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MS-based targeted metabolomics of eicosanoids and other oxylipins: Analytical and inter-individual variabilities

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A B S T R A C T

Oxylipins, including the well-known eicosanoids, are potent lipid mediators involved in numerous physiological and pathological processes. Therefore, their quantitative profiling has gained a lot of attention during the last years notably in the active field of health biomarker discovery. Oxylipins include hundreds of structurally and stereochemically distinct lipid species which today are most commonly analyzed by (ultra) high performance liquid chromatography-mass spectrometry based ((U)HPLC-MS) methods. To maximize the utility of oxylipin profiling in clinical research, it is crucial to understand and assess the factors contributing to the analytical and biological variability of oxylipin profiles in humans. In this review, these factors and their impacts are summarized and discussed, providing a framework for recommendations expected to enhance the interlaboratory comparability and biological interpretation of oxylipin profiling in clinical research.

1. Introduction

Eicosanoids and other oxylipins represent a superfamily of signalling lipids generated from arachidonic acid (AA) and related polyunsaturated fatty acids (PUFAs) through a complex network of biochemical reactions involving over 50 unique and cell-specific enzymes [1]. To regulate a wide array of biological processes, PUFAs are converted to oxylipins *via* four major pathways (Fig. 1): the cyclooxygenase (COX) pathway producing prostanoids such as prostaglandins (PGs) and thromboxanes (Txs); the lipoxygenase (LOX) pathway producing hydroperoxy-PUFAs which are rearranged into monohydroxy-PUFAs or further converted by LOX catalyzed reactions to leukotrienes (LTs) and numerous dihydroxy- and trihydroxy-PUFAs, including specialized pro-resolving mediators (e.g. lipoxins, resolvins, protectins); the cytochrome P450 pathway (CYP), primarily producing epoxy-PUFAs and ω-/ω-1 hydroxy-PUFAs, with the epoxides being further transformed to vicinal (i.e. adjacent or 1,2-) dihydroxy-PUFAs; and the non-enzymatic pathway producing various hydro(pero)xy-PUFAs, epoxy-PUFAs [2] as well as iso- and neuroprostanes [3,4]. In response to external stimuli (e.g. bradykinin, thrombin, inflammatory insult), PUFAs [5] and oxylipins [6] are released from membrane phospholipids by phospholipases including phospholipase A2 (PLA2). Of note, hundreds of structurally and stereochemically distinct oxylipins can be produced from AA and other PUFAs. For instance, depending on the stimuli and the cell type, AA and EPA can be converted into PGE_2 and PGE_3, respectively, through the COX pathway or structurally distinct oxylipins (e.g. LTB_4 and LTB_5) via the 5-LOX pathway (Fig. 1).

The structural specificity of oxylipins leads to specificity in their biological activities, many of which are still being elucidated. Important to clinical research, they are notably involved in the regulation of inflammation, thrombosis, endothelial function, vascular tone and insulin secretion, each of these systems being either stimulated or inhibited by the different oxylipin types as simplified in Fig. 2. Many oxylipins exert their biological effects by binding to cognate receptors, which are members of the G protein-coupled receptor (GPCR). However, for several oxylipins such as epoxy-PUFAs, the receptors characterized to date cannot explain all of the biological effects elicited by these compounds [7]. Other known routes of oxylipin elicited effects include directly influencing the open-state probability of membrane ion channels including the calcium-sensitive potassium channels (K_{Ca}) and transient receptor potential channels (TRPs) [8–10], activation of intracellular transcription factors such as peroxisome proliferator-activated receptors (PPARs) [11,12], and interference with intracellular signalling pathways such as NFκB [13–15].

Oxylipins derived from omega-3 fatty acids can be either more or less potent than or antagonistic to their omega-6-derived analogs [16], e.g. epoxy-omega-3-PUFAs are more potent antihypertensive compounds than their AA-derived counterparts [17]. Even two oxylipins derived from the same PUFA can be antagonists. For instance, TxA_2 and PGI_2, both derived from the oxygenation of AA through the COX-dependent metabolism, respectively activate or inhibit thrombosis. Such regulatory crosstalk among metabolic cascades is common [18–20].

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Abbreviations

(U)HPLC-MS	(ultra) high performance liquid chromatography-mass spectrometry
AA	arachidonic acid
PUFA	polyunsaturated fatty acid
COX	cyclooxygenase
PG	prostaglandin
Tx	thromboxane
LOX	lipoxygenase
LT	leukotriene
CYP	cytochrome P450
EPA	eicosapentaenoic acid
GPCR	G protein-coupled receptor
TRP	transient receptor potential channel
PPAR	peroxisome proliferator-activated receptor
NFκB	nuclear factor kappa B
DHA	docosahexaenoic acid
EpOME	epoxyoctadecenoic acid
HODE	hydroxyoctadecadienoic acid
KODE	ketooctadecadienoic acid
CRP	C-reactive protein
HETE	hydroxyeicosatetraenoic acid
HEPE	hydroxyeicosapentaenoic acid
HDHA	hydroxydocosahexaenoic acid
EDTA	ethylenediaminetetraacetic acid
TriHOME	trihydroxyoctadecenoic acid
BHT	butylated hydroxytoluene
SPE	solid phase extraction
ESI-MS	electrospray ionization-mass spectrometry
GLC-MS	gas liquid chromatography-mass spectrometry
QqQ-MS	triple quadrupole-mass spectrometry
TOF	time of flight
EpETrE	epoxyeicosatrienoic acid
SPM	specialized pro-resolving mediator
LX	lipoxin
MaR	maresin
PD	protectin
LLOQ	lower limit of quantification
LOD	limit of detection
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-CoA reductase
FLAP	5-lipoxygenase-activating protein
CVD	cardiovascular diseases
sEH	soluble epoxide hydrolase
CAD	coronary artery diseases
S/N	signal-to-noise

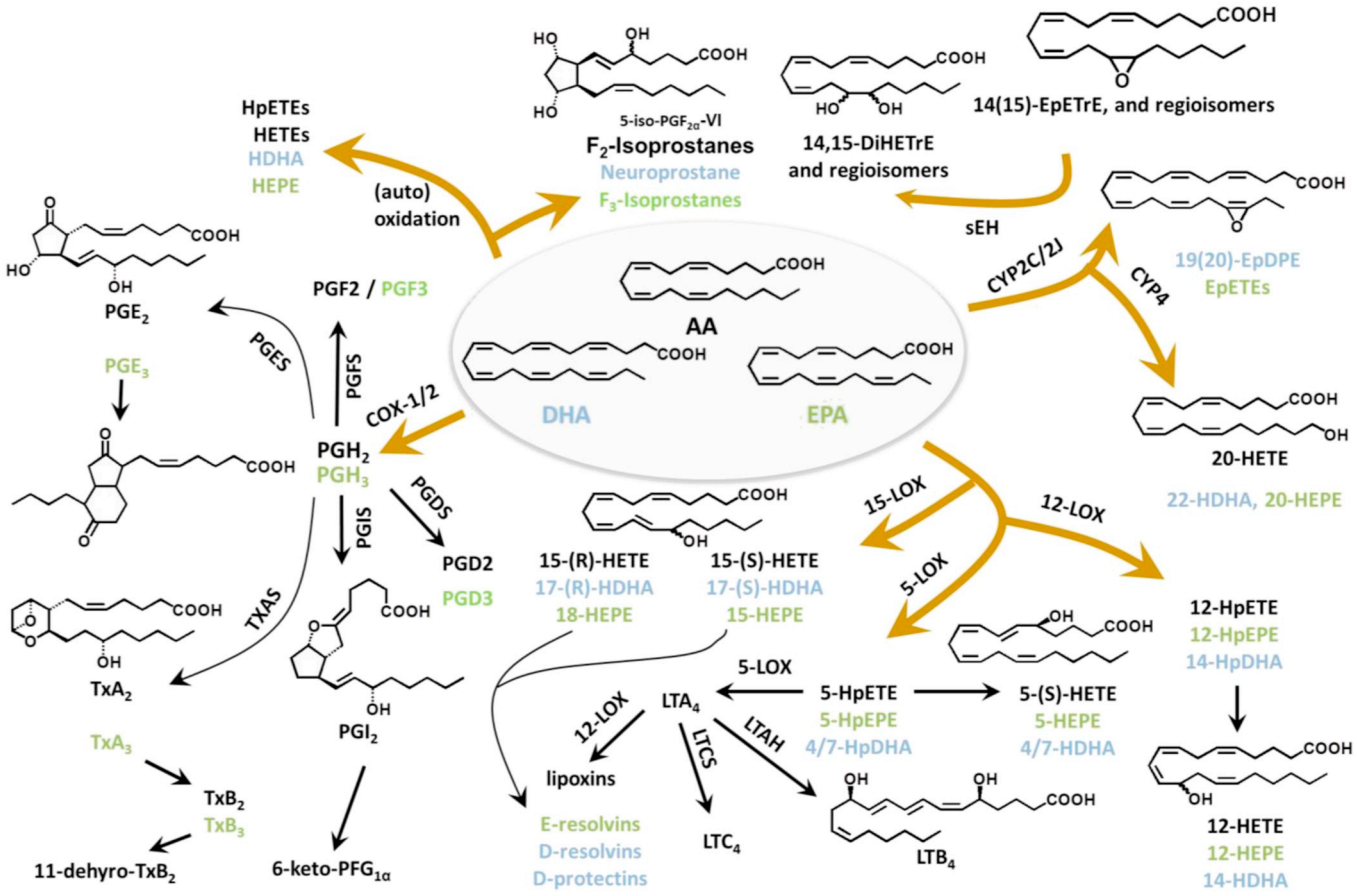

Fig. 1. Overview of the main eicosanoids and other oxylipins produced from AA, DHA and EPA through the COX, LOX, CYP and non-enzymatic pathways.

