 0.013	1.01	Y
		17-HDHA	orange	0.78 ± 0.083	0.86 ± 0.12	1.11	Y
		20-HDHA	orange	0.41 ± 0.037	0.42 ± 0.040	1.03	Y
		21-HDHA	green	2.0 ± 0.17	2.1 ± 0.24	1.06	Y
		22-HDHA	green	2.1 ± 0.27	2.2 ± 0.29	1.03	Y
Oleic Acid	epoxy-PUFA	9(10)-Ep-stearic acid	orange	11 ± 1.2	8.8 ± 0.81	1.23	N
	vic dihydroxy-PUFA	9,10-DiH-stearic acid	green	6.0 ± 0.78	5.9 ± 0.79	1.01	Y

* Higher mean concentration of both time points (t0 and t48) was divided by the lower mean concentration

[+] The factor 'high *vs.* low' was compared against the analytical variance; Y – variation between time points within analytical variance; N – variation between time point not within analytical variance

Tab. S6: Concentration of oxylipins found in plasma of study participants from the study on inter-day and intra-day variation of free oxylipins in plasma on a standardized diet (Study B). Shown are mean ± SEM (n=13) at t24, t48 and t72. The LLOQ is shown in case the analyte's concentration was <LLOQ in more than 50% of the samples. All analytes are shown which were quantified at least at one time point. The color code refers to the analytes' variation in quality control plasma (relative 95%SD <10%: green, 10-20%: orange, >20% red; see Tab. S4). Statistics for normally distributed variables were done with ANOVA with repeated measurements and post-hoc t-Tests for paired samples with Bonferroni-correction and for not-normally distributed variables with Friedman Tests and post-hoc Dunn-Bonferroni tests; significance level p ≤ 0.05.

Analytes			color code (see Table S4)	t24	t48	P t24-48	t72	P t24-72	P Friedman/ ANreM	factor high *vs.* low*	within analytical variance?+
LA	epoxy-PUFA	9(10)-EpOME		1.7 ± 0.36	1.4 ± 0.25	-	1.4 ± 0.26	-	n.s.	1.27	N
		12(13)-EpOME		5.0 ± 1.2	4.8 ± 1.2	-	4.7 ± 1.1	-	n.s.	1.06	Y
	vic dihydroxy-PUFA	9,10-DiHOME		4.3 ± 0.55	4.3 ± 0.62	-	4.2 ± 0.72	-	n.s.	1.04	Y
		12,13-DiHOME		6.6 ± 0.54	6.6 ± 0.97	-	6.6 ± 1.1	-	n.s.	1.01	Y
	hydroxy-PUFA	9-HODE		11 ± 1.1	10 ± 0.93	-	9.8 ± 0.89	-	n.s.	1.17	N
		13-HODE		14 ± 1.2	13 ± 1.2	-	13 ± 1.3	-	n.s.	1.09	Y
	others	13-oxo-ODE		0.20 ± 0.019	0.19 ± 0.036	-	0.19 ± 0.030	-	n.s.	1.05	Y
		9-oxo-ODE		1.2 ± 0.13	1.0 ± 0.064	-	0.95 ± 0.046	-	n.s.	1.29	N
DGLA	prostanoids	13,14-dihydro-15-keto-PGE_1		0.11 ± 0.031	< 0.05	-	0.15 ± 0.059	-	-	1.42	-
	hydroxy-PUFA	5-HETrE		0.13 ± 0.011	0.10 ± 0.009	-	0.11 ± 0.010	-	n.s.	1.22	N
		15-HETrE		0.44 ± 0.021	0.43 ± 0.019	-	0.41 ± 0.020	-	n.s.	1.08	Y
ARA	prostanoids	PGD_2		0.20 ± 0.018	0.19 ± 0.028	-	0.20 ± 0.024	-	n.s.	1.02	-
		PGE_2		0.24 ± 0.030	0.27 ± 0.043	-	0.27 ± 0.037	-	n.s.	1.12	Y
		$PGF_{2\alpha}$		4.7 ± 1.1	4.6 ± 1.1	-	4.6 ± 1.1	-	n.s.	1.02	Y
		13,14-dihydro-15-keto-PGF_{2a}		0.16 ± 0.015	0.17 ± 0.020	-	0.16 ± 0.016	-	n.s.	1.07	Y
		TxB_2		0.46 ± 0.049	0.45 ± 0.068	-	0.40 ± 0.055	-	n.s.	1.14	Y
	isoprostanes	5(*R,S*)-5-F_{2t}-IsoP		0.11 ± 0.006	0.096 ± 0.005	n.s.	0.095 ± 0.007	0.032	0.020	1.13	Y
	epoxy-PUFA	11(12)-EpETrE		0.087 ± 0.007	0.089 ± 0.007	-	0.077 ± 0.006	-	n.s.	1.16	Y
		14(15)-EpETrE		0.20 ± 0.017	0.20 ± 0.015	-	0.18 ± 0.012	-	n.s.	1.12	Y
	vic dihydroxy-PUFA	5,6-DiHETrE		0.30 ± 0.020	0.28 ± 0.022	-	0.28 ± 0.020	-	n.s.	1.07	Y
		8,9-DiHETrE		0.22 ± 0.013	0.21 ± 0.013	n.s.	0.21 ± 0.014	0.024	0.005	1.07	Y
		11,12-DiHETrE		0.56 ± 0.025	0.54 ± 0.024	-	0.53 ± 0.026	-	n.s.	1.05	Y
		14,15-DiHETrE		0.67 ± 0.031	0.67 ± 0.029	-	0.70 ± 0.030	-	n.s.	1.04	Y
	hydroxy-PUFA	5-HETE		0.96 ± 0.11	0.63 ± 0.042	0.032	0.59 ± 0.040	0.003	0.002	1.62	N
		8-HETE		0.39 ± 0.027	0.32 ± 0.015	0.032	0.31 ± 0.018	0.007	0.004	1.27	N
		9-HETE		0.39 ± 0.032	0.30 ± 0.030	-	0.33 ± 0.029	-	n.s.	1.30	-
		11-HETE		0.61 ± 0.035	0.57 ± 0.053	-	0.51 ± 0.037	-	n.s.	1.19	N
		12-HETE		1.5 ± 0.16	1.6 ± 0.12	-	1.4 ± 0.12	-	n.s.	1.08	Y
		15-HETE		1.2 ± 0.075	1.1 ± 0.060	n.s.	1.0 ± 0.082	0.043	0.032	1.15	Y
		20-HETE		0.87 ± 0.048	0.87 ± 0.074	-	0.77 ± 0.050	-	n.s.	1.14	N
		12-HHTrE		0.74 ± 0.079	0.71 ± 0.098	-	0.65 ± 0.078	-	n.s.	1.13	N
		15-oxo-ETE		0.070 ± 0.008	< 0.05	-	< 0.05	-	-	-	

