Quantitation of oxylipins in biological samples, focusing on plasma and urine.

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Abstract

Oxylipins are a large family of oxygenated fatty acids, generated by enzymes that include lipoxygenases, cyclooxygenases and cytochrome P450s. They are formed in almost all cell types and organs and play essential roles in health and disease. Many were discovered in the 1980's, including prostaglandins, thromboxane and leukotrienes. Nowadays, the analysis of these lipids is generally performed using targeted LC-MS/MS methods, which can quantify down to levels of around 1 pg on column, depending on the specific platform. Robust scheduled assays that can analyse up to 150-200 lipids are increasing in popularity but require a very high degree of technical competence and ongoing monitoring to ensure accuracy of their result. Close attention to peak quality and isomer identification is required. ELISAs also exist but there are considerations relating to specificity when applied to complex biological samples. Oxylipins are metabolised by β-oxidation, and many of their tetranor and dinor compounds can be readily detected in urine. Robust assays for some are in clinical use for inflammatory disease and cancer. Last, some are found esterified to phospholipids, glycerides and sterol esters. Quantitative assays for these are less established, due to lack of synthetic standards, but this is a growing area. This chapter will outline the basic principles of oxylipin analysis, focusing on LC-MS/MS, presenting current state of the art in relation to how to approach establishing such methods in the laboratory for plasma and urine.

1. Introduction

Oxylipins are structurally related oxygenated polyunsaturated fatty acids (PUFA) generated by enzymes. Additionally, their stereo- or positional- isomers can be generated non-enzymatically. In the LIPID MAPS classification/nomenclature, they are in the Fatty Acyl (FA) class, represented by: octadecanoids, eicosanoids and docosanoids[1]. They arise by oxygenation of PUFA of usually 18-22 carbons and between 2-6 double bonds and are formed by lipoxygenases (LOX), cyclooxygenases (COX) or cytochromeP450s (CYP450). Sequential metabolism by two or more enzymes can also occur. Here, formation of prostaglandin H2 (PGH_2) by COX is followed by formation of thromboxane A2 (TXA_2) by the CYP450, thromboxane synthase[2]. Prostanoids/prostaglandins (PG) represent oxylipins that contain a 5-carbon prostanoid ring, and include prostaglandins such as PGE_2, PGD_2 and $PGF_{2\alpha}$. Distinct products are formed from different PUFA, based on the substrate and enzyme. For example, while 15-LOX1 oxygenation of arachidonic acid (20:4) generates 15S-hydroperoxyeicosatetraenoic acid (15-HpETE), metabolism of docosahexanoic acid (22:6) results in 17S-hydroperoxydocosahexanoic acid (17-HpDOHE). The major oxylipin biosynthetic pathways are found on LIPID MAPS/Wikipathways (https://www.lipidmaps.org/resources/pathways/wikipathways), summarised in Figures 1,2[3, 4].

Oxylipins are generated in virtually all organs and cells. Many maintain homeostasis, e.g. PGE_2 in the gastric system, or TXA_2 and prostacyclin (PGI_2) in the vasculature. PGE_2 is generated in inflammation, involved in immunity, cancer, infection and pain. During homeostasis, PGE_2 is mainly from COX-1, while during inflammation, high amounts of PGs are generated via COX-2 induced by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). Oxylipins signal by either, (i) high affinity binding and activation of G-protein coupled receptors (GPCRs), or (ii) by low affinity activation of the transcription factor, peroxisomal proliferator activating receptor PPARγ. In the case of GPCRs, specific oxylipins are recognised by their cognate receptors, activating highly controlled signalling in a tissue/cell specific manner. In contrast, PPARγ is activated by many oxylipins, upregulating a transcriptional programme to dampen inflammation and fibrosis.

Nowadays, oxylipin analysis generally utilizes high sensitivity triple quadrupole or Q-Trap MS instruments coupled via electrospray ionization with LC, which have superseded GC/MS, LC/UV and other older methods. The MS is typically operated in *Multiple Reaction Monitoring (MRM)* mode, which may or may not be *Scheduled*, allowing simultaneous detection of large numbers of lipids. The *Scheduled* mode allows the instrument to analyse lipids only around the time when they are expected to elute from the LC column. This relies on the use of *precursor-to-product* ion transitions that are considered sufficiently specific for each lipid analysed, and is also termed selected (SRM). For example, during a 15-20 min separation, each oxylipin will be measured only for up to 2 minutes, meaning that at any particular time, only a small number are simultaneously measured. This allows from 100-200 lipids (depending on platform, chromatographic separation, etc.) to be quantified per sample, since the instrument can acquire sufficient points across a peak (generally around 6-20 or more points per peak is captured) with sufficient dwell time. Here, isotopically labelled (typically deuterated) internal standards are added before extraction, with the most structurally similar being used for each primary (unlabelled) analyte. For all analytes quantified, a primary standard is required. Quantitation relies on generating standard curves for every analyte, plotted against a constant amount of internal standard in serial dilutions. Limit of detection (LOD) and limit of quantitation (LOQ) is defined based on signal:noise (S:N), calculated by height or area, and most vendors' software provide scripts for this. As a general rule, LOQ is used at S:N at least 5-10 in analytical laboratories, with LOD being around 3:1. A clearly discernible peak must be seen above baseline noise, or the lipid is judged to be below the limit of detection/quantitation in the sample. This is in line with guidelines from international bodies[5-14]. Approaches that define LOQ/LOD based on minimum area of integration in the chromatogram, or the presence of ions considered to be diagnostic in an MS/MS spectrum can incorrectly infer lipids are present in baseline noise and should be avoided[15].

2. Analysis of Oxylipins: Plasma, Tissues and Cells

While most PGs are detected at low concentrations, others such as hydroxy-linoleic acid derivatives are often 100-1000 fold higher concentrated. Furthermore, oxylipins can be formed and degraded after and during

sample collection and preparation. Due to this, to ensure a reliable quantitative analysis, all steps from sample collection to MS analysis need to be carefully considered.

2.1 Planning of sample collection preparation and storage

Sample handling needs to be carefully standardized. Inappropriate collection, pretreatment, storage and analytical sample preparation can strongly influence the oxylipin pattern detected[16-19]. This is because PUFA, such as arachidonic and docosahexanoic acids are prone to artefactual oxidation, which is accelerated by free transition metals from disrupted tissue or cells (Fenton-type chemistry). Here, products include not only enzymatically-generated oxylipins, but also large numbers of additional regio- and stereoisomers that can be hard to distinguish. Strategies to minimize this include addition of antioxidants, metal chelators that prevent metal ions from redox cycling, and keeping cell and tissue samples cooled on ice during initial processing. An exception is washed platelets, which need to be isolated at room temperature, to preserve agonist responses.

The test system needs to be carefully considered to decide whether oxylipins are likely to be formed in biologically-meaningful amounts. First, asking if biosynthetic enzymes, e.g. COX, LOX and CYP450s are expressed in the tissue, compartment or matrix at sufficient levels. This can be assessed by prior knowledge from the literature, or screening transcriptional data. For example, the expression of 12-LOX and COX-1 in platelets has been known for decades. Furthermore, species differences exist, with *ALOX15/Alox15* encoding enzymes with different product profiles in mice/rats/pigs (12-lipoxygenating towards arachidonate primarily, and termed 12/15-LOX) versus humans/rabbits (15-lipoxygenating, called 15-LOX)[20, 21]. The impact of activation/stimulation (time, type and dose of stimulus) of the biological sample needs to be considered, since oxylipin patterns change over time, for example induction and activation of COX-2 by pro-inflammatory LPS in macrophages[22]. If using cultured cells, oxylipins are generally enriched in the supernatant since they are secreted to act as paracrine stimuli on other cells[23, 24].

2.2 Consideration of experimental system, focusing on plasma and serum

For the analysis of blood cells, one can choose purified cells, such as platelets, monocytes or neutrophils either alone or in mixed cell populations[25-28]. All have highly specific expression patterns for oxylipin-generating enzymes, and if combined, then transcellular metabolism to secondary products may occur. Whole blood can be analysed, before or after the use of activators such as LPS[29]. Anti-coagulated whole blood can be centrifuged to remove cells, generating plasma. For this, clotting is prevented using anti-coagulants, such as calcium chelators (EDTA, sodium citrate), or heparin. These have distinct effects on oxylipins[30, 31], with chelation being preferred due to its dual action both on coagulation and platelet/leukocyte activation. Heparin prevents coagulation but does not prevent activation of platelets. When whole blood is not anti-coagulated, or is actively coagulated using activators such as glass particles, the removal of cells along with fibrinogen results in serum. Generation of serum represents an *ex vivo* coagulation assay forming high(er) oxylipin levels because of the activation of white cells and platelets during blood clotting. Thus, oxylipin levels are not representative of what was present *in vivo* prior to sampling, and great care would also be needed to ensure that non-enzymatic oxidation to form isoprostanes was prevented. Serum oxylipin analysis can be considered an ex vivo "capacity assay" where the maximum ability of the blood cells to generate the lipids is being measured. This can be useful, for example if testing the ability of an *in vivo* administered COX-1 inhibitor to prevent platelet thromboxane generation, but it doesn't reflect endogenous i*n vivo* circulating oxylipins. Notwithstanding this, serum has been sucessfully used in a large number of published studies[32-34]. Plasma oxylipins can indirectly reflect their formation and action in other tissues such as kidney, liver, lung, etc. The relative contribution of circulating blood cells versus other tissues to measured plasma levels can be difficult to delineate, especially during inflammation.

When isolating plasma or purified cell populations from whole blood, the method of venepuncture needs to be considered. Narrow needles/tubing and vacuum from vacutainers can activate white cells and platelets, and sometimes cause hemolysis. This is a well-recognized "sampling artefact" that causes significant elevations in oxylipins. Another consideration relates to which oxylipin pool is of interest experimentally and biologically. These lipids are generally measured as their free acid forms (unesterified), but they are found esterified into complex lipids. Indeed, the majority of hydroxy-PUFA and epoxy-PUFA present in plasma or

cells appear to be bound to larger lipids, particularly phospholipids (PL) and are termed oxidized PL (oxPL)[16]. Furthermore, these can be generated acutely by activated white cells or platelets[35]. OxPL can be detected either as their intact molecular species (see later section), or as the free acid species generated following hydrolysis. This can be achieved using saponification/base hydrolysis, or by using phospholipaseA2. During saponification, prostanoids such as PGE_2, are destroyed and can't be recovered[36]. For these, enzymatic hydrolysis is preferred[37]. Notably, with this, structural information on the precursor lipids is lost. A detailed protocol for the quantification of "total" oxylipins in blood can be found here[18, 38], and their analysis as intact oxPL is detailed later.

2.3 Obtaining and handling plasma for oxylipin analysis

Many cohorts have stored plasma and serum, thus these tend to be the most commonly analyzed samples in population studies. Oxylipin measurements in plasma/serum from cohorts can be fraught with difficulty since sometimes the sampling methods are not well described, and oxidation can increase in long term storage even at -80 °C[39]. Each step, such as transitory storage, transport or long-term storage can influence the resultant levels of oxylipins detected[16, 17, 19, 31]. Key for successful quantitation is to define all parameters that could cause variability or interferences in advance of starting sample collection. A list of these is provided below, and these have been extensively tested in[16, 17, 19, 31].

1.) Itemize all plasticware (brand and specific type) and test for possible interferences, caused for example by plasticizers. A list of suitable materials are provided here[18].

2.) Collect blood at a defined time (+/- 2 h) of the day, ideally following overnight fasting. Use a needle with a sufficient gauge (20 G or larger) and EDTA collection tubes.

- *Fasting state and/or sampling at different times can lead to high inter-individual variability, while inter-day variability of plasma oxylipins of fasted at the same time of day subjects is low*[16, 40]

3.) During transport, keep the blood cool - but not on ice - and store for maximum 2 h, avoid shaking and minimize transport.

- *Freezing (including freeze/thaw cycles) or shaking can cause hemolysis and/or cell activation, distorting oxylipin levels detected[19].*

4.) To generate plasma, centrifuge at 1,000 g for 15 min at 4 °C, remove plasma from the tube within 1 hour.

5.) Generate aliquots (e.g. 500 µl for free[41, 42] or 100 µl for total oxylipins[38]. Whenever possible generate back-up samples. Pipette the exact volume needed and mix directly with an equal volume of methanol or other organic solvent.

- *Methanol stabilizes the biological samples and including during long term storage[31]*

6.) Freeze the sample immediately, e.g. by snap freezing in liquid nitrogen. Record the time from blood draw till freezing for each sample -(batch). Time to freezing should not exceed 3 hours[17, 19].

7.) Store at -80°C for analyze the samples as soon as possible but within at most 2 years. If the samples need to be shipped send them on dry ice with a temperature logger.

- *Oxylipin levels can change during storage. At temperatures over -30°C significant changes occur, particularly if no methanol is added. In particular, non-enzymatically generated oxylipins, including F2-isoprsotanes (F2-IsoPs), can form from plasma polyunsaturated fatty acids (PUFA) at these temperatures. However, at -80°C, levels of samples prepared as outlined can be regarded as stable for about 2 years, and maybe longer[16, 31].*

2.4. Extraction of oxylipins from plasma

Prior to LC-MS/MS, plasma needs to be processed to remove proteins and other interfering compounds, since electrospray ionization (ESI) and MS detection are strongly influenced by matrix composition. Processing generally uses solid phase extraction (SPE) columns that contain reverse phase LC solid phase, allowing selective elution off the column of oxylipins following removal of polar compounds and neutral lipids. SPE can also enable concentration of samples, e.g. by factor 10[41, 42] facilitating detection of low levels of oxylipins. However, several quantitative oxylipin methods have been described leading to an overall dilution of the samples using rapid sample preparation, without SPE[43] or online-SPE following dilution[44]. Prior to sample clean up, stable isotope-labelled internal standards are added which correct for losses during

extraction, and allow quantitation, as described here[38, 41, 42]. Moreover proteins can be precipitated using by an excess of organic solvent such as methanol, if this has not been added during sample collection (see above)[38, 41].

