Lipid Mediator Profiling in Pulmonary Disease

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Abstract: Oxylipins (*e.g.* eicosanoids) are endogenous signaling molecules that are formed from fatty acids by mono- or dioxygenase-catalyzed oxygenation and have been shown to play an important role in pathophysiological processes in the lung. These lipid mediators have been extensively for their role in inflammation in a broad swathe of respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis and extrinsic allergic alveolitis. Traditional efforts have employed analytical methods (*e.g.* radio- and enzyme-immunoassay techniques) capable of measuring a limited number of compounds simultaneously. The advent of the omics technologies is changing this approach and methods are being developed for the quantification of small molecules (*i.e.* metabolomics) as well as lipid-focused efforts (*i.e.* lipidomics). This review examines in detail the breadth of oxylipins and their biological activity in the respiratory system. In addition, the state-of-the-art methodology in profiling of oxylipins via mass spectrometry is summarized including sample work-up and data processing. These methods will greatly increase our ability to probe oxylipin biology and examine for cross-talk between biological pathways as well as specific compartments in the body. These new data will increase our insight into disease processes and have great potential to identify new biomarkers for disease diagnosis as well as novel therapeutic targets.

Keywords: Inflammation, lung, mass spectrometry, multivariate analysis, oxylipin, eicosanoid, metabolomics, lipidomics, respiratory disease, asthma.

1. INTRODUCTION

Respiratory tract diseases such as asthma and chronic obstructive pulmonary disease (COPD) are common, globally growing disorders that are associated with substantial morbidity, mortality and economic cost [1-5]. Asthma is characterized by airway hyper-responsiveness and chronic airway inflammation [6-9], while COPD is a progressive condition typified by fixed airway obstruction caused by bronchiolitis, small airways disease and emphysema [9-11]. Although treatments such as glucocorticoids and β-2 agonists are available for asthmatics, this type of therapy is symptomatic and not always effective in all patients [12]. There is no definitive cure for either asthma or COPD. Hence, there is an evident need for increased research into these and other respiratory tract diseases to investigate underlying mechanisms in lung pathology. A common denominator in the etiology of many respiratory disorders is a chronic inflammatory component that contributes to disease pathology. Accordingly, biomarkers such as inflammatory cells, cytokines and other inflammatory mediators are commonly studied in order to target disease progress as well as to complement the evaluation and therapy guidance provided by clinical symptoms and lung function tests.

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A new approach in biomedicine is the advent of the omics technologies involving large-scale screening of clinical material [13-15]. In the area of small molecule screening and quantification, metabolomics applications have increased greatly since the inception of the term in 1998 [16]. Metabolite profiling or targeted metabolomics approaches can provide a useful combination of omics-scale screening, while still focusing on biological pathways that are relevant to the pathology of interest [17, 18]. This review focuses on applications of metabolic profiling of inflammatory lipid mediators (oxylipins) as targets for the study of respiratory tract diseases in clinical samples. The term oxylipin was introduced as an encompassing label for oxygenated compounds that are formed from fatty acids by reaction(s) involving at least one step of mono- or dioxygenase-catalyzed oxygenation. Accordingly, this term includes the well-known eicosanoids synthesized from arachidonic acid Fig. (**1**) as well as related compounds formed by oxygenation of a range of polyunsaturated fatty acids of longer and shorter chain length [19]. There are three major enzymatic pathways that initiate oxylipin biosynthesis, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) Fig. (**2**) [20, 21]. Numerous studies have demonstrated the biological importance of lipid metabolic products formed from cell membrane-associated arachidonic acid Fig. (**1**); whereas the biological role of structurally analogous compounds is unclear. The distribution and synthesis of oxylipins are key targets for studies involving a range of inflammatory pathologies, not

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only in the respiratory tract [22-26], but also in nephritis [27], cardiovascular diseases [28-30] and cancer [31]. However, to date, there are no published studies applying large-scale oxylipin profiling methods to investigate respiratory diseases in clinical settings.

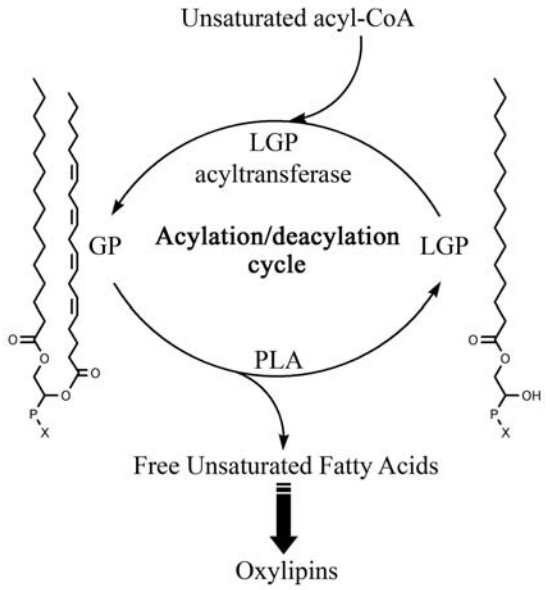

Fig. (1). Unsaturated fatty acids are subject to an acylation/deacylation cycle. Fatty acids are primarily found esterified in glycerophospholipids (GP, where X indicates the polar headgroup) and are liberated by phospholipases A (PLA). Following release from the membrane, unsaturated fatty acids can be converted to oxylipins or, closing the cycle, become associated to coenzyme-A to be acylated to lysoglycerophospholipids (LGP) by acyltransferases.

The oxylipin regulatory networks are complex with cross-talk across multiple biochemical pathways as well as both *in situ* and circulating effects. In addition, molecules generated within the oxylipin network can exert potent and often opposing effects upon multiple physiological processes, including inflammation and immunity [20-23, 32, 33]. In fact, this diversity and the changes of metabolism between different pathways that may occur with disease, pharmacologic treatment, or during physiological responses, is one of the reasons why products of the oxylipin pathways have a central role in medical science.

The following section "Oxylipins in respiratory disease" (Section 2.0) summarizes the current view on the individual role of different oxylipins in respiratory diseases, including biological function and available clinical data. The majority of these data have been obtained using enzyme-immunoassay (EIA)- and high performance liquid chromatography (HPLC)-based techniques and provide valuable information on the biological activity of individual oxylipins in the lung. However, the state-of-the-art approach to oxylipin analysis employs liquid chromatography-mass spectrometry (LC-MS)-based techniques [34-37] that are presented in more detail in the section "Oxylipin profiling" (Secion 3.0), which provides an overview of the field – including applications beyond respiratory disease in order to provide a comprehensive summary of the research area. This section also briefly describes the LC- tandem mass spectrometry (MS/MS) methodology, sample work-up and data interpretation. This review then concludes with a final section on future applications of oxylipin profiling approaches (Section 4.0), with a brief mention of contributions towards personalized medicine.

2. OXYLIPINS IN RESPIRATORY DISEASE

This section focuses on summarizing and highlighting the wealth of clinical data available on individual oxylipins in a range of respiratory diseases. These data are primarily from focused studies on individually targeted oxylipins with distinct hypotheses regarding their role in the observed pathology rather than an omics-based profiling approach as discussed in Section 3.0. The subsections below provide a brief summary of the biological properties of oxylipins originating from arachidonic acid (Section 2.1), linoleic acid (Section 2.2) and ω-3 fatty acids (α-linolenic-, eicosapentaenoic- and docosahexaenoic- acids; Section 2.3) in respiratory pathology. The majority of the research to date has focused on the arachidonates, and in particular the leukotrienes and prostaglandins, with relatively little information available on the ω-3 fatty acid derivatives.

2.1. Arachidonates (Eicosanoids)

Arachidonic acid (20:4 n6, 20:4 ω-6) is a 20 carbon chain polyunsaturated fatty acid (PUFA) with four double bonds (eicosa is 20 in Greek), with the final double bond located 6 carbons from the terminal end of the aliphatic chain opposite the carboxyl group (ω-6 or n6). It is therefore considered to be an ω-6 fatty acid. The terminology ω-6 and n6 are used interchangeably, and are useful to highlight the location of the terminal (omega, ω) double bond, which is important for determining many physiological properties of the PUFA substrate. For the sake of consistency, we primarily employ the "ω" nomenclature in this review. While both eicosapentaenoic acid (EPA, 20:5 ω-3) and dihomo-γ-linolenic acid (DGLA, 20:3 ω-6) derived oxylipins can also technically be referred to as eicosanoids, the classical definition refers to arachidonic acid-derived products. Multiple oxylipins originating from arachidonic acid are involved in respiratory pathology, including leukotrienes (LTs; *e.g.* Cysteinyl LTs and LTB_4), prostaglandins (PGs; *e.g.* PGE_2 and PGD_2) and lipoxins (LXs; *e.g.* LXA_4 and 15-epi-LXA_4) [22, 23]. We review the reported clinical effects of each of these oxylipin groups separately based upon their biosynthetic pathway as shown in Fig. (**2**).