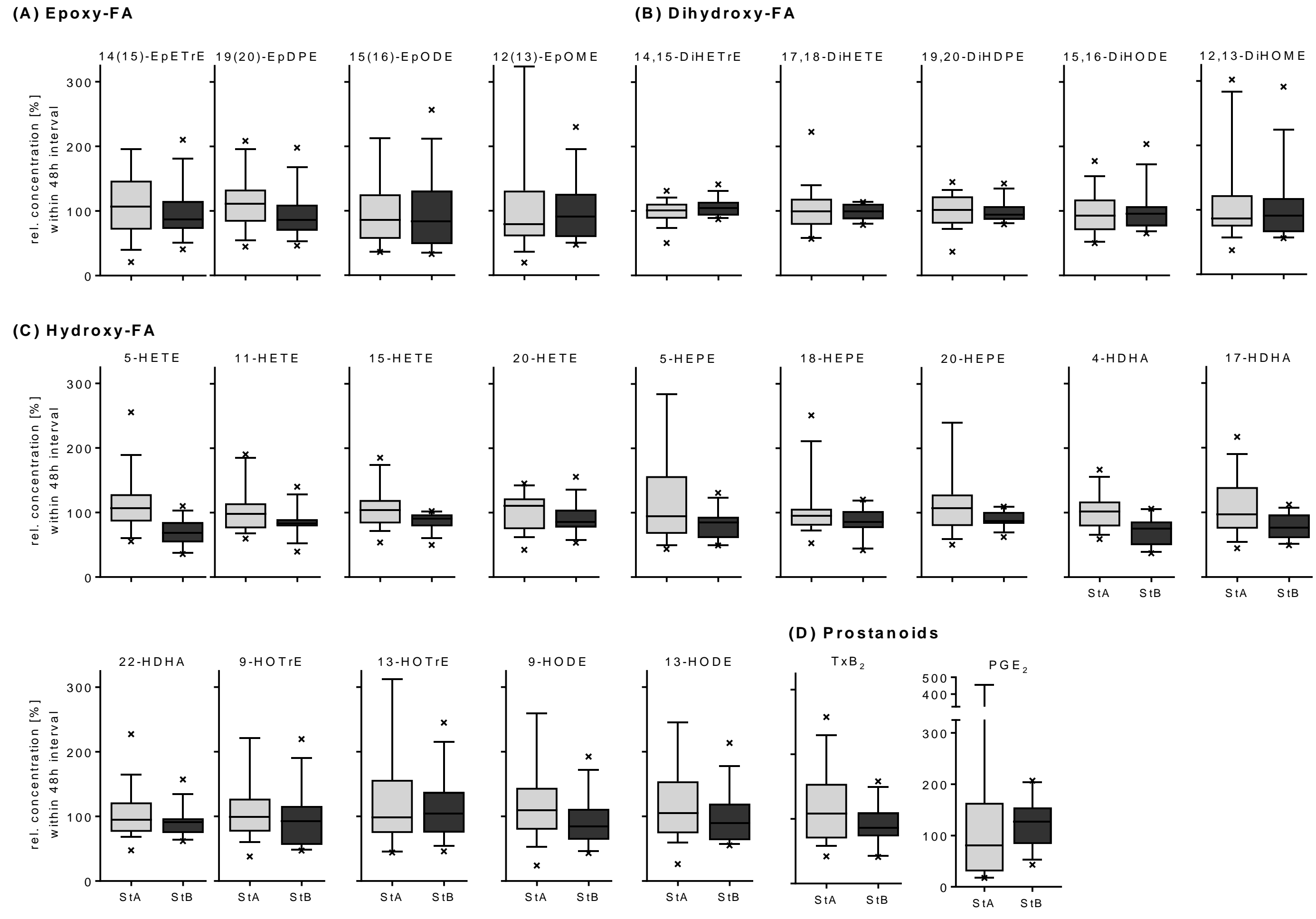
Tab. S6: Continued.