Finally, depending on the receptor, the tissue or the dose, a single oxylipin can also have opposite effects. For example, PGE_2 can exert either pro- or anti-aggregatory effects depending on its dose or the type of EP receptor it binds to Ref. [21]. Similarly, PGE_2 mediates lung inflammation in human cells [22], whereas it inhibits inflammatory signalling in murine peritoneal macrophages [23]. Moreover, while PGD_2 synthesis can have both pro- and anti-inflammatory impacts, and is synthesized by two convergent gene products [24], regulation of this

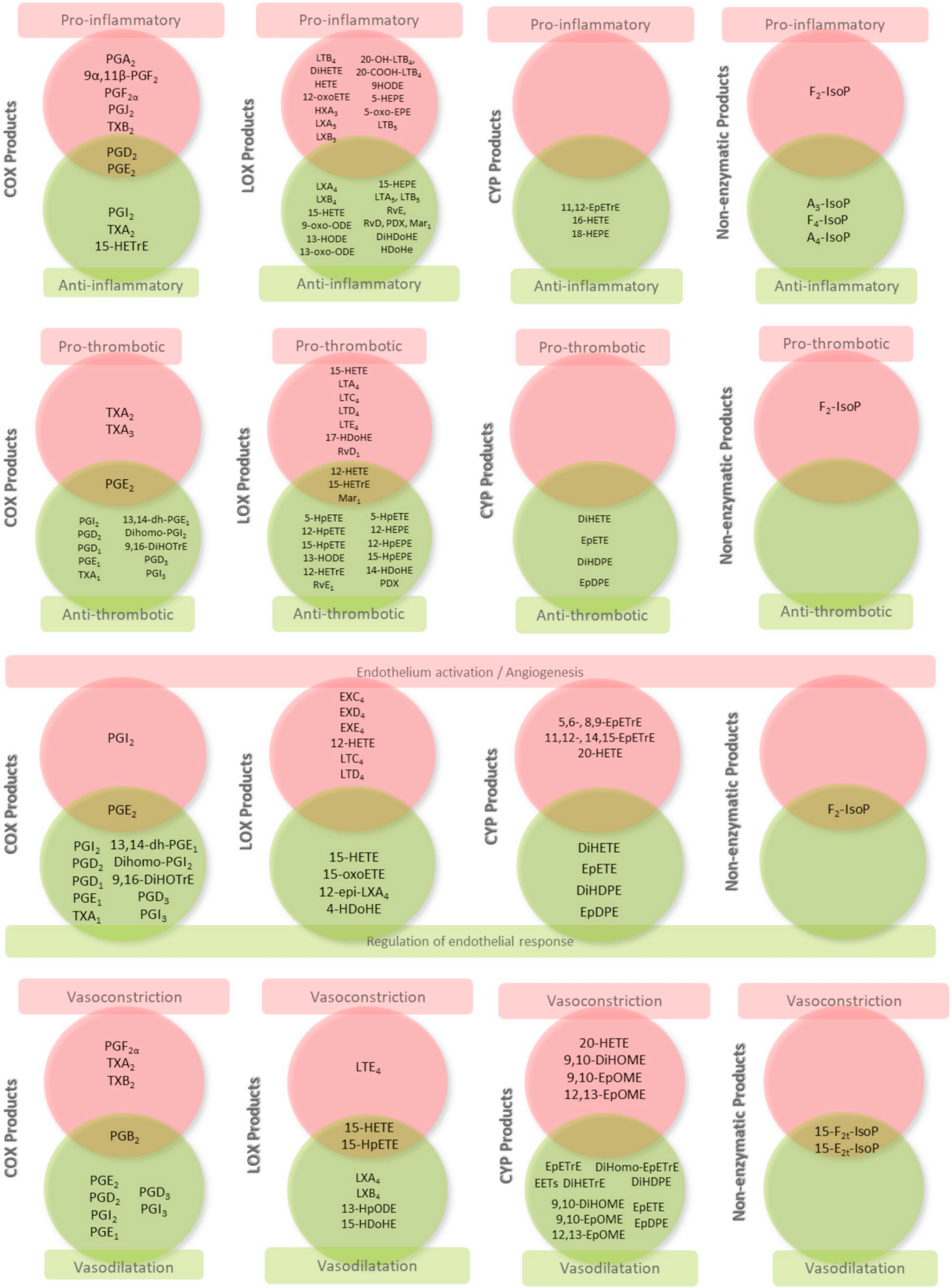

Fig. 2. Overview of the complementary and opposite actions of eicosanoids and other oxylipins on inflammation, thrombosis, endothelial function and vascular tone (*compilation of data from* Refs. [21,200–204]).

system controls the onset and resolution of inflammation in some models [25].

While oxylipins are found in all tissues, cells are highly selective as to the type of oxylipin they synthesize. For instance, TxA_2 is mainly produced by platelets, while the endothelium is a major source of PGI_2. Of note, TxA synthase is also expressed in lung and macrophages and significant levels of PGI synthase is found in smooth muscle cells [26,27]. $PGF_{2\alpha}$ is mainly produced by the uterus, PGE_2 is the major oxylipin generated in kidneys [5] and skin [28], and the hematopoietic form of PGD synthase is highly expressed in immune and inflammatory cells, but also identified in brain and ovary [24,29]. Some LOXs also have preferential cell distribution with 5-LOX being mainly expressed in leukocytes, macrophages and dendritic cells. 12/15-LOX (*ALOX15*) has a broad tissue distribution [30] but is notably abundant in eosinophils and bronchial epithelium and 15-LOX-2 (*ALOX15B*) is highly expressed in the skin and prostate, while 12-LOX (*ALOX12*) is mainly found in platelets [31]. Interestingly, the regiospecificity of products from a single enzyme can differ for individual PUFAs. For example while 12/15-LOX yields the 15-hydroperoxy metabolite of AA, it produces both, 14- and 17-hydroperoxy metabolites of DHA [32]. While CYPs are highly expressed in liver, there are also high levels of expression in brain, lung, kidneys, gastro-intestinal tract and heart [33]. Humans express ~50 CYP isoforms, ~20 of which are shown to biosynthesize oxylipins, notably members of the CYP2C (e.g. *CYP2C8, CYP2C9, CYP2C19*) and CYP2J (e.g. *CYP2J2*) families that are the predominant human epoxygenases, and members of the CYP4A and CYP4F families that have predominantly ω/ω-1-hydroxylase activity [34]. In plasma, the major part of hydroxy-PUFAs and epoxy-PUFAs are found esterified into lipids [35], e.g. the glycerolipids of lipoproteins (> 95% in rats) [36]. Other vectors of circulating oxylipins include albumin that can both passively adsorb oxylipins [37] or form covalent adducts [38] and extracellular vesicles that are both carriers and producers of oxylipins [39]. Free oxylipins are also detected in plasma and are dramatically influenced postprandially by the nature of the diet [40,41].

Oxylipin profiling has the potential to provide a wealth of information regarding global changes in the homeostasis of a vast array of biological processes. Current MS-based oxylipin targeted metabolomics

Table 1
Selected examples of recent applications of the oxylipin profiling to identify biomarkers from human blood samples.

Approach	Method	Main objectives	Reference
Biomarkers of complex disease			
Nested case-control cohort study (n = 375)	EDTA plasma, free oxylipins (n = 36)	• Determine the oxylipin profiles associated with the risk of future cardiovascular events (IMPROVE pan European cohort).	[42]
Nested case-control study (n = 42)	EDTA plasma, free oxylipins (n = 21)	• Screen for metabolic perturbations in umbilical cord blood plasma from preterm infants that did or did not develop subsequent pulmonary hypertension.	[59]
Clinical trial (n = 48)	EDTA plasma, total oxylipins (n = 95)	• Compare the oxylipin profiles of lipoproteins from healthy vs metabolic syndrome.	[45]
Nested case-control cohort study (n = 1488)	EDTA plasma, free oxylipins (n = 12)	• Examine the prospective association between plasma fatty acids, oxylipins, and risk of acute myocardial infarction (Singapore Chinese Health Study).	[43]
Nested case-control cohort study (n = 231)	Serum, free oxylipins (n = 31),	• Study the metabolomic fingerprint in active rheumatoid arthritis irrespective of patients' response (BiOCURA cohort).	[171]
Nested case-control cohort study (n = 479)	EDTA plasma, free oxylipins (n = 35)	• Determine if oxylipins were associated with ventricular arrhythmias and all cause mortality in patients with systolic heart failure (PROSE-ICD prospective cohort).	[172]
Clinical trial (n = 31)	Serum, free oxylipins, n = 37	• Investigate the association between oxylipin profiles and Achilles tendinopathy.	[173]
Nested case-control cohort study (n = 29)	Plasma, free oxylipins (n = 26)	• Identify candidate biomarkers for the noninvasive diagnosis of NASH.	[174]
Nested case-control cohort study (n = 667)	Serum, Free oxylipins (n = 46)	• Investigate the specific signature patterns of oxylipins associated with preclampsia.	[175]
Clinical trial (n = 126)	Plasma, free oxylipins	• Investigate the association between oxylipin profiles and obesity.	[176]
Biomarker of inflammation and oxidative stress			
Clinical trial (n = 14)	EDTA plasma, free oxylipins (n = 51)	• Investigate the alterations of oxylipin profiles in response to biodiesel exhaust exposure.	[177]
Nested case-control study (n = 42)	EDTA plasma, total oxylipins (n = 75)	• Characterization of inflammation and oxidative stress during the early phases of sarcopenia (PROOF cohort).	[47]
Clinical trial (n = 42)	Plasma, free oxylipins (n = 95)	• Evaluate levels of inflammatory markers and blood oxylipins in obese subjects before and after weight reduction.	[178]
Clinical trial (n = 1)	EDTA plasma, free oxylipins (n = 37)	• Investigate postprandial inflammation through changes of oxylipins profiles.	[179]
Clinical trial (n = 10)	EDAT plasma, total oxylipins (n = 27)	• Characterize oxylipin profile and vascular inflammation following a moderately high-fat meal in Alzheimer's patients.	[180]
Biomarkers of nutrition and drug effects			
Dietary intervention (n = 35)	EDTA plasma, free oxylipins(n = 56)	• Determine the impact of long-term treatment with omega-3 fatty acids on plasma oxylipin patterns in patients with severe hyperlipidemia and CVD on standard lipid-lowering and cardioprotective medications.	[181]
Dietary intervention (n = 19)	EDTA plasma, free oxylipins (n = 59)	• Determine the effect of a high-alpha-linolenic acid diet on EPA and DHA levels in red blood cells and their oxylipins in the plasma of subjects with a low EPA and DHA status.	[48]
Dietary intervention	EDTA plasma, total oxylipins (n = 95)	• Compare the oxylipin profiles of lipoproteins from healthy vs. metabolic syndrome subjects with or without supplementation with omega-3 fatty acids.	[45]
Dietary intervention (n = 12)	EDTA plasma, Free oxylipins (n = 46)	• Compare the short and long term effects of DHA supplementation on the oxylipin plasma signature and oxylipin response of stimulated blood cells in healthy men.	[182]
Nested case-control study (n = 231)	Serum, free oxylipins (n = 31),	• Identify predictors for the outcome of TNFi therapy (BiOCURA cohort).	[171]
Ex-vivo experiment	N = 22, Heparin HWB, free oxylipins (n = 122)	• Quantitatively map the effects of small-molecule drugs on the entire arachidonic acid cascade.	[51]
Dietary intervention (n = 40)	Serum, free oxylipins (n = 20)	• Investigate the effects of dietary omega-3 fatty acids on the metabolic signatures of post-menopausal women.	[183]
Dietary intervention (n = 38)	Plasma, free oxylipins (n = 19)	• Evaluate the acute and short-term effects of walnut intake on changes in microvascular function and the relationship of these effects to plasma epoxides.	[184]
Dietary intervention (n = 20)	Plasma, free oxylipins (n = 38)	• Determine if oxylipin concentrations change with the consumption of flaxseed enriched foods or differ with subject age.	[50]

allows the assessment of changes in a vast array of oxylipins simultaneously within acceptable run-times, resulting in increased interest in oxylipin profiling during recent years, notably in the active field of biomarker discovery. This review will present applications of oxylipin profiling over the past several years while describing the main analytical and biological factors contributing to the variability in oxylipin profiles.

