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The role of oxylipins and their validation as biomarkers in the clinical context

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ABSTRACT

Oxylipins are bioactive lipid mediators that participate in a wide range of processes, from blood flow to inflammation. In recent years, their exhaustive characterization as potential biomarkers has been actively pursued. However, several analytical challenges and sources of variability, like the low abundance of oxylipins in samples, their structural diversity, and the lack of harmonized and validated protocols for sample selection, collection, storage, and preparation have hampered their translation into clinical settings. In this review, these sources of variability are addressed and are exemplified with their application in recent clinical studies in different diseases. Our overall analysis highlights the need to achieve harmonized protocols for generating reliable and reproducible results that could be integrated into clinical practice to reach their full diagnostic and prognostic potential.

1. Introduction

Oxylipins are small bioactive molecules derived from the enzymatic or non-enzymatic oxidation of polyunsaturated fatty acids (PUFAs), including linoleic acid (LA, C18:2n-6), dihomo-gamma linolenic acid (DGLA, C20:3n-6), alpha-linolenic acid (ALA, C18:3n-3), arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3). In vivo, oxylipins regulate multiple processes such as blood flow, smooth muscle contractions, paracrine and autocrine cell signaling, inflammation, and the resolution of inflammation [1,2]. They are often referred to as “bioactive lipid mediators” due to their role as effector molecules. Specifically, oxylipins involved in inflammation resolution are called “specialized pro-resolving lipid mediators” or SPMs, and oxylipins derived from 20-carbon fatty acids such as DGLA, AA, or EPA are known as “eicosanoids”.

Enzymes involved in oxylipin synthesis are cyclooxygenase (COX), 15-hydroxyprostaglandin dehydrogenase (15-PGDH), lipoxygenase (LOX), cytochrome P450 (CYP), and soluble epoxide hydrolase (sEH) [3–13]. In general, COX and LOX are involved in hydroxylation reactions, 15-PGDH in the formation of ketones, CYP in epoxidation, and sEH in converting PUFA-epoxides into diols. As shown in Fig. 1, the end-products of PUFA enzymatic oxidation include COX-derived prostaglandins and thromboxanes; LOX-

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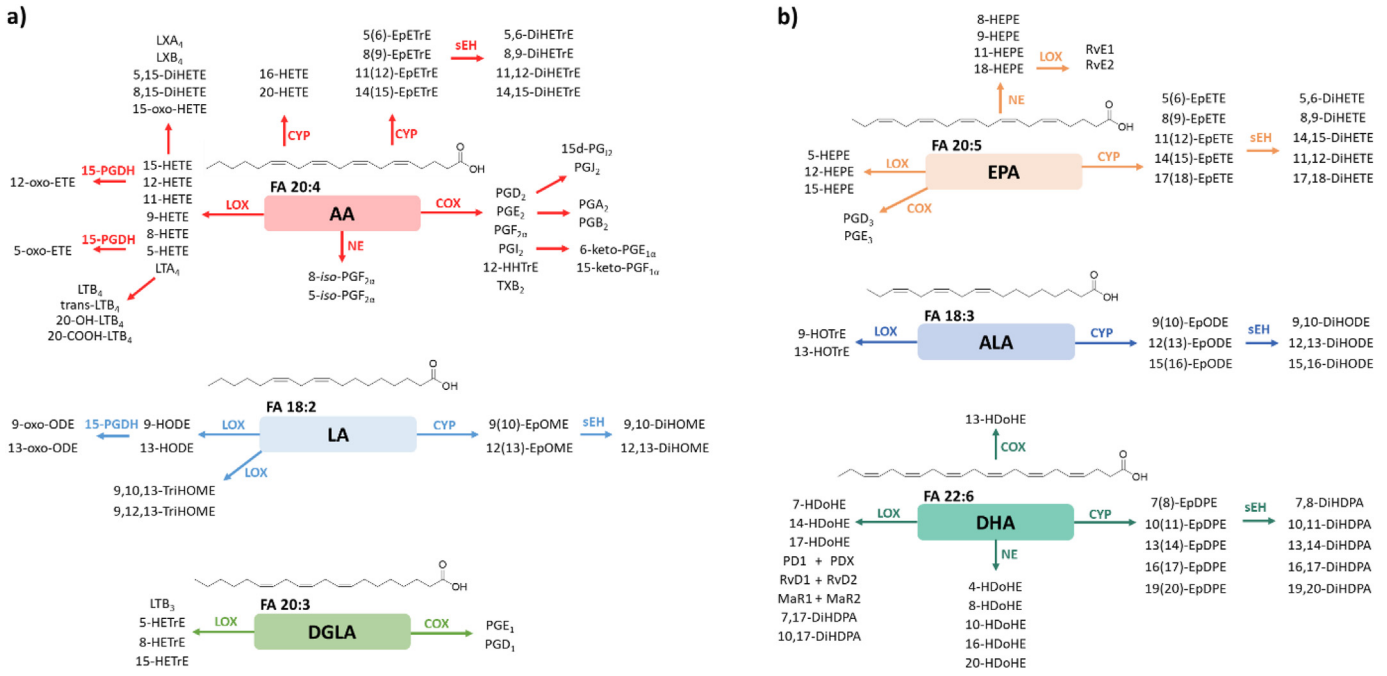

Fig. 1. Schematic representation of oxylipin metabolic pathways covered in this review. The figure shows oxylipins produced from **a)** n-6 PUFA (AA, LA, DGLA), and **b)** n-3 PUFA precursors (EPA, ALA, DHA). It should be noted that several oxylipins (e.g., 13-HDoHE) can be formed by both enzymatic and auto-oxidation pathways. Only the enzymatic pathway is shown in the figure.
Abbreviations: 15-PGDH: 15-hydroxyprostaglandin dehydrogenase, AA: Arachidonic acid, ALA: Alpha-Linolenic Acid, COX: Cyclooxygenase, CYP: Cytochrome P450, DGLA: Dihomo-Gamma Linolenic Acid, DHA: Docosahexaenoic Acid, DiHDPA: Dihydroxy-Docosapentaenoic Acid, DiHETE: Dihydroxy-Eicosatetraenoic Acid, DiHETrE: Dihydroxy-Eicosatrienoic acid, DiHODE: Dihydroxy-Octadecadienoic Acid, DiHOME: Dihydroxy-Octadecenoic Acid, EPA: Eicosapentaenoic Acid, EpDPE: Epoxy-Docosapentaenoic Acid, EpETE: Epoxy-Eicosatetraenoic Acid, EpETrE: Epoxy-Eicosatrienoic Acid, EpODE: Epoxy-Octadecadienoic Acid, EpOME: Epoxy-Octadecenoic Acid, FA: Fatty Acid, HDoHE: Hydroxydocosahexaenoic Acid, HEPE: Hydroxy-Eicosapentaenoic Acid, HETE: Hydroxy-Eicosatetraenoic Acid, HETrE: Hydroxy-Eicosatrienoic Acid, HHTrE: Hydroxy-Heptadecatrienoic Acid, HODE: Hydroxy-Octadecadienoic Acid, HOTrE: Hydroxy-Octadecatrienoic Acid, LA: Linoleic Acid, LOX: Lipoxygenase, LT: Leukotriene, LX: Lipoxin, MaR: Maresin, NE: Nonenzymatic, oxo-ETE: Oxo-Eicosatrienoic Acid, oxo-ODE: Oxo-Octadecadienoic Acid, PD: Protectin, PG: Prostaglandin, Rv: Resolvin, sEH: Soluble Epoxide Hydrolase, TriHOME: Trihydroxy-Octadecenoic Acid, TX: Thromboxane.

derived leukotrienes, lipoxins, hydroxyeicosatetraenoic acids (HETEs), and hydroxyeicosapentaenoic acids (HEPEs); CYP-derived epoxydocosapentaenoic acids (EpDPEs), epoxyeicosatetraenoic acids (EpETEs), and epoxyeicosatrienoic acids (EpETrEs); and sEH-derived dihydroxyeicosatetraenoic acids (DiHETEs), dihydroxyeicosatrienoic acids (DiHETrEs), and dihydroxydocosapentaenoic acids (DiHDPAs).

Prostaglandins (PGs) were the first oxylipins discovered in the 1930s by von Euler, who reported on their vasodilating properties [14]. Follow-up studies by Bergström and Sjövall in 1957 [14] reported the isolation of a 'PG factor' from sheep prostate that stimulated rabbit duodenum contractions at very low levels ($5 \times 10{-}9$ g/mL) [15]. Later studies by the same group identified the chemical structures of several PGs in sheep prostate isolates, including PGE1 and PGF1 from DGLA, and PGE2 and PGF2 from AA [15,16]. Bioactivity was then demonstrated through studies that showed PGE1 was a vasodilator, and PGE2 and PGF2 were promoters of smooth muscle contractions [17,18].

The discovery of PGs is historically unique because they were the first class of oxylipins to be translated to the clinic. PGE1, a vasodilator, was initially sold as alprostadil to treat infants with congenital heart failure, to induce cervical thinning during labor, or to treat erectile dysfunction [19]. A synthetic analogue of PGE1, misoprostol, was later developed and is currently used to induce labor alongside other medications [20,21]. PGE2 (dinoprostone) and PGF have also been shown to promote labor induction by stimulating uterine contractions [22], although less effectively than oxytocin, the current standard of care.

Pro-resolving oxylipins are another class of bioactive lipid mediators that act through specialized G-protein coupled receptors to resolve inflammation [23]. These include LOX-derived mono- and tri-hydroxylated metabolites of EPA (HEPE, resolvin E1) and DHA (resolvin D1 - also known as protectin D1), as well as CYP-derived epoxides of AA (EpETrEs), EPA (EpETEs), and DHA (EpDPEs). Currently, there are no clinically available drugs that target pro-resolving oxylipin metabolism. Candidate drugs such as sEH inhibitors that block the degradation of pro-resolving epoxides into less active diols are in Phase I clinical trials [24].