Analytes			color code (see Table S4)	t24	t48	P t24-48	t72	P t24-72	P Friedman/ ANreM	factor high *vs.* low*	within analytical variance?+
ALA	epoxy-PUFA	9(10)-EpODE		0.20 ± 0.032	0.18 ± 0.037	-	0.17 ± 0.035	-	n.s.	1.17	Y
		12(13)-EpODE		0.20 ± 0.019	0.20 ± 0.037	-	0.19 ± 0.034	-	n.s.	1.09	Y
		15(16)-EpODE		3.7 ± 0.51	3.2 ± 0.70	-	3.1 ± 0.61	-	n.s.	1.03	Y
	vic dihydroxy-PUFA	9,10-DiHODE		0.21 ± 0.019	0.22 ± 0.051	-	0.23 ± 0.057	-	n.s.	1.06	Y
		12,13-DiHODE		0.28 ± 0.030	0.30 ± 0.067	-	0.32 ± 0.077	-	n.s.	1.16	Y
		15,16-DiHODE		12 ± 1.0	12 ± 1.4	-	12 ± 1.5	-	n.s.	1.02	Y
	hydroxy-PUFA	9-HOTrE		0.60 ± 0.061	0.51 ± 0.065	-	0.54 ± 0.066	-	n.s.	1.19	N
		13-HOTrE		0.64 ± 0.064	0.57 ± 0.079	-	0.67 ± 0.085	-	n.s.	1.18	N
EPA	vic dihydroxy-PUFA	8,9-DiHETE		0.056 ± 0.006	< 0.05	-	< 0.05	-	-	-	
		11,12-DiHETE		0.037 ± 0.003	0.034 ± 0.002	-	0.034 ± 0.002	-	n.s.	1.11	Y
		14,15-DiHETE		0.068 ± 0.004	0.066 ± 0.004	-	0.070 ± 0.004	-	n.s.	1.05	Y
		17,18-DiHETE		0.46 ± 0.028	0.44 ± 0.031	-	0.45 ± 0.028	-	n.s.	1.05	Y
	hydroxy-PUFA	5-HEPE		0.15 ± 0.011	0.12 ± 0.009	n.s.	0.11 ± 0.009	0.010	0.005	1.27	N
		9-HEPE		0.067 ± 0.005	0.054 ± 0.006	-	0.055 ± 0.007	-	n.s.	1.23	N
		12-HEPE		0.14 ± 0.018	0.15 ± 0.012	-	0.13 ± 0.012	-	n.s.	1.16	Y
		18-HEPE		0.13 ± 0.007	0.12 ± 0.010	-	0.11 ± 0.011	-	n.s.	1.14	N
		19-HEPE		0.61 ± 0.046	0.60 ± 0.058	-	0.58 ± 0.047	-	n.s.	1.04	Y
		20-HEPE		0.54 ± 0.045	0.51 ± 0.041	-	0.48 ± 0.045	-	n.s.	1.11	Y
DHA	epoxy-PUFA	10(11)-EpDPE		0.082 ± 0.011	0.066 ± 0.006	-	0.064 ± 0.005	-	n.s.	1.24	N
		16(17)-EpDPE		0.059 ± 0.005	0.054 ± 0.006	-	< 0.05	-	-	-	
		19(20)-EpDPE		0.13 ± 0.012	0.12 ± 0.009	-	0.11 ± 0.009	-	n.s.	1.15	Y
	vic dihydroxy-PUFA	4,5-DiHDPE		0.39 ± 0.046	0.35 ± 0.034	-	0.34 ± 0.039	-	n.s.	1.13	Y
		10,11-DiHDPE		0.13 ± 0.014	0.11 ± 0.009	-	0.12 ± 0.011	-	n.s.	1.12	N
		13,14-DiHDPE		0.16 ± 0.015	0.16 ± 0.012	-	0.15 ± 0.013	-	n.s.	1.06	Y
		16,17-DiHDPE		0.20 ± 0.015	0.20 ± 0.012	-	0.21 ± 0.013	-	n.s.	1.04	Y
		19,20-DiHDPE		1.9 ± 0.15	1.8 ± 0.14	-	1.8 ± 0.12	-	n.s.	1.03	Y
	hydroxy-PUFA	4-HDHA		0.18 ± 0.021	0.12 ± 0.008	0.034	0.11 ± 0.006	0.009	<0.001	1.60	N
		8-HDHA		0.37 ± 0.032	0.29 ± 0.019	n.s.	0.27 ± 0.016	0.007	0.004	1.38	N
		10-HDHA		0.083 ± 0.011	0.065 ± 0.005	n.s.	0.060 ± 0.005	0.034	0.009	1.38	N
		11-HDHA		0.13 ± 0.013	0.12 ± 0.007	-	0.11 ± 0.007	-	n.s.	1.15	Y
		13-HDHA		0.16 ± 0.017	0.14 ± 0.015	-	0.12 ± 0.011	-	n.s.	1.35	N
		14-HDHA		0.86 ± 0.14	0.91 ± 0.11	-	0.78 ± 0.078	-	n.s.	1.17	Y
		16-HDHA		0.26 ± 0.019	0.26 ± 0.024	-	0.25 ± 0.029	-	n.s.	1.05	Y
		17-HDHA		0.47 ± 0.033	0.42 ± 0.031	n.s.	0.36 ± 0.031	0.010	0.005	1.31	N
		20-HDHA		0.28 ± 0.018	0.26 ± 0.013	n.s.	0.24 ± 0.013	0.026	0.015	1.15	Y
		21-HDHA		1.6 ± 0.17	1.5 ± 0.12	-	1.5 ± 0.13	-	n.s.	1.08	Y
		22-HDHA		1.3 ± 0.14	1.3 ± 0.11	-	1.2 ± 0.12	-	n.s.	1.13	N
Oleic Acid	epoxy-PUFA	9(10)-Ep-stearic acid		12 ± 2.1	10 ± 1.5	-	9.8 ± 1.5	-	n.s.	1.25	N
	vic dihydroxy-PUFA	9,10-DiH-stearic acid		7.3 ± 0.91	7.7 ± 1.5	-	7.4 ± 1.6	-	n.s.	1.06	Y