Use IS recovery as quality marker for the sample preparation in each sample: Following SPE, the extract is reconstituted in an organic solvent. "Internal standard 2" (IS2) is then added, for example 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, 12-oxo-phytodienoic acid and aleuritic acid[17, 45]. With that, the extraction recovery of the internal standards themselves can be quantified in each sample post extraction. Using that method, only extracted samples with an internal standard recovery of at least 60 % would be used for quantification. If recovery appears lower than expected, adding internal standards after the SPE step and comparing the results allows to pinpoint whether the extraction efficacy of the oxylipins is not sufficient or the signal is reduced due to ion suppression (a matrix effect). This is described in the below paragraph in more detail. Finally, an ion suppression analysis (by post-column addition of an IS solution using a syringe pump and connected via a T-piece between column and MS while monitoring its transition during the injection of a sample) allows a detailed analysis of eluting ion suppressing matrix compounds[38, 46]. For oxylipins, several successful protocols for SPE extraction have been described in the literature, and we refer the reader to detailed procedures here[18, 38, 47].

2.5 Set-up of LC-MS/MS analytical method

Analysis of oxylipins using the newer scheduled MRM methods that can detect and quantify 100-200 analytes are technically demanding, and require significant time, expertise and training. A dedicated postdoctoral scientist, overseeing all analyses, e.g. supervising, training staff and students, and monitoring instrument performance is essential. A number of these methods have been described by several laboratories, as reviewed here.[16, 48] Most often, the LC system uses sub-2-µm particle size filled columns that generate very high backpressure of >500 bar, electrospray ionization in negative ion mode and a triple quadrupole (QqQ)

analyzer operated in SRM/MRM mode (see earlier). Optimised methods for a large number of oxylipins including isoprostanes or so-called "specialist resolving mediators" are described here[17, 41, 42].

The following parameters need to be defined for instrument/method qualification:

- *Which oxylipins should be included?* A quantitative method requires standards for each analyte and an increasing number of oxylipins are commercially available. However, most are not fully characterized regarding purity. If desired, concentrations can corrected by concentration factors determined based on UV-spectroscopy and LC-ESI(-)MS analysis in selected ion monitoring. The detailed procedures can be found here[49].
- *Efficient chromatographic separation*. For UHPLC, column and mobile phase gradient selection should lead to narrow peaks (full width at half maximum (FWHM) height about 3-4 sec[41, 42] to allow sufficient separation of the large number of oxylipins. Several structurally-different oxylipins show identical fragment spectra in mass spectrometry[48] and need to be separated by chromatography. Examples of these, which elute as critical separation pairs are: PGE2/PGD2, PGB2/PGJ2 12(13)-DiHODE/15,16-DiHODE, w-hydroxy-PUFA/(w-1)-hydroxy-PUFA, 8(9)-EpETE/11(12)-EpETE, 11(12)-EpETE/14(15)-EpETE, 8(9)-EpETrE/11(12)-EpETrE, 7-HDHA/11-HDHA[18]. Their efficient separation needs to be visually confirmed using standards.
- *Instability of some lipids*. Most oxylipins, including multiply oxygenated PUFA called SPM, are relatively stable under the analytical conditions outlined here. Exceptions include lipids with hydroperoxide functional groups. These, are highly unstable in disrupted tissue samples due their reactivity towards transitional metal ions. Other lipids that are not possible to analyse are Prostaglandin H2 and Thromboxane A2, which are unstable in aqueous environments generating more stable end products that can be measured instead.
- *Co-elution problems.* Due of the large number of oxylipins generated in a biological system, co-elution with stereoisomers can occur, and this is particularly a problem with multiply oxygenated PUFA. Enantiomers (mirror image) don't separate on reverse phase LC, while diastereomers (non-mirror

image) do. This issue is exemplified with SPM, including PD1[50]. The specific problem with assigning names where there may be co-eluting enantiomers is covered in more detail later.

- *Sensitive and selective mass spectrometric detection*. The LC-MS/MS parameters (source setting, gas flows, temperature, electronical potentials) for the MRM transitions need to be optimized [41, 42]. Each primary standard should be optimized for compound specific parameters such as collision energy.
- *Scheduled MRM.* This approach allows for detection of large numbers of oxylipins, since individual lipids are only monitored around the time when they elute. Here, detection windows need to be narrow, but also wide enough to allow sufficient a background trace to be recorded. This requires a stable retention time, and a cycle time that records sufficient data points per peaks (varying from 6 upwards, with some laboratories using <20) balanced with sufficiently long dwell times (the time during which the instrument is analyzing a transition, ideally 10-50 ms). A starting point, which may need adapting based on the platform and number of analytes, would be 30-60 s window and total cycle time of 0.4 s peaks with FWHM of around 3-4 sec[17, 41, 42]. However, what is critical is that there is sufficient baseline measured to enable robust estimation of background noise, which is used for limit of detection/quantitation (LOD/LOQ) determination as detailed below. Thus the windows must be optimized appropriate to the specific instrument conditions. When measuring very large numbers of lipids, it is generally feasible to only monitor one transition per analyte, due to analytical capacity of instruments. However, if needed, to ensure higher confidence in identification, an additional strategy can involve the monitoring of two per analyte, one being the quantifier and the other the qualifier. The ratio of response of qualifier to quantifier being consistent with a standard would be considered as further evidence, in particular the existence of a chromatographic peak with identical retention time for both transitions. If this is not routinely possible, then it can be built in as part of structure confirmation. MS/MS spectra can also be acquired, however, when lipids are present in low amounts in biological samples, spectra obtained at the expected elution time are generally extremely noisy, and are then not suitable for identification purposes. Only spectra that show a strong visual match to the pure standard provide good evidence for a proposed structure.

- *Define lower limit of detection (LOD).* Using diluted solutions of standards the instrument LOD needs to be defined based on a signal-to-noise ratio (s/n) of ≥3 for each oxylipin with the developed method. With the most sensitive of the current instrumentation, a LOD of >0.1-1 pg on column which means with a typical injection of 10 µl a concentration in an injected solution of 10-100 pg/ml is feasible for most oxylipins[41, 42].
- *Internal standards:* Isotopically labeled internal standards should be chosen to represent the sub-categories of oxylipins being measured, including as many as feasible. These should be added to samples at about 10-30 fold the concentration of the LOD of the analyte (about 50-100 ng/ml). Prepare sufficient amount of IS solution for samples in advance and add to samples prior to extraction.
- *Standard Curves:* A calibration series is generated by sequential dilution of mixtures of primary standards. A detailed pipetting scheme can be found in[17]. Include at least two concentrations of lipids per order of magnitude from the LOD to the highest concentration present in samples, or slightly above the expected upper limit of quantification (about 1 µg/mL). This should remain below the upper limits of dynamic range for the instrument (based on linear response to serial dilutions). Standard solutions are stable for several months at -80°C and freeze/thawing should be avoided by storing in appropriate glass vials or ampules. Unstable oxylipins, such as hydroperoxy-PUFA, should not be included in calibration series.
- *Limits of quantification and calibration*. The LOQ for standards and analytes measured in research laboratory assays is generally defined as a peak exceeding a s/n ratio of ~5-10, in line with recommendations from external agencies, such as FDA, WHO, and others. A linear calibration function can be generated by weighting the concentration $1/x$ or $1/x^2$ (because random variation is relative and thus absolutely smaller for lower concentrations). For all calibrators the accuracy can be calculated, and needs to be within ±15 and (±20% for the calibrator of the LLOQ) which limits the LLOQ. Matrix matched calibration can be helpful in specific cases, is however not required because of the used isotopically labeled standards (so-called isotope dilution analysis), and also feasibility when dealing with many different types of tissues on a routine basis.

- *System Qualification.* It can be helpful, in addition to using the calibration solutions from the MS manufacturer, to make use of a defined solution of oxylipins (e.g. one of the standards) for the purposes of ensuring good system performance prior to analysing samples. Using this approach, a user can confirm specific performance criteria, relating to minimum chromatographic and mass spectrometric performance. These can include background intensity, retention times, peak widths, resolution of critical separation pairs, absolute intensity (peak height, area) of IS, accuracy.

2.6. Quality assessment and control

It's important to closely monitor method performance so that if problems occur, they can be quickly identified and rectified. Variations in instrument performance (sensitivity) and sample preparation (IS recovery) can be routinely monitored. Where laboratories are conducting large scale analyses of cohort samples, it is critical to demonstrate assay stability between batches over time. For this, we recommend a quality control (QS) sample, such as a large volume of (pooled) plasma which is stored in appropriate aliquots. With every analytical batch, such as the 20 samples co-extracted on a SPE manifold, one of the QC samples would be prepared in parallel. Documenting the detected concentration and comparing it with historical controls allows determination of accuracy and precision of the analysis, which is typically ±15 % for most oxylipins.

3. Challenges presented by oxylipin isomers.

3.1. Analytical challenges of isomers

A significant challenge in oxylipin analysis lies in discriminating specific constitutional isomers and in particular stereoisomers, especially when analysing complex biological mixtures. Importantly, bioactivity and (stereo-)chemical structure of oxylipins are inherently intertwined. For example, LTB_4 and its diastereomer 5S,12S-diHETE not only arise from different biochemical pathways but also vastly differ in their bioactivities[51]. LTB_4 is a very strong chemotactic agent, predominantly produced in polymorphonuclear cells by the actions of 5-lipoxygenase and leukotriene A_4 hydrolase, its diastereomer 5S,12S-diHETE on the other hand is a

platelet neutrophil interaction product involving 5- as well as 12-LOX for its production[52, 53]. An oxylipin's stereochemical definition is of great importance to our understanding of its origin and bioactivity. Of note, the actions of the major mammalian enzymes involved in oxylipin production in the immune system are stereochemically S-configuration specific while some skin isomers generate R-enantiomers. However, tandem mass spectra of stereoisomers and in some cases even constitutional isomers are identical. A recent example illustrates co-elution and highly similar MS/MS fragmentation of the constitutional isomers LXA_4 and a 5D2-isoprostane. Using biological interpretation and MS^3 analysis, the authors were able to dissect the two isomers[54]. Nevertheless, this illustrates that even with powerful LC-MS/MS approaches and monitoring presumably analyte specific mass transitions (*m/z* 351 -> 115), likely well separated constitutional isomers can still be mistaken for each other.

The situation becomes even more complicated when taking stereochemistry into consideration. Two major forms of stereoisomers contribute to the stereochemical complexity of oxylipins, geometric double bond isomers and configurational isomers including enantiomers as well as diastereomers (being non-mirror image stereoisomers). Each chiral centre or geometric double bond in an oxylipin can contribute to two stereoisomers, and where there are multiples of these, the theoretical number of isomers increases consecutively by a factor of two. In practice, geometric double bond isomers will likely contribute less since their configurations are fixed, and relatively stable for specific oxylipin substrates, although this is not always the case. As one example, LTB_4 possesses two stereocenters at the hydroxylated positions 5 and 12 of which each can be R or S-configured, additionally LTB_4 possesses four double bonds that can either be E or Z configured, hence $2^6 = 64$ stereoisomers can theoretically be formed. Fortunately, this is a theoretical number and considering "enantiomerically pure biochemical starting materials", such as arachidonic acid (all Z-configured), this number will be much lower. Nevertheless, a significant number of enzymatic and non-enzymatic (stereo-)isomers can be present in biological samples and consequently several analytical approaches have investigated the stereochemical classification of oxylipins. Most important and routinely applied is the separation of diastereomers using reversed phase chromatography. Since physiochemical characteristic of diastereomers differ (unlike enantiomers) many of these species are readily separable using

routine C18 based chromatographic systems. For example, the non-enzymatic hydrolysis products of LTA_4, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 are readily separable on C18-reversed phase columns. In addition, the use of chiral phases has proven highly useful not only for the separation of enantiomers (R versus S) but also diastereomers[31]. Just recently, two reports have shown the applicability of chiral separations for oxylipin analysis[55, 56]. Notably, these have traditionally been restricted to normal phase systems, however novel column chemistries (e.g. the Chiralpak AD-RH and IA-U) allow operation under reversed phase conditions, rendering chiral separations more practical in combination with electrospray ionization tandem mass spectrometry[31, 55]. Nevertheless, the sheer number of possible stereoisomers combined with the complexity of biological sample materials has led to the development and application of additional technologies for the successful stereochemical resolution of oxylipins. Recently, ion mobility separations have been introduced to the field[57]. Originally differential mobility separation was introduced by Kapron et al. for the separation of prostanoids[58] and later adapted by Jonasdottir et al.[52] for the separation of LTB_4 isomers. Lately also, drift tube-based ion mobility has entered the field of oxylipin analysis[59]. All these approaches rely on the availability of sufficiently pure standard materials with stereo-chemical assignments. Many platforms initially assign identity and hence stereochemistry of a specific oxylipin based on reversed phase LC-MS/MS analysis. Particularly for well described diastereomers this is to some extent possible and considering time and budgetary constraints also understandable. However, enantiomers cannot be separated using reversed phase systems, in turn the assignment of a stereo-center being R or S can only be accomplished by chiral chromatography with enantiomerically pure standards at hand. Furthermore, even though diastereomers are, in most cases, well separatable using reversed phase systems, orthogonal chromatographic resolution is key for the unambiguous assignment of a stereo-chemically defined diastereomer. In other words, even though LC-MS/MS analysis results between standard and a specific analyte closely match, this is no guarantee that all geometric and configurational descriptors do so as well. Thus, if unambiguous stereochemical description of an oxylipin is of importance, matching between standard and analyte, applying one or more of the above discussed analytical solutions should be considered. Here, ideally, reversed phase LC-MS/MS results would be confirmed by chiral chromatography or ion mobility separation. An example of this is provided in Figure 3. Here, we see that in peritoneal cells either with or without a 24-hr challenge with

zymosan, a peak around 9.3 min is visible (Figure 3 A). However, this peak comprises both LTB_4 (5S,12R-diHETE) and its diastereomer 5S,12S-diHETE, which don't separate sufficiently on reverse phase LC and thus only one overall peak is seen for both lipids. Analysing the same extract using differential ion mobility (DMS) shows there are different lipids in the samples, with LTB_4 predominating after inflammatory challenge and 5S,12S-diHETE predominating before (Figure 3 B). If sensitivity is an issue, that cannot be met by chiral separation or ion mobility, "orthogonal" eluent systems can be applied to reversed phase columns as an intermediate measure, i.e. swapping methanol for acetonitrile, adding MS compatible buffers, changing the pH. Finally, it is important to realize that all types of LC-MS/MS based stereochemical assignments are of relative nature and a final absolute assignment of stereochemistry can only be made by complimentary technologies such as NMR. In cases where stereochemistry is ambiguous, non-chiral names would be more chemically correct. This is discussed in more detail below.