2.1.1. Prostaglandins (PGs) and Thromboxanes

Prostaglandins (PGs) are produced following sequential oxidation of arachidonic acid by cyclooxygenases (1 and 2; COX-1 or COX-2) and prostaglandin synthases Fig. (**2**), [38-40]). There is an extensive body of literature describing the biological activity of PGs in respiratory disease, and it is beyond the scope of this manuscript to fully summarize the literature. Interested readers are further directed to a number of published studies [41-44] and reviews [23, 45-53]. Below is a selected summary of the biological role of PGs in respi-

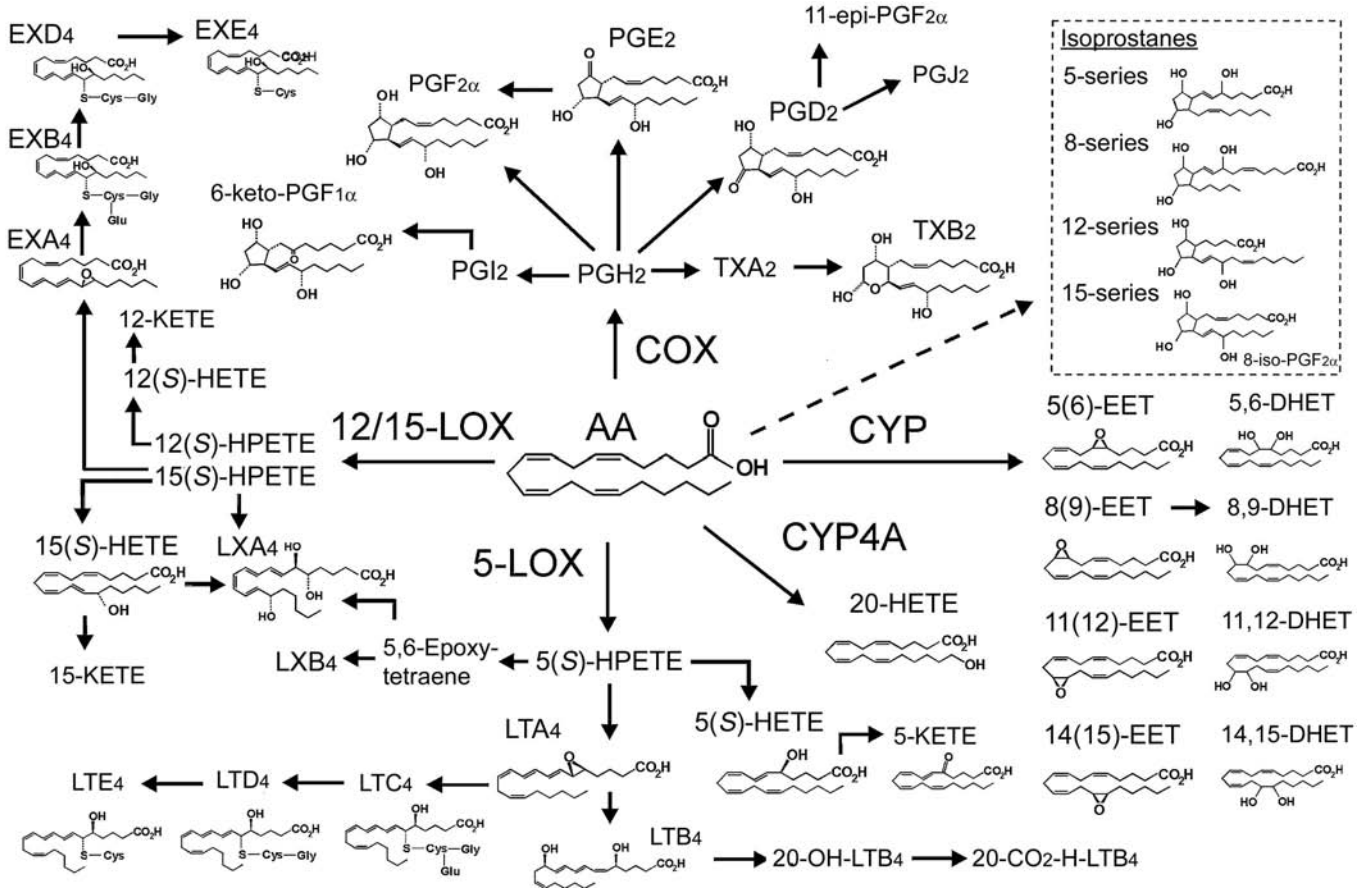

Fig. (2). Overview of the arachidonic acid (AA) cascade highlighting the three predominant enzymatic pathways (LOX, COX and CYP450). The nonenzymatic isoprostanes are also displayed given their prominent role in asthma pathology [360]. It should be stressed that this figure is only an overview and does not represent the full structural diversity of the arachidonic acid cascade, which potentially contains hundreds of primary enzymatic and autoxidation products as well as downstream metabolites.

ratory disease. Arguably the most abundant and also the most well-studied PG is PGE_2, which has a prominent, but complex role in lung physiology and pathology [32]. The mediator possesses multiple functions that are potentially regulated by activation of different receptors and signaling pathways [54, 55]. It has for instance been shown that PGE_2 has a bronchodilatory effect and inhibits both early and late phase responses to allergens and other triggers of bronchoconstriction [42, 48, 56-60]. COX-1 inhibition mediated through nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin can, however, also induce acute bronchoconstriction in the subgroup of asthmatics suffering from aspirin/NSAID intolerance [61-67]. Similarly, in mouse lung, COX-1 activity has been shown to be predominately bronchoprotective, while COX-2 has been indicated to mainly promote infiltration of inflammatory cells (particularly eosinophils) in the lung [54, 55]. Clinical studies have found significantly higher PGE_2 levels and COX-2 expression in sputum obtained from normal smokers and COPD smokers vs. healthy individuals and COPD former smokers [68]. Similarly, PGE_2 levels have also been shown to increase in exhaled breath condensate (EBC) in stable COPD patients and in smokers with asthma in contrast to nonsmokers with asthma [69-72]. One study also indicated that PGE_2 levels were significantly higher in asthmatic smokers compared to smoking controls [72]. This observation might be attributed to differences in the activation state of macrophages in the two groups since cigarette smoke is known to stimulate PGE_2 formation in alveolar macrophage [72]. It has been suggested that the significantly higher PGE_2 levels are due to a protective bronchodilatory mechanism against the increased inflammatory response in asthmatics who smoke [69]. A recent study indicated that PGE_2 levels in EBC from healthy non-smoking subjects were not significantly different compared to current smokers [73].

Thromboxane A_2 (TXA_2), which is generated from the same precursor (PGH_2) as PGE_2 Fig. (**2**), is a potent bronchoconstrictor acting via the TP receptor [74] that has been considered as a target for asthma therapy. TXA_2 is otherwise mainly a pro-aggregatory and vaso-constrictive COX product formed by platelets, and the preventive effect of low dose aspirin treatment in cardiovascular disease is due to inhibition of TXA_2 biosynthesis [75]. In addition to the effects of TXA_2 on platelets, bronchial and vascular smooth muscle, TXA_2 has been implicated in the development of bronchial hyperresponsiveness [76], and inhibitors of TXA_2 are registered for treatment of asthma in some countries.

The non-enzymatic breakdown product of TXA_2, TXB_2, was not detected in EBC from COPD patients, but was in-

creased in EBC of asthmatics [70, 71]. This observation suggests that oxylipin profiles in EBC can potentially differentiate between inflammatory lung diseases and raises the hypothesis that oxylipin profiling could contribute to diagnosis of disease. Furthermore, TXB_2 levels have been found elevated in the urine of cystic fibrosis patients [77]. Increased TXB_2 levels in airways of asthmatics following allergen challenge have also been observed [78]. Several other studies have similarly demonstrated elevated levels of TXB_2 in bronchoalveolar lavage (BAL)-fluid, urine and plasma from asthmatic patients [79-81]. The levels of the enzymatically formed metabolite of TXA_2, 11-dehydro-TXB_2 are however more reliable as indicators of endogenous biosynthesis of the biologically active TXA_2, and, interestingly, this metabolite is increased in the urine of atopic asthmatics following allergen provocation [79, 82]. In contrast to the EBC measurements, it has been shown that urinary excretion of 11-dehydro-TXB_2 in COPD patients is significantly higher than in healthy subjects, suggesting an enhancement of platelet TXA_2 biosynthesis in the patients [83]. It remains to be established if this is due to the common cardiovascular comorbidities of COPD patients or directly relates to lung pathology. It is known that TXA_2 also may be biosynthesized in monocytes, macrophages, neutrophils and lung parenchyma [53, 84-86].

Prostacyclin (PGI_2) is a potent vasodilator that has been used clinically to treat pulmonary hypertension as well as cardiovascular diseases [87]. Because of this dual role, the method of application of PGI_2 (intravenous or inhaled) can affect one or both diseases [88]. Whereas intravenous injection of PGI_2 evidenced efficacy in both cardiovascular and respiratory systems, aerosol inhaled PGI_2 improved pulmonary vasodilation, but did not affect systemic blood pressure [88]. Several studies have reported improvements in acute respiratory distress syndrome (ARDS) following treatment with inhaled PGI_2 [88-90]. PGI_2 has also been studied in the infant respiratory system, with results showing that polymorphism in the promoter region of PGI_2 synthase is associated with severity in syncytial virus lower respiratory tract infections [91]. In addition, inhaled PGI_2 improved oxygenation in a preterm neonate (28 weeks of gestation) with persistent pulmonary hypertension [92]. Although PGI_2 was used in initial clinical studies, the primary drawback in treatment is its chemical instability due to a vinyl ether group that is readily hydrolyzed at low pH. Therefore, for long-term treatments either a continuous supply is required, or more commonly, prostacyclin analogues with increased stability can be used [93, 94].

While monitoring the parent compounds can be useful, it is also important to screen for downstream metabolites of these compounds. A common non-invasive matrix used for this purpose is urine, and PG and thromboxane metabolites have been used as biological markers of pathophysiological processes (*e.g.* respiratory and cardiovascular diseases) [37]. A distinct advantage of analyzing metabolites in urine is the integrative nature of the matrix, providing an indication of whole body production of eicosanoids. Because the lung is a well-perfused system and eicosanoid production in other tissues is relatively low, urine metabolites have been used to trace pulmonary changes in PGs and thromboxanes. On the other hand, studying their levels in other relevant biological matrices, such as BAL-fluid, EBC, saliva and sputum, can yield more specific information about the local biosynthesis and metabolism of these compounds in respiratory diseases.

Several investigations have studied the levels of PG metabolites in asthma, and some studies have aimed to identify predictors of clinical responsiveness to therapy. For example, the effectiveness of pranlukast, a competitive leukotriene receptor antagonist, has been correlated with the urinary levels of eicosanoid metabolites. Patients with a positive response to treatment showed a lower ratio of LTE_4 to the PGI_2 metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$ [95]. In addition, responders to seratrodast, a competitive TXA_2 receptor antagonist, also showed a lower urine ratio of LTE_4 to the TXA_2 metabolite 11-dehydro-TXB_2 [96]. These studies generate interesting hypotheses, but short treatment periods, small groups and considerable overlap in results between groups call for studies to replicate and extend these observations.