* Highest mean concentration from the three time points (t24, t48 and t72) was divided by the lowest mean concentration

+ The factor 'high *vs.* low' was compared against the analytical variance; Y – variation between time point within analytical variance; N – variation between time point not within analytical variance

Fig. S3: Comparison of inter-day variations of circulating oxylipins in subjects on a standardized (StA) and non-standardized (StB) diet. Shown are relative concentrations ± SEM of selected epoxy-PUFA (**A**), dihydroxy-PUFA (**B**), hydroxy-PUFA (**C**), prostanoids (**D**) (n=18 for StA; n=13 for StB). In StA, plasma was collected from study participants at baseline (t0) and after 48h without intervention; in StB plasma was collected after 24 and 72 h on a standardized diet. Relative concentrations of oxylipins for StA were calculated for t48 against baseline and for StB relative concentrations after 72 h on the standardized diet were calculated against 24 h (i.e. 48 h time interval between sample collections).



Tab. S7: Concentration of oxylipins found in plasma of study participants from the study on inter-day and intra-day variation of free oxylipins in plasma on a standardized diet (Study B). Shown are mean ± SEM (n=13) at t0, t2, t4, t6 and t8. The LLOQ is shown in case the analyte's concentration was <LLOQ in more than 50% of the samples. All analytes are shown which were quantified at least at one time point. Statistics for normally distributed variables were done with ANOVA with repeated measurements and post-hoc t-Tests for paired samples with Bonferroni-correction and for not-normally distributed variables with Friedman and post-hoc Dunn-Bonferroni tests; significance level $p \leq 0.05$.