2. Applications of the MS-based profiling of oxylipins

The synthesis of oxylipins and subsequent induction of cell signalling pathways is tightly regulated under normal physiological conditions. Oxylipin synthesizing enzymes and/or receptor dysregulation is associated with a variety of diseases including cardiovascular diseases (CVD) and various immune-related diseases. Moreover, the manipulation of oxylipin synthesis through modulation of precursor PUFAs or enzyme inhibitors *via* nutritional or therapeutic approaches has great potential in the prevention and management of diseases. Therefore, oxylipin profiling of biological fluids is being used to identify potential disease biomarkers, to characterize inflammatory and oxidative status, and to monitor the effects of nutrition or drugs on these regulatory systems. Recent studies addressing one of these goals are presented in Table 1.

Of note, the study design (i.e. power, participant selection and matching), the analytical choices (free or total oxylipins, plasma or serum), and the metabolic coverage of the MS-method applied (number and variety of oxylipins) are very different between the studies and – more importantly – these parameters may have significant consequences on the relevance and robustness of the biomarkers identified. While the natural variance and reference value of oxylipin levels in healthy humans is not clinically established, recent results show that the mean coefficient of variation (CV) of oxylipins in the plasma of clinically healthy humans can reach 87% and the magnitude of variance depends on the type and concentration of oxylipin considered [42] (Fig. 3). This high variance highlights the need for large clinical cohorts of well-selected and matched participants to properly power studies in order to identify discriminant oxylipins. However, while the absolute concentration of individual oxylipins is likely important, shifts in relative abundances or patterns may hold great potential as complex disease biomarkers. Therefore, as assays increase their metabolic coverage of representative oxylipins, they will provide a higher resolution pattern to be considered for biomarker discovery.

In the field of disease biomarkers, most studies applying oxylipin profiling are related to cardiometabolic disorders. For instance, in a recent nested case-control cohort study involving 175 cardiovascular cases and 172 controls (matched for recruitment center, age, sex, diabetes status, insulin use, statin use, and smoking) and covering 36 free oxylipins, one factor containing 10 free fatty acids and 19 oxylipins was significantly associated with cardiovascular events [42]. Of note, this association was only observed in non-diabetic patients, thus highlighting the need for patient stratification in clinical investigation. Moreover, linoleic-acid derived epoxides, alcohols and ketones (i.e. EpOMEs, 9-HODE, 13-HODE and 13-KODE) were the metabolites most strongly associated with changes in inter-adventitia common carotid artery diameter over time. In a Chinese population, a large nested case-control cohort study involving 744 incident acute myocardial infarction cases and 744 controls matched for gender, dialect group, date of birth, date of recruitment and date of blood collection failed to detect significant associations between free oxylipin clusters and acute myocardial infarction (besides TxB_2 which may reflect sample collection, processing or storage) [43]. Interestingly, the authors suggested that examining oxylipins within the esterified pool of lipoprotein particles rather than free oxylipins could provide better predictive biomarkers of coronary heart disease. This lays emphasis on this critical point when focusing on oxylipins as potential disease biomarkers. Although the origin and role of most free and almost all esterified oxylipins are not well understood, assessing the esterified pool could be more relevant in a context of biomarker discovery for three main reasons: (i) It represents the major portion of circulating oxylipins (especially epoxy- and hydroxy-PUFAs); (ii) it is known to be biologically active [44]; and (iii) it fluctuates as a function of lipoprotein particle concentration and composition [45] which is of importance since other factors associated

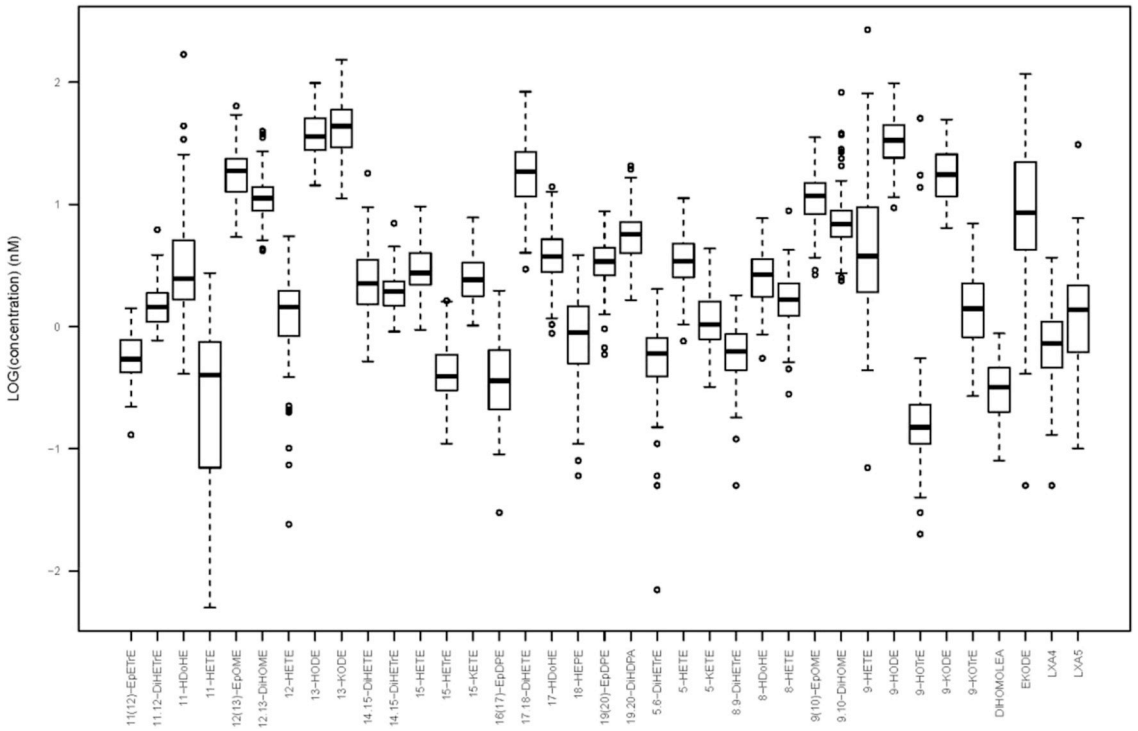

Fig. 3. Box-plots from different oxylipins measured in plasma of 133 healthy individuals from the IMPROVE cohort. Mean CV of oxylipin levels for this sample of the IMPROVE cohort was estimated at 87%. (*Illustration created from supplementary dataset associated with* [42]).

with such lipidomic fluctuations are known disease risk factors (e.g. hypertriglyceridemia, cholesterol distributions). Moreover, one can expect (whereas this has not been assessed) that the esterified oxylipins are more stable and therefore less affected by sample collection, processing or storage.

Another common application for the targeted metabolomics of oxylipins is to generate an integrative assessment of the inflammatory and oxidative stress status of an individual. The utility of such data is also enhanced by broad metabolic coverage to include complementary and opposing oxylipins derived from all pathways and multiple substrates to allow relevant biological interpretation. Inflammation and oxidative stress are the drivers for the onset of many diseases and assessing them accurately is therefore crucial in clinical research. Inflammatory status is usually quantified by markers like C-reactive protein (CRP), interleukin-1, TNF-α, and fibrinogen while the non-enzymatically formed isoprostanes are considered to be the "gold standard" biomarkers of endogenous lipid peroxidation and oxidative stress [46]. However, we recently observed in patients in the early phases of sarcopenia that the classic inflammatory biomarkers do not identify subtle differences in inflammatory and oxidative stress status [47]. Indeed, although CRP and F_2-isoprostanes were similar in patients with different muscle status, several oxylipins (e.g. 15-HETE, 5-HEPE, 9-HETE, 9-HEPE and 14-HDHA) demonstrated subtle differences in inflammation and oxidative stress providing a better characterization of the early phases of sarcopenia.

Targeted metabolomics of circulating oxylipins has also been widely used to monitor and subsequently understand the effect of diet (mainly omega-3 fatty acids) or drugs (e.g. anti-inflammatory drugs) on health. The impacts of these two environmental factors on oxylipin patterns will be detailed later (*see* section 4.1), but important observations arised from studies presented in Table 1. The first is related to the variability of response which is systematically reported by the authors, depending for example on the basal status of omega-3 fatty acids [48,49], participant health status [45], age and sex [50]. The second observation refers to the necessity to assess all pathways of oxylipin biosynthesis to identify unexpected effects of treatments [51]. As with the influence of basal omega-3 fatty acid status on oxylipin responses to fish oil consumption [52], examination of response dynamics through the use of regression analysis can actually take advantage of such variance to provide new information. Similarly, if unique response phenotypes are observed within an experimental population, it may be important to develop new hypotheses surrounding the associations of oxylipins and health risks to truely understand the utility of these potential biomarkers in clinical research.

MS-based targeted metabolomics of oxylipins represents a very interesting tool for various applications in clinical research, but the impact of analytical and biological parameters on the variability of oxylipin patterns needs further understanding to avoid inappropriate study design and subsequent biased biological interpretation. Moreover, expansive oxylipin coverage will increase our ability to interpret the interactions and crosstalk among the various biosynthetic routes and substrate dependent metabolites, improving our understanding and possible treatment of dysoxylipinemic diseases.

3. Analytical variability linked to the MS-based profiling of circulating oxylipins

To enhance our understanding of the physiological roles of oxylipins that influence health and disease, high quality data are required from a wide array of biological matrices. Therefore, robust analytical methods with high sensitivity, accuracy and precision are needed. Due to the complex and interactive nature of lipid mediator signalling, it is now well-accepted that the physiological effects of these compounds result from a shift in the overall oxylipin pattern, e.g. from a pro-to an anti-inflammatory status [53], rather than from the absolute concentration of individual mediators. Thus, state-of-the-art analytical methods aim to precisely detect as many members of the oxylipin cascades (such as the AA cascade) as possible. This broad – and if possible comprehensive –monitoring of such an endogenous pathway is typically referred to as targeted metabolomics [54]. The analysis of oxylipins as a superclass of endogenous compounds presents a number of challenges including a diverse array of compound polarity, stability, and endogenous concentrations that can vary ≥4-orders of magnitude [55,56]. Therefore, to quantify oxylipins with high sensitivity and precision, critical considerations include sample collection and storage, instrument selection, analytical breadth and the biological matrix of concern. Each of these factors will affect method development aspects such as the mode of analyte extraction.