Given the bioactive role of oxylipins, enzymes involved in their synthesis are pharmacological targets of multiple drugs. For instance, non-steroidal anti-inflammatory drugs (NSAIDs) block the synthesis of pro-inflammatory PGs by inhibiting COX-1/2 enzymes [25]. Drugs used to treat asthma target the vasoconstrictive effects of leukotrienes by inhibiting LOX enzymes or leukotriene receptors [19]. Blood thinners, including low-dose acetylsalicylic acid (80–100 mg/day, e.g., Adiro®, Aspirin Cardio®, or Aspirin 81®), target COX-derived thromboxanes to reduce the risk of blood clotting [26,27].

The advent of 'omics' techniques with mass spectrometry (MS) has enabled the discovery and characterization of multiple oxylipin species over the past 25 years. During the 1950s and 1960s, measurements were performed with paper/silica chromatography separation until the development of high-performance liquid chromatography (HPLC) analysis. Currently, over a hundred oxylipins [28] can be identified and measured simultaneously by ultra-HPLC coupled to tandem mass spectrometry (LC-MS/MS), allowing for a more comprehensive network analysis of COX/LOX/15-PGDH/CYP/sEH pathways potentially affected by drugs or disease.

Despite technological advances in oxylipin measurements, not many have moved to the clinic since the discovery of PGs. A possible reason is that the methods used to assess their use as biomarkers are not sufficiently harmonized and adopted due to a lack of analytical validation. Another issue is that clinical biomarker studies are rarely replicated across multiple cohorts.

In view of the diverse and multiple physiological roles of oxylipins, the aim of this review is to highlight their potential as biomarkers and discuss how to achieve their validation as meaningful biomarkers in clinical research. Thus, key considerations for their analysis will be discussed, such as sample collection, sample storage and preparation, and structural complexity, as well as instrumental conditions, analytical validation, and method harmonization. Finally, the clinical applications of oxylipins in different diseases will be discussed, including future directions in their analysis and the long way ahead to achieve their full potential in clinical research.

2. Criteria for oxylipin validation as disease biomarkers

Several studies have explored the role of oxylipins as potential biomarkers of diseases such as cancer [29–31], cardiovascular diseases [32–34], metabolic disorders [35–39], asthma [40], and neurodegenerative disorders [41–44] due to their bioactive roles in vivo. From a strict point of view, the term “biomarker” has been defined as any characteristic that can be objectively measured and used as an indicator of different conditions, such as a biological or pathological process or a pharmacologic response to a therapeutic intervention [45–47]. In this sense, before a compound can reach biomarker status, there are three major steps that have to be fulfilled [46]: 1) biomarker selection; 2) analytical validation of the method for biomarker measurement; and 3) clinical validation in biological samples.

Biomarker selection is often hypothesis-driven because the goal is to identify with confidence which oxylipin(s) can provide maximal discrimination for a specific disease condition. An example is prostaglandins, which have been used as biomarkers of target engagement assays with anti-inflammatory drugs such as NSAIDs [48]. However, biomarker selection is also conditioned by several factors, including the number of tested oxylipins (a more holistic view will always help to select better predictors); the characteristics of patients in a cohort (cohorts that are adequately powered to account for population heterogeneity can lead to more clear changes); the biological matrix (e.g., more oxylipins can be detected in blood versus urine); and the groups to be compared (e.g., healthy vs. disease, mild vs. severe, active vs. placebo). Additionally, it is important to consider using different statistical strategies to select the best oxylipin candidate(s), e.g., the application of receiver operator characteristic (ROC) curves, which are one of the most widely applied methods for statistically determining the specificity and selectivity of a potential biomarker [49–51].

Analytical validation of the method is important for biomarker selection and determination. This step involves the application of different analytical validation tests, including reproducibility between and within days and among different laboratories [52]. However, doing so demands a high workload in the laboratory, detailed knowledge of the analytical methods and biochemical background for interpreting results. Because oxylipins are known to have important roles in inflammatory and immunological pathways, biomarker selection and method validation are often combined iteratively (e.g., for oxylipins, the method is often first developed, validated, and then applied to samples).

The final step in biomarker selection is clinical validation. This step aims to establish the ranges of concentration (cut-off values) of a biomarker to indicate whether a patient suffers from a condition (diagnosis); has worsened or improved disease prognosis paralleled by biomarker changes; or has a positive disease modifying effect after pharmaceutical treatment (clinical response) [53–55]. While two independent cohorts are typically conducted for biomarker discovery, reproducibility of the data within other cohorts is needed to fully establish cut-off values. To fulfill this stage, a multi-site clinical study should be performed in which patients are recruited from different hospitals and samples are analyzed in multiple laboratories following standardized protocols [28,46]. To our knowledge, no oxylipin has achieved this level of clinical validation to diagnose a disease. Thus, the goal of establishing a disease biomarker in the clinic remains unfulfilled.

3. Analytical considerations for the analysis of oxylipins

One of the main challenges with oxylipin quantification is that there are variability between studies. Values can vary by four orders of magnitude for different oxylipins (e.g., 149 ± 54 pM of 14,15-DiHETE versus 158,000 ± 65,000 pM of 9(10)-Ep-stearic acid in plasma [56]), but also variations within the same compound have been reported across studies (e.g., 15-HETE had a 27% coefficient of variation when measured across five different laboratories [52]). This is likely due to multiple factors, including stability, matrix effects leading to ion suppression or enhancement, and handling conditions. In addition, there are three types of oxylipin groups that are typically studied: 1) free circulating oxylipins; 2) esterified oxylipins (oxylipins that are part of complex lipids); and 3) total oxylipins (the sum of free and esterified oxylipins); each requiring a different extraction procedure. For all of these reasons, critical considerations for the reliable analysis (quantification) of oxylipins, such as sample collection, storage, and preparation, instrumental analysis, including analytical complexity, and method validation, will be discussed in this review.

A detailed literature search covering the last 5 years on oxylipin research was carried out with the following terms: “oxylipin OR eicosanoid OR prostaglandin OR isoprostane OR leukotriene OR “protectin” OR “resolvin” OR lipoxin OR maresin OR docosanoids OR specialized pro-resolving mediators (SPM) OR SPM OR oxidized fatty acid*) AND (clinic* OR inflamm* OR cancer* OR neuro* OR cardio* OR metabolic* OR disease* OR biomarker* OR marker* OR mediator* OR target*) AND (“mass spectrometry”) AND (“validated")”, filtering HUMANS during the 2017–2022 period. This also included a reverse search for publications of validated methods without the use of human biological samples that were subsequently applied in human studies. Various search databases, such as PubMed, Scopus, and Google Scholar, were used. The search resulted in 26 original research papers, which will be discussed throughout the next sections and are summarized in Table 1. Additional studies published prior to 2017 have also been included to support our conclusions and provide a more comprehensive view to this review. All in all, we present the published recommendations and complement them with our own experience.

3.1. Sample collection and storage

The type of biological matrix and method of sample collection and handling can be sources of variability in oxylipin analysis. As shown in Table 1, the majority of studied biological matrices for clinical applications are plasma [31,33,34,36,37,41,57–64], serum [35,37,65], and urine [29,30,38,39,42,44,63,66,67], although other matrices such as synovial fluid [68] and saliva [43,69] have been used.

As expected, there are differences in some oxylipins between serum and plasma due to the activation of platelet thromboxane synthase, which converts COX-derived prostaglandin H into

Table 1
Analytically validated methods applied in human biological samples associated with pathologies in the last 5 years.

