Review

Lipidomic methodologies for biomarkers of chronic inflammation in nutritional research: ω -3 and ω -6 lipid mediators

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Abstract

The evolutionary history of hominins has been characterized by significant dietary changes, which include the introduction of meat eating, cooking, and the changes associated with plant and animal domestication. The Western pattern diet has been linked with the onset of chronic inflammation, and serious health problems including obesity, metabolic syndrome, and cardiovascular diseases. Diets enriched with ω -3 marine PUFAs have revealed additional improvements in health status associated to a reduction of proinflammatory ω -3 and ω -6 lipid mediators. Lipid mediators are produced from enzymatic and non-enzymatic oxidation of PUFAs. Interest in better understanding the occurrence of these metabolites has increased exponentially as a result of the growing evidence of their role on inflammatory processes, control of the immune system, cell signaling, onset of metabolic diseases, or even cancer. The scope of this review has been to highlight the recent findings on: a) the formation of lipid mediators and their role in different inflammatory and metabolic conditions, b) the direct use of lipid mediators as antiinflammatory drugs or the potential of new drugs as a new therapeutic option for the synthesis of antiinflammatory or resolving lipid mediators and c) the impact of nutritional interventions to modulate lipid mediators synthesis towards antiinflammatory conditions. In a second part, we have summarized methodological approaches (Lipidomics) for the accurate analysis of lipid mediators. Although several techniques have been used, most authors preferred the combination of SPE with LC-MS. Advantages and disadvantages of each method are herein addressed, as well as the main LC-MS difficulties and challenges for the establishment of new biomarkers and standardization of experimental designs, and finally to deepen the study of mechanisms involved on the inflammatory response.

1. Introduction

Chronic inflammation and metabolic diseases related to diet: role of lipid mediators

The Western pattern diet including high-fat and cholesterol, high-protein, high-sugar, and excess salt intake, promote obesity, metabolic syndrome, and cardiovascular disease, all of them linked to the occurrence of chronic inflammation. The evolution of the human diet towards an increase in the intake of saturated fats and trans fats, ω -6 PUFAs and refined sugars, and a decrease of the amount of ω -3 PUFAs, vitamins, minerals and natural antioxidants raised as one of the main causes of the emergence of new pathologies and metabolic disorders [1,2]. Around 20-30% of adult population suffers Metabolic Syndrome [3], a compilation of risk factors to develop CVD or type 2 diabetes: dyslipidemia, hyperglycemia, hypertension, insulin resistance and obesity [4]. Moreover, CVD and cancer overtake respiratory diseases as the leading causes of death in the EU, according to the Eurostat report of May 2016 [5] or World Health Organization in 2015 [6]. And they have been largely associated with the onset and development of inflammatory processes and oxidative stress [7]. Oxidative stress and inflammation are closely related pathophysiological processes, one of which can be easily induced by another.

As a consequence, interest in better understanding the role of diet components over these metabolic features has increased exponentially in the last decades. Many studies correlate high fat diets with obesity, CVD, diabetes in humans [8–11] or animal models [12–15]. However, fat intake should consider the amount but the type of fat as well. The intake of *trans* fats triggers inflammatory processes, endothelial dysfunction, increases the adiposity index and insulin resistance [16]. An increasing dietary ratio of ω -6/ ω -3 fatty acids correlates with a higher incidence of obesity [17–19], CVD [20], metabolic syndrome [21], insulin resistance [22,23], and the prevalence of inflammatory processes as arteriosclerosis [24], arthritis and asthma [2] in human and animal studies.

On the other hand, diets including high amounts of seafood and fish increase the dietary amount of ω -3 PUFAs and have been linked to reduced risk of CVD, diabetes, Alzheimer's, metabolic syndrome, cancer, Parkinson, and other diseases derived from inflammatory processes and oxidative stress [25–31]. ω -3 PUFAs particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been credited with improving human health [31]. The interest of ω -3 fatty acids and their impact on health and diseases began in the 1970s when it was discovered that the Inuit in

Greenland [32] and Iceland and Alaska Natives [33,34] had a markedly lower incidence of cardiovascular diseases (CVD) than the western population. As consequence, the number of works trying to clarify the nutritional and beneficial role of marine ω -3 PUFA has increased appreciably during the last decades. Studies on the relationship between seafood or EPA + DHA supplements and several diseases have been performed revealing positive effects of a high intake for a range of different medical conditions, in particular inflammatory processes. A recent review made by Khadge et al. [35] provided new insights on the role of PUFAs on inflammation and tumor growth.

However, many studies show conflicting evidence regarding the health benefits of intake of ω -3 from seafood or ω -3 supplements on inflammatory and metabolic diseases [36,37]. Some studies have described a clear healthy effect and in others, the effects could not be clearly established. And, the mechanisms underlying action of these fatty acids need to be fully determined [38]. In the last years, particular attention has been focused on lipid mediators, biomolecules derived from PUFAs which seem to play an essential role on the progression/inhibition of several inflammatory processes.

Lipid mediators from ω -3 and ω -6 PUFAs: synthesis, classification, bioactivity and mechanisms of action

Lipid mediators are a complex family of bioactive compounds derived from the enzymatic and non-enzymatic oxidation of ω -3 PUFAs (e.g.: EPA and DHA) and ω -6 PUFAs (e.g.: arachidonic acid, ARA) as it is shown in **Fig. 1a-b** [39]. Firstly, PUFAs are released from cells membrane into cytosol by phospholipase A₂ (PLA₂) action [40]. Then, these free PUFAs are oxidized to a high number of isomeric compounds called lipid mediators through enzymatic and non-enzymatic pathways. An alternative route occurs via enzymatic or non-enzymatic oxidation of PUFAs esterified to phospholipids, and then followed by PLA₂ release. The enzymatic synthesis of lipid mediators mainly occurs through the action of cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 monooxygenases (CYP450). In addition to these, other enzymes can be involved: cytochrome hydrolase (CyPh), cytochrome epoxigenase (CyPe), desaturases (D), elongases (E), prostaglandin synthases (PGsy), thromboxane synthase (TXsy), glutathione peroxidases (GPX), glutathione transferase (GPT), dehydrogenases (DHO), soluble epoxide hydrolases (sEH), γ -glutamyl transferase (GGT), dipeptidase (CN2), or hydroperoxidase (PGH).

The families of lipid mediators can be classified according to the specific oxidation and functional group. Each family includes several isomers which are differentiated by a number or a letter that indicates the position of the functional group. The

nomenclature and acronyms are not well standardized. So, in **Tab. 1** are summarized the main published families, names, and structures for ARA, EPA and DHA lipid mediators [41].

A significant progress has been done to reveal the mechanisms and routes regulated by these biomolecules. Lipid mediators regulate inflammatory processes [42–46], cell signaling and gene expression [31], lipid metabolism and metabolic diseases [29,31,47]. They have been investigated in the pharmacological field considering their therapeutic application, or in medicine as biomarkers of inflammation; however, little is known about the ability of dietary interventions to modulate the synthesis of these compounds and how they could influence inflammatory processes and metabolic disorders.

Prostaglandins, Thromboxanes, and Isoprostanes from ARA have been associated with hypercholesterolemia [48], liver cirrhosis [49], myocardial reperfusion [50], type-2 diabetes [51], obesity [52], atherosclerosis [52], Crest Syndrome [53], cancer [54], and CVD [29,31]. All these conditions are closely correlated with chronic inflammatory stages. They are considered biomarkers of inflammation, also due to their relatively stability and feasibility to be detected in biological fluids [55]. These lipid mediators can be linked to specific receptors of cell membranes and coupled to membrane G protein [56]. They can activate the cAMP complex pathway which increases the expression of inflammatory cytokines [57] and decrease the ability of leukocytes to phagocyte microorganisms [58]. The clinical importance of these metabolites lies in their effective blocking by exerting an action on their biosynthetic pathways through anti-inflammatory drugs which can react with COX enzymes such as NSAIDs (non-steroidal anti-inflammatory drugs) [41]. In addition, these molecules play an important role in the maintenance of cardiovascular homeostasis by means of the production of vasodilator and vasoconstrictor metabolites [59,60]. On the other hand, the corresponding metabolites produced from EPA which compete with ARA by the same COX enzymes, have been suggested to be less harmful compounds than the ω -6 ones [41].