3.1. Sample collection procedures

A major source of variability in analysis is the formation/degradation of oxylipins during or after sample collection. Oxylipins, particularly COX-1 derived Txs as well as 12-LOX generated hydro(pero)xy-PUFA are formed during blood coagulation [57,58]. Thus, serum has to be regarded as an "*ex vivo*" coagulation assay and it is likely that the time and temperature for coagulation before spinning and freezing affects the formation of oxylipins [59]. The anticoagulant – e.g. ethylenediaminetetraacetic acid (EDTA) or heparin – used for the preparation of plasma has also been shown to modulate the oxylipin pattern [57,60,61]. Thus, levels between differently generated plasmas may vary considerably and should not directly be compared. EDTA is used in most studies investigating the oxylipin profile (Table 1). Best practice is to centrifuge and store samples immediately after collection and define

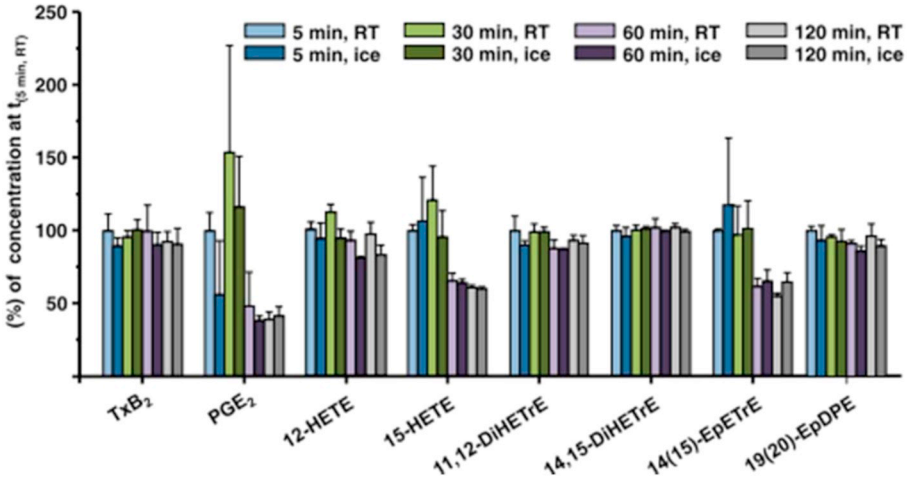

Fig. 4. *Ex vivo* degradation/formation of oxylipins in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 g, 15 min, 4 °C), plasma was immediately frozen (−80 °C) and the oxylipin concentration was analyzed within 5 days. The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5min,\ RT)}$). The results clearly show, that after 60 min storage of whole blood the levels of several oxylipins are massively reduced (e.g. 15-HETE and 14(15)-EpETrE) while other analytes are formed *ex vivo* (e.g. PGE_2). *Reprint with permission from* Ref. [55].

minimal/maximal duration and conditions for transitory storage. Even short periods of storage (1–2 h) of plasma at room temperature or in the refrigerator lead to *ex vivo* changes in the oxylipin profiles [57,60]. For example, Fig. 4 shows the massive variability induced by differences in the handling of whole blood during the first 2 h post-blood collection at different temperatures prior to plasma isolation. Although the direct addition of methanol [57,60] or additives (inhibitors or antioxidants) might have beneficial effects on the stability of analytes, these procedures are seldom feasible in clinical settings since commercially available collection tubes containing such materials are currently not available. Similar considerations are needed in the standardization of post-coagulation freezing delays when serum analyses are considered experimentally relevant [59]. Serial sampling from indwelling catheters offers unique challenges and the process of catheter maintenance must be carefully chosen. If heparin is used for catheter maintenance, or in association with other medical interventions (e.g. heart-lung machine action), large increases in circulating non-esterified oxylipins are observed [61–63]. Although heparin stabilization of lipoprotein lipase can be reversed by the administration of protamine sulfate, great care should be taken when analyzing and interpreting such samples due to the effect heparin can have on the oxylipin profile in these settings.

These data illustrate the importance of standardized procedures and protocols in clinical studies to reduce sample collection induced variability in oxylipin concentrations. Regardless, the analysis of blood samples from cohort studies collected with non-documented and/or variable sample conditions may be valuable to help defining the physiological role of oxylipins. However, researchers should consider that such datasets may have higher than expected variance and interpret data pertaining to metabolites with known instabilities with care. If future studies can identify robust markers of poor sample handling, they may provide non-biased tools for the exclusion of samples with evidence of significant *ex vivo* changes in oxylipin profiles, thus increasing the utility of such studies. In at least 3 studies to date we have found that greatly exaggerated concentrations of 9-HETE and the linoleate-derived trihydroxy metabolites (TriHOMEs) may be such potential markers of poor sample handling (unpublished data). Moreover, in order to correctly interpret the results, quantitative data on the variability induced by transitory storage at each step of plasma generation prior storage at −80 °C is urgently needed.

3.2. Sample storage procedures

Variability of oxylipin levels is also induced by storage time and conditions. In a recent article, Giera and coworkers showed an impressive dataset demonstrating that only 4 weeks of storage at −20 °C leads to artificial oxylipin formation [57]. This is consistent with earlier reports showing that short term storage at 4 °C as well as freeze/thaw cycling massively influence oxylipin patterns [60]. Interestingly, Giera and coworkers also show that the oxylipin pattern in EDTA plasma is not stable when stored at −80 °C [57]. For most of the oxylipins, small changes were observed over the time span of one year. However, two critical oxylipins, i.e. TxB_2 and 12-HETE, were found to increase (2–20 fold and 1.5–15 fold, respectively) under certain conditions (without methanol or butylated hydroxytoluene (BHT, radical scavenger)

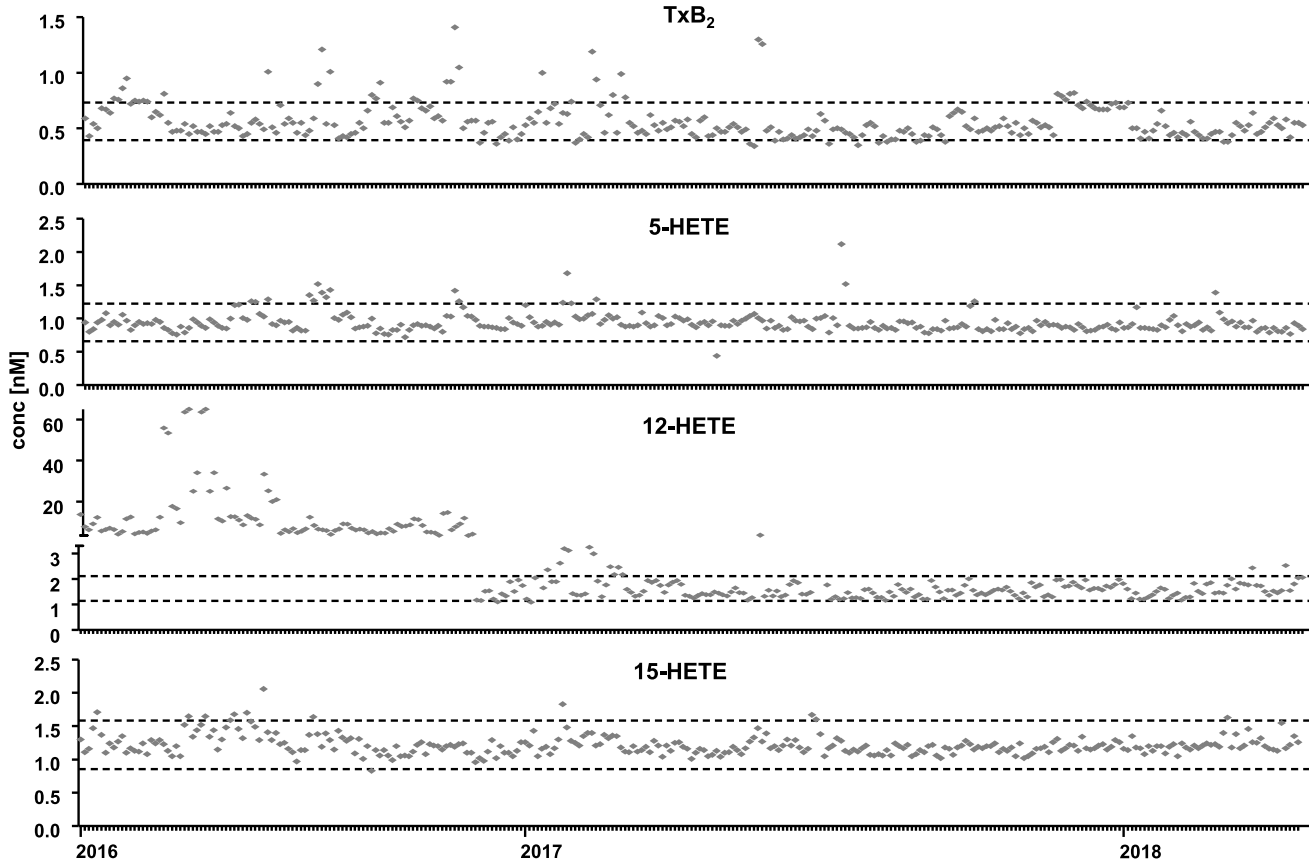

Fig. 5. Stability of oxylipins in EDTA plasma during a storage period of two and a half years. EDTA plasma (pooled from healthy subjects, 3 female and 2 male, 25–38 years) was stored in aliquots at −80 °C. Shown are absolute concentrations of selected oxylipins in plasma (extraction volume: 500 µL). From January 2016 to August 2018 EDTA plasma was repeatedly prepared using the current standard procedure at the time. Since November 2016, protein precipitation by methanol was carried out prior SPE. The dotted lines indicate the mean concentration ± 30% (determined over 32 months). Oxylipin concentrations were determined as described in Ref. [76] till 11/2016 and in 2017–2018 as described in Refs. [4,185].

addition). As these metabolites are hallmark indicators of platelet degranulation [21,64], for long term storage, the preparation of platelet depleted plasma may be advantageous. Taking into account that clinical samples are stored under these conditions, the data for TxB_2 and 12-HETE should be evaluated with caution in samples which have been stored over a long period, e.g. samples from prospective cohort studies. In our hands, the oxylipin pattern in human plasma is stable during storage at −80 °C for 2.5 years as shown for selected oxylipins in Fig. 5. Though this data is only obtained over a period of 2.5 years from a pool of well-prepared EDTA plasma from healthy subjects, it implies that oxylipins can be evaluated in biological samples that have been stored at −80 °C for many years. Overall, this data demonstrates the importance of carefully controlling all aspects of sample collection and storage to minimize oxylipin variability.