Analyte			**t0**	**t2**	**P t0-2**	**t4**	**P t0-4**	**t6**	**P t0-6**	**t8**	**P t0-8**	**P Friedman/ ANreM**
LA	epoxy-PUFA	9(10)-EpOME	2.0 ± 0.32	1.5 ± 0.24	n.s.	2.7 ± 0.29	n.s.	0.97 ± 0.091	n.s.	0.59 ± 0.081	**< 0.001**	**< 0.001**
		12(13)-EpOME	6.1 ± 1.2	17 ± 4.4	**0.005**	23 ± 3.4	**< 0.001**	8.6 ± 1.1	n.s.	3.1 ± 0.40	n.s.	**< 0.001**
	vic dihydroxy-PUFA	9,10-DiHOME	5.7 ± 0.60	9.1 ± 0.72	n.s.	8.9 ± 0.69	n.s.	2.3 ± 0.20	n.s.	2.7 ± 0.57	n.s.	**< 0.001**
		12,13-DiHOME	8.6 ± 0.99	18 ± 1.6	**< 0.001**	16 ± 1.2	**< 0.001**	5.3 ± 0.57	**< 0.001**	4.0 ± 0.72	**0.011**	**< 0.001**
	hydroxy-PUFA	9-HODE	14 ± 1.2	18 ± 1.6	n.s.	28 ± 1.7	**0.006**	13 ± 1.2	n.s.	6.8 ± 0.67	n.s.	**< 0.001**
		13-HODE	16 ± 1.4	38 ± 3.4	**0.029**	42 ± 2.8	**0.005**	16 ± 1.5	n.s.	7.7 ± 0.93	n.s.	**< 0.001**
	others	13-oxo-ODE	0.22 ± 0.022	0.81 ± 0.13	-	0.96 ± 0.088	-	0.40 ± 0.033	-	< 0.1	-	-
		9-oxo-ODE	1.3 ± 0.061	1.0 ± 0.035	n.s.	1.3 ± 0.061	n.s.	1.0 ± 0.058	n.s.	0.82 ± 0.039	**< 0.001**	**< 0.001**
DGLA	prostanoids	13,14-dihydro-15-keto-PGE$_1$	< 0.05	< 0.05	-	0.12 ± 0.036	-	< 0.05	-	< 0.05	-	-
	hydroxy-PUFA	5(S) HETrE	0.12 ± 0.011	0.036 ± 0.006	**< 0.001**	0.072 ± 0.009	n.s.	0.041 ± 0.007	**< 0.001**	0.040 ± 0.008	**< 0.001**	**< 0.001**
		15(S)-HETrE	0.42 ± 0.022	0.22 ± 0.009	**< 0.001**	0.37 ± 0.026	n.s.	0.26 ± 0.016	**< 0.001**	0.32 ± 0.021	**0.020**	**< 0.001**
ARA	prostanoids	PGD$_2$	< 0.1	< 0.1	-	< 0.1	-	0.16 ± 0.016	-	0.19 ± 0.026	-	-
		PGE$_2$	0.088 ± 0.016	0.079 ± 0.014	n.s.	0.10 ± 0.018	n.s.	0.17 ± 0.028	**0.011**	0.24 ± 0.037	**0.019**	**< 0.001**
		PGF$_{2\alpha}$	5.3 ± 1.2	4.1 ± 0.97	n.s.	4.1 ± 0.97	n.s.	3.9 ± 0.98	n.s.	4.0 ± 0.95	n.s.	**0.011**
		13,14-dihydro-15-keto-PGF$_{2\alpha}$	0.14 ± 0.020	< 0.05	n.s.	0.088 ± 0.011	n.s.	0.066 ± 0.005	n.s.	0.074 ± 0.012	n.s.	**0.001**
		TxB$_2$	0.23 ± 0.031	0.19 ± 0.030	n.s.	0.29 ± 0.052	n.s.	0.32 ± 0.046	n.s.	0.48 ± 0.063	**0.029**	**< 0.001**
	isoprostanes	5(*R*,*S*)-5-F$_{2t}$-IsoP	0.10 ± 0.008	0.065 ± 0.006	**0.002**	0.096 ± 0.005	n.s.	0.073 ± 0.007	**0.024**	0.075 ± 0.006	n.s.	**< 0.001**
	epoxy-PUFA	8(9)-EpETrE	0.11 ± 0.015	< 0.1	-	< 0.1	-	< 0.1	-	< 0.1	-	-
		11(12)-EpETrE	0.086 ± 0.008	0.038 ± 0.004	**< 0.001**	0.058 ± 0.005	n.s.	0.043 ± 0.004	**< 0.001**	0.038 ± 0.004	**< 0.001**	**< 0.001**
		14(15)-EpETrE	0.20 ± 0.016	0.11 ± 0.009	**0.001**	0.15 ± 0.012	n.s.	0.10 ± 0.010	**< 0.001**	0.10 ± 0.007	**< 0.001**	**< 0.001**
	vic dihydroxy-PUFA	5,6-DiHETrE	0.29 ± 0.024	0.25 ± 0.020	n.s.	0.30 ± 0.025	n.s.	0.23 ± 0.019	**0.015**	0.23 ± 0.021	**0.026**	**< 0.001**
		8,9-DiHETrE	0.22 ± 0.014	0.19 ± 0.013	n.s.	0.23 ± 0.017	n.s.	0.19 ± 0.013	n.s.	0.18 ± 0.013	**0.008**	**< 0.001**
		11,12-DiHETrE	0.55 ± 0.027	0.40 ± 0.019	**< 0.001**	0.59 ± 0.037	n.s.	0.41 ± 0.028	**0.002**	0.40 ± 0.031	**0.002**	**< 0.001**
		14,15-DiHETrE	0.70 ± 0.036	0.51 ± 0.025	**< 0.001**	0.69 ± 0.036	n.s.	0.54 ± 0.029	**0.001**	0.55 ± 0.033	**0.001**	**< 0.001**
	hydroxy-PUFA	5-HETE	0.80 ± 0.050	0.45 ± 0.036	**< 0.001**	0.55 ± 0.034	**< 0.001**	0.40 ± 0.029	**< 0.001**	0.40 ± 0.029	**< 0.001**	**< 0.001**
		8-HETE	0.38 ± 0.024	0.20 ± 0.015	**< 0.001**	0.24 ± 0.016	**< 0.001**	0.19 ± 0.011	**< 0.001**	0.18 ± 0.017	**< 0.001**	**< 0.001**
		9-HETE	0.27 ± 0.019	< 0.25	-	< 0.25	-	< 0.25	-	0.25 ± 0.027	-	-
		11-HETE	0.38 ± 0.018	0.23 ± 0.014	**< 0.001**	0.33 ± 0.027	n.s.	0.32 ± 0.026	n.s.	0.45 ± 0.041	n.s.	**< 0.001**
		12-HETE	2.1 ± 0.24	0.99 ± 0.14	**< 0.001**	1.4 ± 0.12	n.s.	1.1 ± 0.11	**0.003**	0.96 ± 0.097	**< 0.001**	**< 0.001**
		15-HETE	1.0 ± 0.057	0.63 ± 0.048	**< 0.001**	0.88 ± 0.070	n.s.	0.79 ± 0.077	**0.024**	0.90 ± 0.093	n.s.	**< 0.001**
		19-HETE	< 1.0	< 1.0	-	< 1.0	-	< 1.0	-	< 1.0	-	-
		20-HETE	0.92 ± 0.057	0.49 ± 0.033	**< 0.001**	0.70 ± 0.051	**0.008**	0.45 ± 0.031	**< 0.001**	0.44 ± 0.040	**< 0.001**	**< 0.001**
		12-HHTrE	0.39 ± 0.045	0.31 ± 0.041	n.s.	0.44 ± 0.070	n.s.	0.49 ± 0.064	n.s.	0.71 ± 0.088	n.s.	**< 0.001**
		15-oxo-ETE	0.059 ± 0.009	< 0.05	-	0.053 ± 0.007	-	< 0.05	-	0.050 ± 0.007	-	-

Tab. S7: Continued.