Similarly, ARA **Leukotrienes** are proinflammatory substances that may cause or contribute to the development of arthritis, psoriasis, asthma, rhinitis, or cancer [61,62]. They bind with specific receptors of G protein complex that regulates vasoconstriction, production of NO [63], or activation of NADPH [64]; and lead the activation of the nuclear PPAR α receptor as well [65]. Drugs that inhibit the activity of 5LOX to block

leukotrienes are used to reduce some symptoms and help control some of these diseases [66]. EPA leukotrienes are at least 10 to 30 times less potent vasoconstrictor molecules [67], or 100 times less effective activating nuclear receptors than ARA leukotrienes [68], since EPA derivatives mobilize lower amounts of calcium ions in leukocytes and activate less inflammatory interleukins [69].

Keto-derivates from ARA are a family of lipid mediators still not-well studied. They have been described to activate cellular signaling such as the PI3-AKT pathway or TNF α factor, produce cytokines, or incentive the synthesis of PLA₂ for promoting the synthesis of other proinflammatory lipid mediators [70,71].

However, not all ω -6 mediators are proinflammatory. ARA **Lipoxins** are key in the resolution of inflammation due to the competence for the same enzymes that synthesize leukotrienes [72] and inhibition of NADPH oxidase [73], interleukins [74], nuclear factors like TNF α [75], or adhesion molecule ICAM-1 in cells [76]. Therefore, they have been successfully employed in inflammation models of different diseases such as fibrosis[77], periodontitis [78], asthma [79], colitis [80], or against microbial infections such as tuberculosis [81].

Hydroxy lipids and Hydroperoxy lipids have been described as secondary messengers that may be involved in the regulation of cardiovascular system, arteriosclerosis, corneal lesions, depression, or cancer [43,82,83]. As for the other families, EPA and DHA derivates are less inflammatory than ARA metabolites due to their ability to inhibit the formation of strong proinflammatory substances [84].

Epoxides from ARA, EPA and DHA are metabolized into **Dihydroxy lipids** by the action of sEH enzymes. Epoxides are described as antiinflammatory substances while dihydroxy lipids are described as proinflammatory ones [85]. Then, the use of inhibitors of sEH has been described as a potent antiinflammatory strategy [86]. Epoxides activity is linked with an activation of the PPAR γ factor and an inhibition of TNF α and NF κ B [87]. Consequently, they decrease the production of proinflammatory cytokines, COX expression correlated to prostaglandins synthesis, and decrease adhesion molecules such as ICAM-1 and VCAM-1 [88]. A recent review based on preclinical studies, shows that ω -3 epoxides display cardio protective, vasodilatory, anti-inflammatory, and anti-allergic properties in diverse disease conditions ranging from CVD, bronchial disorders, allergic intestinal inflammation, and inflammatory pain [89].

Finally, specialized pro-resolving mediators (SPMs) are biosynthesized from ω -3 essential fatty acids to **Resolvins**, **Protectins**, and **Maresins** and from ω -6 fatty acids to **Lipoxins**, having a powerful ability to resolve inflammation [83]. These molecules are trihydroxy lipids produced from subsequent oxidation of precursor hydroxy lipids. Such structure modifications provide strong anti-inflammatory properties. They inhibit/enhance cell signaling using G protein-coupled receptors to activate the antiinflammatory and immune response: through the inhibition of NFkB, stimulation of TNF α , inhibition of the synthesis of leukotrienes, or through activation of macrophages [46,90]. Recently, they were effective as antiinflammatory substances in CVD [29], arthritis [91], colitis [92], asthma [93], cancer [94,95], Alzheimer [45,96].

Regulation of the above cited diseases includes a wide range of mechanisms linked to lipid mediators derived from EPA, DHA and ARA as the activation/inhibition of receptors and transcription factors, cytokine synthesis, competition by the same enzymes and others as can be seen in **Fig. 2**.

This review is aimed at show comprehensively the current research which has specifically studied lipid mediators. In a first part, it will review the literature dealing with the correlation between diseases with a high-grade inflammatory state and the synthesis of lipid mediators. Secondly, the review will pay attention on the therapeutic application of lipid mediators as drugs, and the use of new medicines based on controlled lipid mediators biosynthesis. In a third part, the effectiveness of nutritional interventions to modulate the synthesis of lipid mediators towards greater antiinflammatory scenarios will be reviewed. Finally, the review will focus the last findings on the progressions of “omics” techniques to analyze lipid mediators in different matrix so as the main challenges of this subject.

2. Lipid mediator’s research

This section deals with the revision of recently published articles from progress in lipid mediators in relation to:

2.1 Their use as potential biomarkers of inflammation in inflammatory diseases and metabolic disorders.

2.2 Pharmaceutical research for their therapeutic application and drug developments aimed to inhibit the synthesis of proinflammatory lipid mediators.

2.3 “Antiinflammatory nutrition”: nutritional interventions especially based on fishery products with high contents on ω -3 PUFA able to modulate the synthesis of lipid mediators and their benefits for inflammatory processes and metabolic disorders.

2.1 Lipid mediators in inflammatory diseases and metabolic disorders

ARA prostaglandins, thromboxanes and leukotrienes are considered potential biomarkers for diagnosis of chronic **respiratory diseases**, levels of severity, or as a tool to monitor the effectiveness of different treatments. Other families of lipid mediators have also been analyzed in exhaled air, plasma, urine, bronchoalveolar fluids and saliva and related to asbestosis [97,98], asthma [99–103], and pulmonary obstruction in humans [104].

Numerous studies have studied the role of lipid mediators in type 2 **diabetes** [105,106]. Schwartzman et al. [107] correlated diabetes and an increasing LOX activity towards the production of 5HETE and proinflammatory cytokines, reduced amounts of 15HETE and anti-inflammatory epoxides, and production of DHA resolvins and protectins. Increasing levels of LTE₄ in urine have been correlated to type 1-diabetes as well [108].

Lipid mediators have been linked to various types of **cancer** such as lung [54], colon [109], liver [110], ovaries [111,112], breast [113–115], and prostate [62,116,117]. Elevated levels of ARA leukotrienes, prostaglandins and hydroxy lipids have been described in these studies. Additionally, lower levels of resolvers have been depicted. Gleissman et al. [118] found a decrease in resolvins and protectins in neuroblastoma cell lines.

The studies to determine the profile of lipid mediators in **liver diseases** are scarce. Highly oxidative stress markers like hydroxy lipids have been found in plasma from patients with non-alcoholic steatohepatitis [119]. Preliminary data reveal that the eicosanoid profile in plasma reflects tissue conditions; consequently the plasma could be a target matrix to establish biomarkers while avoiding invasive biopsies or more complex operations directly in the organs.

In relation to **cardiovascular health**, many of the lipid mediators have been highlighted for their ability to regulate vascular tone. Arteriosclerosis has been directly correlated with increased ARA hydroxy lipids [43] and decreased EPA and DHA hydroxy lipids. Other studies suggested the ratio between epoxides/di-hydroxy lipids as a marker of inflammation in CDV diseases [120–122]. ARA prostaglandins and leukotrienes have been associated with hypercholesterolemia [48], myocardial reperfusion [50], increased hypertension, obesity [52], heart attacks [123], or ischemic

1 strokes [124]. On the contrary, resolvins and protectins showed beneficial
2 antiinflammatory effectiveness in cardiovascular health [29]. A recent work showed
3 decreased levels of RvD₁ and DHA and increased ARA in the sera of patients
4 presenting an acutely symptomatic carotid plaque rupture event compared to patients
5 with asymptomatic high-grade carotid stenosis [125]. The role of inflammation in the
6 development of diseases such as obesity is not entirely clear, and correlation with lipid
7 mediators profile hasn't studied carefully enough yet.

8
9 In many **other inflammatory conditions**, such as rheumatoid arthritis, asthma,
10 psoriasis, rhinitis, Complex Regional Pain Syndrome, Crest syndrome, or colitis,
11 research have shown high levels of proinflammatory markers as prostaglandins and
12 leukotrienes [53,61,126]. Additionally, several studies have pointed out the
13 antiinflammatory effectiveness of resolvins and protectins in these patients [91,92].
14 Neurovascular diseases such as ischemia [127,128], and other pathologies as
15 depression [129], corneal lesions, or angiogenesis [83] are characterized by increasing
16 levels of ARA hydroxy lipids. Finally, the protective role of resolvins and protectins is
17 being investigated in Alzheimer's and Parkinson's [45,96], and lipoxins in fibrosis [77],
18 periodontitis [78], colitis [80], or tuberculosis [81].