3.3. Sample preparation approach

In general, oxylipins occur in plasma esterified into complex lipids such as phospholipids, triglycerides and cholesterylesters (> 90% of oxylipins (hydroxy- and epoxy-PUFAs)), or as free oxylipins, i.e. in their non-esterified form. Esterified oxylipins are commonly quantified as a sum parameter consisting of esterified and free oxylipins (total oxylipins) following hydrolysis by saponification. It should be noted that alkaline conditions destroy the β-hydroxy-keto PGs (e.g. PGEs, PGDs) and Txs, as well as cysteinyl leukotrienes and ketones but not the β-hydroxy-alcohols (PGFs) [65]. Reported conditions for saponification vary using e.g. potassium hydroxide (0.2–0.5 M in sample [66,67]), sodium hydroxide (0.5–3.75 M in sample [35,68–70]) or sodium bicarbonate (0.1 M in sample [71]). Temperature and duration of hydrolysis range from 4 to 90 °C for 20 min to 18 h [35,66–71]. Moreover, to promote efficient hydrolysis of complex lipids with low solubility including triglycerides and cholesterylesters, transesterification of lipids in methanolic sodium hydroxide in the presence of lipid class surrogates (i.e. phospholipids, triglycerides, cholesterylesters, and free fatty acids) followed by hydrolysis using water has been established [65]. In some protocols an initial liquid-liquid extraction (LLE, e.g. with chloroform/methanol or cyclohexane/isopropanol/ammonium acetate), or protein precipitation with organic solvents has been used to extract the lipids from the biological sample before hydrolysis [65,66,68,70,71]. These different strategies may influence the amount of oxylipins isolated and liberated during hydrolysis and thus directly affect apparent (total) oxylipin concentrations resulting in variations in the reported concentrations. However, only in a few reports data on method optimization are presented – as in Ref. [67] – and thus, so far the effects of different saponification strategies have not been systematically evaluated.

For analysis of free oxylipins in biological samples, solid phase extraction (SPE) is the most commonly applied technique today. SPE materials with different retention mechanisms as well as different elution solvents are used [72–75] and have been summarized and compared in Ref. [76]. SPE allows relative enrichment of analytes from larger sample sizes to lower detection limits. Protein precipitation [57,77] and LLE [78,79] procedures have also been reported. While counterintuitive, the dilution of samples by protein precipitation procedures can effectively lower detection limits by reducing ion suppression/enhancement influences of co-extracted metabolites. In the SPE protocols, sample pre-treatment strategies have to be optimized for each protocol and include protein precipitation followed by dilution of the samples with buffer – often coupled to pH adjustment (e.g. using anion-exchange cartridge material) – or acidification of samples. Following elution with appropriate organic solvents, samples are evaporated and reconstituted to achieve concentration of analytes up to 10-fold (compared to the plasma sample). Differing SPE protocols are most likely one of the most important factors contributing to the high variability in the concentrations of oxylipins in biological samples, such as human plasma of healthy individuals. In a direct comparison of the SPE protocols using the same instrumental setup and the same set of samples [76] it has been shown that apparent recoveries of internal standards added to the samples at the beginning of preparation (i.e. analytical surrogates) varied significantly between protocols. This could be explained by differences in analyte extraction efficiency and/or differential removal of ion suppressing matrix components, such as phospholipids (Fig. 6).

Matrix induced ion suppression/enhancement is a key problem

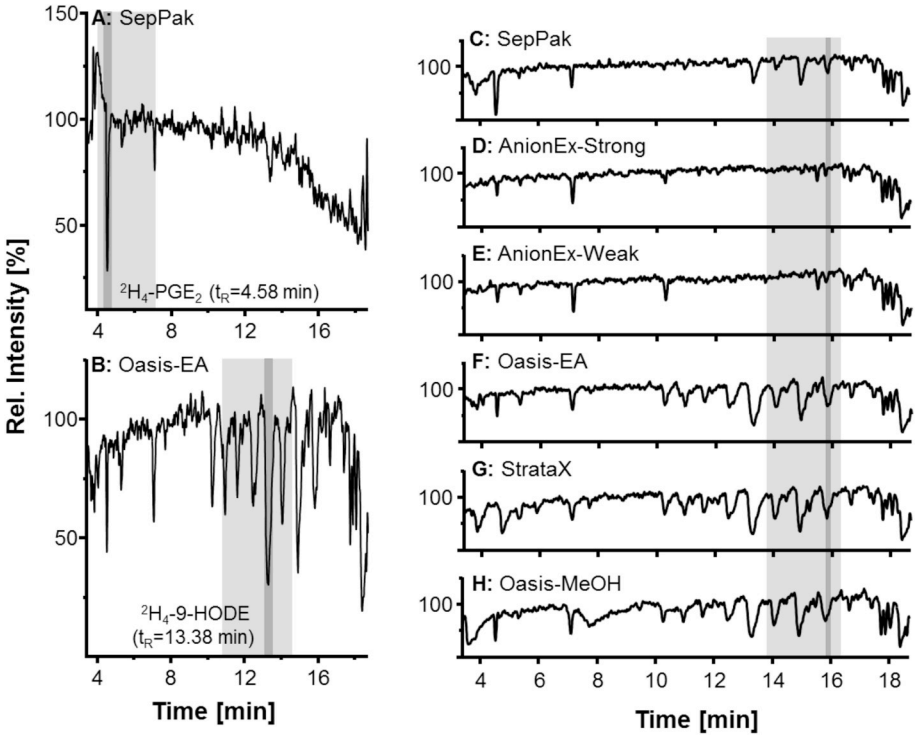

Fig. 6. Ion suppression analysis for (A) $^2H_4$-PGE$_2$ with SepPak-SPE, (B) $^2H_4$-9-HODE with the Oasis-EA-SPE and (C–E) for $^2H_4$-9(10)-EpOME with different SPE protocols. The retention time window of each IS is highlighted in dark grey, the elution window of all analytes using this ISTD is depicted in light grey. The different SPE methods are summarized in Ostermann, 2015. Reprint with permission from Ref. [76].

Table 2
Overview of exemplary instrumental LC-MS-methods covering more than 35 analytes for the quantification of oxylipins. Ionization was carried out by electrospray ionization in negative ion mode on a QqQ instrument and analytes were separated using RP liquid chromatography (C18). Included are sample preparation steps (solid phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation (ProtPrec)), column dimensions, runtime (including equilibration time), lower limit of quantification (LLOQ) or limit of detection (LOD), internal standards (ISTD) and validation parameter.

Lab	Sample Preparation	Column dimensions	Runtime[a]	LLOQ range[b]	No. Analytes	Nb Precursor PUFA	Nb ISTD	Validation	Ref
Schebb, 2018; 2019	ProtPrec, SPE (plasma, serum, other body fluids cell culture)	(150 × 2.1 mm; 1.8 µm)	32	0.5–200	175	10	20	calibration curve, LOD, LLOQ, intra- and interday accuracy and precision, recovery rate, matrix effect	[4,185]
Newman, 2014	LLE, saponification, SPE (plasma)	(150 × 2.1 mm; 1.7 µm)	16	0.4–14	80	6	18	Calibration curve, precision internal standard alkaline stability	[65]
Newman, 2012	LLE, saponification, SPE (plasma)	(150 × 2.1 mm; 1.7 µm)	16	0.5–100	59	6	13	Calibration curve, precision	[49]
Newman, 2002	glucuronidase, LLE (urine)	(150 × 2.1 mm; 5 µm)	28	60–600	13	2	2	Calibration curve, precision, LOD, LLOQ, standard additions	[87]
Hammock, 2009	SPE (serum, BALF)	(150 × 2.0 mm; 5 µm)	21	0.1–100	39	3	8	Calibration curve, LLOQ, precision, accuracy, recovery rate	[74].
Holčapek, 2019	ProtPrec, SPE (plasma)	(150 × 2.1 mm; 1.7 µm)	20	7.5–70	63	6	14	Calibration curve, LOD, LLOQ, carry-over, precision, accuracy, recovery rate, matrix effect	[107]
Dennis, 2007; 2010; 2011	SPE (cell culture media and plasma)	(250 × 2.1 mm; 4 µm)	20	LOD: 3-178	140	7	26	Calibration curve, recovery, LOD	[186–188]
Dennis, 2014; 2018	SPE (saponification) (plasma, cell homogenates)	(100 × 2.1 mm; 1.7 µm)	7	0.3–810	158	9	26	Calibration curve, LOD, LLOQ, recovery rate, matrix effect, accuracy, precision, stability	[67,189]
Dennis, 2011	SPE (cell culture media, rat tissue, mouse tissue)	(250 × 2.1 mm)	25	LOD: 0.3–3.6	141	7	27	Calibration curve, LOD	[72]
Ceglarek, 2013	ProtPrec, online SPE (plasma)	(100 × 2.1 mm; 2.6 µm)	13	5.4–620	94	5	22	recovery, LOD, LLOQ, calibration curve, precision	[190]
Vreeken, 2012	SPE (plasma)	(150 × 2.1 mm; 2.7 µm)	26	1.5–510	104	6	11	Calibration curve, LOD, LLOQ, variation, reproducibility, matrix effect, recovery, stability	[63]
Dalli, 2014	SPE (plasma, serum, spleen, lymph nodes)	(100 × 4.6 mm; 1.8 µm)	> 20	LOD: 0.05–0.66	> 43	3	5	Calibration, ISTD recovery, matrix interferences, LOD, stability	[191]
Dalli, 2018	ProtPrec, SPE (body fluids and tissues)	(100 × 4.6 mm; 2.7 µm)	> 20		41	3	5	Calibration curve	[192]
Ramsden, 2018	SPE (plasma)	(100 × 4.6 mm; 1.8 µm)	35	2.7–17	57	5	9	Calibration curve, LOD, LLOQ, precision, accuracy, reproducibility, matrix effect, recovery, stability	[193]
Nording, 2015	SPE (plasma)	(150 × 2.1 mm; 2.5 µm)	25	0.0016–11	37	5	5	Calibration curve, LOD, LLOQ, precision and accuracy, recovery, stability	[179]
Milla, 2013	SPE (whole blood)	(150 × 2.1 mm; 1.7 µm)	6.5	1.1–1300	122	8	28	calibration curve, LLOQ, matrix effect	[194]
Nicolaou, 2013	SPE (tissue), plasma, other body fluids, cell culture media	(150 × 2.0 mm; 5 µm) (100 × 2.1 mm; 2.6 µm)	30 35	3–600	20 36	3 5	1 1	Calibration, LOD	[195]
Astarita, 2014	SPE (plasma)	(100 × 2.1 mm; 1.7 µm)	> 10	LOD: ~0.1	112	7	15		[196]
Witkamp, 2012	ProtPrec, SPE (plasma, liver, ileum, adipose tissue)	(100 × 2.1 mm; 1.7 µm)	19.75		50	4	15	Calibration curve	[197]
Zhu, 2015	SPE (plasma), LLE (mouse aorta)	(100 × 2.1 mm, 1.7 µm)	18	0.35–12	65	3	10	Calibration curve, LOD, accuracy and precision, stability	[198]
Wheelock, 2018	SPE (plasma)	(150 × 2.1 mm; 1.7 µm)	21		> 46	6		Calibration curve	[42]
Lee, 2018	LLE, Saponification, SPE (plasma, tissue)	(150 × 2.1 mm; 2.6 µm)	20		25	4	4		[199]

[a] Runtime in min.
[b] LOQ range (low-high) [fmol on col.].

when using electrospray ionization-mass spectrometry (ESI-MS) for quantitative analysis in biological matrices [80]. Ion suppression can be particularly problematic in targeted oxylipin metabolomics since only a few isotopically labeled internal standards are used for a large number of oxylipins eluting at different retention times. In this context, it has to be kept in mind that matrix differences between individual samples such as different human plasma samples (i.e. healthy vs. disease, fasting vs. postprandial, normolipidemic vs. hyperlipidemic) may also increase variability in results, and assessing the consistency of suppression across samples within any single study should be considered. Moreover, there is an optimum balance between sample extraction and final concentration that can vary by sample matrix (e.g. adipose vs. liver vs. plasma) and may be influenced by the nature of sample extraction and the total lipid levels of the tissues.