Another recent use of measurements of urinary metabolites was to define pathways for PG biosynthesis in asthmatics [97]. The COX-2 selective inhibitor celecoxib did not inhibit urinary excretion of the major tetranor metabolite of PGD_2. However, in both healthy individuals and asthmatics, celecoxib treatment decreased the levels of the corresponding metabolites of PGE_2. In addition, in comparing asthmatics with controls, baseline urinary levels of PGD_2 metabolites were higher, but levels of PGE_2 did not evidence any change. These data indicate that the bronchoconstrictor PGD_2 is increased in asthmatics and is primarily biosynthesized via COX-1, whereas the bronchodilator PGE_2 is produced primarily via COX-2. Monitoring of metabolites can also be useful for evaluating the effects or selectivity of *in vivo* or *in vitro* interventions (*e.g.* inhibitors, knock-down studies, siRNA). In addition, many times the parent compound is not stable (*e.g.* PGI_2), so it is useful to monitor the downstream metabolites. For example, 6-keto-$PGF_{1\alpha}$ (another stable metabolite of PGI_2) and PGD_2 have been shown to increase in concentration in BAL-fluid following allergen provocation [98] and early measurements of asthmatic responses to allergens have indicated increased levels of the PGD_2 metabolite 9α,11β-PGF_2 in plasma [99] and urine [100]. Since PGD_2 is the main PG generated product in mast cells [101], increased PGD_2 and 9α,11β-PGF_2 levels are apparent indicators of mast cell activation (important in *e.g.* allergic asthma pathology). In contrast, PGI_2 is not a known product of activated mast cells, hence it has been speculated that the abundant levels of PGI_2 produced after provocation reflects a secondary effect of mediators acting on endothelial cells [23].

Similarly to allergic asthma, mast cell infiltration is prominent in acute extrinsic allergic alveolitis (EAA) and elevated urinary levels of 9α, 11β-PGF_2 have been reported in these patients [24]. In addition to interstitial and alveolar inflammation, EAA pathophysiology is also characterized by oxidative lung damage, which can be partially assessed by measuring oxidative species including the isoprostanes [24].

2.1.2. Leukotrienes (LTs)

Similar to the PGs, there is an extensive body of literature describing the biological activity of LTs in respiratory

disease, and interested readers are further directed to a number of published papers [44, 102-105] and reviews [106-111]. We will provide a brief overview of selected papers here. Leukotrienes are formed by liberated arachidonic acid, which is sequentially converted by 5-LOX to LTA_4 via the unstable intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE; Fig. (**3**)) [112]. This conversion requires that 5-LOX is reversibly translocated from its resting position in the nucleoplasm or cytosol to the perinuclear region. 5-LOX and the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family member FLAP (5-Lipoxygenase-Activating Protein) together form a functional complex that drives the catalysis [113]. LTA_4 can then be converted further into two main classes; cysteinyl (Cys)LTs and LTB_4. The classes function synergistically in inflammatory processes and are, for instance, associated with mast cell proliferation and cytokine generation [114-117]. Thus, several LT receptor antagonists have been developed and provide anti-inflammatory effects complimentary to inhaled corticosteroids [118-120].

LTC_4 is formed by conjugation of LTA_4 to reduced glutathione via LTC_4 synthase (integral nuclear membrane protein expressed by eosinophils, basophils, mast cells, monocytes and dendritic cells) and serves as precursor for the other CysLTs [121, 122]. CysLTs are well known as potent contractile agonists on human airway and vascular smooth muscle [25, 123, 124]. LTD_4 is formed via conversion of LTC_4 by cleavage of a glutamic acid residue from the glutathione moiety [125, 126]. The mediator is however short-lived and quickly converted to LTE_4 by a dipeptidase, which releases a glycine residue [127]. LTE_4 is to a large extent excreted in the urine without additional metabolism and hence, increased urinary LTE_4 levels are used as a biomarker of *e.g.* asthma exacerbations [128] and reflect CysLT production *in vivo* [129, 130]. Similarly, increased levels of LTE_4 have been observed in urine from patients with ARDS and EAA [131]. LTE_4 can also be measured in other biological matrices than urine, with aspirin-intolerant asthmatics evidencing higher levels of LTE_4 in saliva, sputum and blood *ex vivo* compared to aspirin-tolerant asthmatics [132]. LTC_4 and LTD_4 can also be measured directly *in vivo*. For example, the BAL-fluid levels of LTC_4 obtained from allergen-challenged atopic asthmatics were significantly increased over pre-challenged levels [133]. A significant challenge in mass spectrometry-based quantification of CysLTs relates to their instability in some matrices, and it can be difficult to accurately measure levels in clinical samples (recoveries as low as 25% have been reported [134]).

The second LT pathway is regulated by LTA_4 hydrolase (LTA_4), which catalyzes the hydrolysis of LTA_4 to LTB_4 Fig. (**3**) [135]. Alveolar macrophages recovered from the BAL-fluid of active sarcoidosis patients release higher quantities of LTB_4 than normal subjects, thus a possible indication of heightened immune response [136]. Patients with COPD have also been reported to have ~2.5-fold higher LTB_4 levels in EBC compared to control subjects [70]. Interestingly, LTE_4 levels are however not increased in COPD patients [70] in contrast to EBC from mild asthmatics, which evidence increased levels for both LTB_4 and LTE_4 [71]. However, the value of LTB_4 measurements in EBC is uncertain as salivary contamination may be a significant source [137].

2.1.3. Lipoxins (LXs)

Lipoxins, or lipoxygenase interaction products, are short-lived eicosanoids that predominantly act to support the resolution of inflammation [22, 138-141]. As shown in Fig. (**2**), there are multiple pathways that may lead to formation of lipoxins. For example, the two main lipoxins, LXA_4 and LXB_4, can be formed in reactions initiated either by action of platelet-derived 12-LOX, or eosinophil-derived 15-LOX. There is also a group of "aspirin-triggered" 15-epimers of lipoxins that are biosynthesized from 15-(*R*)- hydroxyeicosatetraenoic acid (HETE) in cells where COX-2 has been

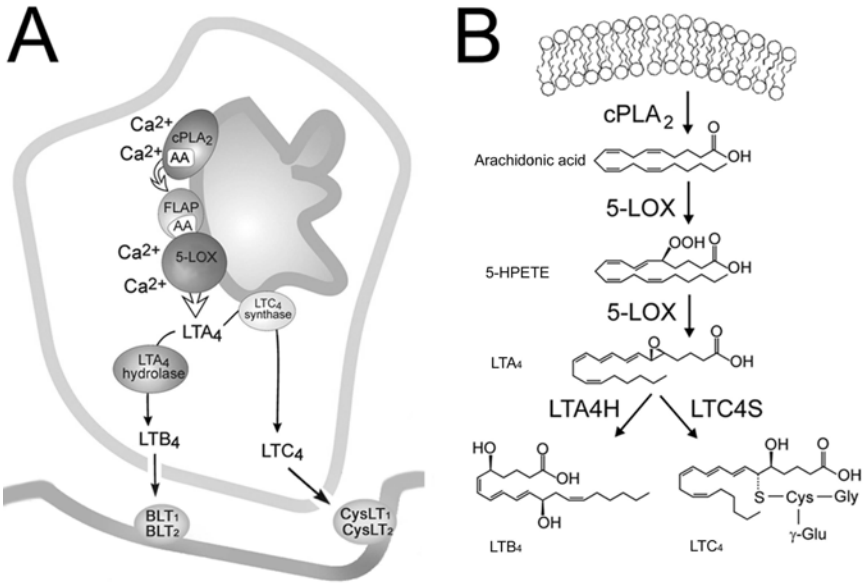

Fig. (3). Leukotriene biosynthesis. (**A**) Scheme for cellular leukotriene biosynthesis at the nuclear membrane. (**B**) Key enzymes and metabolites in the leukotriene cascade. For a complete list of acronyms employed in the figure, please see the accompanying text.

acetylated by aspirin [140, 142, 143]. Observations in experimental models suggest that both the 15-(*S*)- and 15-(*R*)-epimers may participate in a range of physiological and pathophysiological processes and serve as mediators dampening neutrophilic infiltration and initiating resolution [138]. A number of studies measuring lipoxins suggest a protective role for LXA_4 and 15-epi-LXA_4 in asthma [26, 34, 144, 145]. Interestingly, it has been shown that by using a stable analog of LXA_4, airway hyper responsiveness (AHR) and pulmonary inflammation is blocked in a murine model of asthma [144]. Additionally, the same study found that transgenic expression of human LXA_4 receptors in murine leukocytes led to significant inhibition of pulmonary inflammation and eicosanoid-initiated eosinophil tissue infiltration [144]. When comparing LXA_4 levels in activated whole blood from severe and moderate asthmatics, the levels in severe asthmatics were significantly lower [146]. Moreover, an inverse relationship was observed between the levels of LXA_4 versus LTs and airflow obstruction. Similarly, in induced sputum supernatants from severe asthmatics, a deficiency of LXA_4 was observed as compared to mild asthmatics [147]. Interestingly, a study in activated whole blood indicated that aspirin-intolerant asthmatics display a lower biosynthetic capacity to generate lipoxins than their aspirin-tolerant counterparts [34]. It has also been reported that lipoxin concentrations in BAL-fluid from cystic fibrosis patients are significantly suppressed compared to patients with other inflammatory lung conditions (*e.g.* pneumonia, interstitial lung disease and reactive airway disease) [148, 149]. Accordingly, there are extensive experimental reports on a protective role of lipoxins and their derivatives in several models of respiratory disease, indicating the need to further explore the clinical relevance of this biology.

2.1.4. Hydroxyeicosatetraenoic Acids (HETEs) and Oxoeicosatetraenoic Acids (KETEs)

HETEs are monohydroxy fatty acids that are primarily produced via LOX metabolism, but can also be generated non-enzymatically via autoxidation. The main exception is 20-HETE, which is a CYP4 product [150, 151]. 5-HETE is spontaneously formed via reduction from 5-HPETE by peroxidase activity [21], can activate neutrophils independently of receptors of other lipid mediators, and is further converted to 5-oxoeicosatetraenoic acid (5-KETE) via oxidation by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) [152]. This conversion increases the potency ~100-fold for raising cytosolic calcium levels in human neutrophils relative to 5-HETE [153]. 5-KETE may induce bronchodilation [154]. Airway epithelial cells have also been shown to synthesize 5-KETE in response to oxidative stress [155]. 5-KETE exhibits chemoattractant effects on eosinophils and neutrophils *in vitro* and when injected in human skin [153, 156] and there is one report suggesting that this response was greater in atopic asthmatics [157]. A recent study reported elevated levels of 5-HETE in EBC of smokers relative to healthy controls [73]. The same trend has been reported in BAL-fluid from female smokers versus non-smokers [158].