19
20 Fig. 3 shows a summary of the main inflammatory and metabolic diseases in which
21 lipid mediators have been studied for their role promoting or resolving the inflammatory
22 condition.

23 24 **2.2 Lipid mediators in pharmacy**

25 Recent years have witnessed a widespread increase of interest in the administration
26 of specific lipid mediators as antiinflammatory drugs; or finding new substances able to
27 modulate cell signaling, activate or inhibit transcription factors, or block the enzymatic
28 route of synthesis of strong proinflammatory lipid mediators.

29
30 The administration of lipoxins, resolvins, protectins, and epoxides as drugs in animal
31 and cell models has been effective to reduce inflammation of myocardial infarction, or
32 pulmonary fibrosis. An improvement of the systemic state of inflammation through
33 reduction of interleukin synthesis or increase of macrophages activity was noticed
34 [78,80,130,131]. Two recent works [132,133] demonstrated the effectiveness of some
35 prostaglandins such as PGA₂ and PGI₂ for reducing the expression of inflammatory
36 interleukins and inducing membranes protection when they are administrated in cell
37 cultures models of acute lung injury. A recent review shows the use of synthetic

1 analogs to mimic the biological activities of ω -3 epoxides for the treatment of
2 cardiovascular and inflammatory diseases [89].

3
4 There's a lot of interest in developing therapeutic strategies to inhibit the generation
5 of proinflammatory mediators. **NSAIDs** are classical anti-inflammatory drugs able to
6 inhibit the activity of COX and LOX enzymes by limiting the production of
7 prostaglandins, thromboxanes and leukotrienes [41]. High dosages of NSAIDs are
8 required and adverse effects can occur as a collateral or side effect [31]. Moreover,
9 COX and LOX blockage inhibit the synthesis of beneficial resolving lipid mediators.

10
11 sEH, a target enzyme in studies for **new** drug targets in inflammation, regulates the
12 transformation of epoxides (antiinflammatory) into dihydroxy lipids (proinflammatory)
13 [85]. ω -3 PUFAs rich diet in combination with **sEH inhibitors** have showed positive
14 results to increase the level of epoxides and reduce inflammation in mice with hepatic
15 fibrosis [134], and in mice with induced systemic inflammation as well [86]. Additionally,
16 other works have demonstrated the effectiveness of sEH inhibitors in neuropathic pain,
17 arrhythmias, cardiac hypertrophy, arteriosclerosis and ischemia reperfusion [135].

18
19 Leukotriene synthesis pathway through 5-LOX has been highly correlated with
20 inflammatory pathologies [61,123]. **Inhibitors of 5LOX** such as "zileuton" have shown
21 to be effective for the treatment of asthma, but have a pharmacological hepatotoxicity
22 [66]. Moreover, leukotrienes are essential for the inflammatory defense against
23 microorganisms, and animal studies aimed to block 5LOX have resulted in an increase
24 in deaths from infections [136]. Finally, **antipsychotics** as inhibitors of the synthesis of
25 ARA thromboxanes, which regulate the production of serotonin and dopamine,
26 important hormones in schizophrenia have been also suggested [137].

27 28 **2.3 Lipid mediators in antiinflammatory nutritional interventions**

29 The number of publications recorded in the *Scopus* database from 1960s to
30 nowadays, aimed to correlate lipid mediators and inflammatory diseases and metabolic
31 disorders is over 72800 research articles, reviews, book chapters, and so on. The
32 search criteria included all lipid families with possible names found in literature and a
33 huge number of diseases like: inflammation, metabolic disorder, obesity, metabolic
34 syndrome, diabetes, cardiovascular disease, atherosclerosis, asthma, cystic fibrosis,
35 cancer, respiratory disease, cirrhosis, and arthritis. 68% of publications belong to
36 Medicine field, and 20% to Pharmacy. Then, including the criteria of "diet and nutrition"

1 in the *Scopus* search, the number of works that correlate lipid mediators – inflammatory
2 conditions – and nutrition, highly decreased up to five hundred.

3
4 A great interest in lipid mediators has risen during the last decades; however, the
5 analysis of lipid mediators in nutritional research is very scarce. The dietary modulation
6 of lipid mediators profile represents less than 1% of the total studies of lipid mediators
7 and inflammatory diseases. Since the early studies in the Eskimo populations,
8 numerous works have attributed beneficial effects on health to the consumption of fish
9 and fish oils, rich in ω -3 EPA and DHA. It is believed that an "anti-inflammatory
10 nutrition" can interact with the same targets and metabolic pathways as anti-
11 inflammatory drugs like NSAIDs but with no side effects. Then, in this section we will
12 focus the works that investigated the role of ω -3 PUFAs rich diets to modulate the
13 synthesis of lipid mediators in correlation with the enhancement/inhibition of
14 inflammatory and metabolic disorders.

15
16 From a mechanistic perspective, there is substantial evidence that the increase in
17 the content of EPA and DHA in cells membranes and the decline in ARA, moves the
18 pattern of lipid mediators towards the ω -3 series that are less inflammatory or even
19 anti-inflammatory [138–141]. Different nutritional studies have shown the therapeutic
20 efficacy of EPA and DHA consumption by decreasing levels of proinflammatory
21 prostaglandins [142–144], thromboxanes and leukotrienes in patients with rheumatoid
22 arthritis [145] and chronic intestinal inflammation [146]. Caughey et al. [147] and
23 Trebble et al. [148] observed a decrease in interleukins, TNF α and proinflammatory
24 lipid mediators after a 4-week intervention period with a high dosage of 9 g/day of
25 EPA:DHA 3:2, and 12-week period with 2 g/day of EPA:DHA 1:1, respectively.

26
27 Many works include the study of a broader profile of metabolites that can be
28 modulated through the diet, and not only prostaglandins, thromboxanes and
29 leukotrienes. Brahmbhatt et al. [25], or McDaniel et al. [26] suggest that a EPA and
30 DHA intake can modulate the profile of lipid mediators towards a more antiinflammatory
31 situation according to the levels of epoxides, thromboxanes, hydroxy lipids,
32 prostaglandins, and isoprostanes analyzed. Arnold et al. [149] showed how a
33 EPA:DHA rich diet can modulate the sEH enzyme towards the production of epoxides
34 instead of dihydroxy lipids. Westphal et al. [150] correlated this change on sEH with an
35 improvement in cardiovascular health. Finally, Aukema et al. [151] showed that
36 glomerulosclerosis rats fed with fish oils declined the formation of ARA hydroxy lipids,

1 keto-prostaglandins, thromboxanes and prostaglandins by more than 60% through an
2 improvement of the dysfunctional eicosanoid metabolism associated to kidney damage.

3
4 Clinical trials have showed an increment in the synthesis of ω -3 lipid mediators in
5 patients suffering metabolic disorders and in healthy individuals after a 3 week-daily
6 supplementation of 2,4 g of ω -3 PUFAs/day [152]. Schuchardt et al. [153] also
7 observed higher levels of 44 hydroxy, epoxy and dihydroxy lipid mediators in plasma
8 after 12-week daily intake of EPA and DHA in both, hyperlipidemic men and healthy
9 controls. Barden et al. [154] studied the independent and combined action of ω -3
10 PUFAs and Coenzyme Q10 in the diet of patients suffering chronic kidney disease.
11 They observed a significant increase of anti-inflammatory lipid mediators after
12 supplementation whereas proinflammatory leukotrienes were not affected.