Analyte			t0	t2	P t0-2	t4	P t0-4	t6	P t0-6	t8	P t0-8	P Friedman/ ANreM
ALA	epoxy-PUFA	9(10)-EpODE	0.23 ± 0.032	0.12 ± 0.020	n.s.	0.23 ± 0.024	n.s.	0.080 ± 0.009	**0.002**	0.056 ± 0.013	**< 0.001**	**< 0.001**
		12(13)-EpODE	0.20 ± 0.026	0.84 ± 0.14	**0.005**	0.99 ± 0.091	**< 0.001**	0.38 ± 0.031	n.s.	0.090 ± 0.016	n.s.	**< 0.001**
		15(16)-EpODE	4.4 ± 0.48	11 ± 1.3	**< 0.001**	16 ± 1.1	**< 0.001**	6.2 ± 0.56	n.s.	1.9 ± 0.30	**0.005**	**< 0.001**
	vic dihydroxy-PUFA	9,10-DiHODE	0.28 ± 0.046	0.46 ± 0.038	n.s.	0.49 ± 0.041	n.s.	0.16 ± 0.016	n.s.	0.14 ± 0.032	n.s.	**< 0.001**
		12,13-DiHODE	0.38 ± 0.066	0.56 ± 0.046	-	0.70 ± 0.054	-	0.20 ± 0.023	-	< 0.1	-	-
		15,16-DiHODE	15 ± 1.6	29 ± 1.7	**< 0.001**	27 ± 1.6	**< 0.001**	12 ± 0.52	n.s.	8.9 ± 1.3	**0.024**	**< 0.001**
	hydroxy-PUFA	9-HOTrE	0.75 ± 0.082	1.2 ± 0.11	**0.031**	1.7 ± 0.12	**< 0.001**	0.96 ± 0.092	n.s.	0.39 ± 0.043	**0.016**	**< 0.001**
		13-HOTrE	0.71 ± 0.10	2.3 ± 0.19	**0.002**	2.4 ± 0.16	**0.001**	1.5 ± 0.11	n.s.	0.56 ± 0.045	n.s.	**< 0.001**
EPA	vic dihydroxy-PUFA	8,9-DiHETE	< 0.05	< 0.05	-	< 0.05	-	< 0.05	-	< 0.05	-	-
		11,12-DiHETE	0.036 ± 0.003	< 0.025	-	0.038 ± 0.003	-	0.027 ± 0.002	-	< 0.025	-	-
		14,15-DiHETE	0.066 ± 0.005	0.055 ± 0.004	n.s.	0.070 ± 0.006	n.s.	0.061 ± 0.004	n.s.	0.058 ± 0.005	n.s.	**< 0.001**
		17,18-DiHETE	0.46 ± 0.027	0.42 ± 0.025	n.s.	0.53 ± 0.039	n.s.	0.46 ± 0.029	n.s.	0.45 ± 0.030	n.s.	**< 0.001**
	hydroxy-PUFA	5-HEPE	0.15 ± 0.011	0.060 ± 0.007	**< 0.001**	0.088 ± 0.009	n.s.	0.053 ± 0.007	**< 0.001**	0.056 ± 0.008	**< 0.001**	**< 0.001**
		9-HEPE	0.074 ± 0.006	< 0.05	-	< 0.05	-	< 0.05	-	< 0.05	-	-
		12-HEPE	0.18 ± 0.023	0.078 ± 0.014	**< 0.001**	0.11 ± 0.011	n.s.	0.092 ± 0.011	**0.010**	0.075 ± 0.009	**< 0.001**	**< 0.001**
		18-HEPE	0.14 ± 0.012	< 0.1	-	0.11 ± 0.012	-	< 0.1	-	0.097 ± 0.009	-	-
		19-HEPE	0.66 ± 0.054	0.51 ± 0.047	**0.006**	0.68 ± 0.065	n.s.	0.49 ± 0.038	**0.005**	0.45 ± 0.037	**0.002**	**< 0.001**
		20-HEPE	0.60 ± 0.050	0.44 ± 0.041	**< 0.001**	0.52 ± 0.042	**0.046**	0.43 ± 0.041	**< 0.001**	0.48 ± 0.054	**0.007**	**< 0.001**
DHA	epoxy-PUFA	10(11)-EpDPE	0.080 ± 0.010	0.036 ± 0.005	**0.002**	0.064 ± 0.007	n.s.	0.038 ± 0.005	**0.002**	0.026 ± 0.004	**< 0.001**	**< 0.001**
		16(17)-EpDPE	0.051 ± 0.008	< 0.05	-	< 0.05	-	< 0.05	-	< 0.05	-	