3.2. Biological considerations of isomers.

The biology of the tissue can provide important clues to which lipids are present. For example, in the case of PGE_2, if the MRM/SRM channel shows a large number of closely eluting peaks, of fairly similar size, then one would need to consider whether they all originate from non-enzymatic oxidation. On the other hand, if the sample is from inflammatory activated cells, and a prominent signal at the retention time for PGE_2 is seen, with only small peaks for the other related lipids, then it is expected that PGE_2 is the lipid measured and that COX-2 and PGES are involved. Further confirmation can be provided by showing inhibition by indomethacin. For many lipids, unless chiral chromatography is used to verify the structure of the lipid made, or the isomeric composition of the system is well known, stereochemistry can be omitted when reporting structures. In the case of platelets, 12(S)-HETE predominates, due to the well-known high activity of platelet 12-LOX[60]. However, if measuring this lipid elsewhere, it can be called simply 12-HETE. Some oxylipins are named based on their function rather than their structure with these names inferring stereochemistry. These include the DHA and EPA oxygenation products: lipoxins, resolvins, protectins and maresins, commonly called specialist pro-resolving mediators (SPM). Here, the number of stereoisomers that would form via non-enzymatic oxidation of AA, DHA or EPA, considering chirality alone, can be either 4 (with 2 chiral centres, e.g. PD1) or 8

(with 3 chiral centres, e.g. RvD1, D2, D4). Since these lipids have more than one chiral centre, they form groups of non-mirror image stereoisomers that separate on reverse phase (diastereomers) when one or more (but not all) of the chiral centres have different configurations. However, when all the centres have opposite configurations, they are enantiomers (mirror-image stereoisomers) which do co-elute on reverse phase LC-MS/MS. In summary, for all oxylipins, two or more peaks eluting closely together of relatively similar size, rather than a single isomer on its own, should always be seen as suspicion of non-enzymatic oxidation either in a biological system or during sample preparation. Unfortunately, the narrow windows used for scheduled MRM/SRM can potentially lead to these peaks being missed. Even if a single peak is seen, without chiral chromatography on that sample type, one cannot be sure which stereoisomer(s) are present. Labelling the lipid based on what is known, e.g. 7,8,17-triHDOHE, omitting the stereochemistry inferred by the trivial name RvD1, avoids this error. Unfortunately, many studies have used reverse phase chromatography along with stereochemical naming of lipids in complex biological mixtures without confirmation of enatiomeric structure.

4. Analysis of Urine Oxylipin Metabolites

While plasma is more frequently collected in human clinical trials and studies, urine is an alternative biological fluid with distinct advantages for the quantitation of oxylipin biomarkers. Urine collection is non-invasive and can be used in large-scale trials with high participant agreement. Samples can be collected by study participants at home, as many oxylipin urinary metabolites are stable at 4^{o}C (a standard home refrigerator) for a few days. Further, urine can be collected in a sufficient volume that allows for multiple analyses and can be stored for future studies. Important for the analysis of oxylipin metabolites, urine is a filtered fluid with low levels of cell contamination and metals, thus this matrix is less susceptible than plasma to ex vivo oxidation during sample collection and storage[61, 62]. MS methods for oxylipin urinary metabolites are similar to those used for plasma, for example the use of pooled urine QC samples. Analysis of oxylipin metabolites, however, has its own set of considerations regarding sample collection and preparation. First, and most importantly, due to analyte stability and extraction, all urinary metabolites cannot be analyzed in a single

method. Herein, we will first discuss general considerations for urine analysis and then focus on factors specific to individual classes of urine metabolites.

4.1 General considerations

- *Determination of Experimental System*. First, when planning to measure oxylipins and/or their urinary metabolites, it should be determined if urine is an appropriate matrix that will answer the scientific question in the population being studied. Urinary eicosanoid metabolites have often been measured in large-scale clinical trials as biomarkers of disease risk. Additionally, they have been used to assess therapeutic interventions[63-67]. Metabolic enzymes and metabolite formation differ between humans and animals, particularly rodents, and between males and females in both humans and animals[68, 69]. Additionally, there are differences in oxylipin metabolite patterns between pre- and post-menopausal women[70, 71]. Finally, specific pathophysiological conditions, can alter urinary metabolic profiles. For example, kidney dysfunction increases excretion of unmetabolized oxylipins in the urine[72].
- *Planning of sample collection preparation and storage.* Urine samples should be collected at a consistent time of day (ie. first morning void after overnight fasting). If following a therapeutic intervention or procedure, collection should be timed such that metabolites have sufficient time to be excreted. On collection, the sample should be placed on ice or in a refrigerator (4^{o}C) to prevent degradation of metabolites. Samples should remain at room temperature no longer than 1 hour. Multiple aliquots of at least 2 mL should be prepped for storage. The samples should be vortexed prior to aliquoting. We recommend that samples should not be spun to remove particulates as it can lead to unequal distribution of oxylipin metabolite patterns between aliquots[62].
- *Normalization*. The concentration of a urinary metabolite is dependent upon its excretion rate and the urinary flow rate. Urinary biomarkers are commonly reported as a ratio of analyte concentration to urinary creatinine excretion which is typically assumed to be linear to biomarker excretion. Specific gravity and total urine excretion volume are alternative normalization factors. Particular attention to

normalization should be paid when renal function is changing rapidly or in the case of acute kidney injury[61, 62, 73].

4.2 Prostaglandins (PGs)

For decades, it was considered that the most accurate index of endogenous PG production in humans was the measurement of excreted urinary metabolites using MS[68, 74, 75]. The advent of modern, highly sensitive MS has improved our ability to explore local PG formation (ie. CSF, nasal fluid, etc.) and enabled broad spectrum, multi-class oxylipin analysis[76-78]. However, urine still provides a robust option for the evaluation of systemic (whole body) PG production[62]. Quantification of PG urinary metabolites (Figure 4) has allowed for the assessment of PG production in various diseases and intervention strategies (NSAIDs, COXIBs, nutritional supplements[72, 76, 79-85]. In fact, in a recent meta-analysis of urinary biomarkers of colorectal cancer (CRC), measurement of the major urinary metabolite of PGE_2 was shown to be the most clinically promising for detection of CRC risk[86].

- *PGE_2*. PGE-M is the most abundant PG metabolite in human urine. The first method to quantify PGE-M using GC-MS was published in 1976[87]. A second method using GC-MS/MS was published in 1990[88]. However, both are extremely labor intensive, involving multiple purification and derivatization steps. PGE-M is quite unstable, with the two carbonyl groups being susceptible to dehydration and other reactions as is the C-11 hydroxyl group. PGE-M begins to degrade in urine left at room temperature for 1.5 hours (*Milne and Morrow*, unpublished results).

  The development of an LC-MS/MS method for accurate PGE-M quantification was accomplished following the synthesis of analytical standards derivatized with methyloxime HCl or [$^2H_3$]-methyloxime HCl (internal standard)[89]. There are thus several considerations that must be taken into account. First, urine should be derivatized with methyloxime HCl immediately upon thawing. Extraction using a C18 solid phase extraction cartridge is required following derivatization to remove unreacted methyloxime HCl. The internal standard has been derivatized with [$^2H_3$]-methyloxime HCl during synthesis and purified, thus it cannot be added to the sample until after solid phase extraction. (Note: This internal standard is stable at -80^{o}C for 15+ years, but it is not commercially available in

the derivatized form)[68]. Methyloxime HCl reacts with the two keto- groups (C-9 and C-15) to form four (syn/anti) methoxime isomers (Figure 5A). These separate into two distinct peaks when using a shallow gradient on reversed phase LC (C18). The ratio of the peak height of endogenous PGE-M to the peak height of the internal standard is calculated for each methoxime isomeric peak. The ratios of the two PGE-M peaks are averaged for quantification[68].

- *PGD2.* PGD2 is isomeric to PGE2, differing only in the location of the keto- and hydroxyl- groups on the prostane ring. Despite their similarity, the metabolic profile of PGD2 is more varied than PGE2. The two most abundant urinary metabolites of PGD2 are PGD-M and tetranor-PGD-M[90]. PGD-M was identified in 1985 by as the major urinary metabolite of PGD2[91].Due to the proximity of the C-11 hydroxyl- group to the C-15 keto- group, PGD-M readily undergoes cyclization, existing in equilibrium as shown in Figure 5B PGD-M has been quantified using GC-MS following multiple derivatization and purification steps that open up the cyclized form of PGD-M (Figure 5B)[92]. No LC-MS method for quantification of PGD-M is currently available. However, 2,3-dinor-11b-PGF2a, an intermediate metabolite between PGD2 and PGD-M, has been detected in urine using LC-MS/MS[93]. Song, et al identified a tetranor PGD-M as a urinary metabolite of PGD2 in 2008[94]. This metabolite is analogous to PGE-M and formed in concentrations comparable to PGD-M. Tetranor PGD-M is susceptible to the same stability issues as PGE-M. Likewise, reaction of tetranor PGD-M with methyloxime HCl results in formation of four (syn/anti) methoxime isomers. These isomers are separable from those of PGE-M[95, 96].
- *PGJ2 and 15dPGJ2*: PGD2 can also undergo metabolism by dehydration to yield PGJ2, which is further metabolized to 15-deoxy-D12,13-PGJ2 (15dPGJ2). Very little 15dPGJ2 is detected in urine[97]. However, 15dPGJ2 can be metabolized by a human liver cell line (HepG2) to a glutathione conjugate. This is metabolized analogous to leukotriene C4, a glutathione conjugate of leukotriene A4, yielding an end metabolite that is a 15dPGJ2 cysteine conjugate. This may explain why 15dPGJ2 levels in urine are low[98, 99]. When assessing formation of endogenous PGD_2, it is important to consider all routes of metabolism. The biological relevance of these metabolic pathways is incompletely understood.

- *Thromboxane and Prostacyclin*. For over 40 years, the production of prostacyclin (PGI_2) and TxA_2 has been central to the understanding of cardiovascular health[100]. PGI_2 is a product of endothelial cells in the vasculature and is antithrombotic and a vasodilator. TxA_2 is generated in platelets and is pro-thrombotic and a vasoconstrictor[101]. PGI_2 and TxA_2 are rapidly metabolized. The major urinary metabolite of PGI_2 is 2,3-dinor-6-keto-$PGF_1\alpha$ (PGI-M). Major urinary metabolites of TxA_2 are 11-dehydro-thromboxane B_2 ($11dTxB_2$) and 2,3-dinor-TxB_2[102, 103]. Infusion studies that alter levels of PGI_2 and TxA_2 in the circulation have shown that urinary PGI-M and $11dTxB_2$ accurately reflect these changes[104]. These urinary metabolites have been extremely useful in the assessment of therapeutic interventions that modulate PGI_2 and TxA_2 formation. For example, measurement of PGI-M and $11dTxB_2$ contributed to the development of low-dose aspirin for cardio protection[67]. Further, quantification of these metabolites was found to be essential to assessing the role of PGI_2 and TxA_2 in the cardiovascular hazards associated with NSAID usage (ie. rofecoxib)[105]. Interestingly, Nakashima and Schneider have recently reported that $11dTxB_2$ can be formed from PGD_2 via a Baeyer-Village oxidation reaction[106]. The oxidation of PGD_2 to $11dTxB_2$ was accomplished in vitro by oxidation with hydrogen peroxide (H_2O_2), which can be formed endogenously during settings of oxidative stress. These findings provide a potentially interesting link between the metabolism of PGD_2 and TxA_2.
- *Leukotrienes (LTs).* LTs are generated from AA which is first released from cells by the action of cPLA2. The 5-lipoxygenase (LO) enzyme associates with 5-LO activating protein (FLAP) to convert AA into 5-HETE and subsequently leukotriene A4, the 5,6-epoxide of AA. LTA hydrolase converts LTA_4 to LTB_4, a dihydroxy- AA metabolite. Alternatively, LTA_4 can be acted upon by LTC_4 synthase to form the glutathione conjugate LTC_4. LTC_4 is converted to the bioactive LTD_4 and the end product LTE_4 by peptidases. LTs are important in the inflammatory response, particularly in the pathophysiology of asthma and mast cell activation[107]. LTE_4 is readily detectable in the urine. Due to its chemical structure and requirements for extraction and purification, LTE_4 cannot be measured simultaneously with other oxylipin metabolites. LTB_4 metabolism is more complex. Eleven different metabolites of LTB_4 are excreted in human urine following infusion[107]. The major urinary metabolites are LTB_4-

glucuronide and 20-carboxy-LTB_4[108, 109]. These were detected in low levels, but not in the urine of subjects not infused with LTB_4[109]. Interestingly, Morita and colleagues recently reported the detection of LTB_4-ethanolamide in the urine of healthy humans as well as in patients with diabetes mellitus[110]. The authors describe this as a metabolite of arachidonyl-ethanolamine rather than AA. Levels of this metabolite were lower patients with diabetic neuropathy (stage 3-4) than in healthy subjects or in patients with diabetic neuropathy (stage 1-2).