13
14 Since resolvins, protectins and lipoxins may reverse inflammatory processes to
15 normal situations, their occurrence is the subject of latest research. However, they are
16 synthesized at specific moments of the *in vivo* inflammatory process, and their
17 concentration is usually low. So how to detect the minus levels still becomes a key
18 challenge. Recently, levels of LXA₄ and RvD₁ in human breast milk have been detected
19 100 times higher than in plasma, and 10 times higher levels of RvE₁, [155]. Mas et al.
20 [156] identified for the first time RvD₁, RvD₂, PD_x, and PD₁ in human plasma after a
21 nutritional intervention with EPA (1,4g/day) and DHA (1g/day) for 3 weeks. Keelan et
22 al. [157] determined that EPA and DHA supplementation during pregnancy increased
23 placental levels of DHA and 18HEPE and 17HDoHE, precursors of resolvins and
24 protectins. However, these potent antiinflammatory lipid mediators were not detected or
25 were under limit of detection. More studies are still needed to determine these
26 metabolites in other matrix. Nutritional interventions with EPA and DHA in mice
27 suffering from colitis and chronic inflammation in the intestine have shown an
28 increment in the synthesis of resolvins and protectins [158]. Also in healthy rats,
29 lipoxins, RvD₁, PD₁, and RvE₁ were found in bone marrow and levels increased after
30 supplementation with DHA or EPA ethyl ester for 4 months [159].

31
32 Not only the EPA and DHA supplements, but also other functional ingredients have
33 been described for their ability to modulate the profile of lipid mediators. For instance,
34 onion consumption in rats produced a modulation of the profile of lipid mediators
35 towards less inflammatory situation [160]. Mas et al. [161] studied the combined effect
36 of ω -3 PUFAs and Coenzyme Q10 in patients with chronic kidney disease. Authors
37 found that ω -3 PUFA but not Q10 significantly increased 18HEPE and 17HDoHE in

1 plasma, precursors of resolvins and protectins. Barden et al. [162] studied the potential
2 of red wine to moderate chronic inflammation by affecting synthesis of lipid mediators
3 in patients with type 2 diabetes mellitus. Results showed that red wine did not
4 differentially affect any of the metabolites measured when compared with controls;
5 then, elevated levels of lipid mediators in diabetic patients may be related to a
6 homeostatic response to counter ongoing inflammation.

7
8 Since the oxidative stability of EPA and DHA is quite low, and they can be partially
9 degraded in the digestive process [163], Dasilva et al. [164] designed two nutritional
10 interventions to evaluate possible synergistic effects on the inflammatory response
11 associated to a joint intake of EPA:DHA 1:1 and grape seed polyphenols. ω -3 PUFAs
12 supplementation led to anti-inflammatory and antioxidant responses in healthy rats and
13 in an animal model of metabolic disorders induced by hypercaloric nutrition. ω -3
14 PUFAs reduced the production of ARA pro-inflammatory lipid mediators, up-regulated
15 the synthesis of less inflammatory ω -3 derivatives, and increased the production of
16 17HDoHE, precursor of anti-inflammatory resolvins, protectins and maresins.
17 Interestingly, the influence of the polyphenols was different depending on the caloric
18 context of the diet. Polyphenols lowered the parameters of inflammation in healthy rats,
19 depicting the synergistic effect with ω -3 PUFAs. However, polyphenols significantly
20 increased the production of the ω -6 proinflammatory lipid mediators in the high fat high
21 sucrose fed animals and no synergism with ω -3 diets was observed.

22
23 There are several factors in nutritional interventions driving the effectiveness of the
24 intake of ω -3 PUFAs that have not been standardized yet. For instance: appropriate
25 time in interventions, the administered dosage, the age of the individuals, the type of
26 metabolic disorder, or genetic factors. For instance, Rees et al. [165] observed that
27 levels of some lipid mediators in healthy men depended on the amount of supplements
28 and age of the individuals. Massaro et al. [29] suggested a minimum dosage of 3g/day
29 of EPA and DHA to achieve antiinflammatory effects in CVD. And recently, Calder et al.
30 [166] established the minimum dosage in 2g/day of EPA and DHA.

31
32 The relative proportion between the different ω -3 PUFAs seems to be determinant
33 as well. The influence of the dietary proportion of EPA and DHA, on the profiling of lipid
34 mediators and then, the observed regulation of inflammatory and metabolic markers,
35 has been described in a healthy model of Wistar Kyoto rats [167] and in a genetically
36 obese and hypertensive model of Kolestky rats [168]. Concluded results for both
37 models evidenced that the dietary ratios 1:1 and 2:1 EPA:DHA exerted a remarkable

1 healthy effect modulating LOX and COX activities through a decline of pro-
2 inflammatory ARA eicosanoids such as PGE₂ and 11HETE, compared with 1:2
3 EPA:DHA diet. Among mechanisms suggested, a considerable competition for the
4 same enzymes between EPA, DHA and ARA may explain the differences in lipid
5 mediator's profile. When diets are enriched in ω -3 PUFAs, tissues and membranes
6 incorporate these PUFAs; and subsequently, COX and LOX enzymes have more ω -3
7 substrate that competes against ω -6 ARA. A strong modulation of desaturases favoring
8 the synthesis of EPA over ARA was also observed. Accordingly, EPA seems to be
9 more efficient to compete for the same enzyme sites with ARA, than DHA molecule.
10 Finally, the formation of the different lipid mediators was closely correlated with
11 proteomic and metabolic results that confirmed the same response against
12 inflammation depending on the proportion between ω -3 PUFAs, EPA and DHA.

13
14 Accordingly, clinical trials have detected differences in the synthesis of lipid
15 mediators depending on the dietary proportion of EPA and DHA. The modulation of
16 lipid mediators pathways forward EPA derivatives was observed in hyperlipidemic men
17 after 12 week-daily intake of a diet having higher EPA amount than DHA [153].
18 Similarly, a DHA enriched supplementation induced a significant increase of the
19 synthesis of DHA derivatives in plasma of obese women after 3 months of dietary
20 intervention [169].

21
22 Finally, Nording et al. [170] showed how ω -3 interventions induced changes in the
23 total plasma lipid profile and in the synthesis of 87 lipid mediators. However, these
24 authors found a large degree of variation among subjects, pointing out the influence of
25 genetic variability. So, 50% of the subjects showed reduced levels of PGE₂, 12HEPE,
26 and TXB₂ after ω -3 supplementation but there was no changing for others.

27
28 As a conclusion for this section, several evidences have demonstrated the
29 participation of lipid mediators derived from marine ω -3 PUFA in the cell signaling
30 processes that regulate the inflammatory response at different levels and under diverse
31 situations. There has been progress in the knowledge of the mechanisms and routes
32 regulated by the different families of lipid mediators. Some of them have been
33 established as biomarkers of inflammation in different diseases. Pharmacy has also
34 investigated their therapeutic application, and new antiinflammatory drugs are being
35 tested to control lipid mediator synthesis. And, the "antiinflammatory nutrition" seems to
36 be an interesting alternative to modulate lipid mediator profiles in metabolic diseases,
37 although further investigation is needed.

1
2 However, the establishment of lipid mediators as biomarkers for early diagnosis of
3 inflammatory conditions has not been solved yet. The mechanisms underlying the
4 formation of these compounds are interrelated and often involve the simultaneously
5 activity of different families. For example, the same enzyme can regulate the formation
6 of proinflammatory compounds and resolvers of inflammation as well. Then, the
7 mechanisms and complex regulatory networks involved on inflammatory processes
8 cannot be well understanding through the single analysis of a small group of lipid
9 mediators [171]. Instrumental methodologies and bioinformatics must improve to
10 analyze accurately a higher number of compounds. These and other instrumental
11 limitations like standardization of sampling methods, the selectivity in the analysis of
12 isomers, and other issues will be discussed in next section.

13 14 **3. Lipidomic approaches for the study of lipid mediators**

15 In this section, throughout history the different techniques and methodologies used
16 for the analysis of lipid mediators are reviewed. A detailed analysis of the use of SPE
17 and LC-MS based methods is presented. Finally, challenges for the use of Lipidomics
18 and its new approaches to analyze lipid mediators are addressed.

19 20 **3.1 Main Lipidomic difficulties**

21 Systems biology encompasses a wide range of functional areas called "omics",
22 which includes Genomics, Transcriptomics, Proteomics, and Metabolomics [172]. All of
23 them have been significantly developed in the last decades by means of technology
24 and new identification techniques. Lipidomics is a sub-discipline of Metabolomics
25 defined as the large-scale study and detailed characterization of lipid metabolites, their
26 interactions, and the influence on biological systems [173].