		19(20)-EpDPE	0.13 ± 0.012	0.061 ± 0.009	**0.002**	0.12 ± 0.015	n.s.	0.071 ± 0.010	**0.003**	0.059 ± 0.008	**0.001**	**< 0.001**
	vic dihydroxy-PUFA	4,5-DiHDPE	0.35 ± 0.044	0.21 ± 0.029	-	0.27 ± 0.036	-	< 0.2	-	< 0.2	-	-
		10,11-DiHDPE	0.12 ± 0.010	0.082 ± 0.007	**< 0.001**	0.12 ± 0.012	n.s.	0.092 ± 0.010	**0.001**	0.080 ± 0.008	**0.001**	**< 0.001**
		13,14-DiHDPE	0.16 ± 0.013	0.11 ± 0.008	**< 0.001**	0.16 ± 0.012	n.s.	0.12 ± 0.011	**< 0.001**	0.12 ± 0.010	**0.006**	**< 0.001**
		16,17-DiHDPE	0.20 ± 0.013	0.16 ± 0.010	**< 0.001**	0.22 ± 0.015	n.s.	0.19 ± 0.013	n.s.	0.18 ± 0.013	n.s.	**< 0.001**
		19,20-DiHDPE	1.9 ± 0.13	1.5 ± 0.11	**< 0.001**	2.1 ± 0.16	n.s.	1.8 ± 0.16	n.s.	1.7 ± 0.14	n.s.	**< 0.001**
	hydroxy-PUFA	4-HDHA	0.13 ± 0.012	0.078 ± 0.006	**< 0.001**	0.096 ± 0.008	**0.015**	0.070 ± 0.005	0.001	0.072 ± 0.003	**0.006**	**< 0.001**
		8-HDHA	0.39 ± 0.031	0.19 ± 0.018	**0.001**	0.25 ± 0.019	n.s.	0.16 ± 0.016	**< 0.001**	0.16 ± 0.015	**< 0.001**	**< 0.001**
		10-HDHA	0.078 ± 0.008	< 0.05	-	0.052 ± 0.005	-	< 0.05	-	< 0.05	-	-
		11-HDHA	0.14 ± 0.011	0.10 ± 0.007	**0.002**	0.12 ± 0.009	n.s.	0.093 ± 0.008	**< 0.001**	0.083 ± 0.005	**< 0.001**	**< 0.001**
		13-HDHA	0.12 ± 0.012	0.062 ± 0.007	**< 0.001**	0.092 ± 0.008	n.s.	0.079 ± 0.009	**0.019**	0.10 ± 0.009	n.s.	**< 0.001**
		14-HDHA	1.0 ± 0.15	0.52 ± 0.10	**< 0.001**	0.70 ± 0.11	n.s.	0.59 ± 0.082	**0.029**	0.45 ± 0.052	**< 0.001**	**< 0.001**
		16-HDHA	0.15 ± 0.008	0.10 ± 0.007	**0.010**	0.15 ± 0.014	n.s.	0.15 ± 0.015	n.s.	0.21 ± 0.021	n.s.	**< 0.001**
		17-HDHA	0.47 ± 0.048	0.24 ± 0.029	**< 0.001**	0.35 ± 0.036	n.s.	0.30 ± 0.047	**0.024**	0.28 ± 0.039	**0.004**	**< 0.001**
		20-HDHA	0.29 ± 0.020	0.18 ± 0.011	**< 0.001**	0.24 ± 0.013	n.s.	0.19 ± 0.014	**< 0.001**	0.19 ± 0.009	**< 0.001**	**< 0.001**
		21-HDHA	1.7 ± 0.15	0.73 ± 0.070	**< 0.001**	1.2 ± 0.12	**< 0.001**	0.70 ± 0.072	**< 0.001**	0.87 ± 0.12	**< 0.001**	**< 0.001**
		22-HDHA	1.4 ± 0.13	0.47 ± 0.043	**< 0.001**	0.96 ± 0.10	**0.004**	0.47 ± 0.059	**< 0.001**	0.55 ± 0.092	**< 0.001**	**< 0.001**
Oleic Acid	epoxy-PUFA	9(10)-Ep-stearic acid	14 ± 1.8	6.8 ± 0.37	**< 0.001**	9.1 ± 0.68	n.s.	7.2 ± 0.57	**0.001**	6.2 ± 0.47	**< 0.001**	**< 0.001**
	vic dihydroxy-PUFA	9,10-DiH-stearic acid	9.7 ± 1.6	4.2 ± 0.40	**< 0.001**	7.3 ± 1.1	n.s.	3.6 ± 0.35	**< 0.001**	5.2 ± 1.1	**< 0.001**	**< 0.001**