N.	Sample type (disease) and analysis type	Sample collection, storage and number of samples	Sample treatment	LC conditions	MS conditions	Oxylipins measured in analysis and findings	Ref.
1	Plasma (hypertension) lTotal oxylipins (hydrolysis with NaOH)	Blood centrifugation in EDTA Stored at - 80 °C N = 19 (5 cases, 14 controls)	PPT (MeOH) LLE (modified Bligh & Dyer method)	**C**: C18 (PS 2.6 μm) **MP**: (A) H_2O, (B) MeOH **M**: 0.2% FA and 2 mM AmF **CT**: 7.5 min	**MA**: Qtrap **Mode**: MRM **Ion.**: ESI(−)	3 EpETrEs and 3 DiHETrEs Variations of their levels during endothelial stimulation	[33]
2	Plasma (type 2 diabetes) Free oxylipins	Blood centrifugation in EDTA Stored at - 80 °C N = 45 (25 cases, 20 controls)	PPT (MeOH) LLE (EA)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN/H2O (95:5) **M**: 0.1% AmAc **CT**: 10 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	15d-PGJ_2 Understanding of its anti-inflammatory effects in long-term pro-inflammatory disorders	[57]
3	24 h urine (smoking) Free oxylipins	Direct extraction Stored at - 20 °C N = 39 cases	LLE (modified Bligh & Dyer method)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 14 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	9 eicosanoids Partial recovery of smoking-induced alterations in the eicosanoid profile after smoking cessation	[39]
4	Plasma (Alzheimer's disease) Total oxylipins (hydrolysis with KOH)	Blood centrifugation in EDTA Stored at - 80 °C N = 94 (68 cases, 26 controls)	BHT addition SPE: Phenomenex Strata X-AW (WAX) (elution with MeOH + 5% HAc)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.01% HAc **CT**: 8.5 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	18 lipid peroxidation biomarkers Determination of 6 potential predictors of early Alzheimer's disease	[41]
5	Urine (Alzheimer's disease) Free oxylipins	Direct centrifugation Stored at - 80 °C N = 18 (13 cases, 5 controls)	β-glucuronidase addition PPT (MeOH + 37% HCl) SPE: Phenomenex Strata X-AW (WAX) (elution with MeOH + 5% HAc)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) MeOH **M**: 0.01% HAc **CT**: 8 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	17 lipid peroxidation biomarkers Determination of potential predictors of early Alzheimer's disease	[42]
6	Urine (lung cancer) Free oxylipins	Direct extraction Stored at - 20 °C N = 1220 (610 cases, 610 controls)	SPE: Agilent Bond Elut (RP C18) (elution with EA)	**C**: C18 (PS 3.5 μm) **MP**: (A) H_2O, (B) ACN/MeOH (95:5) **M**: 0.15% NH_4OH **CT**: 11 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	8-*iso*-$PGF_{2\alpha}$ High levels of it were associated with more than double risk of lung cancer in former and current smokers	[29]
7	Plasma (hypertension in type 2 diabetes) Total oxylipins (hydrolysis with NaOH)	Blood centrifugation in EDTA Stored at - 80 °C N = 74 (38 cases, 36 controls)	PPT (MeOH) LLE (modified Bligh & Dyer method)	**C**: C18 (PS 2.6 μm) **MP**: (A) H_2O, (B) MeOH **M**: 0.2% FA and 2 mM AmF **CT**: 7.5 min	**MA**: Qtrap **Mode**: MRM **Ion.**: ESI(−)	12 oxylipins Alterations in EpETrEs are associated with conduit artery endothelial dysfunction in type 2 diabetes	[58]
8	Peritoneal dialysate, serum and plasma (peritonitis, septic shock) Free oxylipins	Blood centrifugation in EDTA for plasma Stored at - 80 °C Peritonitis N = 10 (5 cases, 5 controls); Septic shock N = 28 (18 cases, 10 controls)	BHT, IND and *t*-AUCB addition PPT (MeOH) SPE: Agilent Bond Elut Certify II (RP C8 + SAX) (elution with EA/n-hexane (75:25) + 1% HAc)	**C**: C18 (PS 1.8 μm) **MP**: (A) H_2O/B (95:5), (B) ACN/MeOH (80:15) **M**: 0.1% HAc **CT**: 31.5 min	**MA**: Qtrap **Mode**: SRM **Ion.**: ESI(−)	18 SPMs New method developed for quantification of SPMs in different types of samples	[37]
9	Saliva (frontotemporal and vascular dementia and Alzheimer's disease) Free oxylipins	Non-centrifuged spit Stored at - 80 °C N = 30 cases	Two-step LLE (EA), UA-LLsME in the second extraction	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.01% HAc **CT**: 8.3 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	18 lipid peroxidation biomarkers New method developed for quantification of salivary lipid peroxidation compounds	[43]
10	Plasma (age-related sarcopenia) Total oxylipins (hydrolysis with KOH)	Blood centrifugation in EDTA Stored at - 80 °C N = 43 cases	SPE: Agilent Bond Elut Certify II (RP C8 + SAX) (elution with EA/n-hexane (75:25) + 1% HAc)	**C**: C18 (PS 1.8 μm) **MP**: (A) H_2O, (B) ACN/MeOH (80:15) **M**: 0.1% HAc **CT**: 31.5 min	**MA**: Qtrap **Mode**: SRM **Ion.**: ESI(−)	74 oxylipins Identification of early disturbances associated with muscle-loss during aging	[59]

Table 1 (*continued*)

N.	Sample type (disease) and analysis type	Sample collection, storage and number of samples	Sample treatment	LC conditions	MS conditions	Oxylipins measured in analysis and findings	Ref.
11	Urine (Alzheimer's disease) Free oxylipins	No centrifugation Stored at - 80 °C N = 99 (70 cases, 29 controls)	β-glucuronidase addition PPT (MeOH + 37% HCl) SPE: Phenomenex Strata X-AW (WAX) (elution with MeOH + 5% HAc)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.01% HAc **CT**: 8.5 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	18 lipid peroxidation biomarkers Determination of the individual probability of suffering from early Alzheimer's disease	[44]
12	Serum and menstrual fluid (inflammation during period) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 22 (7 treated, 15 controls)	On-line SPE: Waters online Oasis HLB (RP) (elution with H_2O/ACN (55:45) + 0.1% FA)	**C**: C18 (PS 3.5 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 15 min	**MA**: QqQ **Mode**: SRM **Ion.**: ESI(−)	PGE_2 and $PGF_{2\alpha}$ $PGF_{2\alpha}$ levels were higher in the treated group with Tahiti lemon juice, denoting an effect on menstrual inflammation	[35]
13	Urine (smoking) Free oxylipins	Direct extraction Stored at - 20 °C N = 1250 cases	SPE: Agilent Bond Elut (RP C18) (elution with EA)	**C**: C18 (PS 2.5 μm) for 8-*iso*-$PGF_{2\alpha}$/C18 (PS 2.6 μm) for PGE-M **MP**: (A) H_2O, (B) ACN/MeOH (95:5) for 8-*iso*-$PGF_{2\alpha}$/(A) H_2O, (B) MeOH for PGE-M **M**: 0.15% NH_4OH for 8-*iso*-$PGF_{2\alpha}$/15 mM AmAc for PGE-M **CT**: 16 min for for 8-*iso*-$PGF_{2\alpha}$/12.5 min for PGE-M	**MA**: QqQ **Mode**: SRM **Ion.**: ESI(−) for 8-*iso*-$PGF_{2\alpha}$/APCI(−) for PGE-M	8-*iso*-$PGF_{2\alpha}$ and PGE-M Contrary variations in their levels indicate the numerous endogenous and exogenous factors that influence oxidative damage and inflammation	[38]
14	Urine (hepatocellular cancer) Free oxylipins	Direct extraction Stored at - 20 °C N = 1038 (347 cases, 691 controls)	SPE: Agilent Bond Elut (RP C18) (elution with EA)	**C**: C18 (PS 3.5 μm) **MP**: (A) H_2O, (B) ACN/MeOH (95:5) for 8-*iso*-$PGF_{2\alpha}$/(A) H_2O, (B) ACN for PGE-M **M**: 0.15% NH_4OH for 8-*iso*-$PGF_{2\alpha}$/0.1% FA for PGE-M **CT**: 10 min	**MA**: Qtrap **Mode**: MRM **Ion.**: ESI(−)	8-*iso*-$PGF_{2\alpha}$ and PGE-M Risk association between high levels of 8-*iso*-$PGF_{2\alpha}$ and hepatocellular carcinoma independent of major risk factors, establishing it as a biomarker	[30]
15	Urine (baldness) Free oxylipins	Direct extraction Stored at - 20 °C N = 20 (10 cases, 10 controls)	SPE: Agilent Bond Elut (RP C18) (elution with MeOH) + LLE (EA/n-hexane (2:3)) Sample derivatization (2-HP)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 22 min	**MA**: Qtrap **Mode**: MRM **Ion.**: ESI(+)	6 prostaglandins Urinary prostaglandin levels did not change because of the administration of finasteride	[66]
16	Plasma (breast cancer) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 40 (20 cases, 20 controls)	PPT (ACN) SPE: Phenomenex Strata-X (RP) (elution with MeOH)	**C**: C18 (PS 1.7 μm) **MP**: (A) ACN/H_2O (45:55), (B) ACN/IPA (50:50) **M**: 0.02% HAc **CT**: 12 min	**MA**: Qtrap **Mode**: SRM **Ion.**: ESI(−)	63 oxylipins Concentrations of 5 oxylipins were statistically significantly increased in breast cancer	[31]
17	Plasma (Wilson's disease) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 55 (39 cases, 16 controls)	PPT (MeOH) SPE: Waters Oasis PRIME HLB (RP) (elution with ACN/MeOH (50:50))	**C**: C8 (PS 2.6 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 30 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	43 oxylipins Concentrations of 3 oxylipins changed significantly in Wilson's disease	[60]
18	Plasma (type 2 diabetes) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 40 (20 cases, 20 controls)	SPE: Waters Oasis HLB (RP) (elution with MeOH) Sample derivatization (Dns-PP + Dens-PP)	**C**: C18 (PS 1.9 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 60 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	65 eicosanoids Concentrations of 10 eicosanoids changed significantly in type 2 diabetes	[61]
19	Plasma (myocardial infarction) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 53 (22 black, 31 white subjects)	SPE: Waters Oasis PRIME HLB (RP) (elution with MeOH)	**C**: C18 (PS 1.8 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 12 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	40 bioactive lipid mediators Identification of race-based and sex-based SPM signatures following a myocardial infarction	[34]

(*continued on next page*)

Table 1 (*continued*)