The well-known lipid mediator 15-HETE is formed by reduction via glutathione peroxidase (phGPx) of 15-(*S*)-HPETE formed by 12/15-LOX [159] Fig. (**2**), and is the major arachidonic acid metabolite in human bronchi [160, 161]. phGPx is under inverse control by IL-4 and IL-13 in human lung carcinoma cells (A549) and human monocytes [162]. In contrast, 12/15-LOX is up-regulated under the same circumstances, thus this inverse relationship may favor generation of lipoxins following inflammatory activation. Several studies have suggested that high 12/15-LOX activity and 15-HETE levels are mainly indicative of pro-inflammatory responses in asthma [163-165]. 15-HETE can also modulate the activity of the anti-inflammatory peroxisome proliferator-activated receptor (PPAR)-γ, which has been suggested as a new target for regulation of inflammatory states underlying many airway diseases [159, 166, 167]. For instance, the PPARγ agonist rosiglitazone has been shown to display bronchodilator effects in a group of patients with glucocorticoids-resistant asthma [168]. Interestingly, human BAL-macrophages evidenced a decrease in 15-HETE levels in response to lipopolysaccharide in severe asthmatics relative to controls and mild asthmatics [169]. On the contrary, severe asthmatics with persistent airway eosinophils manifest significantly higher levels of 15-(*S*)-HETE in BAL-fluid, both compared to severe asthmatics without persistent airway eosinophils, mild and moderate asthmatics and normal subjects [165]. It has been suggested that this trend may be associated with airway fibrosis. Patients with chronic bronchitis have also shown significantly elevated concentrations of 15-(*S*)-HETE in induced sputum compared to control subjects [170]. Noteworthy is that the levels were negatively correlated with the percentage of neutrophils. Similarly to 5-HETE, 15-HETE can be oxidized to 15-KETE [171]. However, whether this process is a major pathway of 15-HETE metabolism has yet to be established.

20-HETE is generated via the cytochrome P450 pathway by ω/ω-1-hydroxylases [172]. This HETE has been shown to induce concentration dependent relaxations of human bronchi [173] and a recent study in a murine model of ozone-induced airway hyperresponsiveness supports the hypothesis that 20-HETE is a mediator of this response [174].

2.1.5. Cis-epoxyeicosatrienoic Acids (EETs) and Dihydroxyeicosatrienoic Acids (DHETs)

Cis-epoxyeicosatrienoic acids are derived from the CYP monooxygenase pathway. CYP metabolism of arachidonic acid forms a series of regiospecific and stereospecific fatty acid epoxides (5,6-, 8,9-, 11,12-, and 14,15- EETs) [175-177]. The EETs can further be transformed to their corresponding diols, dihydroxyeicosatrienoic acids (DHETs) [178] via the soluble epoxide hydrolase (sEH) [179-181]. Compared to the COX and LOX arachidonic acid-derived pathways, the role of CYP-derived eicosanoids in lung physiology and pathophysiology is less well known. However, given the abundance of CYP2J2 expression in epithelial airway cells (or CYP2C29 in mice), bronchial and vascular smooth muscle cells, as well as endothelium and alveolar macrophages [182, 183] in combination with the detection of message for CYP enzymes in human lung RNA [184], it is tempting to speculate that EETs may contribute to important events in the lung. There are indeed observations suggesting that 11,12- and 5,6-EET are involved in regulation of bronchomotor tone [185-187], hypoxic pulmonary vasoconstriction [188, 189], control of the composition of airway lining fluid and limitation of pulmonary inflammation [175]. Inter-

estingly, similar to 15-HETE, EETs have been shown to be PPARγ ligands with κ_d values in the μM range [190].

It has been demonstrated that EETs have anti-inflammatory properties in particular in vascular inflammation [191-193]. Thus, given that CYP2J2 is prominent in pulmonary artery endothelial cells, it is likely that EETs have similar properties in the lung. It has for instance been shown that acute tobacco smoke induced inflammation in rats is significantly decreased by sEH inhibition (as observed by lower levels of neutrophils, alveolar macrophages, and lymphocytes in BAL-fluid) [194]. The same study also demonstrated that the combination of sEH inhibitor and EETs implantation was more significant in reducing inflammation. The combination of EETs and DHETs were found to be the predominant eicosanoids in perfused human lung upon stimulation with the Ca^{2+} ionophore A23187 [195] and after *Escherichia coli* hemolycin and formyl-methionyl-leucyl-phenylalanin challenge [196]. To date the majority of studies targeting EET and DHET distribution have been performed in animals [194, 197, 198], thus, there is a need to investigate EET and DHET alterations in humans *in vivo*.

2.1.6. Eoxins (EX)

In addition to undergoing reduction to 15-HETE, it has also been shown that 15-HPETE can be dehydrated to 14,15-epoxy-eicosatetraenoic acid (14,15-LTA_4) [199, 200]. Similarly to the CysLTs, 14,15-LTA_4 can be transformed further to 14,15-LTC_4, 14,15-LTD_4 and 14,15-LTE_4. Since eosinophils are an abundant source of these compounds [201] and in order to clearly distinguish them from their 5-LOX derived counterparts, the 14,15-LT series are referred to as eoxins (EX) [201, 202]. It has for instance been shown that human eosinophils, cord blood-derived mast cells and nasal polyps from allergic subjects can all convert arachidonic acid via the 15-LOX-1 pathway to EXC_4, EXD_4 and EXE_4 [201]. The same study also indicated that EXs induced increased permeability of the endothelial cell monolayer *in vitro*. Interestingly, the EXs were shown to be 100 times more potent than histamine and almost as potent as LTC_4 and LTD_4. However, the corresponding synthetic compounds were found to be almost inactive when tested in peripheral airway models where CysLTs are potent agonists [203]. Little work has been performed to investigate the biological function of these compounds, but a recent study reported BAL-fluid concentrations of EXC_4 for patients with a range of diseases (Churg-Strauss syndrome, eosinophilic pneumonia, sarcoidosis, lung cancer, idiopathic pulmonary fibrosis and asthma) [202]. Eoxins have also been found in EBC from healthy subjects [73] and a recent study reported significantly elevated levels of eoxins in EBC from asthmatic children and children with bronchial hyperresponsiveness compared to healthy children [204]. These results suggest that further efforts should be made to elucidate the potential role(s) of these compounds in respiratory diseases.

2.1.7. Isoprostanes

Isoprostanes, which are primarily found in the sn-2 position of glycerophospholipids, were described *in vivo* in the early 1990's [205]. When cells are under oxidative stress, different reactive oxygen species (collectively known as ROS's) may react with arachidonic acid to yield the prostaglandin-like compounds that are collectively termed isoprostanes [206, 207]. Whereas enzymatically mediated reactions are stereo- and enantioselective; radical driven reactions yield a wealth of diastereoisomers structurally similar to PGs. According to this structural analogy, isoprostanes receive the prefix iso- respective to their enzyme-mediated counterparts, showing rings similar to PGs D, E and F that give name to the corresponding isoprostane series (*e.g.* iso-$PGF_{2\alpha}$). Because of the lack of selectivity of the radical-driven reaction, every isoprostane series covers up to 64 diastereoisomers. Notably, whereas the stereochemistry of the ring in prostaglandins is "*trans*", isoprostanes show both configurations, but predominantly "*cis*". Due to their ROS-mediated origin, isoprostanes have been primarily studied as oxidative stress markers in lung diseases [208-210]. Oxidative stress and isoprostanes are influenced by several exogenous factors, including smoking, exercise and diet. Although they are not enzymatic products, they do possess distinct biological activity, that can be potentially important in respiratory diseases [211, 212]. For example, among five isoprostanes, only 12-iso-$PGF_{2\alpha}$ activates the prostaglandin F receptor (FP) [213]. Therefore, as this receptor may be involved in respiratory diseases (*e.g.* idiopathic pulmonary fibrosis [214]), it can be speculated that there is a relationship between oxidative stress and distinct lipid mediator species in respiratory diseases. In addition, depending upon their stereochemistry, isoprostanes are catabolized via specific pathways [215], suggesting that, whereas their generation is not enzyme-mediated, their presence and biological activity can be enzyme-regulated.

Isoprostanes have been monitored in several clinical studies. For example, patients with pulmonary hypertension evidenced increased levels of isoprostanes in contrast to controls, and the response to inhaled NO correlated to the basal levels of these compounds [216]. Nevertheless, isoprostanes constitute a large family related by a similar structure. Consequently, the oxidative stress component in disease has been monitored by delimiting the analysis to a few or even individual compounds. Among oxidative markers of lung diseases, 8-iso-$PGF_{2\alpha}$ is a good candidate for studying the influence of oxidative stress in respiratory diseases, as it shows strong constriction properties in smooth muscle *in vitro* through activation of several prostanoid receptors [217]. Accordingly, 8-iso-$PGF_{2\alpha}$ has emerged as the "gold standard" to study the oxidative stress in pathophysiological processes. Thus as expected, high urinary levels of 8-iso-$PGF_{2\alpha}$ have been documented in extrinsic allergic alveolitis patients [24, 218]. On the other hand, as smoking is associated to oxidative stress and is a risk factor for COPD, 8-iso-$PGF_{2\alpha}$ levels have also been studied in the EBC of smoker and non-smoker COPD patients [219]. Interestingly, there was no difference in EBC from heavy smoker and ex-heavy smoker COPD patients, suggesting that giving up smoking by heavy smoker COPD patients has negligible effect on oxidative stress. Furthermore, 8-iso-$PGF_{2\alpha}$ levels in sputum were found to correlate with disease severity [220]. Patients with pulmonary sarcoidosis showed an increased level of EBC 8-iso-$PGF_{2\alpha}$ compared with controls, pointing to an oxidative stress component in this disease. Furthermore, the levels of 8-iso-$PGF_{2\alpha}$ in sarcoidosis also correlated with the severity of the pathophysiological process [221].