- *Cytochrome P450 Oxylipins*. Cytochrome P450 (CYP) products of AA oxidation include the EETs (CYP2C, CYP2J), which are metabolized by soluble epoxide hydrolase (sEH) to dihydroxy- molecules (DHETs), and 20-HETE (CYP4A, CYP4F). The specific CYP enzymes as well as the generated oxidation products vary by species, sex, and organ[111]. While EETs have been detected in urine (primarily kidney derived), they are generally excreted as the DHET metabolite conjugated with glucuronide. 20-HETE is also excreted as a glucuronide conjugate. Thus, urine must be treated with glucuronidase enzymes prior to LC-MS analysis to ensure complete capture of total DHET or 20-HETE[112, 113] Importantly, unlike PGs and LTs, urinary DHETs and 20-HETE do not necessarily reflect systemic levels of these molecules. Intravenous administration of radiolabeled 14,15-EET to dogs found a significant increase in 14,15-DHET in the plasma, but little radioactivity was detected in the urine[114] thus, implying that urinary CYP oxylipins originate in the kidney. Urinary DHETs, however, are increased in diabetic kidney disease, diet-induced hypertension, and pregnancy-induced hypertension[113-116].
- *Isoprostanes and related metabolites.* F_2-Isoprostane (F_2-IsoPs) are a well-studied class of non-enzymatically generated oxylipins. F_2-IsoPs are formed via autoxidation by different types of free radicals[77] and have been shown to be reliable biomarkers of endogenous lipid peroxidation and oxidative stress. These molecules are chemically stable in biological fluids when stored correctly[117]. F_2-IsoPs are isomeric to $PGF_{2\alpha}$ [117]. F_2-IsoPs can form four classes of regioisomers depending on the position where the oxygen molecule is inserted on the arachidonic acid carbon backbone. The four sub-families consist of 16 diastereoisomers, since the hydroxy group on the cyclopentane ring can be arranged in eight different configurations, resulting in total 64 F_2-IsoPs[117]. Multiple nomenclature systems for F_2-IsoPs exist. Despite the potential existence of 64 isomers, the most widely measured

F_2-IsoP is 15-F_{2t}-IsoP (also known as 8-iso-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III) as it is one of the most abundant of the 64 stereoisomers in vivo and was the first to be synthesized[90]. Even though 15-F_{2t}-IsoP is commonly measured in urine in its free form, Yan et al. (2010) demonstrate a 40 % increase in the IsoPs concentration in urine after treating samples with glucuronidase prior to MS analysis[73]. Moreover Li et al. (1999) demonstrated that the 15-F_{2t}-IsoP is not the most abundant isomer in urine; with a range of 1.11 ± 0.45 pg/mg creatinine for 15-F_2-IsoP but a range of 8.33 ± 3.17 pg/mg creatinine for 5-F_2-IsoPs (5-*epi*-5-F_2-IsoP). This has also observed by others[72] (Milne and Yang, 2021, unpublished results). Although 5-F_2-IsoPs might be found in higher concentration in human urine, little is known about their biological activities[118]. Two major urinary metabolites of 15-F_{2t}-IsoPs have been identified, 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (Figure 6). In addition to 2,3-dinor- and 2,3-dinor-5,6-dihydro metabolites, 13,14-dihydro-15-keto- and 2,3,4,5-tetranor-derivatives of 15-F_{2t}-IsoP have been identified[117]. 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP is not subject to autoxidation nor renal production and may be a more sensitive marker of endogenous oxidative stress in urine than intact F_2-IsoPs[70]. It is possible that glucuronide conjugates of 15-F_{2t}-IsoP-M could exist since it has a similar structure to 15-F_{2t}-IsoP, however these compounds have not been studied to date. Since their discovery over 30 years ago, GC-MS has been the method most commonly used to quantify F2-IsoPs. The use of LC-MS/MS for their analysis is still in its infancy, hindered until recently by technology and the availability of internal standards. Durand and colleagues have synthesized many isomers, thus the field is ripe for exploration of their biological activity[119, 120]. The ability to quantify individual isomers and metabolites will certainly expand our understanding of IsoP formation in human physiology and pathophysiology.

- *Non-AA derived IsoPs, and isomers of other PGs.* It is worth mentioning that PUFA other than AA can generate IsoPs. Adrenic acid (AdA), eicosapentenoic acid (EPA), docosapentaenoic acid, and docosahexaenoic acid (DHA) generate F_{2t}-dihomo-isoprostanes (F_{2t}-dihomo-IsoP), F_{3t}-isoprostanes (F_{3t}-IsoP), and F_{4t}-neuroprostanes (F_{4t}-NeuroP). These IsoPs have been proposed as biomarkers of neurological diseases such as Alzheimer's, Rett Syndrome, epilepsy, and age-related macular degeneration (AMD)[121-124], and were proposed to predict neonatal morbidity in preterm infants [125,

[126]. When conducting EPA and DHA supplementation studies, the analysis of urinary F_3-IsoP and F4-NeuroPs could be considered, since supplementation of these fatty acids can alter the fatty acid composition of plasma, cells, and tissues in humans[127, 128]. In addition to F-ring molecules, compounds isomeric to PGE2, PGD2, and TxB2 can be generated via non-enzymatic lipid peroxidation. 15-E2t-IsoP (also referred to as 8-iso-PGE2 or iPE2-III) was found to have both vasoconstrictive and vasodilatory effects, suggesting a biological activity of this molecule in the cardiovascular system. 15-E2t-IsoP has been detected in the urine of triathlon elite athletes[82].

5. Analysis of oxylipins attached to phospholipids.

There is increasing evidence that considerable amounts of oxylipins are rapidly generated attached to complex lipids such as phospholipids, sterol esters and glycerides[129]. This generally involves initial enzymatic generation of oxylipins from free FA, followed by their fast esterification into lysoPL via Lands cycle enzymes, although in the case of 15-LOX1, direct oxygenation of the PL occurs[130]. LC-MS/MS analysis of esterified oxylipins is hampered by the relative lack of synthetic standards, thus structures are often presented as incompletely annotated forms based on the information available, and changes expressed as fold change differences. This pragmatic approach that allows biology to be characterised without overstatement of findings in relation to amounts or structures. The exception to this is HETE-PE/PC, where a limited number of synthetic standards are available including commercially[131]. Furthermore, standards for other monohydroxy-oxylipins such as HDOHE or HODEs attached to complex lipids can be generated using air or LOX-mediated oxygenation[131]. In biological samples and cells, there are often many different molecular species seen, co-eluting closely together on reverse phase LC-MS/MS. One example is platelets where the prominent forms are phosphatidylethanolamines (PE) or -cholines (PC) with 12S-HETE attached[132]. While these lipids can be quantified, many others formed in the same cells can only be expressed as fold-changes, using comparison with an internal standard[133]. Relating to this, early work used di-myristoyl-PE/PC, since it was absent in isolated cells, however for plasma, since there is a low endogenous signal, we instead use 15:0/18:1-*d7*-PE/PC. As discussed earlier, a different approach to analysing oxidized PL was taken by Dennis et al who hydrolysed chemically, and measured total released oxylipins instead of the intact PL[36]. The

advantage is that it allows quantitation of a higher number of oxylipins, since there are synthetic standards for the free acid forms. However, the precise esterified structures are missed. Since analysis of esterified oxylipins is complex, a number of informatics tools were recently developed to assist, e.g. a computational database on LIPID MAPS (https://www.lipidmaps.org/tools/ms/gp_ox_form.php) LPPTiger, an informatics tool from the Fedorova group that predicts structures from MS/MS data[134], and a library of structures[135]. Analysis of PL-esterified oxylipins generally relies on precursor-to-product ion MRM transitions in negative ion mode, where the carboxylate anion of the FA generated from *Sn2* is used as the product ion. While this works very well for phospholipids, where the precursor generates a strong negative ion, it is less useful for glycerides or sterol esters which rely on positive ion mode. As oxylipins are rather unstable during collision induced dissociation, they readily generate internal daughter ions that can inform on positional isomers. In the case of HETEs, a strong ion at *m/z* 319.2 is seen for the intact FA, along with smaller ions that indicate the position of oxygenation, e.g. *m/z* 115 for 5-HETE. Using reverse phase HPLC/UPLC, positional isomers of HETEs separate slightly, allowing quantitation of the individual isomers, when using their distinct internal product ions[131]. A useful mode for "fishing" for esterified oxylipins in new sample types is precursor scanning LC-MS/MS, in negative ion mode. This approach allowed initial identification of many HETE-PE and -PC in blood cells such as neutrophils, monocytes and platelets, as well as esterified PGE_2, and could easily be applied to additional structures in other cell types[37, 130, 132]. It is relatively insensitive however and won't detect many of the lower abundant multiply oxygenates species present. There, prediction of structures using informatics or manual approaches, followed by scanning predicted MRM transitions can work well. For general methods for preparation of oxPL standards and analysis of these lipids in tissues using LC-MS/MS, specifically mono-oxygenated forms, see Morgan et al[131].

6. Conclusions

Methods that allow the quantitation of large numbers of oxylipins and their metabolites in a single run are increasing in popularity. This is in part driven by instrumentation advances such as the ability to conduct chromatography at ever increasing pressures, improving peak resolution and reducing analysis time; combined with MS instruments that scan extremely fast and are highly sensitive. However, a note of caution

is that these assays are technically demanding to set up and run, and their routine implementation requires highly trained technical support and expert oversight. It is also important to keep biology and biochemistry in mind when interpreting data, with the pattern of products and their isomers guiding the interpretation of findings, e.g. whether the lipids were generated via enzymes, which enzymes were involved, etc. Consideration of sampling: blood or urine, how and where it will be obtained, transported and stored is essential, and this should ideally happen prior to initiation of the study. This review summarises all these issues and should be a useful guide to those new to the field as well as others seeking guidance around detailed aspects of interpretation of complex data from analysis of biological samples.

Funding acknowledgement

Funding to LIPID MAPS from the Wellcome Trust is gratefully acknowledged (203014/Z/16/Z). NHS is supported by a grant (SCHE 1801) of the German Research Foundation (DFG).

References

1. https://lipidmaps.org/databases/lmsd/browse.
2. Moncada S and Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol Rev*. 1978;30:293-331.
3. Fahy E, Slenter, D, Willighagen, E, Weitz, E, Miller, R. . Eicosanoid metabolism via cytochrome P450 monooxygenases (Mus musculus).
4. Kwa A, Slenter, D, Pico, Alexander, Hanspers, K, Kutmon, M, Pirola, CJ, Kelder, T, Chichester, C, Willighagen, E, Weitz, E. Eicosanoid synthesis (Homo sapiens).
5. Research CfDEa and Research CfBEa. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics. *https://wwwfdagov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics*. 2015.
6. Araujo P. Key aspects of analytical method validation and linearity evaluation. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877:2224-34.
7. Commission E. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044) *https://opeuropaeu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en*. 2002.
8. Desimoni E, Brunetti, B. Signal to noise ratio; Limit of detection; Standard error of the regression; Chromatographic/Voltammetric/Spectroscopic signals. *PHARMACEUTICA ANALYTICA ACTA* 2015;6.

9. FDA U. Bioanalytical Method Validation Guidance for Industry. *FDA-2013-D-1020*.
10. Heyden YVB, R. *The limit of detection*. https://www.researchgate.net/profile/Stefano_Polesello/post/How_to_calculate_LOD_and_LOQ_of_analyte_by_hplc/attachment/59d6356379197b8077992f7e/AS:384058174525440@1468578012222/download/The+Limit+of+Detection-LCGC.doc: LCGC Europe; 2009.
11. Wenzl T, Haedrich, J, Schaechtele, A, Robouch, P, Stroka, J. Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. *EUR 28099; Publications Office of the European Union: Luxembourg,*. 2016;https://publications.jrc.ec.europa.eu/repository/bitstream/JRC102946/eur%2028099%20en_lod%20loq%20guidance%20document.pdf.
12. http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1225.html.
13. https://www.ema.europa.eu/en/ich-q2-r1-validation-analytical-procedures-text-methodology.
14. https://www.who.int/medicines/areas/quality_safety/quality_assurance/28092018Guideline_Validation_AnalyticalMethodValidation-Appendix4_QAS16-671.pdf.
15. O'Donnell V, Schebb, NH, Milne, GL, Murphy, MP, Thomas, CP, Steinhilber, D, Wendell, SG, Kühn, H, Jakobsson, PJ, Blair, I, Murphy, RC, Freeman, BA, Brash, AR, FitzGerald, G. Failure to apply standard limit-of-detection or limit-of-quantitation criteria to specialized pro-resolving mediator analysis incorrectly characterizes their presence in biological samples. *Zenodo*. 2021.
16. Gladine C, Ostermann AI, Newman JW and Schebb NH. MS-based targeted metabolomics of eicosanoids and other oxylipins: Analytical and inter-individual variabilities. *Free Radic Biol Med*. 2019;144:72-89.
17. Koch E, Mainka M, Dalle C, Ostermann AI, Rund KM, Kutzner L, Froehlich LF, Bertrand-Michel J, Gladine C and Schebb NH. Stability of oxylipins during plasma generation and long-term storage. *Talanta*. 2020;217:121074.
18. Mainka M, Dalle C, Petera M, Dalloux-Chioccioli J, Kampschulte N, Ostermann AI, Rothe M, Bertrand-Michel J, Newman JW, Gladine C and Schebb NH. Harmonized procedures lead to comparable quantification of total oxylipins across laboratories. *J Lipid Res*. 2020;61:1424-1436.
19. Rund KM, Nolte F, Doricic J, Greite R, Schott S, Lichtinghagen R, Gueler F and Schebb NH. Clinical blood sampling for oxylipin analysis - effect of storage and pneumatic tube transport of blood on free and total oxylipin profile in human plasma and serum. *Analyst*. 2020;145:2378-2388.
20. Kutzner L, Goloshchapova K, Heydeck D, Stehling S, Kuhn H and Schebb NH. Mammalian ALOX15 orthologs exhibit pronounced dual positional specificity with docosahexaenoic acid. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2017;1862:666-675.
21. Ivanov I, Kuhn H and Heydeck D. Structural and functional biology of arachidonic acid 15-lipoxygenase-1 (ALOX15). *Gene*. 2015;573:1-32.
22. Dennis EA, Deems RA, Harkewicz R, Quehenberger O, Brown HA, Milne SB, Myers DS, Glass CK, Hardiman G, Reichart D, Merrill AH, Jr., Sullards MC, Wang E, Murphy RC, Raetz CR, Garrett TA, Guan Z, Ryan AC, Russell DW, McDonald JG, Thompson BM, Shaw WA, Sud M, Zhao Y, Gupta S, Maurya MR, Fahy E and Subramaniam S. A mouse macrophage lipidome. *J Biol Chem*. 2010;285:39976-85.
23. Ebert R, Cumbana R, Lehmann C, Kutzner L, Toewe A, Ferreiros N, Parnham MJ, Schebb NH, Steinhilber D and Kahnt AS. Long-term stimulation of toll-like receptor-2 and -4 upregulates 5-LO and 15-LO-2 expression thereby inducing a lipid mediator shift in human monocyte-derived macrophages. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2020;1865:158702.