27
28 The underlying strategy in Lipidomics involves the isolation of biological samples,
29 and the extraction, fractionation and characterization of the different lipid families.
30 Lipidomics applied to nutrition research, and especially applied to lipid mediators, has
31 to face several difficulties:

- 32
33 - The lack of simpler and standardized experiment design procedures: the duration of
34 the nutritional interventions, administered dosages, age of the individuals, genetic
35 factors, and so on, as have been pointed in previous section.
36 - The opposing bioactivity showed by the different families of lipid mediators
37 (antiinflammatory vs proinflammatory). Additionally, the synthesis of these conflicting

compounds is sometimes mediated by the same enzymes. Then, drug mediated metabolic activation or inhibition of a single pathway may simultaneously promote the development of inflammation and favor their resolution.

- Complex networks are involved in the inflammatory response. Therefore, to understand the mechanisms that are being regulated for a supplemented diet, the single analysis of a small group of compounds can hide the whole mechanism. It is necessary to obtain the widest profiling of all the groups of compounds involved in a given pathway.

- From an analytical point of view, there are certain limitations and significant challenges related to the combined determination of a large number of compounds belonging to different families concurrently in one analysis. Briefly, it can be highlighted the scarce standardization of the sampling methods, the interferences in the analysis due to sample matrix effects, the instability and easy degradation of the compounds, the low LOD of important families such as resolvins and protectins that hinder their determination *in vivo*, or the combined analysis of families of isomers chemically very similar, which are difficult to resolve chromatographically and hard to identify unequivocally using mass spectrometry.

In spite of these difficulties, the interest of the scientific community in Lipidomics has been progressively growing, in parallel with the increase of selectivity and sensitivity of the new mass spectrometers.

3.2 Lipidomics applied to lipid mediators in nutrition and other areas

3.2.1 Sample preparation methodologies for lipid mediators

Sample preparation is a crucial step for good analytical practices in lipid mediator analysis. The effective extraction of analytes from the matrix, together to the elimination of interferences, and signal enhancement for trace analytes, are issues to carefully optimize. Sample storage is also critical due to the high oxidative susceptibility of lipid mediators at room temperature or the enzymatic residual activity. Then, samples are usually stored at -80°C and AEDT to inhibit PLA₂ activity [174] or antioxidants like BHT are added [124,125,175].

Liquid-Liquid Extraction (LLE) has been widely used for the extraction of lipid mediators. Many authors have proposed alternative protocols to the use of chloroform-methanol, like ether [176], acetone-chloroform [177], hexane-isopropanol [178], or ethyl acetate-ethanol and ethyl acetate-hexane [179]. LLE has been validated in biological samples such as urine [180,181], brain tissue [177,182], or cell cultures [176,179].

However, the most commonly used technique for sample preparation of lipid mediators is Solid Phase Extraction (SPE). It provides a fast and proficient extraction with greater selectivity, major capacity for concentrating analytes, less solvent spending and reduced sample handling steps. Both methodologies can be combined to maximize the purification of particularly complex samples and reduce matrix effects in the subsequent LC-MS analysis [105,124,125,183–187]. SPE stage is also critical for some lipid mediators which are highly unstable like hydroperoxy lipids. Therefore, in order to achieve good recoveries for these metabolites low working temperatures may be important [188].

Critical pre-treatment of samples before the SPE procedure is needed to solubilize the lipid mediators in a selective solvent and then, eliminate surfactants, proteins, salts, and other interfering molecules. Liquid samples as plasma or urine, must be incubated into a methanol solution and subsequently centrifuged to precipitate protein residues. Acidification is also necessary before adding to the SPE cartridges. Matrix effects in tissues samples are also important due to ion suppression in LC-MS; this fact limits the amount to be analyzed [42]. Prior to centrifugation, these samples are homogenized in methanol solution by physical methods such as a mortar [42,124], ultra-turrax [189], or ultrasonic assisted extraction [175,190]. During the conditioning or solvation of the cartridges, pH can play an important role. Lipid mediators are compounds with moderate polarity, so the applications found in literature, either use neutral pH [52,83,183,191,192] or a formic acid derived pH between 4 and 5 [60,124,193,194]. In both cases, similar recovery percentages are obtained. Regarding stationary phases, extraction of lipid mediators are commonly executed in reverse phase; therefore, silica C-18, or polymeric Oasis HLB and Strata X are the most commonly used as shown in **Tab. 2**. Washing solvents may reduce matrix effect as they eliminate interferences but not elute the metabolites. **Tab. 2** illustrates the most commonly SPE conditions for lipid mediators described in literature.

Lipid mediators	Stationary phase	Conditioning	Matrix	Washing	Elution	%Recovery	Ref.
PGE1, PGE2, 8isoPGF2α	C18 (100mg/1mL)	Cl ₂ CH ₂ :MeOH(1:1) + MeOH30%	Urine	MeOH 30%	Cl ₂ CH ₂ :MeOH (1:1)	80-84%	[195]
HEPE, HETE, HODE, LTB ₄ , RvD1, RvE1, PD1	C18 (500mg/6mL)	MeOH + Water	Brain, liver, plasma	MeOH 15%, Water, Hexane	Methyl methanoate	76-122%	[83]
PGD1, PGE1, PGF1α, PGB2, PGD2, PGE2, PGF2α, PGD3, PGE3, PGF3α, TXB2, TXB3, ...	C18 (500mg/6mL)	MeOH + Water	Brain, liver, plasma	MeOH15%, Water, Hexane	Methyl methanoate	83-116%	[183]
PG, TX, HETE, HEPE, LT, EET, HDoHE, Rv, PD	C18 (100mg/1mL)	MeOH + Water	Cell culture	Water, Hexane	MeOH		[196]

PGF2 α , PGF1 α , TXB2 PGE, PGF	C18 (200mg/6mL)	MeOH:FA 98:2+Water	Urine	Water, MeOH 5%, Hexane	Ethil acetate	59-67%	[60]
PGF2 α , TXB2	C18 (200mg/6mL)	MeOH + Water	Urine	Water, MeOH 5%, Hexane	Ethil acetate		[52]
PG, TX, HETE, EET, HDoHE, PD, DiHETE, LX, HETrE, LT	C18 ODS (500mg)	MeOH + Water	Cornea tissue	Water, Hexane	MeOH		[197]
RvD ₁ , DHA, ARA	C18 (500mg/6mL)	MeOH + Water	Serum	MeOH, Water,	MeOH		[125]
IsoP, PG	Strata X (100mg/3mL)	MeOH + Water	Urine		MeOH 80% (HAc 1%)	80-100%	[198]
PGF2 α , PGF1 α , TXB2 PGE, PGF	Strata X (100mg/3mL)	MeOH:FA 98:2+Water	Urine	Water, MeOH 25%, ACN	MeOH	93-107%	[60]
122 lipid mediators	Strata X (60mg)	MeOH, Water	Plasma	MeOH 10%	MeOH		[199]
PG, TX	Strata X (500mg/6mL)	MeOH, Water	Plasma, cell culture	Water, MeOH 15%, hexane	Methyl Formate		[42]
PGF2 α	Strata X (100mg/3mL)	MeOH:FA 98:2+Water	Urine	Water, MeOH 25%, ACN	MeOH		[194]
158 lipid mediators from ARA, EPA, DHA, DGLA,...	Strata X (100mg/3mL)	MeOH + Water	Plasma	MeOH 10%	MeOH	54-112%	[200]
PG, HETE	Strata X (10mg/1mL)	MeOH+Water:FA	Brain tissue	MeOH+ Water:FA	MeOH	50-98%	[124]
PG	Oasis HLB (60mg/1mL)	MeOH, Water, ACN	Urine	ACN 5%	ACN		[201]
PG, EET, DiHETE, HETE	Oasis HLB (60mg/1mL)	MeOH, Water, Acetone, Hexane	Brain	MeOH 10%, Water	ACN	67-117%	[190]
PGF2 α , PGF1 α , TXB2 PGE, PGF	Oasis HLB (200mg/6mL)	MeOH:FA 98:2+Water	Urine	HCl 0.1M, MeOH 5%, Hexane	MeOH	60-71%	[60]
8isoPGF2 α	Oasis HLB (30mg)	MeOH + Water	Urine	HCl 0.1M, MeOH 5%, Hexane	MeOH	86%	[191]
ω 6 lipid mediators	Oasis HLB (60mg/1mL)	AcEt, MeOH, Water:MeOH 95:5	Plasma	Water:MeOH 95:5 + 0.1%HAc	MeOH + AcEt	88-127%	[193]
EETs	Oasis HLB	MeOH + Water	Arteria		ACN		[189]
DiHETE, HETE, EET, HpETE, LT, LX, PG, TX	Oasis HLB (60mg/3mL)	MeOH + Water	Plasma		Ethil acetate	45-84%	[202]
DiHETE, HETE, EET, oxoETE, LX, PG, TX	Oasis HLB	MeOH, Water, AcEt	Plasma	MeOH 5% (0.1%HAc)	Ethil acetate	88-127%	[193]
PG, TX, HETEs, LT, ...	Intercambio iónico	MeOH + Water	Cells	MeOH 10%	MeOH	25-100%	[192]

Tab. 2: Bibliographic compilation of the main SPE conditions for lipid mediators

described in literature. Information is organized according to the stationary phase used.