N.	Sample type (disease) and analysis type	Sample collection, storage and number of samples	Sample treatment	LC conditions	MS conditions	Oxylipins measured in analysis and findings	Ref.
20	Plasma (obesity and type 2 diabetes) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 30 (11 cases, 19 controls)	BHT addition SPE: Waters Oasis PRIME HLB (RP) (elution with ACN + 1% FA)	**C**: PFP (PS 1.8 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.01% FA **CT**: 8 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	7 oxylipins Discrimination between type 2 diabetes patients and lean and obese controls with 4 oxylipins	[62]
21	Plasma and urine (age-related macular degeneration) Total oxylipins (hydrolysis with KOH)	Blood centrifugation for plasma/no centrifugation for urine Stored both at - 80 °C N = 99 cases	BHT addition to plasma/β-glucuronidase addition to urine PPT (MeOH) in urine samples LLE (Folch method)	**C**: C18 (PS 2.6 μm) **MP**: (A) H_2O, (B) MeOH **M**: 0.1% HAc **CT**: 21 min	**MA**: QTOF **Mode**: MRM-based software **Ion.**: ESI(−)	28 oxylipins New method developed for quantification of oxylipins in different types of samples	[63]
22	Plasma (type 2 diabetes) Total oxylipins (hydrolysis with KOH)	Blood centrifugation in EDTA Stored at - 80 °C N = 1161 (310 cases, 851 controls)	BHT and TPP addition LLE (modified Bligh & Dyer method) + SPE: Biotage Evolute Express ABN (RP) (elution with EA/MeOH (2:1)) Sample derivatization (AMPP)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 10 mM (~0.05%) FA **CT**: 18 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(+)	4 EpETrEs and 4 DiHETrEs No significant association of total EpETrE levels with diabetes, but 14,15-DiHETrE did have association with insulin and glucose levels	[64]
23	Saliva (oxidative stress in preterm infants) Free oxylipins	Non-centrifuged saliva from blotter swabs Stored at - 80 °C N = 36 (23 cases, 13 controls)	Two-step LLE (EA), UA-LLsME in the second extraction	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.01% HAc **CT**: NS	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(−)	4 prostaglandins Determination of lipid peroxidation biomarkers in preterm infants (e.g., PGE_2)	[69]
24	Serum (hepatitis B) Free oxylipins	Blood centrifugation Stored at - 80 °C N = 41 (19 cases, 22 controls)	PPT (ACN) Sample derivatization (dansyl chloride)	**C**: C8 (PS 1.7 μm) **MP**: (A) H_2O, (B) ACN/H_2O (95:5) **M**: 5 mM AmF **CT**: 12 min	**MA**: QqQ **Mode**: MRM **Ion.**: ESI(+)	45 immunity-related metabolites (9 oxylipins among them) 5 oxylipins increased in hepatitis, whereas PGD_2 decreased allowing better knowledge of the disease	[65]
25	Urine (type 2 diabetes with nephropathy) Free oxylipins	Direct urine centrifugation Stored at - 80 °C N = 144 (111 cases, 33 controls)	SPE: GL Sciences Monospin (RP C18) (elution with MeOH + 0.1% FA)	**C**: C8 (PS 2.6 μm) **MP**: (A) H_2O, (B) ACN **M**: 0.1% FA **CT**: 30 min	**MA**: QqQ **Mode**: SRM **Ion.**: ESI(−)/ESI(+)	196 oxylipins Tetranor-PGEM as a novel biomarker of diabetic nephropathy	[67]
26	Plasma (knee and hand osteoarthritis) Free oxylipins	Blood centrifugation in EDTA Stored at - 80 °C N = 216 cases	PPT (MeOH) Two-step LLE (modified Matyash method)	**C**: C18 (PS 1.7 μm) **MP**: (A) H_2O, (B) MeOH **M**: 0.01% HAc **CT**: 11.5 min	**MA**: Qtrap **Mode**: SRM **Ion.**: ESI(−)	28 oxylipins Eicosanoids may be involved in osteoarthritis severity	[36]

Abbreviations: 2-HP: 2-Hydrazinopyridine, ACN: Acetonitrile, AmAc: Ammonium Acetate, AmF: Ammonium Formate, AMPP: *N*-(4-Aminomethylphenyl)-Pyridinium, APCI: Atmospheric Pressure Chemical Ionization, BHT: Butylated Hydroxytoluene, C: Column, CT: Chromatographic Time per Sample, Dens-PP: (Diethylamino) Naphthalene-1-Sulfonyl Piperazine, DiHETrE: Dihydroxy-Eicosatrienoic Acid, Dns-PP: 5-(Dimethylamino) Naphthalene-1-Sulfonyl Piperazine, EA: Ethyl Acetate, EDTA: Ethylenediamine Tetraacetic Acid, EpETrE: Epoxy-Eicosatrienoic Acid, ESI: Electrospray, FA: Formic Acid, HAc: Acetic Acid, HCl: Hydrochloric Acid, IND: Indomethacin, Ion.: Ion Source, IPA: Isopropanol, KOH: Potassium Hydroxide, LC: Liquid Chromatography, LLE: Liquid-Liquid Extraction, M: Modifiers (additives + buffer salts), MA: Mass Analyzer, MeOH: Methanol, MP: Mobile Phases, MRM: Multiple Reaction Monitoring, MS: Mass Spectrometry, NaOH: Sodium Hydroxide, NH_4OH: Ammonium hydroxide, NS: Not Stated, PFP: Pentafluoro-Phenyl, PG: Prostaglandin, PPT: Protein Precipitation, PS: Particle Size, QqQ: Triple Quadrupole, QTOF: Quadrupole Time-of-Flight, Qtrap: Quadrupole-Linear Ion Trap, SAX: Strong Anion Exchange, SPE: Solid Phase Extraction, SPM: Specialized Pro-resolving Lipid Mediators, SRM: Selected Reaction Monitoring, *t*-AUCB: *Trans*-4-[4-(3-Adamantan-1-yl-Ureido)-Cyclohexyloxy]-Benzoic Acid, TPP: Triphenylphosphine, UA-LLsME: Ultrasound-Assisted Liquid-Liquid Semi-Microextraction, WAX: Weak Anion Exchange.

thromboxanes that act as blood coagulants, and the activation of COX and LOX during clotting. When compared to plasma, this enzymatic activation has been shown to increase the serum levels of thromboxane B2, 12-HHTrE and some HETEs [70,71]. This issue has made serum a less studied matrix than plasma [71].

Blood plasma is generated by the use of anticoagulants such as ethylenediamine-tetra acetic acid (EDTA) or heparin. As shown in Table 1, EDTA, the most commonly used anticoagulant [72], chelates calcium and, in doing so, inhibits multiple calcium-dependent clotting factors in platelets, thus inhibiting thromboxane formation [73]. Heparin prevents coagulation by inhibiting antithrombin III, another enzyme involved in blood clotting [74]. Differences in oxylipin concentrations have been reported in heparin versus EDTA plasma. In this regard, heparin was shown to increase platelet-derived oxylipins compared to EDTA-plasma [75,76]. The use of EDTA plasma is therefore preferred over heparin.

Another critical step in sample collection for oxylipin analysis is the time it takes for the whole blood sample to be processed into plasma, as well as the surrounding temperature when doing so. In general, whole blood should not be left at room temperature for more than 2 h before being processed. This is because residual platelet activity can affect the concentration of several oxylipins, as

evidenced by the increase in 12-LOX and thromboxane-derived oxylipins in plasma obtained from blood kept for 2 h at room temperature versus on ice [56,77]. However, if whole blood is left on ice (4 °C), oxylipin concentrations can remain stable for 2–4 h [71,75,77,78].

After the samples are collected and plasma or serum is prepared, they are stored at low temperatures until oxylipins are determined by liquid chromatography coupled to mass spectrometry (LC-MS). Collecting all the samples required to carry out a research study might take several months or even years. Therefore, it is important to understand how storage conditions, such as temperature and the use of additives, might influence oxylipin concentrations.

Storing serum or plasma samples after protein precipitation in methanol (MeOH) or other organic solvents has been shown to stabilize oxylipins. This occurs when enzymes involved in oxylipin formation and degradation are removed during protein precipitation, thus quenching metabolism. According to our experience, this can be achieved by adding MeOH to the sample, centrifuging, and storing the supernatant containing the oxylipins in −80 °C. It has been shown that oxylipins are stable in heparin-plasma for up to 24 h at 6 °C, up to 1 week at −20 °C in EDTA-plasma after protein precipitation with MeOH [76], and even up to 7 days at 4 °C in EDTA-plasma [71]. There is no consensus on the amount of solvent needed to increase plasma stability, as some authors add 60% (v/v) of MeOH [71], while others add 300% (v/v) [76]. However, the key point in this strategy is to add enough MeOH to ensure that enzymatic activity is stopped [71]. Other tested strategies include the addition of antioxidants, such as butylated hydroxytoluene (BHT), to plasma samples before storage. Nonetheless, some studies have shown that BHT does not affect oxylipin concentrations [71,76]. When longer storage times are needed, colder temperatures (−80 °C) have been shown to maintain most oxylipin levels in plasma samples for 12–15 months [56,75,76], with only a handful of oxylipins being affected because of autoxidative processes or residual platelet activity. Stability was further improved when plasma samples were stored in MeOH [76]. It should be noted that during long-term cold storage, new oxylipin species may appear either due to residual platelet activity or release from esterified lipid pools in plasma. Both of these mechanisms are halted when samples are stored in MeOH through the inactivation of participating enzymes [76]. Moreover, total oxylipins have been found to be less affected by sample collection and storage than free oxylipins, which makes them robust candidates for biomarker studies [56,78]. With this in mind, it is important to point out that most of the articles in Table 1 (21 out of 26) analyzed free oxylipins rather than esterified or total oxylipins. With recent data demonstrating their role in regulating the turnover and bioavailability of free oxylipins in vivo [79], and emerging evidence of their possible involvement in neurodegenerative disorders such as multiple sclerosis [80], esterified oxylipins represent a promising opportunity for new lines of research.