24. Willenberg I, Meschede AK, Gueler F, Jang MS, Shushakova N and Schebb NH. Food Polyphenols Fail to Cause a Biologically Relevant Reduction of COX-2 Activity. *PLoS One*. 2015;10:e0139147.
25. Cooper BA and Rosenblatt D. Folate coenzyme forms in fibroblasts from patients deficient in 5,10-methylenetetrahydrofolate reductase. *Biochem Soc Trans*. 1976;4:921-2.
26. Crescente M, Menke L, Chan MV, Armstrong PC and Warner TD. Eicosanoids in platelets and the effect of their modulation by aspirin in the cardiovascular system (and beyond). *Br J Pharmacol*. 2019;176:988-999.
27. Gijon MA, Zarini S and Murphy RC. Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells. *J Lipid Res*. 2007;48:716-25.
28. McAdam BF, Mardini IA, Habib A, Burke A, Lawson JA, Kapoor S and FitzGerald GA. Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. *J Clin Invest*. 2000;105:1473-82.
29. Schuchardt JP, Ostermann AI, Stork L, Fritzsch S, Kohrs H, Greupner T, Hahn A and Schebb NH. Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood. *Prostaglandins Leukot Essent Fatty Acids*. 2017;121:76-87.
30. Goodfriend TL, Pedersen TL, Grekin RJ, Hammock BD, Ball DL and Vollmer A. Heparin, lipoproteins, and oxygenated fatty acids in blood: a cautionary note. *Prostaglandins Leukot Essent Fatty Acids*. 2007;77:363-6.
31. Jonasdottir HS, Brouwers H, Toes REM, Ioan-Facsinay A and Giera M. Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2018;1863:1511-1522.
32. Lundstrom SL, Yang J, Brannan JD, Haeggstrom JZ, Hammock BD, Nair P, O'Byrne P, Dahlen SE and Wheelock CE. Lipid mediator serum profiles in asthmatics significantly shift following dietary supplementation with omega-3 fatty acids. *Mol Nutr Food Res*. 2013;57:1378-89.
33. Schuchardt JP, Schmidt S, Kressel G, Dong H, Willenberg I, Hammock BD, Hahn A and Schebb NH. Comparison of free serum oxylipin concentrations in hyper- vs. normolipidemic men. *Prostaglandins Leukot Essent Fatty Acids*. 2013;89:19-29.
34. Schuchardt JP, Schmidt S, Kressel G, Willenberg I, Hammock BD, Hahn A and Schebb NH. Modulation of blood oxylipin levels by long-chain omega-3 fatty acid supplementation in hyper- and normolipidemic men. *Prostaglandins Leukot Essent Fatty Acids*. 2014;90:27-37.
35. O'Donnell VB and Murphy RC. New families of bioactive oxidized phospholipids generated by immune cells: identification and signaling actions. *Blood*. 2012;120:1985-92.
36. Quehenberger O, Dahlberg-Wright S, Jiang J, Armando AM and Dennis EA. Quantitative determination of esterified eicosanoids and related oxygenated metabolites after base hydrolysis. *J Lipid Res*. 2018;59:2436-2445.
37. Aldrovandi M, Hammond VJ, Podmore H, Hornshaw M, Clark SR, Marnett LJ, Slatter DA, Murphy RC, Collins PW and O'Donnell VB. Human platelets generate phospholipid-esterified prostaglandins via cyclooxygenase-1 that are inhibited by low dose aspirin supplementation. *Journal of lipid research*. 2013;54:3085-97.
38. Ostermann AI, Koch E, Rund KM, Kutzner L, Mainka M and Schebb NH. Targeting esterified oxylipins by LC-MS - Effect of sample preparation on oxylipin pattern. *Prostaglandins Other Lipid Mediat*. 2020;146:106384.
39. Meckelmann SW, Hawksworth JI, White D, Andrews R, Rodrigues P, O'Connor A, Alvarez-Jarreta J, Tyrrell VJ, Hinz C, Zhou Y, Williams J, Aldrovandi M, Watkins WJ, Engler AJ, Lo Sardo V, Slatter DA, Allen SM, Acharya J, Mitchell J, Cooper J, Aoki J, Kano K, Humphries SE and O'Donnell VB. Metabolic Dysregulation of the Lysophospholipid/Autotaxin Axis in the Chromosome 9p21 Gene SNP rs10757274. *Circ Genom Precis Med*. 2020;13:e002806.

40. Ostermann AI, Greupner T, Kutzner L, Hartung Nicole M, Hahn A, Schuchardt JP and Schebb NH. Intra-individual variance of the human plasma oxylipin pattern: low inter-day variability in fasting blood samples versus high variability during the day. *Analytical Methods*. 2018;10:4935-4944.
41. Kutzner L, Rund KM, Ostermann AI, Hartung NM, Galano JM, Balas L, Durand T, Balzer MS, David S and Schebb NH. Development of an Optimized LC-MS Method for the Detection of Specialized Pro-Resolving Mediators in Biological Samples. *Front Pharmacol*. 2019;10:169.
42. Rund KM, Ostermann AI, Kutzner L, Galano JM, Oger C, Vigor C, Wecklein S, Seiwert N, Durand T and Schebb NH. Development of an LC-ESI(-)-MS/MS method for the simultaneous quantification of 35 isoprostanes and isofurans derived from the major n3- and n6-PUFAs. *Anal Chim Acta*. 2018;1037:63-74.
43. Pedersen TL, Gray IJ and Newman JW. Plasma and serum oxylipin, endocannabinoid, bile acid, steroid, fatty acid and nonsteroidal anti-inflammatory drug quantification in a 96-well plate format. *Anal Chim Acta*. 2021;1143:189-200.
44. Jonasdottir H, Ioan-Facsinay A, Kwekkeboom J, Brouwers H, Zuurmond A-M, Toes R, Deelder A and Giera M. An Advanced LC–MS/MS Platform for the Analysis of Specialized Pro-Resolving Lipid Mediators. *Chromatographia*. 2014;78:1-11.
45. Yang J, Schmelzer K, Georgi K and Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem*. 2009;81:8085-93.
46. Ostermann AI, Willenberg I and Schebb NH. Comparison of sample preparation methods for the quantitative analysis of eicosanoids and other oxylipins in plasma by means of LC-MS/MS. *Anal Bioanal Chem*. 2015;407:1403-14.
47. Giera M.
Clinical Metabolomics
Methods and Protocols
. 2018.
48. Willenberg I, Ostermann AI and Schebb NH. Targeted metabolomics of the arachidonic acid cascade: current state and challenges of LC-MS analysis of oxylipins. *Anal Bioanal Chem*. 2015;407:2675-83.
49. Hartung NM, Mainka M, Kampschulte N, Ostermann AI and Schebb NH. A strategy for validating concentrations of oxylipin standards for external calibration. *Prostaglandins Other Lipid Mediat*. 2019;141:22-24.
50. Rund KM, Peng S, Greite R, Claassen C, Nolte F, Oger C, Galano JM, Balas L, Durand T, Chen R, Gueler F and Schebb NH. Dietary omega-3 PUFA improved tubular function after ischemia induced acute kidney injury in mice but did not attenuate impairment of renal function. *Prostaglandins Other Lipid Mediat*. 2020;146:106386.
51. Lee TH, Menica-Huerta JM, Shih C, Corey EJ, Lewis RA and Austen KF. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene B5 and the double lipoxygenase product. *J Biol Chem*. 1984;259:2383-9.
52. Jonasdottir HS, Papan C, Fabritz S, Balas L, Durand T, Hardardottir I, Freysdottir J and Giera M. Differential mobility separation of leukotrienes and protectins. *Anal Chem*. 2015;87:5036-40.
53. von Schacky C, Marcus AJ, Safier LB, Ullman HL, Islam N, Broekman MJ and Fischer S. Platelet-neutrophil interactions. 12S,20- and 5S,12S-dihydroxyeicosapentaenoic acids: two novel neutrophil metabolites from platelet-derived 12S-hydroxyeicosapentaenoic acid. *J Lipid Res*. 1990;31:801-10.
54. Chakraborty T, Thuer E, Heijink M, Toth R, Bodai L, Vagvolgyi C, Giera M, Gabaldon T and Gacser A. Eicosanoid biosynthesis influences the virulence of Candida parapsilosis. *Virulence*. 2018;9:1019-1035.

55. Cebo M, Fu X, Gawaz M, Chatterjee M and Lammerhofer M. Enantioselective ultra-high performance liquid chromatography-tandem mass spectrometry method based on sub-2microm particle polysaccharide column for chiral separation of oxylipins and its application for the analysis of autoxidized fatty acids and platelet releasates. *J Chromatogr A*. 2020;1624:461206.
56. Blum M, Dogan I, Karber M, Rothe M and Schunck WH. Chiral lipidomics of monoepoxy and monohydroxy metabolites derived from long-chain polyunsaturated fatty acids. *J Lipid Res*. 2019;60:135-148.
57. Liakh I, Pakiet A, Sledzinski T and Mika A. Methods of the Analysis of Oxylipins in Biological Samples. *Molecules*. 2020;25.
58. Kapron J, Wu J, Mauriala T, Clark P, Purves RW and Bateman KP. Simultaneous analysis of prostanoids using liquid chromatography/high-field asymmetric waveform ion mobility spectrometry/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2006;20:1504-10.
59. Hinz C, Liggi S, Mocciaro G, Jung S, Induruwa I, Pereira M, Bryant CE, Meckelmann SW, O'Donnell VB, Farndale RW, Fjeldsted J and Griffin JL. A Comprehensive UHPLC Ion Mobility Quadrupole Time-of-Flight Method for Profiling and Quantification of Eicosanoids, Other Oxylipins, and Fatty Acids. *Anal Chem*. 2019;91:8025-8035.
60. Hamberg M and Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc Natl Acad Sci U S A*. 1974;71:3400-4.
61. Il'yasova D, Scarbrough P and Spasojevic I. Urinary biomarkers of oxidative status. *Clin Chim Acta*. 2012;413:1446-53.
62. Khamis MM, Adamko DJ and El-Aneed A. Mass spectrometric based approaches in urine metabolomics and biomarker discovery. *Mass Spectrom Rev*. 2017;36:115-134.
63. Bain G, King CD, Rewolinski M, Schaab K, Santini AM, Shapiro D, Moran M, van de Wetering de Rooij S, Roffel AF, Schuilenga-Hut P, Milne GL, Lorrain DS, Li Y, Arruda JM, Hutchinson JH, Prasit P and Evans JF. Pharmacodynamics and pharmacokinetics of AM103, a novel inhibitor of 5-lipoxygenase-activating protein (FLAP). *Clin Pharmacol Ther*. 2010;87:437-44.
64. Balgoma D, Larsson J, Rokach J, Lawson JA, Daham K, Dahlen B, Dahlen SE and Wheelock CE. Quantification of lipid mediator metabolites in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects. *Anal Chem*. 2013;85:7866-74.
65. Drew DA, Chin SM, Gilpin KK, Parziale M, Pond E, Schuck MM, Stewart K, Flagg M, Rawlings CA, Backman V, Carolan PJ, Chung DC, Colizzo FP, 3rd, Freedman M, Gala M, Garber JJ, Huttenhower C, Kedrin D, Khalili H, Kwon DS, Markowitz SD, Milne GL, Nishioka NS, Richter JM, Roy HK, Staller K, Wang M and Chan AT. ASPirin Intervention for the REDuction of colorectal cancer risk (ASPIRED): a study protocol for a randomized controlled trial. *Trials*. 2017;18:50.
66. Duffield-Lillico AJ, Boyle JO, Zhou XK, Ghosh A, Butala GS, Subbaramaiah K, Newman RA, Morrow JD, Milne GL and Dannenberg AJ. Levels of prostaglandin E metabolite and leukotriene E(4) are increased in the urine of smokers: evidence that celecoxib shunts arachidonic acid into the 5-lipoxygenase pathway. *Cancer Prev Res (Phila)*. 2009;2:322-9.
67. FitzGerald GA, Oates JA, Hawiger J, Maas RL, Roberts LJ, 2nd, Lawson JA and Brash AR. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *J Clin Invest*. 1983;71:676-88.
68. Murphey LJ, Williams MK, Sanchez SC, Byrne LM, Csiki I, Oates JA, Johnson DH and Morrow JD. Quantification of the major urinary metabolite of PGE2 by a liquid chromatographic/mass spectrometric assay: determination of cyclooxygenase-specific PGE2 synthesis in healthy humans and those with lung cancer. *Anal Biochem*. 2004;334:266-75.
69. Saude EJ, Adamko D, Rowe BH, Marrie T and Sykes BD. Variation of metabolites in normal human urine. *Metabolomics*. 2007;3:439-451.