Other parameters compiled are: families of lipid mediators extracted, conditioning of cartridges, washing and elution conditions, matrix analyzed, and percentages of global recovery.

3.2.2 Analytical methodologies for the identification and quantification of lipid mediators

Different techniques have been applied coupled to the extraction procedure for the identification and quantitative analysis of lipid mediators. Here a summary of the main analytical platforms used for this issue is presented. Undoubtedly, LC-MS is the selected technique for most of the researches.

Immunoassay techniques were extensively used in the early research into lipid mediators [48,203,204]. They require enzymes and antibodies specific for the quantification of each metabolite and are limited to the quantification of one single compound. Then, these methods became expensive and inefficient. Blaho et al. [205] determined the COX-2 contribution in the regulation of the inflammatory response in an animal mouse model of induced arthritis. For such study two of their main metabolites, the prostaglandins PGE₂ and PGJ₂, were determined using commercial ELISA kits. Authors observed a direct correlation between the enzymatic activity and the resolution of the inflammation. However, other lipid mediators involved in the inflammatory process could not be detected by these immunoassay tests. Other authors have suggested ELISA to determine isoprostanes in urine as oxidative stress markers [206,207]. However, antibodies are not capable to discriminate between different isomers so the wide use of these procedures is limited.

Through gas chromatography-mass spectrometry (GC-MS), the limitations of the immunoassay techniques were overcome and simultaneous analysis of multiple compounds could be performed. Complete analysis of ARA derived isoprostanes in plasma and urine were analyzed in different subjects: non-insulin-dependent diabetes patients [51], diabetic patients [208,209], and smokers [210]. In these methodologies, plasma samples are derivatized to produce pentafluorobenzyl-esters and trimethylsilyl-ethers from lipid mediators. The derivatives are then useful for subsequent analysis by GC-MS-negative chemical ionization (NCI). In NCI, gas molecules ionized by electron ionization react with metabolites of the gas phase in order to achieve ionization. Other authors have also used GC-MS for the determination of isoprostanes, prostaglandins, or thromboxanes [211,212]. GC-NCI-MS was also applied to identify other lipid mediators groups like monohydroxy lipids, dihydroxy lipids, and epoxides in mouse brain tissue as indicators of oxidative damage and mitochondrial dysfunction [213]. It has been also used for understanding the regulation of vascular tone in myocardial damage studies of ischemia-reperfusion [214]. GC-MS offers high sensitivity and high selectivity; however, lipid mediators are not volatile and a derivatization step is needed. Such step is not always effective for all families of compounds. Additionally, many lipid mediators are thermally unstable and derivatization may induce changes in their structure.

Due to the limitations of GC, researches prefer liquid chromatography (LC) since lipid mediators are relatively soluble in the most common mobile phases and no

1 volatilization is needed. Some studies coupled LC-fluorescence detection for the
2 analysis of lipid mediators. Previously, samples must be derivatized and then detected
3 at 365/447 nm [189], or 260/396 nm [190]. Some authors have suggested coupled LC-
4 UV detection, but in most of these works this technique is not properly considered as
5 the method of analysis. It is used for a first fractionation of the sample and then, a
6 subsequent method for identification is needed. Waddington et al [43] separated
7 compounds by LC-UV; then, they derivatized extracts and analyzed hydroxy lipids in
8 patients with arteriosclerosis by GC-MS. Although these hydroxy lipids can be detected
9 by the absorbance of their conjugated dienes and trienes at 235 and 270 nm
10 respectively, the sensitivity and selectivity of the analysis was much lower than that
11 achieved through the use of MS. Other researchers have also used LC-UV coupled to
12 LC-MS [46] or NMR [215] to identify lipid mediators.

13
14 The majority of the works use LC coupled to Mass Spectrometry (MS), since it
15 combines the advantages described for LC with the high selectivity and sensitivity of
16 MS analysis. Reverse phase retention columns are normally used [195,216,217] for the
17 chromatographic separation of lipid mediators. Nevertheless, certain applications use
18 chiral columns for the specific separation of R/S stereoisomers [176,192]. Concerning
19 mobile phases, some authors proposed MeOH: water with formic acid [195] or
20 ammonium acetate [46], acetonitrile with formic [190,216,218] or acetic acid
21 [183,200,219] have been applied as well. An acidic additive could seem inefficient for
22 ESI in negative mode since the positive charges are unfavorable for ionization.
23 However, they improve the chromatographic separation helping to activate the silanol
24 groups of the columns and then, increasing the Van der Waals interactions with the
25 analytes [39]. Although some applications describe the use of isocratic elution
26 [179,197,220], most of the works have utilized chromatographic gradient conditions for
27 the analysis of a high number of compounds with an enhanced global sensitivity. For
28 instance, Wang et al. [200] proposed an LC-MS method for the determination of 158
29 lipid mediators in less than 5 minutes. The methodology should consider several
30 factors as the number of target compounds to be separated and the presence of
31 isomers with identical fragmentation pattern. Therefore, an excellent chromatographic
32 resolution is required to identify them [183].

33
34 MS offers a highly sensitive and selective detection of lipid mediators. The coupling
35 with LC needs an ionization interface to convert liquid output of the column into
36 desolvated ions. This is achieved through different ionization systems. As for lipid
37 mediators, many of them are relatively unstable compounds, and the ionization process

1 can provoke decomposition and loss of its chemical integrity. Electrospray Ionization
2 (ESI) is a so-called 'soft ionization' technique, since there is very little fragmentation
3 and it's the most commonly used for lipid mediators. Although the general mode of
4 ionization for organic molecules is positive, lipid mediators are generally analyzed
5 using negative mode motivated by the presence of the carboxylic group. Different
6 works have analyzed lipid mediators using negative ESI in plasma, liver, brain, cells,
7 urine, and other tissues [60,83,175,183,192]. Kretscher et al. [195] have used positive
8 ESI for the determination of prostaglandins as oxidative stress markers in urine.
9 Positive ESI was also applied by Yue et al. [190] who analyzed prostaglandins, hydroxy
10 lipids and epoxides in brain tissue, and recently Yang et al [124] for the analysis of
11 prostaglandins in brain tissue. One of the disadvantages of the positive mode is the
12 need of derivatization, and the decline of sensitivity compared to negative as several
13 studies have demonstrated [221][222].

14
15 Chemical Ionization at Atmospheric Pressure (APCI) is other ionization system to be
16 applied for lipid mediator analysis. This ionization mode is not widely used since it is a
17 stronger ionization process that can degrade analytes, and generally require a previous
18 derivatization step. Some examples are registered in the work by Santiago-Vázquez et
19 al. [217], in which ARA hydroxy lipids are determined using APCI in positive mode prior
20 derivatization to methyl esters. Lee et al. [176] increased the sensitivity of the
21 technique by derivatizing into the pentafluorobenzyl esters. Using this derivatization,
22 they analyze hydroxy lipids, prostaglandins and isoprostanes through the use of APCI
23 in negative mode. They suggested that the same methylation process stabilizes the
24 metabolites and prevents their oxidative degradation.