Other sampling techniques include dried blood spot (DBS) collection and solid phase microextraction (SPME). DBS consists of the application of blood droplets to a paper, from which analytes can be extracted with a solvent. DBS has several advantages, including easy collection, reduced sample volumes, simplified transport and storage, and easy oxylipin extraction with organic solvents (e.g., MeOH). A specific DBS collection kit for oxylipins has been developed by Xerion Limited, VIC, Australia. This includes a lipid-free paper called PUFAcoat™ used for measuring omega-3 and omega-6 PUFA-derived oxylipins in DBS. When using this paper, oxylipins are protected from oxidation since enzymes are inactivated upon dehydration of the blood spot, and its use allows the sample to be stable at room temperature for up to 2 months [81]. SPME combines sample collection, extraction, and concentration in one step. So far, it has been used in the in vivo oxylipin analysis of mouse brain and blood eicosanoids [82,83]. This strategy, which is yet to be used in human clinical settings, has the benefits of requiring less solvent and sample volume, as well as the ability to provide real-time information on the in vivo roles of oxylipins. A limitation of the technique is that it may not be as sensitive as other methods such as liquid-liquid extraction (LLE) or solid phase extraction (SPE), and any readout may be attributed to tissue damage caused by the brain implant consisting of a SPME coated with a biocompatible material [83–85].

To our knowledge, no studies have addressed the influence of sample collection and storage on other biological matrices such as urine or saliva. However, these are interesting matrices since their collection is non-invasive and the volumes of sample used are typically low, from 40 μL to 3 mL for urine [29,30,38,39,42,44,63,66,67], and from 50 μL to 150 μL for saliva [43,69]. Although the majority of studies have focused on blood plasma or serum, there is a lack of standardized protocols across laboratories for sample collection and storage, which would be critical for clinical applications.

3.2. Sample preparation

Sample preparation involves the efficient extraction of oxylipins from a biological matrix prior to instrumental analysis. Multiple approaches have been published in this regard, but in general, the steps for unesterified (free) oxylipin extraction typically are: 1) protein precipitation (PPT), and 2) oxylipin purification from the biological matrix either by LLE or SPE alone or applied consecutively. Extraction of total or esterified oxylipins involves the additional step of hydrolyzing with a base (e.g., sodium or potassium hydroxide, sodium carbonate, or sodium methoxide) for the release of oxylipins from complex lipids [86].

PPT is usually done using cold organic solvents (−20 °C) that disrupt non-covalent bonds between proteins and oxylipins. The most common organic solvents used are acetonitrile (ACN), isopropanol (IPA), MeOH, and ethanol [31,33,57,65,84,86]. Alcohols, such as MeOH and ethanol, are the best solvents for oxylipin extraction as they can cover a wide range of polarities, including hydroxy-PUFAs (more polar) and epoxy-PUFAs (less polar) [84,86]. After solvent extraction, oxylipins still need to be separated from matrix components that may interfere with LC-MS analysis (e.g., other lipids, vitamins). This typically involves subsequent LLE or SPE steps that achieve the dual purpose of removing co-eluting matrix components and concentrating oxylipins, thereby increasing the signal-to-noise ratio during LC-MS analysis.

The most common LLE methods for lipids are the Folch method [63] (2:1 chloroform/MeOH [87]) and the Bligh and Dyer method [33,39,58,64] (2:1 MeOH/chloroform [88]), but other solvent combinations can be used, such as methyl *tert*-butyl ether/MeOH [35] (Matyash method [89]), or ethyl acetate [43,57,69]. The use of acidified solvents with acetic acid has also been tested in LLE, and some authors have commented on its benefits in recovery. However, oxylipin yields have been shown to be comparable between the different LLE methods [86].

Most protocols use SPE to perform a further sample clean-up of extracts of oxylipins isolated from a matrix [29–31,34,35,37,38,41,42,44,59–62,64,66,67]. A sample extract is applied to a stationary phase, made of C8 and C18 for reverse phase or with an adsorbent for anion exchange, that enables different interactions and oxylipin coverages. Then, oxylipins are purified and eluted with specific solvents that detach them from the stationary phase. SPE is the extraction method of choice for the majority of studies listed in Table 1, although both SPE and LLE are widely used. Each has a different mechanism of extraction that

comes with differences in recovery [84]. For instance, the recovery of hydrophilic compounds like prostaglandins and leukotrienes when using LLE can be lower than that of SPE. Nonetheless, losses of analytes can be corrected with the use of labeled internal standards (e.g., d4-8-iso-PGF2α, d5-LTE4, d8-12-HETE [39]) as well as to monitor the extraction efficiency.

During sample preparation, the use of additives has been considered to avoid auto-oxidation and/or enzymatic degradation. To prevent oxidation, several additives have been tested, such as triphenylphosphine (TPP) [63] and BHT (a radical scavenger that reduces the autoxidation of PUFAs) [37,41,62–64]. Furthermore, EDTA [33,37,41,57–59], COX-1/-2 inhibitor indomethacin [37], and soluble epoxide hydrolase inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic (*t*-AUCB) have been used for enzymatic inhibition [37]. However, in a recent study, it was seen that only BHT was able to decrease the formation of hydroxy-PUFAs (i.e., 15-HETE), whereas EDTA, indomethacin, and *t*-AUCB did not have any effect on oxylipin concentrations [56]. The study suggests that some oxylipins are affected mainly by free radicals rather than by enzymatic activity during sample preparation. Various studies, however, found that BHT had no protective effect on oxylipin concentrations during storage, which means that any storage-related changes were likely due to residual enzymatic activity [71,76]. As evidenced by the previous examples, there are inconclusive results on the real usefulness of BHT and even on the source of oxylipin variability (enzymatic or non-enzymatic). However, the addition of alcohols or organic solvents for PPT has been proven to be sufficient for stopping enzymatic activity and thus preventing enzymatic oxylipin degradation [56,76].

Regarding other biological matrices from Tables 1 and in the case of urine, it is important to know that oxylipins might be bound to glucuronic acid as glucuronides or other conjugates. As a result, during sample preparation, β-glucuronidase is added to this type of sample to hydrolyze glucuronic acid conjugates [84]. This strategy was used in three of the studies compiled in Table 1 [42,44,63].

3.3. Isomeric complexity

The analysis of oxylipins holds a significant analytical challenge since one of their most essential characteristics is their isomeric heterogeneity. This is due to the enzymatic processes and autooxidation reactions producing a variety of isomeric species with different biological functions, e.g., 20-HETE and 15-HETE pair, with predominantly proinflammatory [90] and anti-inflammatory activities [91], respectively. The complexity of these mixtures, comprising constitutional or structural isomers (same formula but different structure) and stereoisomers (same formula and structure but different spatial conformation; *R*- and *S*-, *cis*- and *trans*-, diastereomers), adds further obstacles to their analysis. These limitations can be overcome by derivatization, the use of chromatographic strategies to separate the isomeric structures (e.g., chiral columns), or the use of MS in tandem mode to cover the wide range of fragmentation patterns.

One example of the determination of regioisomers is the case of 8-HETE and 12-HETE, which can be detected by means of the specific transitions of their fragments following electrospray ionization and collision-induced dissociation (CID) [92]. This is also the case for 5-HETE and 8-HETE, 9-HETE and 15-HETE, 8(9)-EpETrE and 11(12)-EpETrE, and 7-HDoHE and 10-HDoHE pairs [93], which also differ in their functional group position. However, in the event that the fragmentation pattern of regioisomers is the same, sufficient chromatographic separation is necessary, e.g., 12-HETE and 9-HETE [92], LXA4 and LXB4, RvD1 and RvD2, and RvD3 and RvD4 [93], which can be resolved using reversed-phase LC (RPLC). In the case of isomers with similar transitions and chromatographic coelution, some minor fragments could help, as is the case for 9-HODE and 9(10)-EpOME; their additional and distinct transition (m/z 295 → 195) allows them to be distinguished from their 13-counterpart isomers [93]. These examples give an insight into the analytical complexity of oxylipin analysis. With these strategies, validated methods for lipidomic profiling have recently characterized up to 130 oxylipins in human plasma [94] by relying on the unique separation and fragmentation patterns of structural isomers.

In the case of stereoisomers, recent studies have focused on their separation. Oxylipins containing epoxy groups with different cis/trans configurations have been resolved using conventional RPLC [92]. However, due to the interest in studying enantiomerically selective enzymatic pathways, the majority of the work has focused on the separation of enantiomers (*R*- and *S*- separations), which involves a higher degree of complexity [95]. The complexity of the analysis lies in the fact that, by conventional achiral RPLC-MS techniques, enantiomers are indistinguishable. The main efforts are focused on achieving satisfactory and selective chromatographic resolution by employing polysaccharide-based chiral columns [96], which increase oxylipin coverage. Validated methods have been used to resolve enantiomers of trihydroxyoctadecenoic acids in bronchoalveolar lavage fluid (BALF) [97] and pro-resolving oxylipins in commercial standards [98] using amylose with a particle size (PS) of 3 μm as the stationary phase. Isobaric compounds have also been deciphered with the use of these columns, e.g., LXA4 and 15-epi-LXA4 [98]. In chromatographic separations, the smaller the particle size, the better the resolution achieved. The sub-2 μm particle size amylose column allowed an excellent peak resolution of 19 enantiomeric pairs from different oxylipins (e.g., HODEs, HETEs, HEPEs, HDoHEs, RvD1s, HETrEs, HOTrEs, and DiHETEs) in platelet releases, allowing the establishment of an elution order in this method, with *R*-enantiomers eluting before their *S*-equivalents [99].

A recently published method provided improved chromatographic resolution and better coverage of both constitutional isomers and stereoisomers [95]. This consisted of using small achiral guard columns, for the separation of structural isomers, attached upstream to chiral columns for the separation of enantiomers, e.g., enantiomers of 15-HETE and 14(15)-EpETrE. In this analysis, two approaches were required to successfully resolve different subsets of oxylipins in human plasma: an amylose column for mid-chain monoepoxides and a cellulose column for all monohydroxides [95]. Chiral derivatization is also a possibility. Derivatization of oxylipins containing a 3-hydroxyalkanoate moiety using 3,5-dimethylphenyl isocyanate (3,5-DMPI) creates a urethane derivative, which can be resolved on a chiral column [100]. Moreover, further characterization of complex isomers can be achieved by coupling LC-MS analysis to ion mobility spectrometry (IMS) [101]. LC-IMS-MS analyses offer extra separation of isomers based on their spatial conformation [102], which can be sufficient for detecting them without the use of chiral derivatization or chiral columns [101,103].