70. Dorjgochoo T, Gao YT, Chow WH, Shu XO, Yang G, Cai Q, Rothman N, Cai H, Li H, Deng X, Shrubsole MJ, Murff H, Milne G, Zheng W and Dai Q. Obesity, age, and oxidative stress in middle-aged and older women. *Antioxid Redox Signal*. 2011;14:2453-60.
71. Helmersson J, Mattsson P and Basu S. Prostaglandin F(2alpha) metabolite and F(2)-isoprostane excretion rates in migraine. *Clin Sci (Lond)*. 2002;102:39-43.
72. Medina S, De Las Heras-Gomez I, Casas-Pina T, Bultel-Ponce V, Galano JM, Durand T, Martinez-Hernandez P, Ferreres F, Jimeno L, Llorente S and Gil-Izquierdo A. Urinary oxylipin signature as biomarkers to monitor the allograft function during the first six months post-renal transplantation. *Free Radic Biol Med*. 2020;146:340-349.
73. Yan W, Byrd GD and Ogden MW. Quantitation of isoprostane isomers in human urine from smokers and nonsmokers by LC-MS/MS. *J Lipid Res*. 2007;48:1607-17.
74. Catella F, Nowak J and Fitzgerald GA. Measurement of renal and non-renal eicosanoid synthesis. *Am J Med*. 1986;81:23-9.
75. Nugteren DH. The determination of prostaglandin metabolites in human urine. *J Biol Chem*. 1975;250:2808-12.
76. Gomes JA, Milne G, Kallianpur A and Shriver L. Isofurans and Isoprostanes as Potential Markers of Delayed Cerebral Ischemia Following Aneurysmal Subarachnoid Hemorrhage: A Prospective Observational Study. *Neurocrit Care*. 2021.
77. Liakh I, Pakiet A, Sledzinski T and Mika A. Modern Methods of Sample Preparation for the Analysis of Oxylipins in Biological Samples. *Molecules*. 2019;24.
78. Scott WC, Cahill KN, Milne GL, Li P, Sheng Q, Huang LC, Dennis S, Snyder J, Bauer AM, Chandra RK, Chowdhury NI and Turner JH. Inflammatory heterogeneity in aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol*. 2021;147:1318-1328 e5.
79. Chan MV, Hayman MA, Sivapalaratnam S, Crescente M, Allan HE, Edin ML, Zeldin DC, Milne GL, Stephens J, Greene D, Hanif M, O'Donnell VB, Dong L, Malkowski MG, Lentaigne C, Wedderburn K, Stubbs M, Downes K, Ouwehand WH, Turro E, BioResource N, Hart DP, Freson K, Laffan MA and Warner TD. Identification of a homozygous recessive variant in PTGS1 resulting in a congenital aspirin-like defect in platelet function. *Haematologica*. 2021;106:1423-1432.
80. Drew DA, Schuck MM, Magicheva-Gupta MV, Stewart KO, Gilpin KK, Miller P, Parziale MP, Pond EN, Takacsi-Nagy O, Zerjav DC, Chin SM, Mackinnon Krems J, Meixell D, Joshi AD, Ma W, Colizzo FP, Carolan PJ, Nishioka NS, Staller K, Richter JM, Khalili H, Gala MK, Garber JJ, Chung DC, Yarze JC, Zukerberg L, Petrucci G, Rocca B, Patrono C, Milne GL, Wang M and Chan AT. Effect of Low-dose and Standard-dose Aspirin on PGE2 Biosynthesis Among Individuals with Colorectal Adenomas: A Randomized Clinical Trial. *Cancer Prev Res (Phila)*. 2020;13:877-888.
81. Edelman MJ, Wang X, Hodgson L, Cheney RT, Baggstrom MQ, Thomas SP, Gajra A, Bertino E, Reckamp KL, Molina J, Schiller JH, Mitchell-Richards K, Friedman PN, Ritter J, Milne G, Hahn OM, Stinchcombe TE, Vokes EE and Alliance for Clinical Trials in O. Phase III Randomized, Placebo-Controlled, Double-Blind Trial of Celecoxib in Addition to Standard Chemotherapy for Advanced Non-Small-Cell Lung Cancer With Cyclooxygenase-2 Overexpression: CALGB 30801 (Alliance). *J Clin Oncol*. 2017;35:2184-2192.
82. Garcia-Flores LA, Medina S, Gomez C, Wheelock CE, Cejuela R, Martinez-Sanz JM, Oger C, Galano JM, Durand T, Hernandez-Saez A, Ferreres F and Gil-Izquierdo A. Aronia-citrus juice (polyphenol-rich juice) intake and elite triathlon training: a lipidomic approach using representative oxylipins in urine. *Food Funct*. 2018;9:463-475.
83. Lara-Guzman OJ, Medina S, Alvarez R, Oger C, Durand T, Galano JM, Zuluaga N, Gil-Izquierdo A and Munoz-Durango K. Oxylipin regulation by phenolic compounds from coffee beverage: Positive outcomes from a randomized controlled trial in healthy adults and macrophage derived foam cells. *Free Radic Biol Med*. 2020;160:604-617.

84. Montine TJ, Sonnen JA, Milne G, Baker LD and Breitner JC. Elevated ratio of urinary metabolites of thromboxane and prostacyclin is associated with adverse cardiovascular events in ADAPT. *PLoS One*. 2010;5:e9340.
85. Reyes-Uribe L, Wu W, Gelincik O, Bommi PV, Francisco-Cruz A, Solis LM, Lynch PM, Lim R, Stoffel EM, Kanth P, Samadder NJ, Mork ME, Taggart MW, Milne GL, Marnett LJ, Vornik L, Liu DD, Revuelta M, Chang K, You YN, Kopelovich L, Wistuba, II, Lee JJ, Sei S, Shoemaker RH, Szabo E, Richmond E, Umar A, Perloff M, Brown PH, Lipkin SM and Vilar E. Naproxen chemoprevention promotes immune activation in Lynch syndrome colorectal mucosa. *Gut*. 2021;70:555-566.
86. Altobelli E, Angeletti PM and Latella G. Role of Urinary Biomarkers in the Diagnosis of Adenoma and Colorectal Cancer: A Systematic Review and Meta-Analysis. *J Cancer*. 2016;7:1984-2004.
87. Seyberth HW, Sweetman BJ, Frolich JC and Oates JA. Quantifications of the major urinary metabolite of the E prostaglandins by mass spectrometry: evaluation of the method's application to clinical studies. *Prostaglandins*. 1976;11:381-97.
88. Schweer H, Meese CO and Seyberth HW. Determination of 11 alpha-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostan oic acid and 9 alpha,11 alpha-dihydroxy-15-oxo-2,3,4,5,20-pentanor-19-carboxyprostanoi c acid by gas chromatography/negative ion chemical ionization triple-stage quadrupole mass spectrometry. *Anal Biochem*. 1990;189:54-8.
89. Taber DF and Teng D. Total synthesis of the ethyl ester of the major urinary metabolite of prostaglandin E(2). *J Org Chem*. 2002;67:1607-12.
90. Milne GL. Lipid Biomarkers of Inflammation. In: S. Roy, Bagchi, D, Raychaudhuri, SP., ed. *Chronic Inflammation: Molecular Pathophysiology, Nutritional and Therapeutic Interventions*: CRC Press; 2016: 275.
91. Liston TE and Roberts LJ, 2nd. Transformation of prostaglandin D2 to 9 alpha, 11 beta-(15S)-trihydroxyprosta-(5Z,13E)-dien-1-oic acid (9 alpha, 11 beta-prostaglandin F2): a unique biologically active prostaglandin produced enzymatically in vivo in humans. *Proc Natl Acad Sci U S A*. 1985;82:6030-4.
92. Morrow JD, Prakash C, Awad JA, Duckworth TA, Zackert WE, Blair IA, Oates JA and Roberts LJ, 2nd. Quantification of the major urinary metabolite of prostaglandin D2 by a stable isotope dilution mass spectrometric assay. *Anal Biochem*. 1991;193:142-8.
93. Kolmert J, Gomez C, Balgoma D, Sjodin M, Bood J, Konradsen JR, Ericsson M, Thorngren JO, James A, Mikus M, Sousa AR, Riley JH, Bates S, Bakke PS, Pandis I, Caruso M, Chanez P, Fowler SJ, Geiser T, Howarth P, Horvath I, Krug N, Montuschi P, Sanak M, Behndig A, Shaw DE, Knowles RG, Holweg CTJ, Wheelock AM, Dahlen B, Nordlund B, Alving K, Hedlin G, Chung KF, Adcock IM, Sterk PJ, Djukanovic R, Dahlen SE, Wheelock CE and U-Biopred Study Group obotUBSG. Urinary Leukotriene E4 and Prostaglandin D2 Metabolites Increase in Adult and Childhood Severe Asthma Characterized by Type 2 Inflammation. A Clinical Observational Study. *Am J Respir Crit Care Med*. 2021;203:37-53.
94. Song WL, Wang M, Ricciotti E, Fries S, Yu Y, Grosser T, Reilly M, Lawson JA and FitzGerald GA. Tetranor PGDM, an abundant urinary metabolite reflects biosynthesis of prostaglandin D2 in mice and humans. *J Biol Chem*. 2008;283:1179-88.
95. Mohebati A, Milne GL, Zhou XK, Duffield-Lillico AJ, Boyle JO, Knutson A, Bosworth BP, Kingsley PJ, Marnett LJ, Brown PH, Akpa EG, Szabo E and Dannenberg AJ. Effect of zileuton and celecoxib on urinary LTE4 and PGE-M levels in smokers. *Cancer Prev Res (Phila)*. 2013;6:646-55.
96. Zhang Y, Zhang G, Clarke PA, Huang JT, Takahashi E, Muirhead D, Steenwyk RC and Lin Z. Simultaneous and high-throughput quantitation of urinary tetranor PGDM and tetranor PGEM by online SPE-LC-MS/MS as inflammatory biomarkers. *J Mass Spectrom*. 2011;46:705-11.

97. Bell-Parikh LC, Ide T, Lawson JA, McNamara P, Reilly M and FitzGerald GA. Biosynthesis of 15-deoxy-Δ12,14-PGJ2 and the ligation of PPARγ. *The Journal of Clinical Investigation*. 2003;112:945-955.
98. Brunoldi EM, Zanoni G, Vidari G, Sasi S, Freeman ML, Milne GL and Morrow JD. Cyclopentenone prostaglandin, 15-deoxy-Delta12,14-PGJ2, is metabolized by HepG2 cells via conjugation with glutathione. *Chem Res Toxicol*. 2007;20:1528-35.
99. Hardy KD, Cox BE, Milne GL, Yin H and Roberts LJ, 2nd. Nonenzymatic free radical-catalyzed generation of 15-deoxy-Delta(12,14)-prostaglandin J(2)-like compounds (deoxy-J(2)-isoprostanes) in vivo. *J Lipid Res*. 2011;52:113-24.
100. Lands WE. The biosynthesis and metabolism of prostaglandins. *Annu Rev Physiol*. 1979;41:633-52.
101. Imig JD. Eicosanoid blood vessel regulation in physiological and pathological states. *Clin Sci (Lond)*. 2020;134:2707-2727.
102. Mitchell JA, Knowles RB, Kirkby NS, Reed DM, Edin ML, White WE, Chan MV, Longhurst H, Yaqoob MM, Milne GL, Zeldin DC and Warner TD. Kidney Transplantation in a Patient Lacking Cytosolic Phospholipase A2 Proves Renal Origins of Urinary PGI-M and TX-M. *Circ Res*. 2018;122:555-559.
103. Morrow JD and Minton TA. Improved assay for the quantification of 11-dehydrothromboxane B2 by gas chromatography-mass spectrometry. *J Chromatogr*. 1993;612:179-85.
104. Grosser T, Naji A and FitzGerald GA. Urinary Prostaglandin Metabolites: An Incomplete Reckoning and a Flush to Judgment. *Circ Res*. 2018;122:537-539.
105. McAdam BF, Byrne D, Morrow JD and Oates JA. Contribution of cyclooxygenase-2 to elevated biosynthesis of thromboxane A2 and prostacyclin in cigarette smokers. *Circulation*. 2005;112:1024-9.
106. Nakashima F and Schneider C. Transformation of Prostaglandin D2 to 11-Dehydro Thromboxane B2 by Baeyer-Villiger Oxidation. *Lipids*. 2020;55:73-78.
107. Murphy RC and Gijon MA. Biosynthesis and metabolism of leukotrienes. *Biochem J*. 2007;405:379-95.
108. Berry KA, Borgeat P, Gosselin J, Flamand L and Murphy RC. Urinary metabolites of leukotriene B4 in the human subject. *J Biol Chem*. 2003;278:24449-60.
109. Mita H, Turikisawa N, Yamada T and Taniguchi M. Quantification of leukotriene B4 glucuronide in human urine. *Prostaglandins Other Lipid Mediat*. 2007;83:42-9.
110. Morita Y, Kurano M, Sakai E, Sawabe M, Aoki J and Yatomi Y. Simultaneous analyses of urinary eicosanoids and related mediators identified tetranor-prostaglandin E metabolite as a novel biomarker of diabetic nephropathy. *J Lipid Res*. 2021:100120.
111. Capdevila JH and Falck JR. The CYP P450 arachidonic acid monooxygenases: from cell signaling to blood pressure regulation. *Biochem Biophys Res Commun*. 2001;285:571-6.
112. Kirchheiner J, Meineke I, Fuhr U, Rodriguez-Antona C, Lebedeva E and Brockmoller J. Impact of genetic polymorphisms in CYP2C8 and rosiglitazone intake on the urinary excretion of dihydroxyeicosatrienoic acids. *Pharmacogenomics*. 2008;9:277-88.
113. Santos JM, Park JA, Joiakim A, Putt DA, Taylor RN and Kim H. The role of soluble epoxide hydrolase in preeclampsia. *Med Hypotheses*. 2017;108:81-85.
114. Catella F, Lawson JA, Fitzgerald DJ and FitzGerald GA. Endogenous biosynthesis of arachidonic acid epoxides in humans: increased formation in pregnancy-induced hypertension. *Proc Natl Acad Sci U S A*. 1990;87:5893-7.