Hence, a carefully optimized and well-characterized extraction protocol leading to reproducible analyte concentrations in the analyzed matrix is of utmost importance for the production of biologically meaningful oxylipin data. If ion suppression is ignored or not characterized apparent changes in oxylipin concentration may result from differences in the matrix and not from changes in the associated metabolic cascades. Therefore, ideal methods will limit the observable matrix-dependent ionization effects across the entire chromatographic run.

3.4. Analytical hardware

While gas liquid chromatography-MS (GLC-MS) methods were commonly used in the early days of oxylipin quantification, these have been almost completely replaced by (U)HPLC-MS methods on reversed phase (RP) columns, which can cover > 170 analytes from up to six PUFA precursors including linoleate (C18:2n6), alpha-linolenate (C18:3n3), dihomo-gamma-linolenate (C20:3n6), arachidonate (C20:4n6), eicosapentaenoate (C20:5n3) and docosahexaenoate (C22:6n3) (Table 2). Rare analyses of oxylipins from adrenic acid (C22:4n6) [4,81,82] or n3-or n6-docosapentaenoic acid (DPA, C22:5n3/n6) [4,83], and very long chain elovanoids [84] have also been reported. Recent articles describe the use of super-critical-fluid chromatography [85]. Because oxylipins in their non-esterified form bear the acidic carboxy-moiety of the polyunsaturated fatty acid precursor, ionization is dominantly carried out by ESI in negative ionization mode. Only in a few applications different techniques are used, e.g. atmospheric pressure chemical ionization following derivatization [86] (Table 2).

Today, oxylipins are most often quantified using triple quadrupole-MS (QqQ-MS) (Table 2). This type of analyzer is well suited for the detection of oxylipins because of its high ion transmission and matrix independence (compared to Paul traps or linear ion traps). In addition, these systems are generally coupled with either photomultipliers or electron multipliers, thus providing linear detector responses over concentration ranges of 5-6 orders of magnitude. The latter is crucial for oxylipin quantification because levels of analytes differ in the very same sample by well over three orders of magnitude, e.g. in plasma of healthy humans from 0.05 to 11 nM (11,12-DiHETE and 15,16-DiHODE [41]) or in healthy mouse plasma (following feeding with omega-3 fatty acids) from 0.064 to 495 nM for PGE_2 and 12-HEPE [56] (data obtained from the mean reported concentraton within one group). Pathophysiological conditions induce strong changes where a linear range of at least four orders of magnitude is needed [45,55]. A key feature of QqQ-MS is that oxylipins are detected following fragmentation. Given that oxylipins are formed by oxygenation of unsaturated bounds within the PUFA backbone, their (exact) molecular masses cannot distinguish regioisomeric structures. Thus, only fragmentation makes a specific MS-detection of isobaric compounds possible. It is important to remember, however, that oxylipin regioisomers can often generate non-specific fragments, and care must be taken in both quantitative ion selection and chromatographic resolution of certain spieces [87]. However, QqQ-MS only allows the analysis of pre-selected species, i.e. “targeted metabolomics”, and today analyses of 50–200 oxylipins are common (Table 2). Using time of flight (TOF) or orbitrap mass analyzers equipped with a quadrupole collision cell (e.g. qTOF or Q Exactive™) allow acquisition of full (fragment) spectra making simultaneous targeted and non-targeted oxylipin metabolomics/lipidomics possible. To date, few applications with these instruments have been published, most being qualitative [88,89]. At present, using two methods for non-targeted (orbitrap) and targeted (QqQ) metabolomics, as recently described by Wheelock and coworkers [42], is the most promising approach for gaining both, lipidomics data and quantitative information on oxylipins.

3.5. Isomeric and enantiomeric complexity

Due to the complexity of oxylipin isomer profiles present in biological samples, careful selection of collision induced mass transitions along with high chromatographic resolution and stable retention times are required to yield the selectivity and sensitivity needed to correctly identify and quantify oxylipins. Thus, effort is required to optimize both, the mass spectrometric parameters and the chromatographic method, to avoid peak misalignment and/or mass spectrometric overlap of secondary ions which can lead to the generation of inaccurate data and thus to false conclusions. The importance of selective mass transitions is illustrated in Fig. 7. Two transitions of the hydroxylated DHA 17-HDHA were used to quantify the analyte in human plasma and rat liver. In both matrices, the selective transition yields clean chromatograms with only one peak while using the unspecific transition results in several peaks. These interfering peaks hamper peak integration and thus might lead to variations in apparent concentrations.

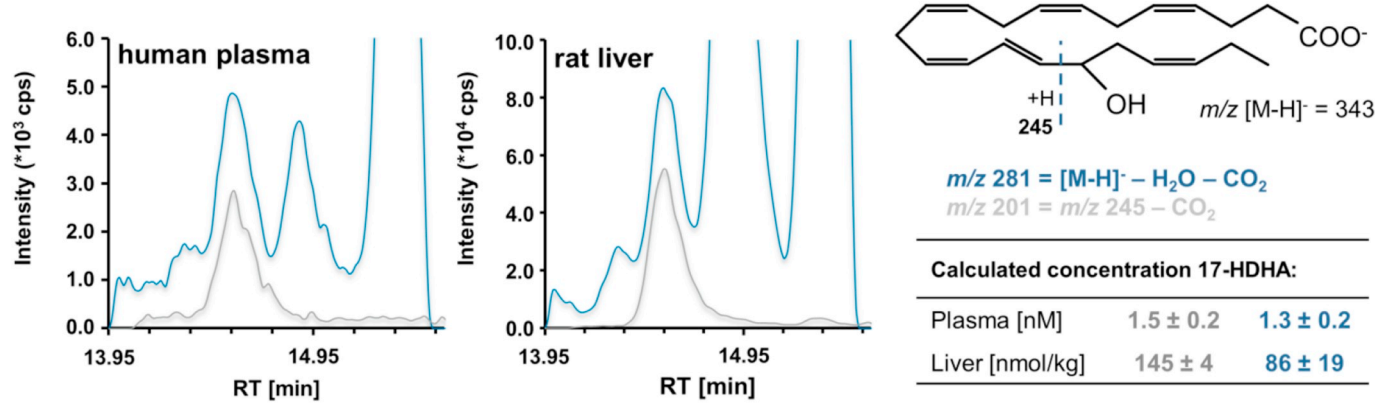

Fig. 7. Quantification of 17-HDHA in human plasma and rat liver tissue using different MS-transitions. LC-MS based oxylipin analysis and sample preparation was carried out according to Ref. [76].

In addition to selective MS detection, the large number of isomeric oxylipins requires excellent chromatographic separation. Currently, separation is typically carried out by reversed phase chromatography often using columns filled with sub-2-μm or fused core particles (Table 2). The fragment spectra of several oxylipins, particularly regio- and stereoisomers, are often too similar to be resolved spectrally. An example of such a critical separation pair, i.e. two oxylipins with the same precursor mass giving rise to similar fragment spectra, are the hydroxylated AA metabolites 9-HETE and 12-HETE [90]. Similarly, the most sensitive transition for 8(9)-EpETrE also shows signals for 11(12)-EpETrE [87] making these two epoxy-AAs another critical pair requiring chromatographic baseline separation. Even using optimized MS-transitions and optimal (state-of-the-art) RP-LC, isobaric interferences from the sample matrix could overlap with the oxylipin peak. This is particularly a challenge in the detection of multiple-hydroxylated PUFAs such as specialized pro-resolving mediators (SPMs), with a large number of possible regio- and stereoisomers. Well characterized examples for this which – if not recognized – lead to strong variations in the apparent concentration (i.e. poor accuracy), are the interference between LTB_4 and 5(*S*),12(*S*)-diHETE [91] and different isobaric compounds mimicking lipoxin A4 (LXA_4) [79]. Similarly, interfering peaks for maresin 1 (MaR1), and the protectins PD1 and PDX occur in improperly stored plasma [57]. Complicating matters, *trans*-double bond containing oxylipins also exist, produced by either the metabolism of trans-fatty acids or the rearrangement of formed oxylipins [92–94].

Another significant consideration in oxylipin analyses is the chirality of the detected species. In particular, independent detection and quantification of enantiomers can aid in interpretation of results in a biologically relevant context. For instance, a variety of oxylipins can be produced by the direct interaction of reactive oxygen species with unsaturated lipids, with the resulting products having no enantiomeric enrichment, while enzymatic reactions generally have high degrees of enantiomeric enrichment [95–102]. Notably, the clotting process yields dramatic shifts in oxylipin enantiomeric patterns [99]. In addition, some enzymes yield the opposite enantiomers of the same regioisomers, for example 12(*R*)-HETE production by CYPs and psoriatic lipoxygenases, and 12(*S*)-HETE production by the *ALOX12* gene product [103,104]. Historically, analysis of these enantiomeric compounds has relied upon normal phase chromatography of pentafluorobenzyl ester derivatives, however, new reversed phase chiral materials are opening the door to routine application of these methods in the future [100–102]. Recently developed approaches couple a non-chiral reversed phase-column with a chiral amylose-based column that can be used with reversed phase solvents [101,105]. These were used e.g. to successfully unveil the stereospecific formation/degradation of epoxy- and hydroxy-PUFAs [101] as well as to characterize the configuration of SPMs [105].

3.6. Accessibility of analytical calibrants and internal standards

To accurately quantify oxylipins, a calibration with authentic reference compounds is required, typically carried out by external calibration with internal standards introduced to the samples, thus allowing correction for methodological variance (e.g. sample loss, ion suppression). Currently, several hundred non-esterified oxylipins are commercially available through a small handful of companies. However, oxylipins esterified into complex lipids including triacylglycerides, phospholipids, cholesterylesters and appropriate isotopically labeled analogs are essentially unavailable through the commercial market place and thus only available to those scientists with the skill and resources to synthesize and purify them. With respect to the available standards, varying purities of these reference compounds have been reported [106], which is likely one cause of the massive differences in reported concentrations [52,107]. The limited availability of isotopically labeled structural analogs and their non-uniform application in these analyses likely exacerbates this problem. Recently, verified-concentration standards, i.e. quantitative grade standards, became available for selected compounds, such as MaxSpec™ (Cayman Chemicals, Ann Abor, MI, USA) and are supplied with batch-specific certificates of analysis. However, up to now only a few standards are available and we recently suggested a strategy to quantify non-verified material with these few verified-concentration standards [106].