The inflammatory immune process in the lungs of cystic fibrosis patients is also associated with severe oxidative stress. This fact can explain the significantly higher 8-iso-$PGF_{2\alpha}$ levels observed in cystic fibrosis patients as compared to healthy controls in urine [77], EBC [222, 223] and plasma [224, 225]. These levels were even higher in patients with unstable cystic fibrosis compared to patients with stable cystic fibrosis [223]. In asthma, baseline levels of 8-iso-$PGF_{2\alpha}$ in EBC also correlated with the severity of the disease as it was increased in severe compared with mild and moderate asthma [226]. A higher level of 8-iso-$PGF_{2\alpha}$ in EBC from asthmatic children contrasted with controls points to an increase in oxidative stress in childhood asthma [227]. Also in asthmatic children, treatment with prednisone decreased the level of EBC 8-iso-$PGF_{2\alpha}$, although not to the level of control baseline values. Consistently, several other studies have reported a low influence of corticosteroids in the increased 8-iso-$PGF_{2\alpha}$ levels presented in childhood asthma [227, 228], suggesting that this treatment is not completely effective for the oxidative component of the disease [229-231]. In one study of exercise induced bronchoconstriction in asthmatic children, EBC baseline 8-iso-$PGF_{2\alpha}$ levels correlated with the fall in expiratory volume in one second (FEV_1), despite the fact that levels of 8-iso-$PGF_{2\alpha}$ did not evidence any change during the challenge [232]. In addition to the studies in respiratory related samples, oxidative stress in asthma can also be studied via the urinary levels of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, which is an 8-iso-$PGF_{2\alpha}$ metabolite. This metabolite showed an increase at 2 hours following allergen challenge, but did not change after challenge with inhaled methacholine [233]. Accordingly, there are many interesting observations reported, but there are also shortcomings with regard to both analytical methods and clinical study designs that warrant inclusion of isoprostanes in profiling methods in order to understand their role in airways disease.

2.2. Linoleates

Linoleic acid (18:2 n6, 18:2 ω-6) is an essential fatty acid that cannot be synthesized in mammals, and is therefore an important dietary component with a prominent role in the modern Western diet [234]. Linoleic acid can further be metabolized by Δ^6 and Δ^5 desaturases and elongases, forming the ω-6 series of fatty acids (γ-linoleic → dihomo-γ-linolein → arachidonic acid) [234-236]. The same metabolic route is taken by the parent essential ω-3 fatty acid, α-linolenic acid (18:3 ω-3); however, a high linoleic acid intake can interfere with desaturation and elongation of α-linolenic acid [237]. In addition to functioning as precursor to other ω-6 fatty acids, linoleic acid can be directly metabolized via the same enzymatic pathways as arachidonic acid [238, 239]. Accordingly, there is a linoleic acid pathway analogous to the arachidonic acid pathway shown in Fig. (**2**). The primary enzymatic pathways in the linoleic acid cascade are 1) CYP activity, producing leukotoxins, *i.e.* 9(10)- and 12(13)- epoxyoctadecenoic acids (EpOME) [178, 240] and 2) lipoxygenation by 12/15-LOX, which produces 13-(*S*) and 9-(*S*)-hydroperoxyoctadecadienoic acid (HPODE) [159, 241]. The products from both pathways as well as their corresponding enantiomers can also be produced non-enzymatically by autoxidation. We discuss the main linoleic acid-derived oxylipins on a pathway-specific basis in the subsections below.

2.2.1. Leukotoxins

The linoleic acid epoxides 9(10)-EpOME and 12(13)-EpOME, and their corresponding diols 9,10- and 12,13- dihydroxyoctadecenoic acid (DiHOME) [178], are generally referred to as leukotoxin/iso-leukotoxin and leukotoxin-diol/iso-leukotoxin-diol, respectively, due to their biological properties and production in leukocytes [242]. Animal studies have for instance shown that injected leukotoxin causes acute lung injury associated with neutrophil infiltration [243, 244]. When comparing EpOMEs and DiHOMEs, it was reported that the DiHOMEs were more potent [243]. The leukotoxins have in particular been associated with multiple organ failure and ARDS, commonly seen in severe burn patients. It has been suggested that this often fatal condition is attributed to the observed high concentrations of leukotoxins produced by recruited leukocytes to the burned skin in order to control infection [243]. For instance, BAL-fluid obtained from ARDS patients contains elevated levels of leukotoxin [243]. Other factors may also enhance leukotoxin biosynthesis. For example, in rats exposure to NO_2 and other oxidants initiates auto-oxidation products of the leukotoxins in the lung [245]. Moreover, elevated biosynthesis and metabolism of linoleic acid epoxides due to induction of CYP enzymes and epoxide hydrolases have been shown in mice [240].

2.2.2. Hydroxyoctadecadienoic Acids (HODEs), Oxooctadecadienoic Acids (KODEs) and Trihydroxyoctadecenoic Acids (TriHOMEs)

Similarly, to the synthesis of 15-HETE from 15-HPETE, the HPODEs are reduced by phGPx to form their corresponding hydroxyl lipid counterparts, hydroxyoctadecadienoic acids (HODEs). The HODEs can be transformed further to their corresponding keto-dienes, oxooctadecadienoic acids (KODEs). In addition, 9- and 13-HODE can also be metabolized to 9,12,13- trihydroxyoctadecenoic acid (TriHOME) via 12,13-epoxy-9-hydroxyoctadecenoic acid, and 9-HPODE can likewise give 9,10,13-TriHOME via 9,10-epoxy-13-hydroxyoctadecenoic acid. Similarly to 15-HETE, 13-HODE has also shown binding affinity to and activation of PPARγ [159, 246]. 13-HODE can also inactivate the receptor by up-regulation of the mitogen-activated protein kinase (MAPK) signaling pathway, which induces PPARγ phosphorylation with subsequent decrease in transcriptional activity [247]. These complex up and down regulatory mechanisms are poorly understood, and the local concentration of 13-HODE may dictate the nature of the response [247]. A recent study found that 13-(*S*)-HODE and 15-(*S*)-HETE levels are significantly reduced in human lung cancer tissue compared with non-tumorous lung tissue [248]. Furthermore, data from mice in the same study indicated that this reduction correlates with decreased PPARγ activity in lung tumors and contributes to the development of lung tumors induced by tobacco smoking [248]. Interestingly, three other linoleic acid metabolites, 9-HODE, 9-KODE and 13-KODE have also demonstrated binding affinity to PPARγ [246, 249, 250]. It could be speculated that alterations in the levels of these compounds and in particular 13-HODE may be expected in some respiratory sensitive subgroups (*e.g.* smokers). Accordingly, this group of oxylipins warrants further investigation for their involvement in respiratory diseases.

Little is known about possible biological functions of the TriHOMEs. Generally, accurate measurements of these compounds are not trivial since they have high background levels in many biological matrices. However, these compounds warrant analysis because alterations may occur that are due to active biological functions or effects caused by alterations in the 13- and/or 9-HODE precursors. We have for instance observed elevated levels of these compounds in BAL-fluid from healthy individuals following subway air exposure that correlate with shifts in HODE and KODE levels (unpublished data [251]).

2.3. ω-3 Fatty Acid-Derived Oxylipins

In recent years, the focus in inflammatory research has expanded from investigating pro-inflammatory functions to understanding the underlying mechanisms of the resolution phase. Thus, searching for oxylipins that can control the duration and magnitude of inflammation is a significant area of interest. It has been found that oxylipins derived from ω-3 fatty acids can be active during the resolution process, providing increased evidence for the beneficial health effects of these fatty acids in the diet [252-258]. ω-3 fatty acids are defined as fatty acids that contain a double bond three carbons from the terminal methyl group of the fatty acid backbone (analogous to the ω-6 fatty acids, which have the final double bond six carbons from the terminal methyl group as described in Section 2.0). This group of fatty acids have been shown to have beneficial effects in inflammatory [259] and cardiovascular disease [260-263], but have been less well-studied in respiratory disease. The majority of studies focus on the essential fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid, which cannot be directly synthesized in mammalian systems. However, similarly to linoleic acid, α-linolenic acid can be transformed further by Δ^6 and Δ^5 desaturases and elongases to EPA and further to DHA [234-236]. The main dietary sources of long chain ω-3 fatty acids are from cold water oily fish such as salmon [234], although there are significant botanical sources of the shorter chain α-linolenic acid (*e.g.* flax or linseed). There is a current debate that a shift from a diet rich in EPA and DHA to ω-6 fatty acids has contributed to the increasing incidence of a number of diseases including atherosclerosis, coronary heart disease, hypertension, obesity and possibly cancer [234]. This theory remains to be substantiated, but there is evidence of the health benefits of the ω-3 fatty acids [264, 265]. However, questions remain as to the appropriate level of consumption as well as the relative balance of which ω-3 fatty acid species should be provided.

In terms of ω-3 fatty acid-derived oxylipins, there are analogous cascades to that shown in Fig. (**2**). Recent work has demonstrated that a number of alcohol, epoxide and diol ω-3-derived oxylipins can be detected in human plasma following a diet of ω-3 fatty acid ethyl esters [266]. The ω-3 fatty acid-derived oxylipins that so far have received the most attention are the EPA- and DHA-derived resolvins [252]. The resolvin (Rv)E series are formed when EPA is converted to 18-(*R*)-hydroperoxyeicosapentaenoic acid (HPEPE) by CYP or aspirin acetylated COX-2 [252, 267]. The intermediate can then be transformed further by 5-LOX to 5-(*S*)-18-(R)-HEPE for subsequent enzymatic oxidation to RvE_1 or reduction to RvE_2 [268]. CYP and aspirin acetylated COX-2 can similarly convert DHA to the stereoselective intermediate 17-(*R*)-hydroperoxydocosahexaenoic acid (17-(*R*)-HpDoHE) for subsequent enzymatic conversion to the 17-(*R*)-RvD series [253, 269]. Additionally, DHA can also be converted to 17-(*S*)-HpDoHE via 12/15-LOX, which results in the 17-(*S*)-RvD series [253].