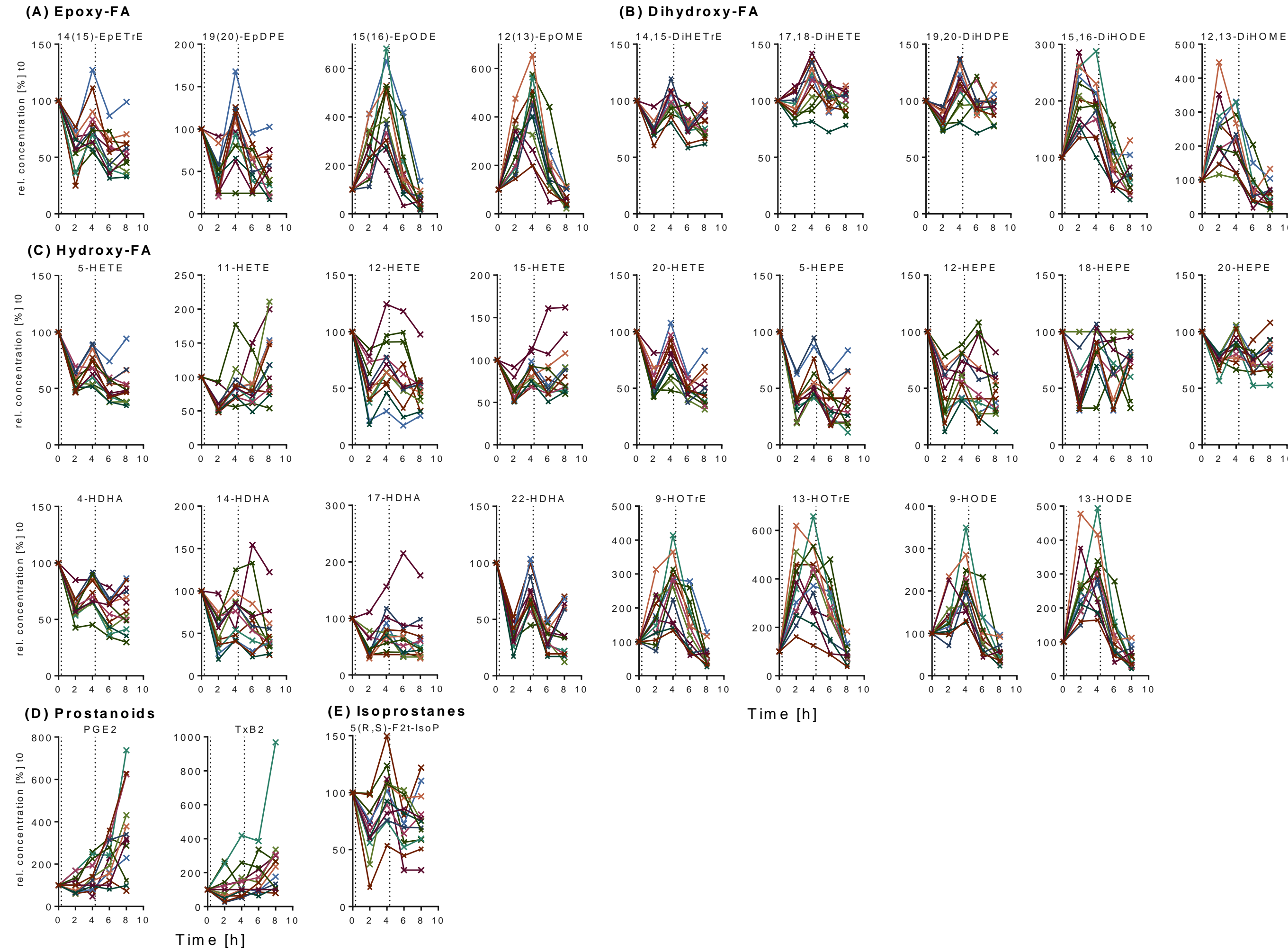


Fig. S4: Individual values of intra-day variation of circulating oxylipins in plasma of subjects on a standardized diet. Shown are relative individual concentrations of selected epoxy-PUFA (**A**), dihydroxy-PUFA (**B**), hydroxy-PUFA (**C**), prostanoids (**D**) and isoprostanes (**E**) (n=13). Plasma was collected from study participants on a standardized diet at baseline and t2, t4, t6 and t8. Relative concentrations of oxylipins were calculated against baseline. Dotted lines in the diagrams indicate food intake (20 min post sample collection at t0 and t4).

Appendix Acknowledgements

Diese Arbeit ist zwischen Januar 2016 und Oktober 2018 am Institut für Lebensmittelwissenschaft und Humanernährung der Leibniz Universität Hannover entstanden. Ich möchte mich an dieser Stelle bei allen bedanken, die mich während der Anfertigung meiner Dissertation begleitet und unterstützt haben.

Allen voran möchte ich mich bei meinem Doktorvater PD Dr. Jan Philipp Schuchardt bedanken, der mich in dieser Zeit hervorragend betreut hat und maßgeblich zum Gelingen der Dissertation und der in deren Rahmen entstandenen Publikationen beigetragen hat.

Ein ganz besonderer Dank gilt zudem Professor Dr. Andreas Hahn für sein Vertrauen bei der Überlassung des Themas, zahlreiche Gespräche mit wichtigen Denkanstößen und für die Übernahme des Prüfungsvorsitzes meiner Disputation. Außerdem bin ich dankbar für die Möglichkeit, meine Arbeit auf dem ISSFAL Kongress 2018 in Las Vegas präsentiert haben zu dürfen.

Bei Professor Dr. Thomas Scheper bedanke ich mich für das Interesse an meiner Thesis und die Übernahme des Korreferates.

Dr. Inga Schneider danke ich für die geduldige Beantwortung meiner Fragen und ihre Hilfestellung bei der Planung und Durchführung der Humanstudien.

Bei Kristina Wachau bedanke ich mich für die Übernahme zahlreicher bürokratischer Aufgaben und Heike Kohrs danke ich für die tatkräftige Unterstützung bei der Präanalytik.

Bedanken möchte ich mich auch bei Josefine Nebl, Paulina Wasserfurth, Katharina Mansouri und Dr. Sandra Gellert für die hilfreichen Gespräche in fachlicher aber auch persönlicher Hinsicht. Josefine Nebl gilt ein ganz besonderer Dank für die gewissenhafte Durchsicht meiner Dissertation und zahlreiche hilfreiche Anregungen bei der Finalisierung der Arbeit.

Außerdem möchte ich allen wissenschaftlichen Hilfskräften danken, die mich bei der Studienvorbereitung, an den Studientagen und bei der Dateneingabe unterstützt haben: Merle Armgard, Lea Weber, Holger Bartlau, Erik Dietrich, Carina Seipelt, Greta Teltemann, Hilke Maria Schönwald, Vivian Gaida und Kirsten Tinsen.

Dank gilt auch der kooperierenden Arbeitsgruppe von Professor Dr. Nils Helge Schebb, insbesondere Dr. Annika Ostermann und Laura Kutzner, die in den verschiedenen Publikationen dieser Arbeit mit mir zusammengearbeitet haben.

Ein großer Dank gilt meinem Freund Valentin, der mich immer unterstützt und ermutigt hat und mir bei zahlreichen Fragen mit seiner Außensicht weitergeholfen hat. Auch unserem Sohn Julius bin ich dankbar für die immer willkommene Ablenkung, die mir geholfen haben, den Blick fürs Wesentliche nicht aus den Augen zu verlieren.

Abschließend möchte ich mich bei meiner Mutter und ihrem Partner Sven für den Rückhalt und die Unterstützung mit Julius bedanken.

Appendix Curriculum vitae

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