Despite optimizing and standardizing sample collection, preparation and analysis, inter- and intra-day variation will remain. Recent studies show that an intra-day variation of < 20% can be achieved for most analytes in human plasma when present at concentrations above the lower limit of quantification (LLOQ) [41]. However, variability increases as analyte concentrations approach the LLOQ. Therefore, methodological robustness should be considered when interpreting results, particularly when the null hypothesis is not rejected. Such robustness factors should include method variability along with other validation criteria such as internal standard recoveries, detected matrix effects, sensitivity (i.e. LLOQs), and calibration stability and accuracy. To this end, we would encourage the routine reporting of these robustness factors which are rarely reported outside of method development studies at this time.

When comparing oxylipin concentrations from different laboratories (Table 2), it is important to consider the analytical choices within each method. Round-robin trials comparing methods established and routinely implemented in different laboratories will be the key to identify strengths, weaknesses, and intercomparability of different approaches. Ultimately, such efforts will allow the implementation of internationally agreed upon sample preparation procedures and/or a routine performance standard that will allow gaining the highest confidence in these important datasets and promote the direct comparison of studies performed at multiple locations. Ongoing efforts in different national projects e.g. German Research Foundation 41696725 (http://gepris.dfg.de/gepris/projekt/4169672511) or international projects such as the EU JPI-HDHL OXYGENATE project (https://www6.inra.fr/jpi-hdhl-biomarkers-oxygenate/) aim to address these questions. However, until these procedures have been established a comparison of the (absolute) reported concentration of oxylipins should be performed carefully and only the relative change within the studies seems to be a measure which can be robustly compared.

Accurate analyte quantification requires signal-to-noise-ratios (S/N) to exceed a defined threshold. This is comprehensively discussed in internationally accepted guidelines on method validation (European Medicines Agency, US Food and Drug Administration, US Environmental Protection Agency, etc.). With an S/N ratio of at least 5, the typically reported LLOQ for oxylipins on state-of-the-art QqQ instruments is ~0.5–50 fmol on column (Table 2). The LLOQ varies about 10-fold between different oxylipins based on their ionization and fragmentation behavior. Proper definition of analyte specific LLOQs in the methods used is crucial because it impacts the breadth and number of oxylipins that can be reported in a biological sample. Reported levels below the LLOQ need to be identified as they are prone to high variation and inacceptable accuracy. It should be stressed, however, that while measures below the limit of detection (LOD) are not an indication of the presence or absence of a compound and those below the LLOQ (above the LOD) are subject to higher false positive/false negative rates [108], they may have statistical value in maintaining the number of observations across all analytes and may be better than removal and imputation or arbitrary replacement by the LLOQ, procedures that should be implemented with great care [109–111]. Taking an injection volume of 5–10 μL and an average molecular mass of 300 Da into account, as a rule of thumb, the LLOQ for oxylipins in the injected solution is between 0.1 and 10 nmol/L (0.03–3 ng/mL). The detection of lower concentrations of oxylipins becomes possible by pre-concentration during sample preparation if a concurrent increase in ion suppression is not observed.

4. Inter-individual variability of plasma oxylipin patterns

As illustrated in Fig. 3, plasma concentrations of oxylipins are highly variable in clinically healthy humans and each oxylipin has its

own variability. Both, environmental and intrinsic factors can contribute to variance in measured oxylipin patterns which are independent of analytical variability. Such natural variance of systemic oxylipins can stem from changes in either substrate availability or modulation of the expression and/or activity of responsible biosynthetic enzymes.

4.1. Influence of environmental factors on oxylipin patterns

Oxylipin patterns reflect the integration of many factors, most notably the availability of unsaturated fatty acid precursors, the relative abundance and activity of specific enzymes, and the degree of oxidative stress present in the individual at the time of sample collection. Each of these factors is influenced by the environment.

Diet constitutes the single largest environmental factor that modulates both the type and dose of PUFAs available for oxylipin biosynthesis. Oxylipins can be produced directly in phospholipid membranes (e.g. non-enzymatically or via 15-LOX pathways) [112,113], however for most enzymes, the esterified PUFAs have to be released from membranes by the action of PLA2 [114]. In most human tissues and circulating cells, the omega-6 PUFAs (i.e. AA and linoleic acid) are the most abundant PUFAs stored in membranes [115,116] and therefore the main substrates for oxylipin biosynthesis. However, changing dietary PUFAs (type and dose) can readily modify the fatty acid composition of cellular lipids. This has been well-demonstrated for increased intake of long chain omega-3 PUFAs (i.e. EPA and DHA) from fish or fish oil based supplements which leads to dose- and time-dependent incorporation of both fatty acids in blood lipids, blood cell lipids, and many tissue pools [49,117,118] (and *see review* [119] *for more details*). The incorportion of long chain omega-3 PUFAs is usually accompanied by a decrease in omega-6 PUFAs including linoleic, dihomo-gamma-linolenic and AA [119], but the rate and magnitude of these changes are dependent on an individuals basal omega-3 status [49]. Changing the relative proportion of omega-3 and omega-6 PUFAs in membranes influences oxylipin patterns because of both, shifts in substrate availability and variable substrate affinity to the different enzymes. For instance, cytosolic PLA2 has a strong affinity for AA and EPA but very weak interaction with DHA which is preferentially hydrolyzed by the calcium-independent PLA2 (iPLA2, Type VIA) [120]. A difference in substrate affinity was also reported for COX-2, which preferentially oxidizes AA while having a much weaker interaction with EPA, and almost no affinity for DHA [121]. Concerning the CYP pathway, the affinitiy of CYP isoforms with PUFAs depends on the carbon chain length as well as the type and number of unsaturations. Interestingly, double bonds in the omega-3 position offer a preferential epoxidation site for many CYPs including CYP1A, CYP2C, CYP2J and CYP2E, while CYP4A also predominantly hydroxylates the terminal methyl-group of EPA and DHA (*see* Ref. [122] *for more details*). Consistently, Fischer et al. demonstrated that CYP dependent-epoxide formation from EPA and DHA were 8.6-fold and 2.2-fold more efficient than from AA, respectively [123]. Basically, in healthy subjects, long chain omega-3 PUFA supplementation can elevate total EPA and DHA oxylipins at the expense of total AA oxylipins [45,49,52]. Similar, but less consistent results have been reported for free oxylipins [52]. Relative changes in EPA and related oxylipins are generally more pronounced. Crossvalidating the effects of omega-3 PUFA supplementation on the different pathways of biosynthesis between different studies produces no systematic trends [52]. Several authors, however, reported predominant changes in the CYP pathways [123] consistent with a higher affinity of CYP enzymes with omega-3 PUFAs. Recent data demonstrate that the plasma levels of hydroxy-PUFAs, epoxy-PUFAs and dihydroxy-PUFAs derived from n3-PUFAs increase in a linear fashion with the intake of n3-PUFAs [124].

While PUFA intake can alter substrate availability, other factors can alter oxylipin patterns through modification of the expression and/or activity of enzymes of the oxylipin pathways. The first environmental factor to consider when interpreting oxylipin profiles should be the use of drugs that specifically target oxylipin pathways. These notably include non-steroidal anti-inflammatory drugs (NSAIDs), which act *via* the inhibition of COX isoenzymes and are one of the most widely used drugs in global pharmacological management of acute and chronic pain [125]. Other examples are 5-LOX inhibitors (e.g. Zileuton) in the treatment of allergy and asthma, or antithrombotic agents (e.g. Dazoxiben, Camonagrel, Picotamide) that inhibit Tx synthases. Of note, even for these widely used and well characterized drugs, responses are complex and impacts on oxylipin patterns are often broader than expected [51]. For instance, the use of celecoxib, a specific COX-2 inhibitor, in patients with colon polyp, was associated with increased levels of CYP- and LOX-derived oxylipins [126] which could be a result of substrate shunting into these alternative pathways. Other drugs not specifically designed to modulate enzymes of the oxylipin pathways can also influence oxylipin patterns. This is notably the case for statins, inhibitors of HMG-CoA reductase, which are widely used in the treatment of hypercholesterolemia. Statins have been shown to increase the production of COX-2-derived oxylipins in various tissues *via* S-nitrosylation of COX-2 [127–130], while only modest changes in 5-LOX metabolites appeared in the non-esterified plasma pool with high-dose simvastatin [131]. Phytochemicals in medicinal herbs or plant-based foods (i.e. alkaloids, polyphenols, terpenoids and plant-derived lipids) also influence the activity of oxylipin enzymes. Among them, polyphenols are representative and nutritionally significant compounds investigated for the last 30 years. Numerous *in vitro* cell-based or enzyme-based assays support the inhibitory effects of various dietary polyphenols including flavonoids, curcumin, stilbenes and secoiridoids on PLA2, COX, 5-LOX and CYP gene expression and/or activity [132–137]. Of note, some of this *in vitro* evidence should be interpreted with caution since they often arise from experiments conducted using native compounds rather than circulating forms of polyphenols and at high doses not achievable through diet. However, short term or long term controlled dietary interventions have reported significant modulation of systemic COX- and LOX-derived oxylipin levels following intake of polyphenol-rich foods in healthy humans [138,139] even though interindividual variability sometimes hampers such demonstration [140]. Other general lifestyle habits such as smoking [141,142], alcohol consumption [143] or physical activity [144,145] can also influence systemic oxylipin patterns through an increased inflammatory state caused by the combined effects of inhaled mediators and/or oxidative stress.

4.2. Influence of intrinsic factors on oxylipin patterns

Intrinsic factors that can modulate oxylipin profiles include such variables as age, sex, and genetic polymorphisms, all of which have the potential to modulate biosynthetic enzyme expression and/or activity. Various disease states such as infections or cardiometabolic disturbances (e.g. obesity, type 2 diabetes) also influence oxylipin biosynthesis, but this will not be discussed here.