Several experimental studies have indicated that RvE_1 is a potent anti-inflammatory mediator. For example, RvE_1 decreases polymorphonuclear leukocytes (PMNs) tissue accumulation by blocking human PMN transendothelial migration [267] and facilitates apical PMN clearance from mucosal epithelial cells [270], binds to the LTB_4 receptor BLT_1 as a partial agonist and consequently attenuates pro-inflammatory signaling (*i.e.* NF-κB activation) [271], stimulates macrophage phagocytosis of apoptotic PMNs (108) and inhibits dendritic cell migration and cytokine release [272, 273]. Similarly to RvE_1, RvD_1 has been shown to regulate PMN by limiting PMN infiltration in a dose dependent fashion [274]. The same study also indicated that RvD_1 is more resistant to metabolic inactivation than LXA_4 [274].

The majority of work to date has been performed in animal models; however, RvE_1 has been detected in human plasma [272]. There are to date no published reports on resolvin levels in BAL-fluid or bronchial wash. However, due to the demonstrated anti-inflammatory properties of resolvins in experimental models, it is likely that these compounds would have a protective role in the lung, promoting the resolution of inflammation and airway injury.

DHA also serves as a precursor for the biosynthesis of protectins. The lead member of this family, protectin D_1 (PD_1) is generated through conversion of DHA by 15-LOX to 17-(*S*)-HpDoHE via an epoxide intermediate at the 16(17) position [275]. Protectin generation is known to occur in, for instance, human peripheral blood mononuclear cells (PBMCs) and Th2 $CD4^+$ T-cells [275, 276]. PD_1 and its precursor 17-S-HDoHE (formed following17-HpDoHE) have been observed in EBC from healthy subjects [277]. Furthermore, compared to the healthy individuals, PD_1 levels in EBC collected during clinical asthma exacerbations were significantly reduced to trace amounts [277]. Thus, these results suggest that asthma exacerbations are associated with reduced airway levels of PD_1. Exogenous PD_1 administered prior to aerosol allergen challenge in allergen sensitized mice, has also been shown to block leukocyte infiltration and airway hyperresponsiveness. Furthermore, when administered after established allergic airway inflammation, PD_1 also accelerated the clearance of eosinophils [277]. Other *in vivo* effects observed for PD_1 include decreased T-cell migration, tumor necrosis factor (TNF) and interferon-γ signaling and promotion of T-cell apoptosis [278]. Accordingly, there is emerging evidence that PD_1 should be further investigated in clinical studies in order to elucidate its role in allergic airway inflammation.

In addition to resolvins and protectins, DHA and EPA can be the source of other oxylipin mediators, for example the DHA-derived maresins that are formed in murine macrophages via 12/15-LOX [279]. EPA can also be converted into hydroxyeicosapentaenoic acid (HEPEs) via LOX activity, for example, 5-HEPE, which similarly to 5-HETE is produced by 5-LOX [280], and 15-HEPE, generated by 12/15-

12/15-LOX [281] similarly to 15-HETE. Little is known about the potential roles of these compounds in lung pathology, but available data indicate that they merit further investigation. For example, we have observed significantly higher levels of 12- and 15-HEPE in BAL-fluid obtained from allergic asthmatics compared to healthy individuals (unpublished data [282]).

In combination with exploring the formation and action of new families of compounds biosynthesized from EPA and DHA, more efforts should be placed on mediators from the α-linolenic pathway. In particular, the 12/15-LOX derived 9- and 13-hydroxyoctadecatrienoic acid (HOTE) and their corresponding keto-trienes, oxooctadecatrienoic acids (KOTEs) could be of interest. We observed that the levels of 9- and 13-HOTE in BAL-fluid were significantly increased in healthy individuals compared to mild asthmatics following exposure to subway air, suggesting that these compounds may potentially play a role in the inflammatory response and/or subsequent resolution, and that this response is distinct in healthy individuals versus asthmatics (unpublished data [251]).

2.4. Abnormalities in Polyunsaturated Fatty Acid (PUFA) Turnover and Dietary Effects

Several studies indicate that an imbalance in the ratio between PUFAs can trigger and cause exacerbations in disease [283-286], creating an interest in the role of lipid supplements in nutritherapy. Epidemiological studies have for instance demonstrated that ω-3 PUFAs are dysregulated in respiratory tract diseases such as asthma and cystic fibrosis [283, 287]. Interestingly, linoleic acid levels in lung tissue obtained from patients with cystic fibrosis and in particular those patients with malabsorptions have been reported as significantly lower compared to healthy subjects [288]. Furthermore, it has been shown that cystic fibrosis patients without clinically evident malabsorption or irregular fat absorption have better mean clinical scores and milder pulmonary disease [288, 289]. A recent study on F508del homozygous cystic fibrosis mice showed markedly decreased linoleic acid levels and high levels of arachidonic acid and γ-linolenic acid in cystic fibrosis affected tissue (lung, intestine and pancreas) compared to wild type mice [290]. Interestingly, following a 6 week low dose supplementation of glycerophospholipid-liposomes obtained from DHA enriched eggs (GPL-DHA), cystic fibrosis mice showed restored linoleic acid levels in intestines and lung, but not in pancreas and diminished arachidonic acid biosynthesis in all 3 organs [290]. Thus, GLP-DHA supplementation could potentially be promising for nutritherapy in cystic fibrosis patients similarly to GLP-DHA supplementation in elderly [291]. An additional study indicated improvement in lung function following 8 months of supplementation with both DHA and EPA [292]. However, there are also studies performed with DHA supplementation of cystic fibrosis patients that have indicated no clinical improvement [293, 294] or even negative effects [295]. Thus, the observed PUFA abnormalities in cystic fibrosis patients are complicated and require additional research. It is most likely that the effects of any PUFA feeding studies are complex and based upon multiple genetic and environmental factors in combination with disease heterogeneity. Interested readers are further directed to reviews on the subject [296, 297].

Beneficial effects related to increased dietary intake of EPA and γ-linolenic acid in ARDS patients have also been observed. In a randomized double blind study, patients with EPA and γ-linolenic acid supplements showed significant decreases (2.5-fold) in the number of total cells and neutrophils per mL of BAL-fluid compared to those patients fed on control diet [298]. Overall the study improved clinical outcomes including time to liberation from mechanical ventilation and discharge from the intensive care unit. In a follow-up study performed on BAL-fluid from the same patients, decreased levels of IL-8, and LTB_4 were identified and related to the decreased neutrophils and alveolar membrane protein permeability with the EPA and γ-linolenic acid diet [299].

There are indications that intake of ω-3 PUFAs from fish oils can be beneficial and that ω-6 and trans-fatty acids may be detrimental for asthma treatment [284, 286, 300-304]. A randomized study in which children with bronchial asthma were given fish oil capsules (containing 84 mg EPA and 34 mg DHA) or control capsules (containing 300 mg olive oil) for 10 months indicated decreased asthma symptom scores and airway responsiveness in the fish oil group [287]. It has also been reported that fish oil supplementation has a protective effect on exercise induced bronchoconstriction in elite athletes and asthmatics [305, 306]. However, there are also studies arguing that a high intake of ω-3 fatty acids does not appear to protect against asthma [307, 308]. In the MORGEN-EPIC study, which was a cross sectional investigation of ~14,000 subjects, it was concluded that higher intake of ω-6 fatty acids was associated with lower FEV_1 and that this observation was most prominent for current smokers [307]. The study could not find a correlation between a high intake of ω-3 fatty acids and protective effects against asthma or COPD. A case control asthmatic study of dietary and erythrocyte membrane fatty acids (used as an objective biomarker for intake) conversely associated higher levels of linoleic acid with lower risk of asthma [308].

It is of interest that both ω-3 and ω-6 fatty acids can serve as substrates for formation of a great number of lipid mediators that have the potential to play important roles in respiratory health and disease states. It is possible that the balance between ω-3 and ω-6 fatty acids and their downstream metabolic pathways are connected to disease etiology. However, more research is needed to investigate the relative effects caused by shifts in diet, malabsorption and genetic variants in the metabolism of both ω-3 and ω-6 fatty acids. Accordingly, targeted oxylipin profiling will be a new powerful research tool to assist in addressing these questions. Given the increase in nutritherapy and so-called functional foods, it will be useful to have analytical methods capable of simultaneously quantifying shifts in the ratio of ω-3/ω-6 and their downstream metabolites. It is particularly important that oxylipin profiling methods are applied in these feeding studies in order to more fully elucidate mechanisms. For example, given the range of downstream metabolites of ω-3 and ω-6 fatty acids, only quantifying levels of the parent PUFAs most likely does not provide sufficient information to associate shifts in levels with disease.

3. OXYLIPIN PROFILING

As described in the preceding section, numerous studies support that fundamental mechanistic questions may be addressed by measurements of oxylipins in patients with respiratory disease. However, few studies have been performed in which human clinical samples were subjected to large-scale oxylipin profiling, aiming to provide an overview of global trends and cross talk between pathways in the cascades constituting this complex regulatory network. This section of the review will briefly present the state-of-the-art in analytical methodology, and then summarize the oxylipin profiling studies performed to date in both respiratory and non-respiratory systems. The section will conclude with a discussion of methodology limitations and approaches for data analysis.