25
26 Regarding the mass analyzers, the choice between Quadrupoles (Q), Linear Ions
27 Traps (LIT), or Time of Flight (TOF) depends on the type and number of compounds
28 to be analyzed, the sensitivity and accuracy needed, or the required structural
29 information of the metabolites. The main type of analyzer used in the analysis of lipid
30 mediators is type Q [60,83,176,183,192,195,217,223]. LIT [200,218,224] and TOF
31 [225–227] have been also used in various applications with lipid mediators. In general,
32 MS equipment's combine two or more analyzers to increase sensitivity and accuracy.
33 The most common combinations for the analysis of lipid mediators are QqQ
34 [191,194,199,202,221,223,228,229], QqTOF [225–227,230], and QqLIT [200,218,224].

35
36 QqQ are the common choice for lipid mediators, working in MS/MS and *multiple*
37 *reaction monitoring (MRM)* mode. This is probably the most sensitive technique and

allows determining lipid mediators according to their specific fragments of MS/MS. Although QqLIT are still less popular than QqQ, one of the advantages is that QqLIT allow working in *full scan acquisition mode*, which offers more structural information of metabolites than *MRM*, and with higher sensitivity too [231]. First, QqLIT uses the Q1 to select the precursor ion; then, the precursor ion is fragmented in Q2; finally, LIT scans all product ions. A similar strategy is the *Parallel Reaction Monitoring (PRM)*, based on Q-Orbitrap instruments that perform a full scan of each transition, monitoring all fragments from the precursor ion. Therefore, *full scan acquisition mode* and *PRM* not only have the *MRM* target quantitative analysis capabilities. They also offer structural information that may help to distinguish between isomers in samples, and screen to search metabolites not included in the *MRM* list.

Masoodi et al. [232] developed and validated a method for the analysis of more than 100 lipid mediators combining initial screening in full scan and non-direct MS/MS. They grouped the compounds according to their molecular ion and recorded the full spectrum of MS/MS. Based on the study of the fragmentations produced and the retention times, they proposed an identification that was later validated using commercial standards and *MRM*. More recently, Panthi et al. [187] developed an analytical strategy to analyze isoprostanes and prostaglandins in human tear film samples; initially, they performed an untargeted analysis of the extracts by a TOF instrument to identify any lipid mediators. Then, they followed a targeted *MRM* analysis by a QqLIT to quantify compounds and verify previous identifications. Other works have analyzed more than 100 lipid mediators by a QqLIT [200,232,233]. **Tab. 3** summarizes literature related to the extraction and determination techniques for the analysis of lipid mediators in different samples.

Lipid mediators	Matrix	Extraction procedure	Determination technique	Ref.
8isoPGF2 α , 11hidro-TXB2	Urine	SPE	Immunoassay	[48]
8isoPGF2 α	Pericardoidal fluids		Immunoassay	[203]
PGE ₂	Tissues y fluids	LLE/SPE	Immunoassay	[204]
PGE ₂ , PGJ ₂	Tissues	Fractioning HPLC	Immunoassay	[205]
8isoPGF2 α , 11hidro-TXB2	Urine	SPE + TLC	Immunoassay	[206]
8isoPGF2 α	CLLE cultures	SPE	Immunoassay	[227]
20-HETE	Plasma	SPE + derivatization	GC-NCI-MS	[214]
15 and 11 HETE	Arteriosclerosis plaques	LLE + HPLC/UV	GC-MS	[43]
HETE, isoP	Mitochondria	SPE + derivatization	GC-NCI-MS	[213]
8isoPGF2 α , 5transPGF2 α , PGF2 α , PGF2 β	Urine, plasma	LLE + derivatization	GC-NCI-MS	[210]
8isoPGF2 α	Plasma	Derivatization	GC-NCI-MS	[51]
IsoP	Urine	LLE + TLC	GC-NCI-MS	[208]

IsoP	Plasma	SPE + derivatization	GC-NCI-MS	[209]
PG, TX, LT, IsoP	Urine	SPE + derivatization	GC-NCI-MS	[211]
PG, TX, IsoP	CLLE cultures	SPE + derivatization	GC-MS	[212]
PD	CLLE cultures	LC-UV + SPE	LC-LIT GC-MS NMR	[46]
HETE, HpETE	Protozoa	Solid-liquid extraction	LC-APCI(+)-Q	[217]
HETE, PG, isoP	Intestine cLLEs	LLE	LC-APCI(-)-QqQ	[176]
F2-IsoP	Plasma, urine	Online-SPE	LC-APCI(-)-QqQ	[229]
LTB4	Exhaled air		LC-ESI(-)-QqQ LC-ESI(+)-QqQ LC-APCI-QqQ	[221]
90 ω-6 lipid mediators	CLLE cultures	SPE	LC-ESI(-)-QqLIT LC-APCI(-)-QqLIT	[192]
PGE1, PGE2, 8isoPGF2α	Urine	SPE	LC-ESI(+)-QqQ	[195]
PG, LT, TX, LX, HETE,...	Exhaled air	LLE	LC-ESI(-)-QqQ LC-ESI(+)-QqQ	[222]
HEPE, Rv, PD, PG, TX	Plasma, liver, brain	SPE	LC-ESI(-)-QqQ	[83,1 83]
PGF2α, PGF1α, TXB2, PGE, PGF	Urine	SPE	LC-ESI(-)-QqQ	[60]
8isoPGF2α	Urine	SPE	LC-ESI(-)-QqQ	[191]
8isoPGF2α, TXB2	Urine	SPE	LC-ESI(-)-QqQ	[52]
PGF2α	Urine	SPE	LC-ESI(-)-QqQ	[194]
PG, TX, LT, HETE, diHETE, EpETE	Plasma	SPE	LC-ESI(-)-QqQ	[202]
PG, TX, LT, HODE, HETE	Colon mucosa	LLE	LC-ESI(-)-QqQ	[109]
IsoP	Plasma, urine	SPE	LC-ESI(-)-QqQ	[234]
6 HETE, IsoP, HODE	Plasma	LLE	LC-ESI(-)-QqQ	[120]
PG, TX	Tissues	LLE	LC-ESI(-)-QqQ	[235]
TX, PG, LT, LX, HETE, HEPE, RV, HDoHE	Plasma	SPE	LC-ESI(-)-QqQ	[236]
PG, IsoP; TX, LT, HETE	Urine	LLE	LC-ESI(-)-QqQ	[180]
PG	Tissues	LLE	LC-ESI(-)-QqQ	[113]
PG	Urine	Derivatization + Online-SPE	LC-ESI(-)-QqQ	[237]
PG, TX, isoP	CLLE cultures	LLE	LC-ESI(-)-QqQ	[179]
IsoP, PG	Urine	SPE	LC-ESI(-)-QqQ	[198]
PG, TX, HETE, HEPE, LT, EET, HDoHE, Rv, PD	CLLE cultures	SPE	LC-ESI(-)-QqQ	[196]
PG	Urine	SPE	LC-ESI(-)-QqQ	[201]
122 lipid mediators	Plasma	SPE	LC-ESI(-)-QqQ	[199]
PG, TX	Plasma, cLLE cultures	SPE	LC-ESI(-)-QqQ	[42]
104 lipid mediators	Plasma	SPE	LC-ESI(-)-QqQ	[202]
DHET, EET, HETE, LX, oxoETE, PG, TX	Plasma	SPE	LC-ESI(-)-QqQ	[193]
PG, TX, HETE, EET, HDoHE, PD, DiHETE, LX, HETrE, LT	Cornea tissue	SPE	LC-ESI(-)-QqQ	[197]
HETE, EET, PG	Urine	SPE	LC-ESI(-)-QqQ	[117]
HETE	CLLE cultures	SPE	LC-ESI(-)-QqQ	[220]
HEPE, Rv, PD, PG, TX, HETE, HODE, EET, LT, Lx, HDoHE, DiHETrE	Plasma, liver, adipose, intestine	LLE SPE	LC-ESI(-)-QqQ	[175]
HETE, EET, oxoETE, PG	Tissues	LLE SPE	LC-ESI(-)-QqQ	[105]
PG, Rv, Lx, TX, LT, PD, HEPE, HETE, EET	CLLE cultures	SPE	LC-ESI(-)-QqQ	[238]
HETE, PG	Plasma, cLLEs	SPE	LC-ESI(-)-QqQ	[219]
HETE, HODE, PG, EET, oxoETE	Plasma, urine	LLE	LC-ESI(-)-QqQ	[223]
RvD ₁ , DHA, ARA	Blood serum	SPE	LC-ESI(-)-QqQ	[125]
PG, HETE	Brain tissue	SPE	LC-ESI(+)-QqQ	[124]
PG, TX, isoP, HETE	Plasma	SPE	LC-ESI(-)-QqLIT	[218]