The combination of selective mass transitions and chromatographic separation is crucial for achieving the required selectivity and sensitivity in the analysis of oxylipins, as well as for their independent quantification. It is highly advisable to optimize the chromatographic conditions by selecting the most suitable column, mobile phases, additives, and gradients. Therefore, there is no single methodology suitable for the analysis of multiple oxylipins in human plasma, and this would also be the situation for other biological matrices; hence, several strategies should be combined to obtain the highest possible oxylipin coverage.

3.4. Experimental and instrumental conditions

The majority of oxylipins have a carboxylic acid group in their structure, which allows them to be ionized in MS using electrospray ionization (ESI) in negative mode. In addition, different strategies to enhance their analysis include derivatization, which increases sensitivity and allows ESI in positive mode. This could be achieved with charge-switch derivatization using *N*-(4-amino-methylphenyl)-pyridinium (AMPP) as employed in plasma for characterizing total oxylipins in triple quadrupole-MS (QqQ) [64]. However, an independent comparative study using the same instrument showed that oxylipins derivatized with this methodology presented similar sensitivity in ESI(+) to non-derivatized products in ESI(−) in human plasma (e.g., 11-HETE, 5-HEPE, and 15-HEPE) [104]. On the other hand, IMS quadrupole time-of-flight mass spectrometry (IMS-QTOF-MS) analysis of oxylipins treated with this derivatization method revealed comparable sensitivity to that of other state-of-the-art instruments such as QqQ. RPLC-IMS-QTOF-MS was able to resolve 52 different oxylipins in human plasma, serum, and cells [103]. Other chemical derivatization procedures utilized to increase sensitivity in ESI(+) analyses are the use of 2-hydrazinopyridine (HP) [66] and dansylation [65], with the latter method providing better chromatographic resolution. Finally, twin derivatization has demonstrated improved sensitivity and resolution in ESI(−). This type of derivatization refers to the use of two reagents, one for the independent derivatization of the internal standards and a second reagent to derivatize the sample. In the twin derivatization approach, a 5-(dimethylamino) naphthalene-1-sulfonyl piperazine (Dns-PP)-derivatized plasma sample is mixed with an equal volume of (diethylamino) naphthalene-1-sulfonyl piperazine (Dens-PP)-derivatized eicosanoid internal standards. In this way, internal standards showed different retention times from those biological eicosanoids present in the sample [61]. Only 4 derivatization strategies have recently been applied in validated methods, including Dns-PP + Dens-PP [61] as well as AMPP [64], dansyl chloride [65], and 2-hydrazinopyridine (2-HP) [66] (Table 1). It is important to consider that oxylipins can degrade during the derivatization process, which is usually time-consuming, making clinical implementation less likely.

RPLC is the most commonly used chromatography method for the analysis of oxylipins, as seen in the selected articles from Table 1. The chromatographic columns used were made of alkyl C18 (22 out of 26) and C8 (3 out of 26) or pentafluoro-phenyl (PFP) (1 out of 26) as silanol derivatives, with different particle sizes, the majority being smaller than 2 μm (17 out of 26) as they provide better resolution of isobaric oxylipins [105]. As for chiral analysis (not included in Table 1 as applications did not fulfill our search criteria), the enantiomeric resolution employed specific polysaccharide chiral columns [93,95,97–99].

Another key chromatographic condition for oxylipin analysis is the mobile phase. These are different from those of other lipidomic analyses due to the higher relative polarity of oxylipins compared to other more complex lipids (e.g., phospholipids). Suitable mobile phases are H_2O as phase A and ACN as phase B, which are the most commonly used (50% of papers in Table 1). In a comparative study of organic solvents for mobile phase B, the use of ACN showed the advantage of better peak shapes in the chromatograms and provided more flexibility in terms of adjustments for analyte mobile phase elution times [99]. However, if changes in mobile phase composition or polarity are desired for the resolution of specific oxylipins, the addition of IPA, MeOH, or H_2O is possible. For pH adjustments and promotion of specific ionization forms, both formic acid (FA) and acetic acid (HAc) have been equally used as modifiers to solutions in low percentages (from 0.01% to 0.2%) (Table 1). Neither compromised the resolution of oxylipins, but HAc appeared to slightly increase the sensitivity [99]. Only three studies in Table 1 used ammonium hydroxide (NH_4OH) at 0.15% in the mobile phases [29,30,38]. Other modifiers such as ammonium formate [33,58,65] or ammonium acetate [38,57] have been used to separate isomers. For example, ammonium acetate at a concentration of 12 mM showed the highest separation of isomers of hydroxy-epoxy-octadecenoic acids (H-E-LAs) or keto-epoxy-octadecenoic acids (K-E-LAs) [93].

As shown in Table 1, ESI was the main ionization source used for oxylipin analysis in MS except for PGE-M analysis, in which negative atmospheric pressure chemical ionization (APCI) coupled to RPLC was used [38]. Interestingly, one recently validated method for the chiral analysis of derivatized HETEs in serum employed normal phase LC (NPLC) coupled to electron capture APCI (ECAPCI) [106]. This is a coupling that cannot be carried out with ESI. The study showed a substantial increase in sensitivity when compared with conventional APCI but did not reach sufficient sensitivity compared to ESI [104].

Currently, due to their versatility, specificity, sensitivity, and large dynamic range, tandem LC-MS methodologies are chosen for oxylipin quantification [93,107]. Targeted detection on QqQ was used in 17 out of 26 publications in Table 1, and quadrupole linear ion trap (Qtrap) instruments were used in 8 out of 26, both operating in multiple reaction monitoring (MRM) mode. This can be explained by the increased selectivity when a precursor-product transition in MRM is used for coeluting isomers, since isomers with the same mass can produce different fragments due, for example, to the position of the −OH group, which can lead to different MRM transitions. Precursor-product transitions can also be used in QTOF mass analyzers, as shown in Ref. [63] with the advantage of improved mass resolution. However, not all QTOF instruments can work in MRM, as they need specific software to control this function. As a result, conventional QTOF mass analyzers are rarely used in the study of oxylipins. This can be due to their reduced sensitivity compared to QqQ mass analyzers as well as their inability to unequivocally measure isomeric coeluting compounds when using MS1 mode only. Regardless of MS detection methods, optimizing various MS parameters such as the declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) may lead to better resolution and selectivity of oxylipins [93].

Using a method from our laboratory in QqQ, up to 72 oxylipins were independently detected with their specific transitions even though coelution occurred (Fig. 2, unpublished data). Some of these coeluting isomers could not be resolved using QTOF, such as 9-HODE and 13-HODE (Fig. 2), and 8-HETE and 12-HETE (data not shown).

To sum up, there are different chromatographic and MS conditions optimized in each method for target oxylipins depending on the desired sensitivity and resolution. Therefore, efforts should be focused on establishing harmonized methods so that optimized parameters can be extrapolated to multiple laboratories. A summary scheme of the analytical considerations shown in this section for the analysis of oxylipins is presented in Fig. 3.

3.5. Analytical validation and method harmonization

Reporting accurate concentrations of oxylipins in biological matrices is important because they can be used to establish what is a normal biological state (i.e., a range of concentrations of metabolites that are not associated with altered biological conditions) or to detect disturbances in metabolic pathways, serving as disease biomarkers. Such reference values can also be used to characterize the therapeutic efficacy and safety of drugs that regulate oxylipin metabolism (e.g., COX inhibitors). To reach these goals, analytical

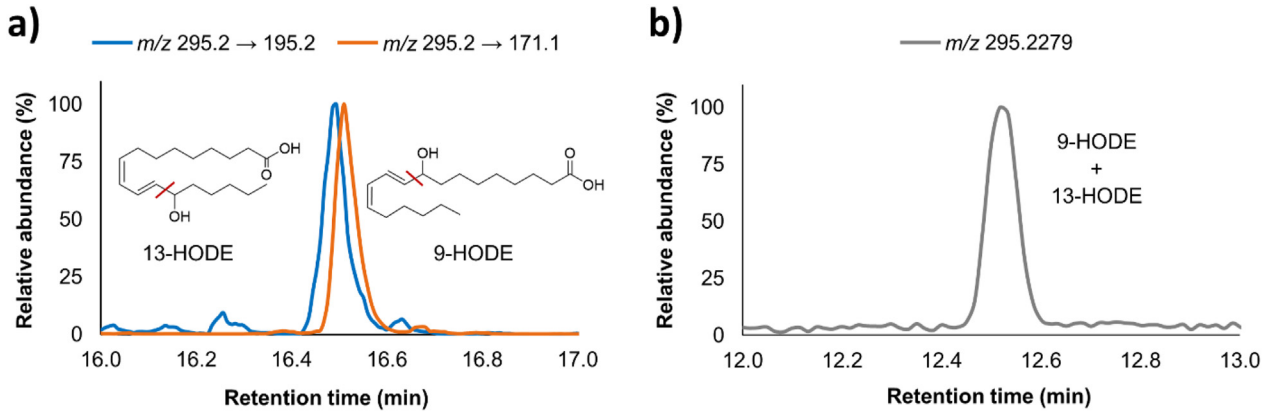

Fig. 2. Comparison of the selectivity for two isomeric oxylipins between **a)** HPLC-ESI(−)-QqQ-MS/MS (Agilent® 6470) and **b)** UHPLC-ESI(−)-QTOF-MS (Agilent® 6546). Chromatographic conditions: Agilent ZORBAX Eclipse Plus C18 (2.1 mm × 150 mm x 1.8 μm) column, mobile phases: A water solution of 0.1% HAc and B 80:15 ACN/MeOH with 0.1% HAc. The chromatography gradient started at 35% of B at 0–2 min, 85% at 12–15 min, 100% B at 15.10–17 min, and 35% at 17.10–19 min [108]. Experimental data in our laboratory.

validation and method harmonization are needed. The validation of bioanalytical methods aims to ensure the quality and certainty of the reported concentration values in biological samples and to define the limitations and suitability of the method. Validated methods are a prerequisite for protocol harmonization because they would have been tested and approved by the scientific community to achieve uniformity and interchangeability of results between different laboratories [109]. Currently, there are two main documents describing the validation of bioanalytical methods, which are in constant revision: The "Bioanalytical Method Validation" [110] from the Food and Drug Administration (FDA), latest version from 2018, and "The ICH Guideline M10 on Bioanalytical Method Validation" [111] published by the European Medicines Agency (EMA) and harmonized by the International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), latest version released in 2019. Both report in detail the characteristics that should be evaluated in the validation of ligand binding assays and chromatographic methods and should be considered the gold standard for the validation of oxylipin bioanalytical methods for clinical applications.