115. Mota-Zamorano S, Robles NR, Lopez-Gomez J, Cancho B, Gonzalez LM, Garcia-Pino G, Navarro-Perez ML and Gervasini G. Plasma and urinary concentrations of arachidonic acid-derived eicosanoids are associated with diabetic kidney disease. *EXCLI J*. 2021;20:698-708.
116. Wang MH, Smith A, Zhou Y, Chang HH, Lin S, Zhao X, Imig JD and Dorrance AM. Downregulation of renal CYP-derived eicosanoid synthesis in rats with diet-induced hypertension. *Hypertension*. 2003;42:594-9.
117. Milne GL, Dai Q and Roberts LJ, 2nd. The isoprostanes--25 years later. *Biochim Biophys Acta*. 2015;1851:433-45.
118. Pandya BA and Snapper ML. A cross-metathesis route to the 5-F(2)-isoprostanes. *J Org Chem*. 2008;73:3754-8.
119. Cuyamendous C, de la Torre A, Lee YY, Leung KS, Guy A, Bultel-Ponce V, Galano JM, Lee JC, Oger C and Durand T. The novelty of phytofurans, isofurans, dihomo-isofurans and neurofurans: Discovery, synthesis and potential application. *Biochimie*. 2016;130:49-62.
120. Galano JM, Lee YY, Oger C, Vigor C, Vercauteren J, Durand T, Giera M and Lee JC. Isoprostanes, neuroprostanes and phytoprostanes: An overview of 25years of research in chemistry and biology. *Prog Lipid Res*. 2017;68:83-108.
121. Leung HH, Leung KS, Durand T, Galano JM and Lee JC. Measurement of Enzymatic and Nonenzymatic Polyunsaturated Fatty Acid Oxidation Products in Plasma and Urine of Macular Degeneration Using LC-QTOF-MS/MS. *Lipids*. 2020;55:693-706.
122. Miller E, Morel A, Saso L and Saluk J. Isoprostanes and neuroprostanes as biomarkers of oxidative stress in neurodegenerative diseases. *Oxid Med Cell Longev*. 2014;2014:572491.
123. Pena-Bautista C, Vigor C, Galano JM, Oger C, Durand T, Ferrer I, Cuevas A, Lopez-Cuevas R, Baquero M, Lopez-Nogueroles M, Vento M, Hervas-Marin D, Garcia-Blanco A and Chafer-Pericas C. New screening approach for Alzheimer's disease risk assessment from urine lipid peroxidation compounds. *Sci Rep*. 2019;9:14244.
124. Signorini C, De Felice C, Galano JM, Oger C, Leoncini S, Cortelazzo A, Ciccoli L, Durand T, Hayek J and Lee JC. Isoprostanoids in Clinical and Experimental Neurological Disease Models. *Antioxidants (Basel)*. 2018;7.
125. Kuligowski J, Aguar M, Rook D, Lliso I, Torres-Cuevas I, Escobar J, Quintas G, Brugada M, Sanchez-Illana A, van Goudoever JB and Vento M. Urinary Lipid Peroxidation Byproducts: Are They Relevant for Predicting Neonatal Morbidity in Preterm Infants? *Antioxid Redox Signal*. 2015;23:178-84.
126. Pena-Bautista C, Durand T, Vigor C, Oger C, Galano JM and Chafer-Pericas C. Non-invasive assessment of oxidative stress in preterm infants. *Free Radic Biol Med*. 2019;142:73-81.
127. Calder PC and Yaqoob P. Omega-3 polyunsaturated fatty acids and human health outcomes. *Biofactors*. 2009;35:266-72.
128. Skarke C, Alamuddin N, Lawson JA, Li X, Ferguson JF, Reilly MP and FitzGerald GA. Bioactive products formed in humans from fish oils. *J Lipid Res*. 2015;56:1808-20.
129. O'Donnell VB, Aldrovandi M, Murphy RC and Kronke G. Enzymatically oxidized phospholipids assume center stage as essential regulators of innate immunity and cell death. *Sci Signal*. 2019;12.
130. Maskrey BH, Bermudez-Fajardo A, Morgan AH, Stewart-Jones E, Dioszeghy V, Taylor GW, Baker PR, Coles B, Coffey MJ, Kuhn H and O'Donnell VB. Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. *The Journal of biological chemistry*. 2007;282:20151-63.
131. Morgan AH, Hammond VJ, Morgan L, Thomas CP, Tallman KA, Garcia-Diaz YR, McGuigan C, Serpi M, Porter NA, Murphy RC and O'Donnell VB. Quantitative assays for esterified oxylipins generated by immune cells. *Nat Protoc*. 2010;5:1919-31.

132. Thomas CP, Morgan LT, Maskrey BH, Murphy RC, Kuhn H, Hazen SL, Goodall AH, Hamali HA, Collins PW and O'Donnell VB. Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *The Journal of biological chemistry*. 2010;285:6891-903.
133. Slatter DA, Aldrovandi M, O'Connor A, Allen SM, Brasher CJ, Murphy RC, Mecklemann S, Ravi S, Darley-Usmar V and O'Donnell VB. Mapping the Human Platelet Lipidome Reveals Cytosolic Phospholipase A2 as a Regulator of Mitochondrial Bioenergetics during Activation. *Cell metabolism*. 2016;23:930-44.
134. Ni Z, Angelidou G, Hoffmann R and Fedorova M. LPPtiger software for lipidome-specific prediction and identification of oxidized phospholipids from LC-MS datasets. *Sci Rep*. 2017;7:15138.
135. Matsuoka Y, Takahashi M, Sugiura Y, Izumi Y, Nishiyama K, Nishida M, Suematsu M, Bamba T and Yamada KI. Structural library and visualization of endogenously oxidized phosphatidylcholines using mass spectrometry-based techniques. *Nat Commun*. 2021;12:6339.

Figure 1

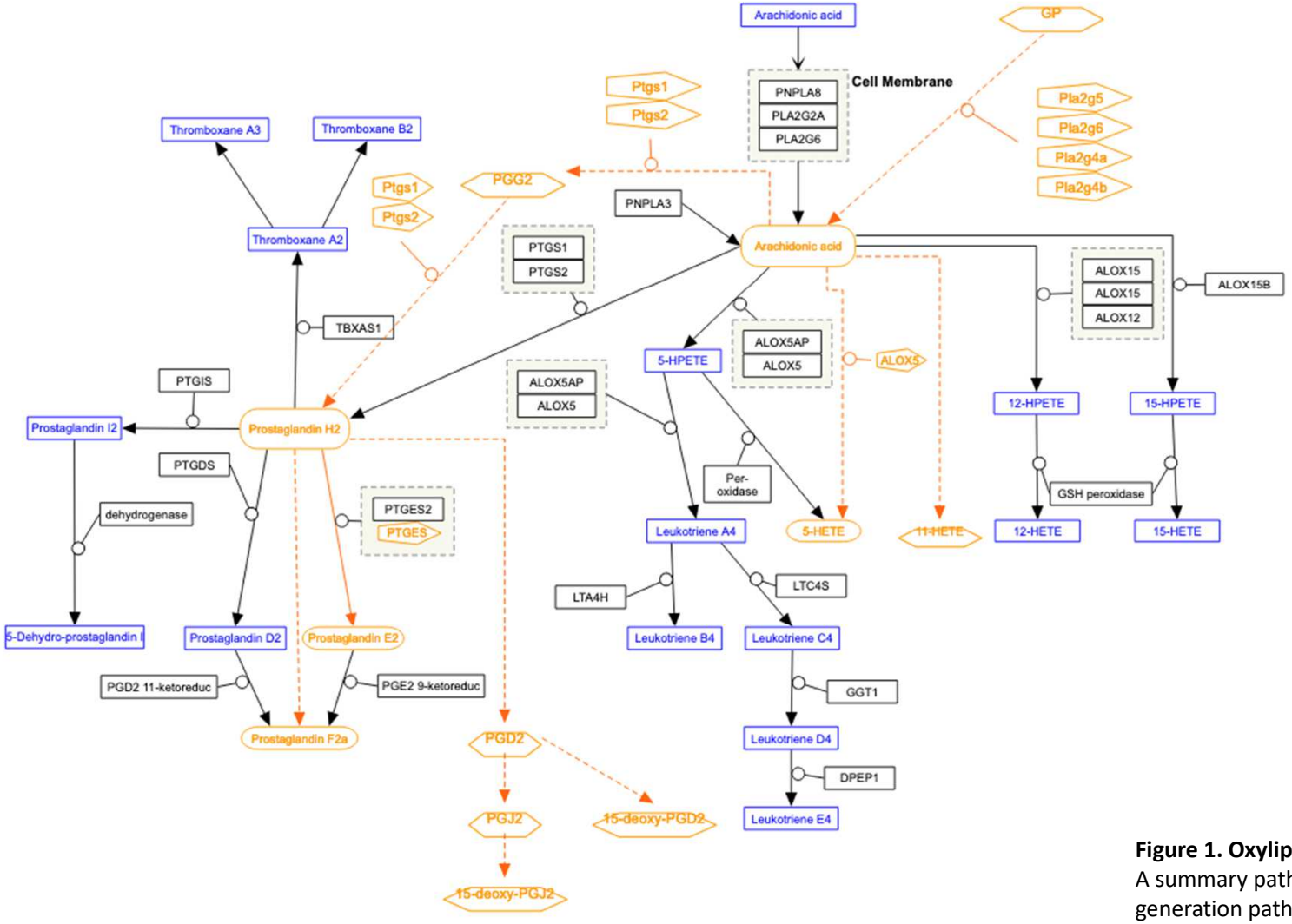
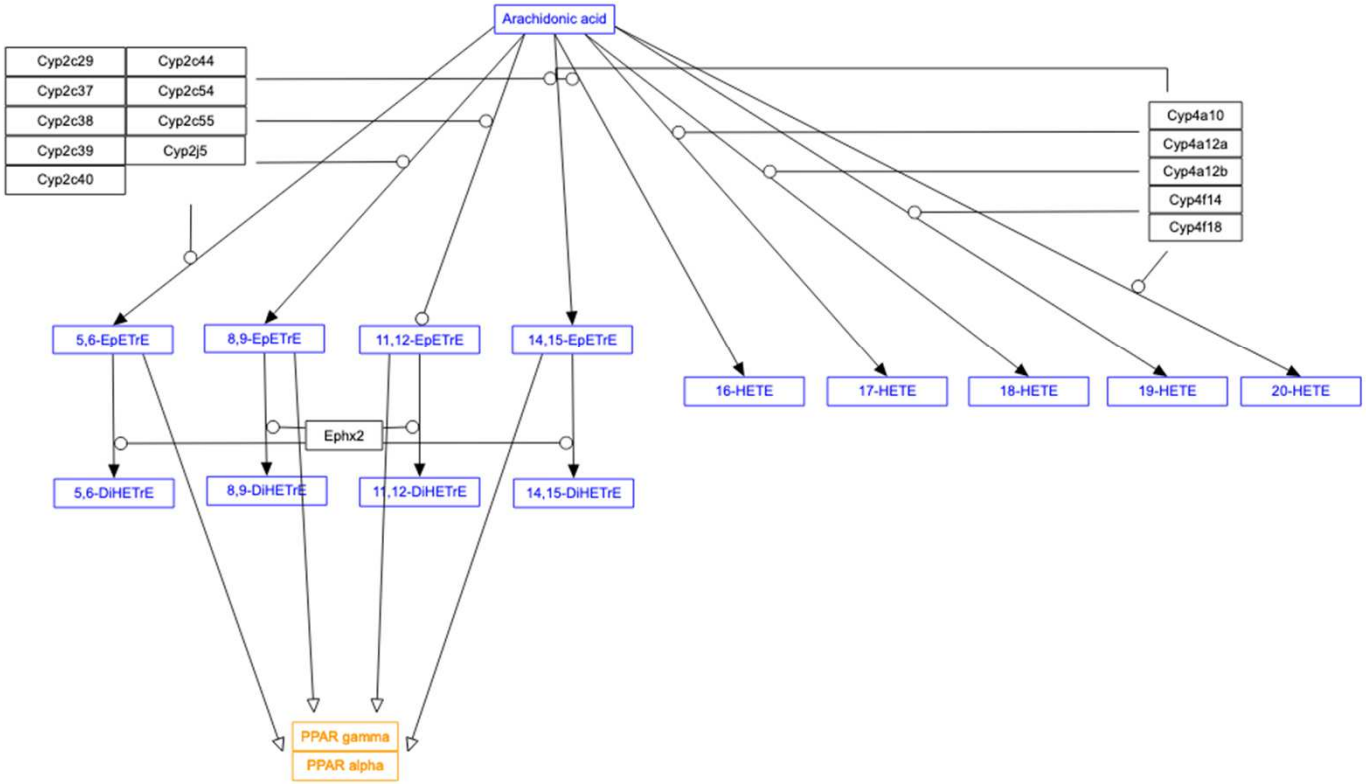


Figure 1. Oxylipin generation from arachidonic acid (homo sapiens)
A summary pathway showing the main cyclooxygenase and lipoxygenase generation pathways in mammals. Taken from WikiPathways/LIPID MAPS
https://www.lipidmaps.org/resources/pathways/wikipathways/WP167

Figure 2

Figure 2. Oxylipin generation via the cytochrome P450 pathway (mus musculus)
Taken from WikiPathways/LIPID MAP. Links on the pathway provide more information including to additional resources and databases.
https://www.lipidmaps.org/resources/pathways/wikipathways/WP4349



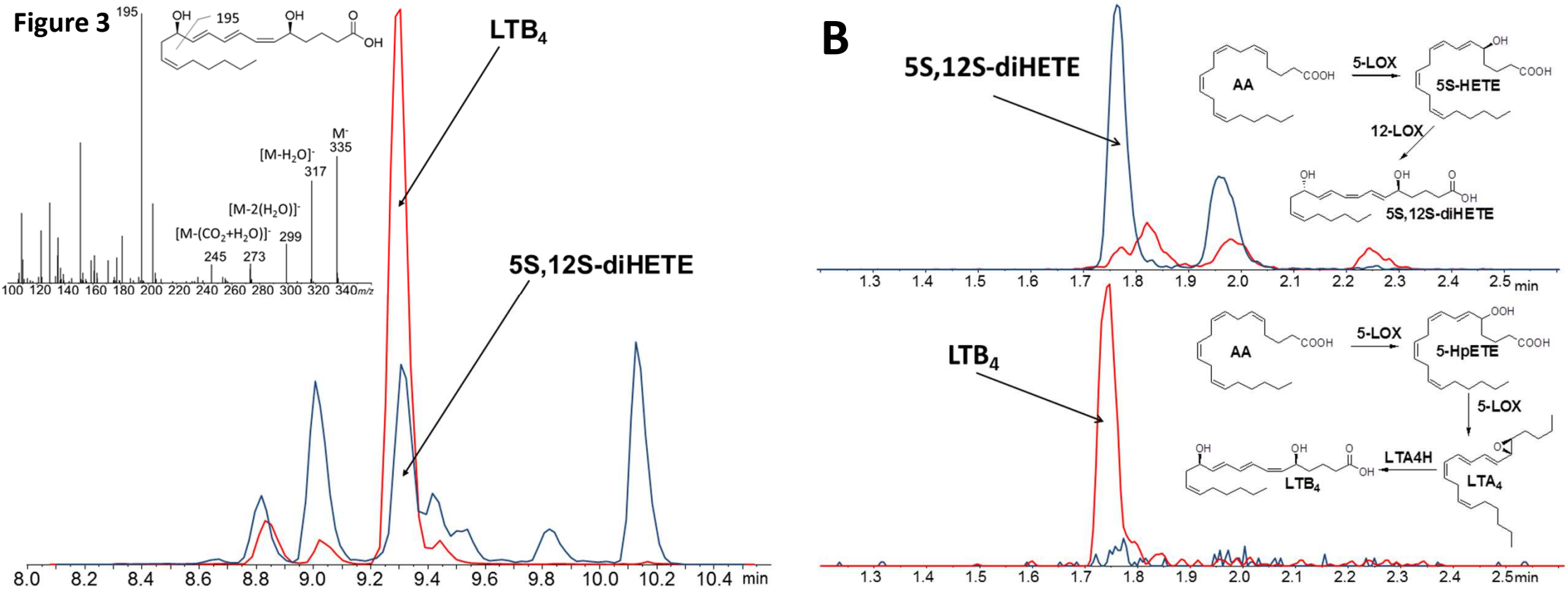


Figure 3. LTB4 and its enantiomer, 5S,12S-diHETE are not separated on reverse phase LC-MS/MS, but can be separated using differential ion mobility analysis.