While not comprehensively investigated, several independent studies in both, animal models and humans have reported a significant influence of age and sex on oxylipin levels (*see* Refs. [146,147] *for recent compilation of the literature*). Aging is generally associated with increased levels of COX-derived oxylipins including PGE_2, 6-keto-$PGF_{1\alpha}$, TxB_2 and their stable metabolites 2,3-dinor TxB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$. Levels of pro-inflammatory leukotrienes (e.g. LTB_4, LTC_4) and hydroxy-PUFAs derived from the LOX-pathway (e.g. 5-HETE) are also higher in biological fluids or tissues from aged individuals compared to younger ones. On the contrary, pro-resolving LOX-derived oxylipins (e.g. LXA_4, LXB_4, MaR1, RvD1) are generally found in lower abundance in aged individuals with the exception of PD1 which was reported to be increased in the brain of aged mice [146]. Other oxylipins produced by sequential conversion by LOX and CYP enzymes or autoxidation of linoleic acid [98,148], namely 9,10,13- and 9,12,13-TriHOME, were also higher in plasma of old healthy men

and women in comparison with younger controls [146]. The effects of aging on CYP-derived oxylipins in humans are more scarce and contradictory [149]. Most studies have investigated the expression or activity of CYPs at the hepatic level, but the difficulty in accessing human liver samples hampered the demonstration of a significant effect of aging on human CYPs and its consequences on the production of CYP-derived oxylipins. According to the free radical theory of aging [150] and knowing the role of reactive oxygen species [151] and lipid peroxides [3] in the regulation of the expression/activity of COX and LOX, oxidative stress is likely a contributing factor to the effect of aging on oxylipin patterns.

Modulation of oxylipin biosynthesis by sex was reported more than 50 years ago [152]. Sexual dimorphism of oxylipin pathways depends on the type of tissue investigated as well as the hormonal status of individuals which is itself age related. However, males generally have higher levels of PGE_2 and Txs derived from the COX pathway, while females have higher levels of leukotrienes but lower levels of pro-resolving oxylipins, both produced by LOX enzymes (*See detail in* Ref. [147]). This is consistent with the higher preponderance of leukotriene-related diseases such as asthma and rheumatoid arthritis in women. Sex is a known factor influencing CYP-dependent drug metabolism [153], but conflicting results were reported in relation to oxylipin metabolism and it is difficult to ascertain if gender has a significant influence on the expression/activity of oxylipin generating CYPs [149]. However, there are clear sex differences in the levels and distribution of the soluble epoxide hydrolase (sEH), an important epoxy-PUFA converting enzyme [154]. It should be noted that, when looking at comprehensive oxylipin profiles, which has been rarely done so far, the overall effect of sex and its interaction with other factors makes interpretation more complicated. Recent studies in rats showed that ~40% of oxylipins (from profiles including 60–71 oxylipins) were influenced by sex and almost all were higher in male rats [155,156]. These studies also showed that sex differences are influenced by diet (e.g. male rats have higher levels of adipose oxylipins when fed a high DHA diet, while females have higher oxylipins when fed a high EPA diet), by the type of precursor PUFA (e.g. rat kidney DHA-derived oxylipins are higher in females, while AA-derived oxylipins are more often higher in males) and by tissue (e.g. in kidney, DHA oxylipins are influenced by diet and sex whereas no change is observed in liver). Mechanisms underlying the influence of sex on enzymes of the oxylipin biosynthetic pathways logically include a regulation of expression/activity by sex hormones. Their role as modulators of COX pathways remains unclear, with investigations mainly focused on primary enzymes of the pathway (i.e. COX). On the other hand, differences in LOX-derived oxylipins have been directly related to variant androgen levels in men and women [147]. This can be the result of a direct modulation of LOX enzymes by sex hormones but other regulatory aspects of the LOX-dependent oxylipin formation including interaction with other proteins (e.g. 5-lipoxygenase-activating protein or FLAP), subcellular localization, phosphorylation and other factors (e.g. ATP, glyceride, redox tone, Ca^{2+}) [3] may also be affected by sex and sex hormones.

Genes of enzymes involved in the biosynthesis of oxylipins are highly polymorphic which may contribute to the variability of oxylipin patterns in healthy humans. However, human genetic studies mostly focused on clinical outcomes rather than oxylipin metabolism. Several large studies investigated the associations between the polymorphism of primary oxylipin enzymes (i.e. COX, LOX and CYP) and the risk of diseases involving oxylipins, such as CVD and cancer. For instance, Ross et al. conducted a remarkable study to prospectively explore the association of a COX-2 variant (i.e. rs20417) in 49,232 participants. The results showed that the rs20417 variant was associated with a reduced risk of major cardiovascular events [157]. Common polymorphisms in the 5-LOX pathway including variants of 5-LOX, FLAP, LTA_4H and LTC_4S were associated with myocardial infarction in a recent case-cohort study conducted in 3000 participants of the Danish Diet, Cancer and Health study [158], and promoter region variants in the *ALOX5* have been linked to the efficacy of fish oil modulation on cardiovascular risk [159]. Genetic variability of 5-, 12- and 15-LOX enzymes and FLAP may also affect colorectal neoplasia as reported in three US population-based case-control studies of colorectal cancer involving 5625 subjects [160]. CYP metabolites (i.e. EpETrE) have recognized cardioprotective effects [161] and associations between CYP and sEH polymorphisms and CVD have been intensively investigated. Even though some studies detected significant associations between several variants of CYP and CVD, contradictory results, probably due to ethnic variability, environmental factors (e.g. smoking) and gender specific effects, have been reported and hamper conclusions of significant associations [162,163]. Associations between sEH polymorphisms and CVD have been investigated in case-cohort samples of the Atherosclerosis Risk in Communities (ARIC) study. In a first sample batch of 1336 participants, two common haplotypes with opposing effects showed significant associations with the risk of ischemic stroke in African American subjects [164]. In a second study involving 2065 participants of the ARIC cohort, Caucasians with the K55R polymorphic variant allele of sEH were found to have a higher risk of CAD and an increase in the relative abundance of linoleate-derived diols relative to their epoxide precursors [165]. Similarly, the most common epoxygenase gene polymorphism has been associated with a significant decrease of enzymatic activity and consequently EpETrE biosynthesis [162]. Other evidence of a polymorphism-dependent effect on oxylipin levels were reported by Ross et al. [157] in which COX-2/rs20417 carriers had significantly lower urinary levels of Tx and prostacyclin (PGI_2) compared to non-carriers. Concerning the LOX pathway, common variants generally have no functional consequences and loss-of-function variation of the human LOX genes are rare (global allele frequency < 0.1%) [166]. Nevertheless, an intervention study conducted in 116 healthy adults reported that 5-LOX gene variants were associated with altered production of AA, EPA and DHA derived LOX-metabolites (i.e. hydroxy and oxo-PUFAs but not leukotrienes) and different response to omega-3 (i.e. fish oil) supplementation [159].

While poorly studied and being more a concern of intra-individual variability, temporal changes in both plasma and urinary oxylipins have been reported. Temporal changes in plasma oxylipins may be linked to meal induced changes [41], however, circadian rhythms in AA metabolism have also been reported [167] and circadian effects on other aspects of lipid metabolism are well documented [168]. Similarly, urinary CYP-dependent metabolites were found to oscillate independent of meals in free feeding men and women [87]. As oxylipins are also important regulators of renal water retention and as urinary but not plasma levels are dramatically influenced by sodium intake and depuration [169,170], these are also important factors to consider when urinary oxylipin profiling is considered.

5. Summary and outlook

Oxylipins represent an important regulatory cascade and continuing to unravel this system and its intricate interactions has the potential to offer great value to society. To maximize the utility of this information and to accelerate our gain in knowledge, it will be critical (i) to promote our ability to directly compare results generated by independent research teams and (ii) to be able to distinguish aberrant oxylipins pattern from natural variance. To do this, sources of variance, both analytical and biological, must be identified, minimized where possible, and considered during data analysis and interpretation.

The preanalytical sources of variance are large and best practices along with international guidelines regarding standardization in sample collection, storage and preparation should be established. Based on our own understanding of the current literature, we would argue for the use of EDTA plasma and to centrifuge and store samples at −80 °C immediately after collection. A first important point to consider for sample preparation is the choice of analyzing total (i.e. free and esterified) or only free oxylipins, as this choice will have important consequences on

the oxylipin profiles and subsequent biological interpretation. Of note, total oxylipins represent the major portion of circulating oxylipins and may be less affected by sample collection, processing or storage, but their quantitative anaysis currently requires hydrolysis which destroys several COX-derived oxylipins. Subaliquots of 100 µL of plasma for total (free and esterified) oxylipin analysis or of 500 µL for free oxylipin analysis should be stored at −80 °C in methanol pre-cleaned polyethylene tubes until use. This procedure should reduce artifacts associated with freeze/thaw cycling and platelet activation after prolonged storage.

Differences in sample preparation procedures (i.e. SPE, LLE, protein precipitation), the application of analytical internal standard corrections (i.e. which isotope labeled internal standard is being used to correct for which specific analytes) and the purity of calibration standards are likely sources of variance when comparing results from different laboratories. In particular, matrix effects resulting in ion suppression of analytical internal standards that do not coelute with their analytical target may lead to inaccurate, while reproducible results (systemic error). Therefore, researchers should take care in assessing the stability of their internal standard recoveries across all samples within their study. First, they should be sure that ion suppression does not segregate by experimental group and they should report all internal standard recoveries as metadata to the study so that others can assess to what degree corrections for loss/suppression are being applied to the reported data, as this may influence the accuracy and thus global comparability of the results.

In terms of analytical variability, in general, modern analytical hardware – if well maintained – is exceptionally stable. Much more important are the standards used to calibrate these instruments and to control over factors including ion suppression/enhancement. The greatest needs in the scientific community are the availability of analytical standards and an increased number of isotopically labeled analogs, including those which allow the direct quantitative analysis of oxylipins within complex lipids. It would be valuable for the research community to establish priorities for the production of new analytical resources for this field. Moreover, the concentration of all standards used should be validated based on concentration-verified standard material. Until certified standards are available for all oxylipins quality assessment strategies as described by Hartung et al. [106] have to be implemented in each analytical lab.

Beyond these procedural and analytical factors, environmental and intrinsic factors tremendously contribute to the natural variability of oxylipin profiles. Care should be taken during the analysis of human studies to truely test assumptions of population normality prior to committing to simple mean testing as a primary outcome. While the accumulating literature shows that dietary and pharmacological components together with age, sex and genetics all have the potential to modulate substrate PUFAs and/or enzyme activities and thus to shift oxylipin profiles, the natural variance of each circulating oxylipin has not been established so far. This will be crucial to appropriately power experimental designs and to enhance the identification of reliable and relevant biomarkers of disease. Increasing our knowledge regarding the natural variance of oxylipin biosynthesis is also of great interest in personalized nutrition or medicine. These efforts will provide new understanding regarding the variability of individual responses to dietary factors and therapeutics, thus offering insights into diseases susceptibility that may allow for patient stratification and personnalization of disease management. Similarly, if unique response phenotypes are observed within an experimental population, it may be important to develop new hypotheses surrounding the associations of oxylipins and health risks to truely understand the utility of these potential biomarkers in clinical research.

Therefore, moving forward, if we consciously control analytical variance while embracing biological variance as a research community, we are sure to enhance the impact of our efforts and accelerate our discovery of new knowledge regarding the biological regulation of these important regulatory factors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.05.012.

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