3.1. LC-MS/MS Quantification and Sample Preparation

Due to the potentially large number of oxylipins that are present and their short half-life, it is challenging to isolate and detect these compounds. The development of LC-MS approaches has provided a method in which a large number of lipid mediators (≥150 at the present date) can be quantified simultaneously without derivatization [35, 134, 197, 309-312]. The details of the method and MS parameters have been described in detail elsewhere and are only briefly reviewed here. The LC-MS/MS method is based on chromatographic separation of compounds due to their physicochemical properties, followed by unambiguous identification based upon the characteristic product ions in the MS. The majority of oxylipin profiling methods use electrospray ionization (ESI). With the exception of CysLTs and EXs, most oxylipins are suitable for detection in negative ionization mode [36, 73], however there are examples in which CysLTs also have been screened as negative ions [311]. The most frequently used MS analyzers are triple quadrupole- [36, 313] or hybrid quadrupole ion trap- instruments [73, 309, 311], which both provide excellent quantification for complex sample analyses. However, the linear ion trap has the advantage that it can also perform multiple stages of tandem MS (MS^n) and can be useful for the identification of unknown compounds. The acquisition rate as well as the sensitivity and selectivity provided by selective reaction monitoring (SRM; Fig. (**4**)) enables the screening of high quality data for more compounds at faster rates [310]. While current oxylipin methods are capable of quantifying ≥150 compounds, there is no theoretical limit. Instead, the number of compounds that can be identified in a single run depends upon chromatographic separation in combination with the ion dwell times (based on the number of analytes in each SRM, the inter-scan delays and the peak width of the analytes). It is expected that the coverage of oxylipin profiling methods will continue to increase with advances in mass spectrometry in combination with chromatography. Ideally oxylipin methods could be combined with screening for other biologically active lipid mediators such as the endocannabinoids.

Several different methods for sample preparation are available for analyzing oxylipins in biological matrices [35, 37, 309, 314-317] and generally only require a simple extraction step [315, 318, 319]. The primary extraction method used in our laboratory uses C18 solid phase extraction (SPE) cartridges and is described in detail in recent publications [266, 309, 310]. It should be stressed that the majority of these methods focus on isolating and quantifying oxylipins in the free fatty acid form. However, there are significant levels esterified to membrane lipids and recent work has reported a base hydrolysis step to analyze these esterified oxylipins [266]. Oxylipin quantification is performed with a series of internal, technical and external calibration standards [35, 134, 309, 310] involving isotopically labeled internal standards (usually deuterated). Interested readers are further directed to the published literature for specific details of the analytical methods.

3.2. Oxylipin Profiling In Pulmonary Disease

Few studies have examined in depth oxylipin profiles in pulmonary research. However, there are data available in the literature, both from animal [36, 197, 214] and human clinical studies [73, 214]. For example BAL-fluid from rats following 2, 6 and 24h exposure to 1-nitronaphthalene (1-NN) with and without prior long-term exposure to ozone has been investigated [36]. Using this approach the inflammatory stages (*i.e.* injury and repair) during the time course were targeted and variations between the groups were compared with cytokine and chemokine levels. Long-term ozone exposure was associated with increases in PGE_2 and 12-HETE. Furthermore, long-term ozone exposure causes a remodeling of the airways that renders the rats more susceptible to other air pollutants, including 1-NN. In terms of oxylipins, these synergistic alterations included increased levels of CysLTs. Correlation with alterations in cytokine levels indicated that the observed synergism may be related to a shift from a Th1

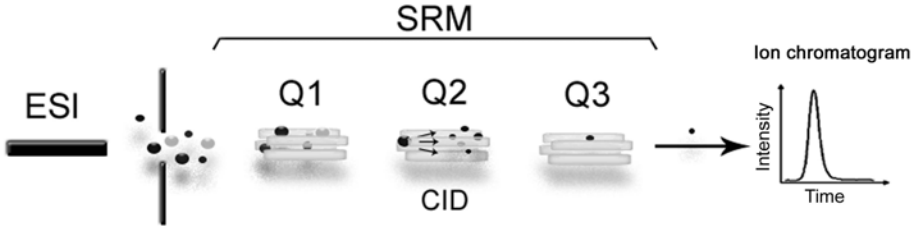

Fig. (4). Example of selective reaction monitoring (SRM) using a triple quadrupole mass spectrometer. Following LC-based separation, oxylipins are ionized by electrospray ionization (ESI). The molecular ion of interest is selected in the first quadrupole (Q1) and fragmented by collision induced dissociation (CID) in the second quadruple (Q2). The third quadrupole (Q3) monitors selected product ions that can be identified and quantified in the ion chromatograms.

to a Th2 mediated inflammatory response in the airways. In a different study, the oxylipin profiles of BAL-fluid obtained from patients with idiopathic pulmonary fibrosis (IPF) were compared to sarcoidosis patients [214]. High levels of $PGF_{2\alpha}$ were observed in the IPF patients, supporting the hypothesis that $PGF_{2\alpha}$ stimulation via the $PGF_{2\alpha}$ receptor causes proliferation and collage production of lung fibroblasts (which are characteristics for IPF) and that this stimulation occurs independently of the transforming growth factor (TGF)-β pathway. Another study found elevated levels of 5-HETE and 8-iso-$PGF_{2\alpha}$ in EBC of smokers relative to healthy never smokers [73], while both 5-HETE and $PGF_{2\alpha}$ were found to increase under hypoxic conditions in rabbit lung homogenates [197].

We recently examined BAL-fluid from healthy individuals and mild asthmatics following exposure to air in the Stockholm subway system relative to a control environment (unpublished data [251]). The oxylipin profiles between the two populations showed distinct differences in their response to subway air. The most prominent differences were significantly alterations in 12/15-LOX metabolites from the linoleic and α-linolenic acid pathways, with levels increasing in the healthy individuals, but not in the asthmatics following subway air exposure. Noteworthy is also that we observed the same trend in PGE_2 alterations. It can be speculated that these observations indicate a defense mechanism triggered in the healthy individuals, which is absent or repressed in the asthmatics. Another ongoing study in our group has shown that the profiles of healthy and mild allergic asthmatics differ significantly in a number ω-3 related metabolites (unpublished data [282]). Fig. (**5**) displays a total ion chromatogram (TIC) from BAL-fluid from one asthmatic and healthy individual, demonstrating that the profiles are similar, but that there are distinct differences in the EPA derivatives 5-, 12- and 15-HEPE.

3.3. Oxylipin Profiling in Non-Respiratory Systems

There is a growing body of research in the literature that provides data from oxylipin profiling in cell studies [312, 313, 320], animal models [311, 321, 322] and clinical investigations in other areas than pulmonary disease [266, 323]. Oxylipin profiling has been used in studies investigating deletion and/or overexpression of a gene, which can give valuable pathway information [311, 321, 324]. For example, this approach was taken in a study with transgenic rabbits overexpressing human 15-LOX-1 in leukocytes [321]. When the profiles were compared directly to non-transgenic controls, it was observed that 15-LOX-1 overexpression leads to enhanced LXA_4 and 5,15-DiHETE formation and reduced LTB_4 formation; leading to generally reduced inflammation

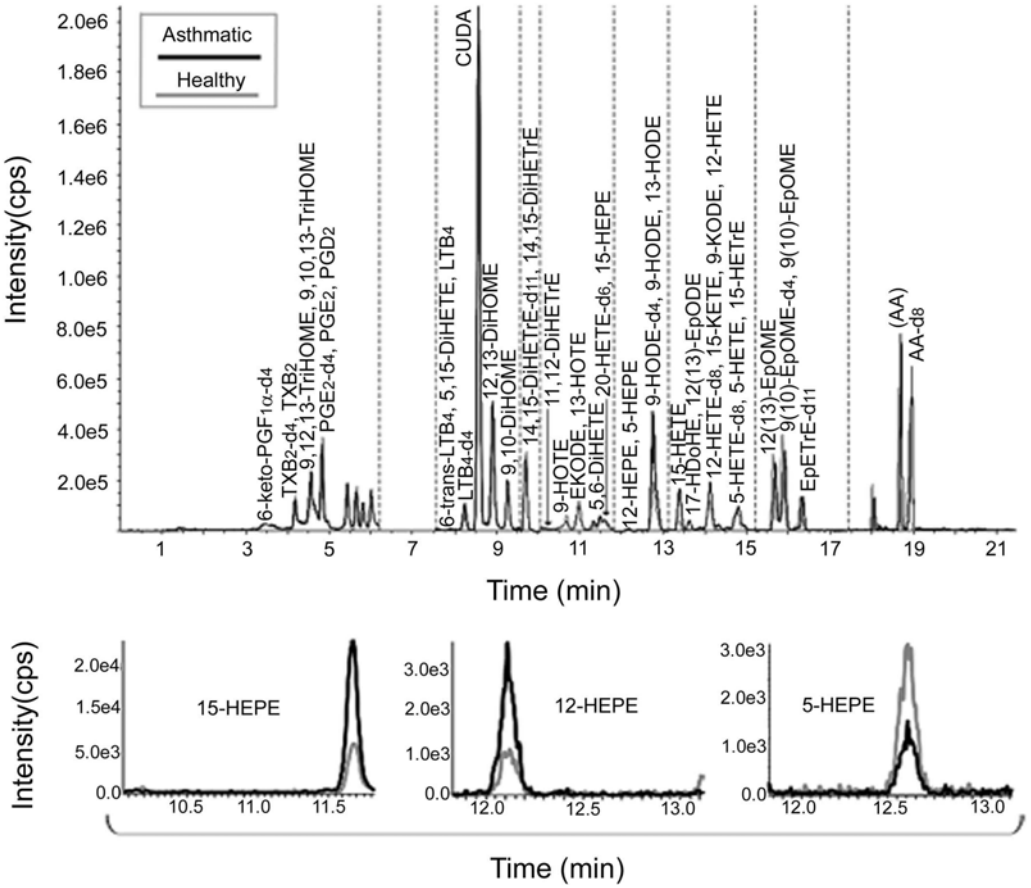

Fig. (5). Oxylipin profiling performed via LC-MS/MS of BAL-fluid from a patient with mild asthma vs. a healthy individual. (**A**) Total ion chromatogram (TIC). The 32 oxylipins detected above the limit of quantitation, the technical standard 12-[[(cyclohexylamino)-carbonyl] amino]-dodecanoic acid (CUDA) and the deuterated internal standards are indicated in the figure. (**B**) Selected ion chromatograms of 15-, 12- and 5-HEPE. 12- and 15-HEPE were detected in higher concentrations and 5-HEPE in lower concentrations in the asthmatic individual.

and protection from tissue damage in the transgenic rabbits. Another more recent study characterized oxylipin production during experimental Lyme arthritis in arthritis susceptible- and resistant mice infected with *Borrelia burgdorferi* [311]. Temporal and quantitative differences in the profiles were observed between the mouse strains, which correlated with differences in arthritis development. Additionally, the study investigated the profiles of *B. burgdorferi*-infected COX-$2^{-/-}$ mice, which revealed not only reductions in COX-2 products, but, interestingly enough, also significant reductions in 5-LOX metabolites. Thus, fluctuations in the global oxylipin profile were observed beyond the COX-2 pathway, demonstrating the utility of simultaneously monitoring multiple oxylipin biosynthetic pathways. Work in cell lines has been particularly useful for characterizing the kinetics of lipid mediator production. Such an approach can serve as a strategy for understanding the regulatory mechanism of lipid mediator production. For example, the time course of oxylipin mediator production after stimulation with calcium ionophore has been shown to vary with both macrophage type and timeline of lipid species production [320].