101 lipid mediators	Plasma	SPE	LC-ESI(-)-QqLIT	[233]
ω-6 lipid mediators	Plasma	SPE	LC-ESI(-)-QqLIT	[224]
158 lipid mediators from ARA, EPA, DHA, DGLA,...	Plasma	SPE	LC-ESI(-)-QqLIT	[200]
100 lipid mediators	CLLE cultures	SPE	LC-ESI(-)-LITOrbitrap LC-ESI(-)-QqLIT	[232]
LT, PG	Standards		LC-ESI(-)-LIT LC-ESI(-)-QTOF	[239]
FFA	Plasma	LLE	LC-ESI(-)-QTOF	[225]
HODE, EpETE, HETE, LT, TX, PG, isoP	Plasma, cLLE cultures	LLE	LC-ESI(-)-QTOF	[226]
MaR1, PD1	CLLE cultures	SPE	LC-ESI(-)-QTOF	[227]
EET	Plasma	SPE	LC-ESI(-)-QTOF	[230]
Rv, PG, HETE, HEPE, HDoHE, EET, ...	Cells	SPE	LC-ESI(-)-QTOF LC-ESI(-)-Orbitrap	[240]
EET	Renal arteries	SPE + derivatization	LC-fluorescence	[189]
PG, EET, DiHETE, HETE	Brain	SPE + derivatization	LC-fluorescence + LC-ESI(+)-QqQ	[190]

Tab. 3: Bibliographic compilation of the main extraction and determination techniques applied for the analysis of lipid mediators. Information is organized according to the determination technique used. Families of lipid mediators analyzed are specified as well as the matrix where they were extracted.

3.2.3 Limitations and new challenges of Lipidomics applied to lipid mediators

In recent years, the number of applications for the detection and quantification of lipid mediators by LC-MS has exponentially increased, especially due to the development of new spectrometers with greater sensitivity, resolution and data acquisition capacity [171]. That fact has allowed the implementation of methodologies for the analysis of complex biological matrices, and the quantification of an elevated number of lipid mediators in the same analytical process. However, the number of publications found in literature which incorporates Lipidomics for the study of lipid mediators in nutrition is still scarce.

Published methods range from the identification of a few number of lipid mediators [52,191,195,229] to the simultaneously analysis of more than 100 compounds [199,200,202,233]. When the number of target metabolites is low, *MRM* methods are highly sensitive techniques able to provide more accurate identifications and data processing platforms that are user-friendly and easier to set-up. However, for studies aimed to understand inflammatory processes associated to diseases or the dietary influence on systemic inflammation, it is important to obtain as much information as possible. Thus, information about all routes and metabolic pathways that are being modulated is needed. The analysis of a high number of lipid mediators may help to better understand the complex regulation of the inflammatory response, therapeutic effectiveness or the influence of a dietary intervention.

1
2 Despite the great technological advances and the new mass spectrometer
3 equipment's, the analysis of lipid mediators still entails certain difficulties. Some of the
4 most reported are:

5
6 - The optimization of the sample treatment is crucial to minimize the degradation of
7 these analytes, the generation of new compounds different from the original matrix, or
8 to avoid losses due to solubility during manipulation or vaporization steps [241].

9 - The low limits of *in vivo* detection of some families of lipid mediators as the
10 interesting resolvins, protectins or maresins. Although they have been identified *in vitro*,
11 there are not many works which could detect these compounds at *in vivo* inflammatory
12 processes.

13 - The high instability and fast degradation of some metabolites can lead to false
14 identifications and loss of sensitivity.

15 - The matrix effect. This effect generally consists in the increase/decrease of the real
16 quantification for a compound due to the presence of co-eluting interferences. These
17 interferences may come from the mobile phase modifiers, or from the sample matrix
18 [242,243]. Matrix effect produces a systematic error. If interferences can be ionized in a
19 easier process than lipid mediators, they will hinder the ionization of lipid mediators
20 causing a signal decrease. On the other hand, there may be interferences that
21 enhance the ionization of lipid mediators, increasing its signal as well.

22 - The high chemical similarity between the lipid mediators and the large number of
23 isomers found in samples make difficult their accurate and robust identification. There
24 are groups of isomers with identical fragmentation pattern, and if conventional
25 chromatographic methods cannot resolve them, identification becomes complex or not
26 possible. For instance, **Fig. 4** shows the mass spectra of two typical co-eluting isomers
27 under common chromatographic conditions, PGE₂ and PGD₂. These compounds have
28 the same precursor ion (351 m/z) and the same main product ions (271 and 189 m/z)
29 since they only differ in the position of the ketone and hydroxyl group within the ring.
30 Therefore, the MS characteristics are almost identical and only a good
31 chromatographic resolution would allow their identification [183]. As we pointed above,
32 some authors report chiral chromatographic separations to separately determine the R
33 and S enantiomers of hydroxy lipids and hydroperoxy lipids [27,244,245], epoxy-
34 enantiomers [246,247], or the stereoisomers of prostaglandins/isoprostanes [182].
35 However, these are applications for the analysis of a low number of analytes, and they
36 are not methods with a wide range of applicability [39].
37

The development of chromatographic equipment's that can operate at higher pressures have allowed the use of columns with smaller particle sizes (<2µm) [248–250]. By decreasing particle size, peaks are narrower, which provides a higher resolution (separation of critical compounds), and a decrease in the limits of detection and in the time of analysis, even less than 5-10 minutes [251]. Therefore, the use of more packed columns and lower chromatographic flows, combined with mass spectrometry, may reduce the cases of false positives occurring in the identification of lipid mediators [180,252]. And finally, the analysis using *scan mode* instead of *MRM*, provides the complete information of the mass spectrum allowing the accurate identification of isomers [232]. Then, an alternative to QqQ may be the combination of high-resolution mass devices such as Orbitrap and QqLIT hybrid equipment's for *full scan acquisition* methods [192,233].

Here we summarized some of the main challenges that Lipidomics still has to face in the analysis of lipid mediators:

- Solving the problems of sample treatment, limits of detection, instability, matrix effect and identification of isomers described above.

- The standardization of experimental design: the extension of nutritional interventions, administered dosages, age and characteristics of individuals and, genetic factors as well. It is necessary to understand how these factors may influence lipid mediator's synthesis.

- The rigorous analysis of lipoxins, resolvins, protectins or maresins that have been discovered in the last decade and whose complete biochemical characterization is still unknown [253–256].

- The identification of the new lipid mediators derived from plants with antimicrobial activities [257].

- A systematic approach for developing robust LC-MS methods for lipid mediators as routine analysis in clinical laboratories [258–260].

- The combination of Lipidomics and other “omics” techniques, to generate a greater knowledge of biological systems and understand how genetic polymorphisms could influence the absorption, transport, biosynthesis and metabolism of lipid mediators [115,261–264]. Consequently, to gain advanced knowledge in the role that lipid mediators play in health, nutrition, and pharmacy.

4 Conclusions

In this review, we showed that Lipidomics approaches combine the advantages of SPE and LC with the high selectivity and sensitivity of MS and constitute a robust and valuable tool to analyze the profile of lipid mediators in different matrix. Such Lipidomics platforms can be significantly useful for the establishment of new biomarkers of chronic inflammation. LC-MS has been widely applied for the analysis of these compounds in different fields. As the bioactivity of these metabolites has been strongly correlated with the development or resolution of the complex inflammatory response, we summarized the main works where lipid mediators were analyzed in the context of inflammatory and metabolic disorders. Their potential in new therapeutic options and the use of the antiinflammatory nutrition to revert or prevent inflammation in several metabolic disorders have been also discussed. The establishment of biomarkers for early diagnosis of inflammatory diseases has not been solved yet. There is a need to improve methodologies and bioinformatics in order to increase the number of target compounds in a single robust and precise analysis. These and other challenges still remain for Lipidomics applied to lipid mediators such as the identification of new metabolites, or the combination with other “omics” techniques.

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