The two guides exhaustively describe the validation steps based on the evaluation of the following characteristics and provide the specific procedures to perform them: selectivity, specificity, matrix effect, recovery, calibration curve and range, accuracy and precision, carryover, dilution integrity, stability, and reinjection reproducibility. Because of their nature, some of these characteristics are particularly important in the study of oxylipins. For example, specificity is an important characteristic to evaluate in the validation of the method, not only for the study of all the oxylipins present in a sample but also because of the high number of isomers that can be found. Also, since the concentration of oxylipins can differ between normal and pathological conditions, the calibration curves should be properly designed to capture all possible concentration ranges. Moreover, the lower limit of quantification (LLOQ), the upper limit of quantification (ULOQ), and the limit of detection (LOD), if declared, should be experimentally confirmed with spiked samples, ideally with labeled standards in the biological matrix. Finally, and of particular interest in oxylipin analysis, stability should be evaluated under different conditions, including freeze-thaw experiments, short-term stability under bench-top conditions, long-term stability in the freezer, stability of the processed sample in storage conditions (i.e., freezer or fridge), and the stability of the sample in the autosampler.

The EMA and FDA guidelines also describe the importance of using suitable and high-purity reference standards with adequate certificates of analysis for their use in quality control samples and calibration curves in chromatographic methods. They also outline the need to use stable isotope-labeled standards that do not undergo isotope exchange reactions in MS methods. However, although the availability of native free oxylipin standards covers the biological variability fairly well and many labeled standards are commercially available, this is not the case for esterified oxylipins, for which the availability of standards is very limited due to the large number of potential complex oxidized lipid species present in biological matrices [28].

Among the works retrieved from the current literature on clinical applications of oxylipins, 26 studies met all the search criteria, including the validation of the LC-MS method used, as listed in Table 1. Most of the validations performed included selectivity, specificity, linearity, precision, and accuracy; however, only a few studies reported the evaluation of matrix effects and standard recovery. Carryover, dilution integrity, or reinjection reproducibility were typically not mentioned. Although LOQ and LOD were frequently reported, they were not experimentally confirmed. The fact that complete validation of the methods used for the application of oxylipins for clinical purposes has not been fully achieved makes it imperative to further pursue the harmonization of methods across laboratories. A 2020 study assessed the possible sources of variations between laboratories in an attempt to harmonize the analytical protocols [109]. This was a five interlaboratory study that used standardized sample preparation and analysis protocols for each laboratory. The study concluded that using the same protocol in different laboratories led to reproducible and reliable results for many oxylipins [109]. Nonetheless, it was noticed that sample handling and technical expertise were the main sources of variation than differences in the MS instruments or oxylipin stability. On the other hand, specific oxylipins like epoxy and oxo-PUFA appeared to be more sensitive to protocol deviations. The study indicates that it is possible to obtain reproducible results when studying oxylipins, particularly if efforts are made to ensure consistent sample handling and technical training between labs [109]. There is still a lot of work to be done on establishing standard operating procedures (SOP), involving more laboratories, and using good-quality reference materials to obtain consensus values for oxylipin content in different biological matrices.

4. Confirmation of findings, the middle step towards clinical validation

Biomarker validation entails analytical validation and confirmation of findings within and between studies. Clinically, this would entail demonstrating the potential utility of a biomarker in different

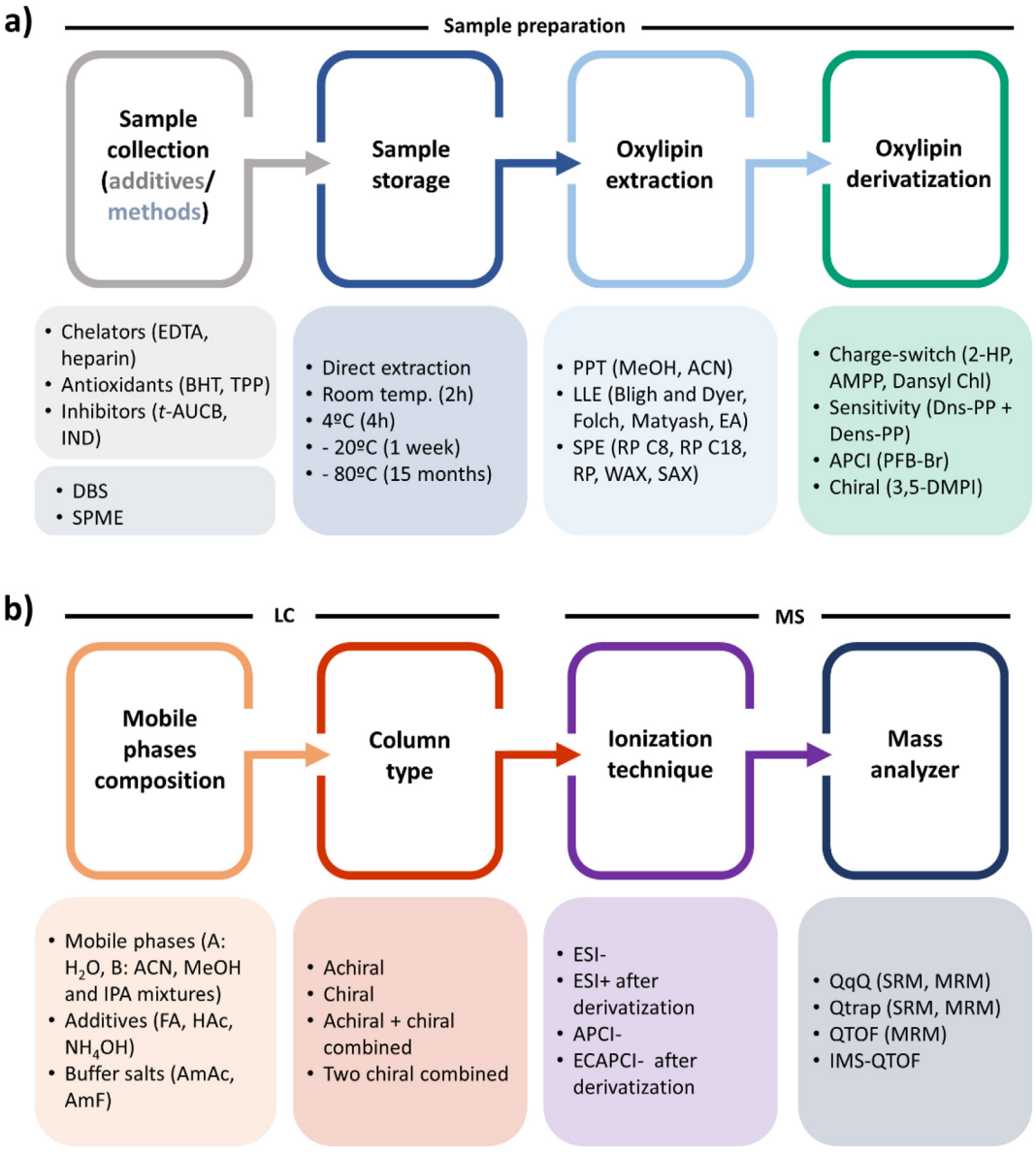

Fig. 3. Workflow for oxylipin analysis. The possibilities presented here are described in the articles reviewed in this work. a) Methodologies applied in the sample preparation prior to analysis. **b)** Instrumentation and analytical conditions for the analysis, including the resolution of the isomeric complexity present in samples.
Abbreviations: 2-HP: 2-Hydrazinopyridine, 3,5-DMPI: 3,5-Dimethylphenyl Isocyanate, ACN: Acetonitrile, AmAc: Ammonium Acetate, AmF: Ammonium Formate, AMPP: *N*-(4-Aminomethylphenyl)-Pyridinium, APCI: Atmospheric Pressure Chemical Ionization, BHT: Butylated Hydroxytoluene, DBS: Dried Blood Spot, Dens-PP: (Diethylamino) Naphthalene-1-Sulfonyl Piperazine, Dns-PP: 5-(Dimethylamino) Naphthalene-1-Sulfonyl Piperazine, EA: Ethyl Acetate, ECAPCI: Electron Capture Atmospheric Pressure Chemical Ionization, EDTA: Ethylenediamine Tetraacetic Acid, ESI: Electrospray, FA: Formic Acid, HAc: Acetic Acid, IMS: Ion Mobility Spectrometry, IND: Indomethacin, IPA: Isopropanol, LC: Liquid Chromatography, LLE: Liquid-Liquid Extraction, MeOH: Methanol, MRM: Multiple Reaction Monitoring, MS: Mass Spectrometry, NH_4OH: Ammonium Hydroxide, NP: Normal Phase, PFB-Br: Pentafluorobenzyl Bromide, PPT: Protein Precipitation, QqQ: Triple Quadrupole, QTOF: Quadrupole Time-of-Flight, Qtrap: Quadrupole-linear Ion Trap, RP: Reversed Phase, SAX: Strong Anion Exchange, SPE: Solid Phase Extraction, SPME: Solid Phase Microextraction, SRM: Selected Reaction Monitoring, *t*-AUCB: *Trans*-4-[4-(3-Adamantan-1-yl-Ureido)-Cyclohexyloxy]-Benzoic Acid, TPP: Triphenylphosphine, WAX: Weak Anion Exchange.

cohorts (separate from the discovery cohort) [55,112]. Such studies would represent a first step on the long path towards reaching reference ranges for a clinical biomarker. To our knowledge, there is no oxylipin that is routinely used as a biomarker in the clinic yet. However, there are few studies in which the authors have confirmed oxylipin changes in a different cohort of samples separate from the discovery cohort. In the last 5 years, up to 6 confirmatory studies in different cohorts have been carried out and are listed in Table 2.