A) LC–MS/MS analysis of an ethanol extract from peritoneal cells monitoring the SRM transition m/z 335 → 195. Red trace, 24 h after zymosan A challenge; blue trace, 2 h after PBS injection (control group). Upper left corner, MS/MS spectrum of LTB_4 and its isomers, showing the typical fragment ion m/z 195

B) Analysis of murine peritoneal cell ethanol extracts using µLC–DMS-MS/MS. Upper panel, control mice (PBS injection). Lower panel, zymosan A challenged mice. Red traces, SV 4500 V and COV 17.9 V; blue traces, SV 4500 V and COV 20.3 V. Additionally the biochemical pathways leading to LTB_4 (lower panel) and 5S,12S-diHETE (upper panel) are shown. Abbreviations: AA, arachidonic acid; 5S-HETE, 5(S)-hydroxyeicosatetraenoic acid; 12-LOX, 12-lipoxygenase; 5HpETE, 5-hydroperoxy-eicosatetraenoic acid; LTA4, leukotriene A4. Unknown: undefined isomer in the trace of 5S,12S-diHETE. Taken from Anal. Chem. 2015, 87, 10, 5036–5040

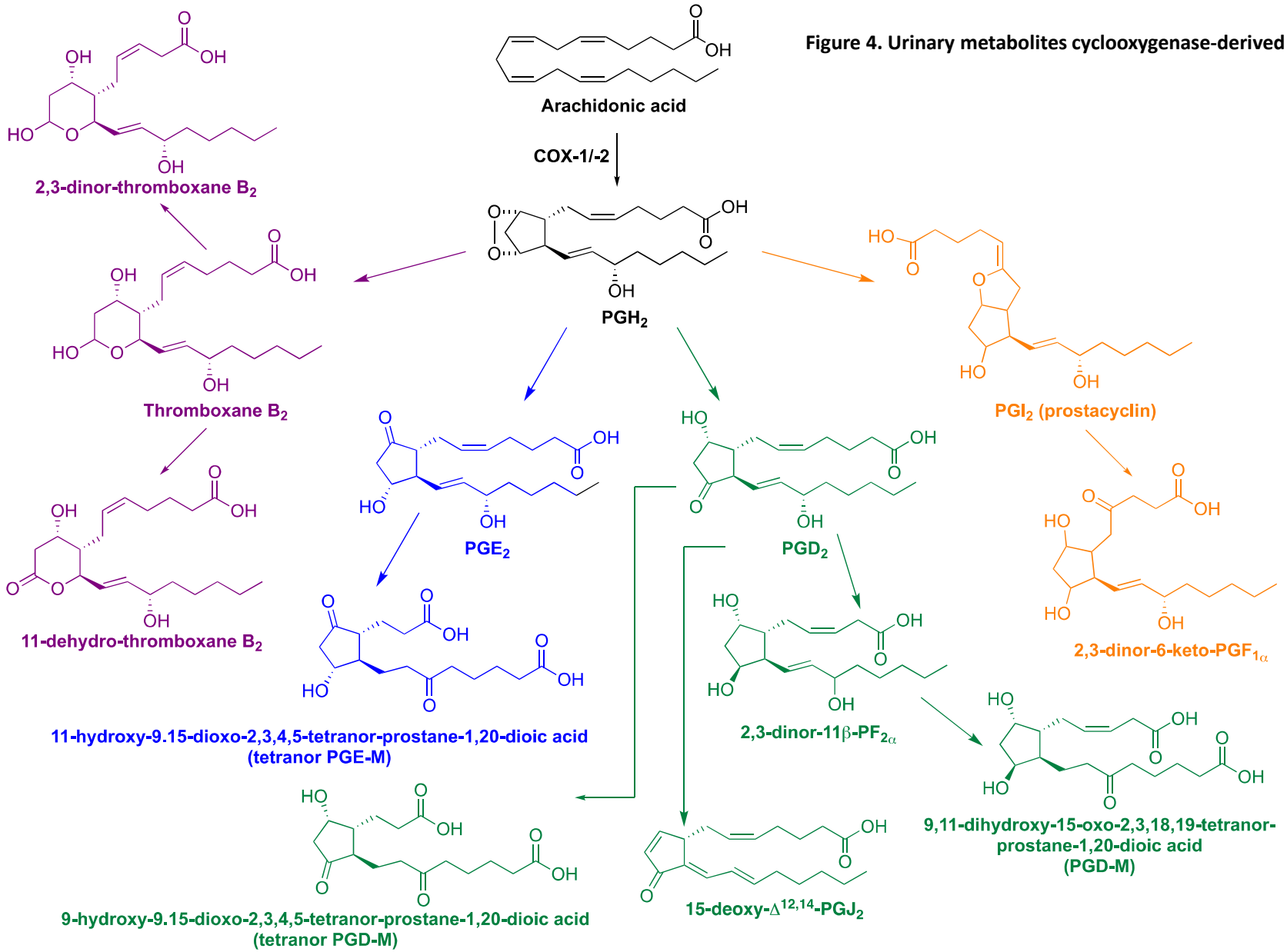


Figure 4. Urinary metabolites cyclooxygenase-derived prostaglandins.

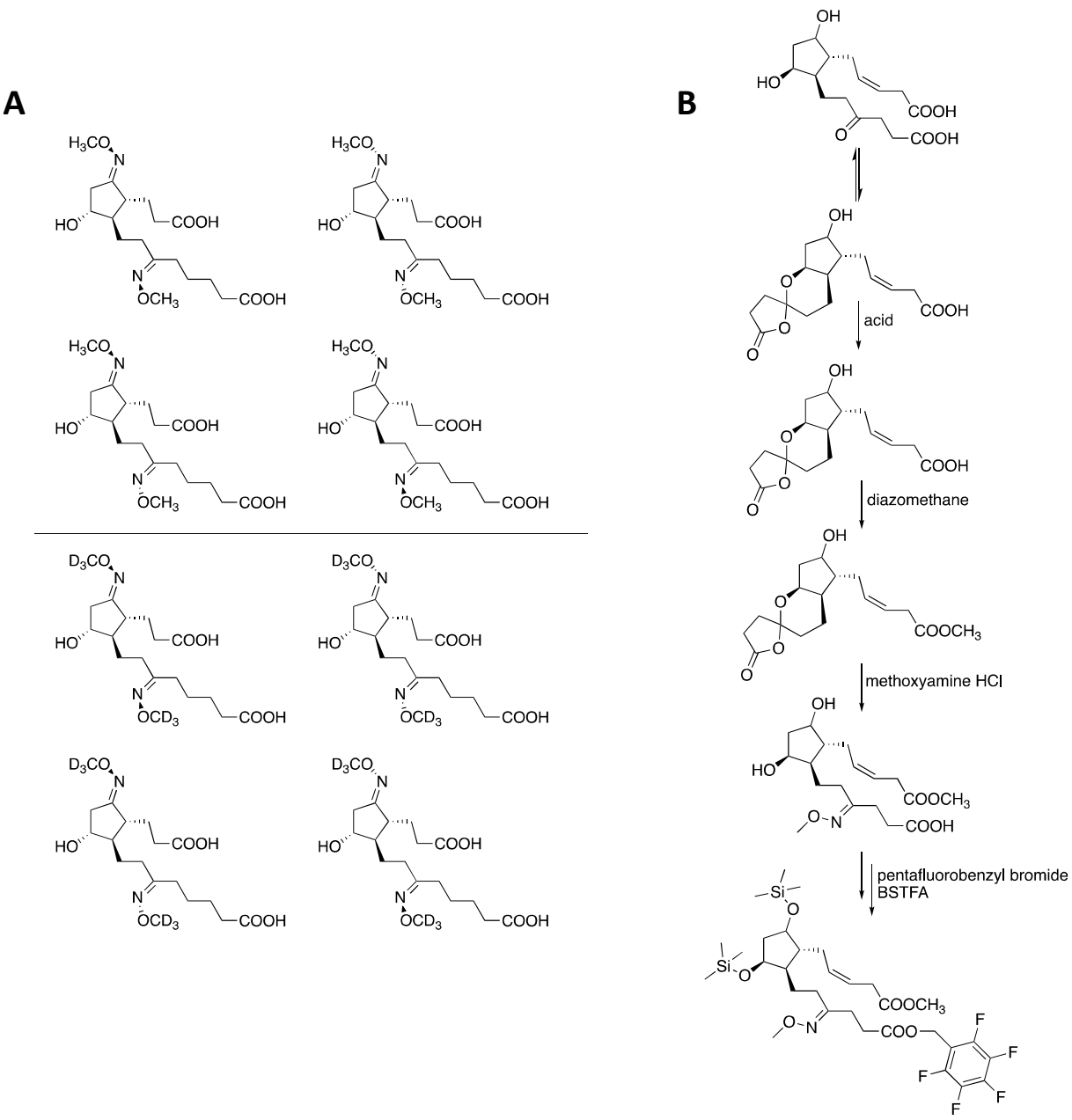


Figure 5. Derivatization strategies are used to stabilize urinary metabolites of PGE_2 and PGD_2 for MS analysis.
(A) Syn- and anti- methoxyime isomers of PGE-M formed during derivatized with methyloxime HCl or [$^2H_3$]-methyloxime HCl. (B) Cyclization of PGD-M and stabilization by derivatization for GC-MS analysis.

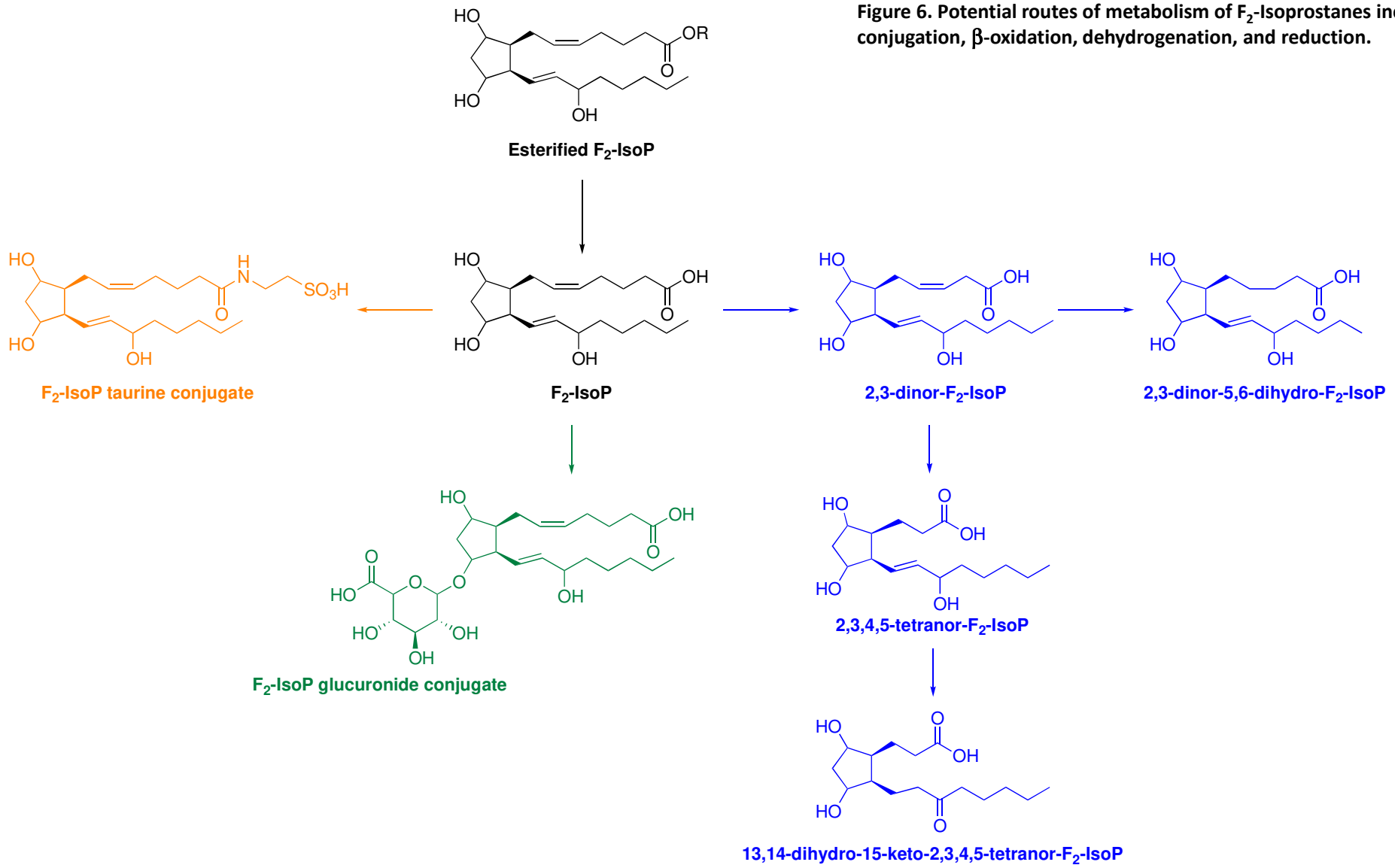


Figure 6. Potential routes of metabolism of F_2-Isoprostanes include conjugation, β-oxidation, dehydrogenation, and reduction.