Due to the efforts needed to obtain samples, the majority of biomarker discovery in metabolomics often relies on a limited number of case and control participants [113]. Therefore, subtle differences such as diet, age, gender, race/ethnicity, and socio-economic factors might influence biomarker performance in a specific disease, leading to non-generalizable conclusions. Therefore, proper clinical validation should involve large, adequately powered cohorts of samples from diverse patient groups to overcome the biological variability between patients, and to validate the consistency of the identified candidate oxylipins [28]. In Table 2, the confirmation of findings from a first discovery cohort was not always feasible for some analytes in the replication cohort. However, it is worth pointing out a study of biomarkers for hypertension, which included thousands of patients, provided a high degree of

Table 2
Confirmatory studies of putative oxylipin biomarkers in independent cohorts applied in human biological samples in the last 5 years. ‡Referred to in the article as "eicosanoids and related oxylipin mediators".

N.	Disease (sample)	Discovery cohort size	Validation cohort size	Oxylipin profiling	Potential biomarkers	Importance of biomarkers	Ref.
1	Renal dysfunction with uremia (plasma)	34 cases, 46 controls (N = 80)	10 cases, 10 controls (N = 20)	59	5,6-DiHETrE, 5-HETE, 9(10)-EpOME, 12(13)-EpOME	Diagnosis of renal dysfunction	[116]
2	Gouty arthritis (plasma)	26 cases, 26 controls (N = 52)	20 cases, 20 controls (N = 40)	68	LTB_4	Diagnosis of gouty arthritis Therapeutic target by the inhibition of 5-LOX	[120]
3	Hypertension (plasma)	4532 cases, 3567 controls (N = 8099)	1186 cases, 1673 controls (N = 2859)	545‡	11-dehydro-2,3-dinor-TXB_2, 12-HHTrE, tetranor-12(*R*)-HETE, 5,6-EpETrE	Diagnosis of high systolic blood pressure by the eicosanoid risk score made of 6 metabolites	[114]
4	Rheumatoid arthritis under DMARD (plasma)	30 responders, 24 non-responders (N = 54)	36 responders, 22 non-responders (N = 58)	55	RvD_4, LXA_4, MaR1, 10(*S*),17(*S*)-DiHDPA	Treatment responsiveness prediction at 6 months after its initiation	[121]
5	Diabetic macular edema in type 2 diabetes (plasma)	30 cases, 30 controls (N = 60)	43 cases, 43 controls (N = 86)	60	12-oxo-ETE, 15-oxo-ETE, 9-oxo-ODE, 20-COOH-LTB_4	Diagnosis of macular edema in diabetic patients	[117]
6	Metabolic syndrome (plasma)	137 cases, 137 controls (N = 274)	101 cases, 101 controls (N = 202)	133	4 epoxy-PUFAs (*e.g.*, 9(10)-EpOME), 2 ketone-PUFAs (*e.g.*, 9-oxo-ODE), 12 mid-chain hydroxil-PUFAs (*e.g.*, 5-HETE), 5 vicinal dihydroxy-PUFAs (*e.g.*, 9,10-DiHOME)	Diagnosis of metabolic syndrome and determination of its phenotype by a consistent oxylipins signature (29 oxylipins) Prediction of cardiometabolic diseases risk	[115]

Abbreviations: DiHDPA: Dihydroxy-Docosapentaenoic Acid, DiHETrE: Dihydroxy-Eicosatrienoic Acid, DiHOME: Dihydroxy-Octadecenoic Acid, DMARD: Disease-Modifying Antirheumatic Drugs, EpETrE: Epoxy-Eicosatrienoic Acid, EpOME: Epoxy-Octadecenoic Acid, HETE: Hydroxy-Eicosatetraenoic Acid, HHTrE: Hydroxy-Heptadecatrienoic Acid, LOX: Lipoxygenase, LT: Leukotriene, LX: Lipoxin, MaR: Maresin, oxo-ETE: Oxo-Eicosatrienoic Acid, oxo-ODE: Oxo-Octadecadienoic Acid, PUFAs: Polyunsaturated Fatty Acids, Rv: Resolvin, TX: Thromboxane.

confidence in 6 biomarkers, among which 4 oxylipins have been identified [114]. The next steps for this line of research into clinical validation would be longitudinal follow-up studies that could capture the progression of the disease. This is difficult to achieve clinically but is needed to understand how a biomarker changes with disease state. This approach may provide a way to reproduce biomarker findings longitudinally within a cohort.

From the results of Table 2, we can conclude that changes in circulating oxylipins may indicate the presence of several diseases with overlapping pathways. For instance, the same pro-inflammatory oxylipins may be altered across multiple diseases where inflammation plays a role. This is the case of the metabolic syndrome [115], where the characterized biomarkers are 9(10)-EpOME, 5-HETE, and 9-oxo-ODE, the first two coinciding with renal dysfunction [116] and the last with diabetic macular edema [117]. This is why looking for a single oxylipin as a biomarker might be impractical, but rather a distinct subset of oxylipins might be more useful in discriminating one inflammatory condition from the another. This is the case for the metabolic syndrome, which has a distinct "oxylipin signature" that comprises variations in 29 oxylipins (including 5-HEPE, 5-HETrE, and 5,6-DiHETrE, among others) that can be used to diagnose it and determine the patient's phenotype. However, the identified oxylipin signature would require further validation in various and larger cohorts to confirm and expand these findings into the clinic [115]. It remains to be determined whether a similar or different network of oxylipins is altered by other metabolic disorders.

5. Discussion and future work

To date, there are more than a hundred oxylipins reported in the literature [28]. From these, specific sets of oxylipins have been described after untargeted or targeted analysis in several diseases (e.g., Tables 1 and 2). Regardless of the way specific oxylipins are selected, their quantification in biological samples remains an analytical challenge. Several key steps are not yet standardized between laboratories, such as the target approach to be studied (free, esterified, or total oxylipins) and the protocols to be followed in the workflow from sample collection to their analysis. However, significant efforts have been carried out to test the conditions of sample collection and addition of reagents, storage conditions to maintain their stability, analytical solutions for their isomeric complexity, and the use of novel analytical techniques and mass analyzers (e.g., QqQ or TOF). Future applications involving the use of chiral columns or the application of IMS-MS [101,103] could overcome isomer resolution problems associated with coelution. In addition, the lack of commercially available isotope-labeled standards for method development and quantification, particularly in the case of esterified oxylipins, has limited their analysis in biological samples. The use of at least one internal and/or external standard per oxylipin is a prerequisite [109]. The ideal internal standard should have the same chemical and physical properties as the oxylipin of interest while still being distinguishable on MS [107,118]. As a result, for accurate quantification, a stable isotope-labeled internal standard for each oxylipin is the preferred option. However, external calibration curves with internal standards have also been used as an approach for the analysis of oxylipins [109].

In everyday clinical practice, the use of oxylipins as disease biomarkers remains in its infancy due to the lack of standardized procedures and validation across multiple cohorts. In this regard, future efforts should be invested in standardizing multiple protocols between laboratories (e.g., PPT, LLE, and SPE) to demonstrate that they are optimized and tested for rigor, simplicity, and reproducibility. For clinical translation, the participation of several hospitals and analytical centers may be required. Furthermore, the development of new tools such as biosensors, that could carry out the analysis at the time the sample is taken is promising. This is the

case for a biosensor designed to measure PGE2 for the rapid diagnosis of urinary tract infection [119].

6. Conclusions

There is still a long way to go before oxylipins can be used in the clinical environment. According to our research and experience, there is an urgent need for harmonized protocols for multiple critical steps prior to further innovation of methodologies such as biosensors. These include sample collection, storage, and preparation procedures, as well as analytical resolution of isomeric oxylipins. Inter-laboratory validation of multiple analytical protocols may be a practical way forward in order to obtain reliable data from studies carried out in different hospitals and health centers with different methods. However, the task of doing so is not simple, as it requires dedicated funding mechanisms and inter-disciplinary collaborations between researchers and industry (particularly for stable isotope-labeled internal standard synthesis for esterified oxylipins). Future exploration and validation of point-of-care devices such as biosensors may also represent a novel and rapid approach that can be easily used in clinical settings.

Author contributions (CRediT)

Sandra M. Camunas-Alberca: Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Maria Moran-Garrido:** Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Jorge Sáiz:** Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Alma Villaseñor:** Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Ameer Taha:** Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Coral Barbas:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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