In the drive towards integrative systems biology studies, the combination of oxylipin profiling with other "omics" based datasets can provide key mechanistic insight. A recent study used an integrative omics approach to characterize the modulation of inflammation in overweight men during an intervention with the NSAID diclofenac. Data were acquired from multiple analytical platforms including 80 plasma proteins, >300 plasma metabolites (lipids, free fatty acids, oxylipins and polar compounds) and an array of PBMC gene expression products. Multivariate and correlation analysis were then applied to the full dataset to construct biological response networks. Out of this large array of components measured, a panel of genes, proteins and metabolites, including PGE_2 and TNF-α, were identified that described a diclofenac-response network, with the arachidonic acid metabolite 5,6-DHET identified as a novel candidate marker of inflammatory modulation [323]. This study demonstrated the power of integrating data from multiple platforms, while focusing on the utility of including an oxylipin panel. It is expected that the studies of this nature will increase due to their ability to capture biological information over a complex response network.

3.4. Intra-Individual Correlations of Oxylipins and other Inflammatory Markers

In order to examine respiratory pathology, oxylipin levels have been measured in a number of different biological matrices including BAL-fluid, bronchial wash, sputum, saliva, EBC, blood and urine. However, since there are to date no comprehensive data on profiles obtained simultaneously from these different matrices, it is difficult to estimate intra-individual correlations. For example, there is an important question on the level of cross-talk between oxylipin levels produced in different biological compartments. A recent study observed globally higher baseline levels of CysLTs in saliva, induced sputum, *ex vivo* stimulated blood and urine from subjects with aspirin intolerant asthma compared to subjects with aspirin tolerant asthma [132]. Similarly, globally elevated levels of 8-iso-$PGF_{2\alpha}$ in cystic fibrosis patients have been observed in urine, EBC and plasma [77, 222, 223, 225, 325]. CysLT and 8-iso-$PGF_{2\alpha}$ levels in EBC and BAL-fluid from sarcoidosis patients have also been shown to correlate with each other as well as with a high percentage of eosinophils [326]. Accordingly, there is evidence for alterations in oxylipin metabolism between multiple biological compartments. However, it is likely that the local oxylipin profile measured in different bodily compartments also evidence distinct differences, similar to what has been observed in cell content and other inflammatory mediators. For example, the cell composition in induced sputum is different from bronchial wash and BAL-fluid, suggesting an origin of sputum mainly from the larger airways [327-329]. Additionally, sputum and BAL-fluid from mild asthmatics have shown to be different from blood with higher proportions of T-cells and lower proportions of CD19+ T-lymphocytes [330]. The same study also reported significantly higher proportions of CD4+ T-cells, lower proportions of CD8+ cells and a higher CD4+/CD8+ ratio in sputum as compared to blood. These results indicate that sputum, blood and BAL-fluid reflect different compartments and/or components of inflammation. However, there are also examples of global effects observed in induced sputum, bronchial wash and BAL-fluid eosinophilia in subjects with eosinophilic bronchitis or asthma [331]. Fig. (**6** displays the multivariate analysis (MVA) of a recent study in our group on oxylipin profiling from BAL-fluid, bronchial wash and serum of healthy individuals (unpublished data [332]). As expected, when analyzed on a concentration-specific basis, oxylipin profiles of BAL-fluid and bronchial wash clustered tightly, compared to those in serum. In addition, serum evidenced a significant spread among individuals when analyzed on a concentration-specific level Fig. (**6A**). In contrast, analyzing the data as percent oxylipin composition (based upon the total number of oxylipins quantified) notably reduced the inter-individual variability observed in the serum, showing that while specific oxylipin levels demonstrate significant inter-individual variability, the relative relationships in the overall composition are similar Fig. (**6B**). Interestingly, the oppostive effect was observed in the BAL-fluid and bronchial wash samples. This observation demonstrates the specificity of the oxylipin profiles in the different matrices. It also raises the question of how best to perform inter-individual comparisons, because the results are specific for the matrix of interest as well as the data analysis method. These data unsurprisingly suggest that biological fluids from the same compartment exhibit cross-talk and/or have similar biosynthetic pathways, whereas global integration of tissues such as occurs in serum are less likely to evidence a correlated oxylipin signal. It is therefore important to examine for distinct patterns of cross-talk across biological compartments between healthy individuals and those demonstrating symptoms of disease.

3.5. Methodology Limitations

While oxylipin profiling methods are generally robust, there are a number of issues that should be considered by interested users. For example, while the availability of internal standards for the range of compounds discussed in this review is increasing, there are still multiple compounds for which labeled standards are not available. In addition, calibration standards are not available for many of the newer oxylipins of interest, including resolvins, protectins and

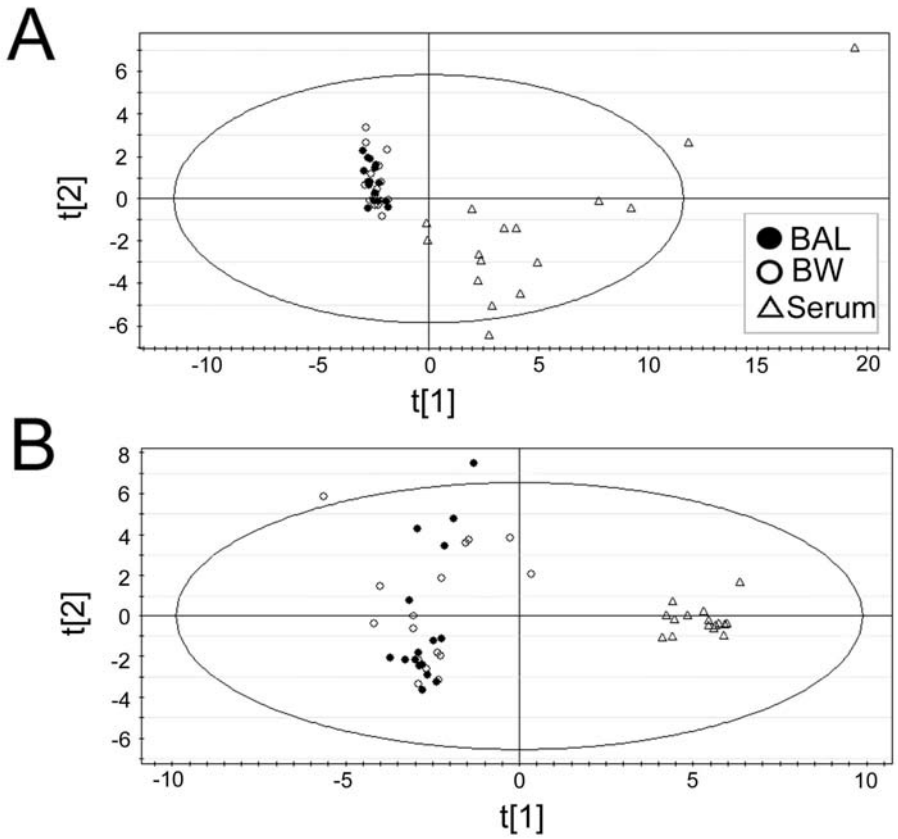

Fig. (6). PCA scores plots of oxylipin profiling data from BAL-fluid, bronchial wash (BW) and serum of healthy individuals (n=16). Each individual is represented in the plot by 3 symbols, one each from oxylipin levels in BAL-fluid, BW and serum. (**A**) Model based on oxylipin concentrations in nM (R2(cum)=0.82, Q2(cum)=0.59). Oxylipin profiles of BAL-fluid and BW cluster tightly, thus indicating similar oxylipin concentrations compared to those in serum, which are ~100 times higher and evidence a significant spread among individuals. (**B**) Model based on the relative composition (%) of quantified oxylipins (R2(cum)=0.71, Q2(cum)= 0.51). The relative oxylipin composition in BAL-fluid and BW also cluster in the 16 individuals, indicating that they are similar relative to those in serum. However, analyzing the data as percent composition notably reduces the inter-individual variability observed in the serum, showing that while specific oxylipin levels demonstrate significant inter-individual variability, the relative relationships in the compositon are similar. Interestingly, the oppostive effect is observed in the BAL-fluid and BW. Accordingly, alternate forms of data analysis can be useful to examine different endpoints.

maresins, greatly limiting the progress of the field. One issue that is rarely discussed is the stability and reproducibility of the analytical standards. In our laboratory, we have observed significant batch-to-batch variability (as high as 200%) in analytical standards that can have serious repercussions on the accuracy of these methods. These issues are further exasperated by the instability of many of these oxylipin standards, which should be stored at low temperatures in an inert atmosphere. This point should be further addressed by the research community in terms of suggesting minimal qualifications for reporting standards and providing a repository of "gold standard" analytical standards for cross-laboratory method standardization.

A particular limitation of many of the current oxylipin profiling methods is the lack of information on stereochemistry. The majority of oxylipins are synthesized enzymatically in an enantioselective process, but autoxidation leads to equal production of both diastereoisomers. Accordingly, the oxylipin synthetic route can be assessed by the stereochemistry. This is the case of HPETEs, which can be produced by two enzymatic pathways [333] and via autoxidation [334]. For example, 15-(*S*)-HPETE is synthesized via 15-LOX, COX-2 and autoxidation; whereas 15-(*R*)-HPETE is produced via COX-2 and autoxidation. It would be particularly useful to perform an enantioselective analysis of the plethora of diastereoisomers in the isoprostane group, as many of them have biological activity and are metabolized via specific pathways [215]. Accordingly, there is a distinct need for chiral liquid chromatography-based methods in oxylipin profiling [335, 336], but there are as of yet no comprehensive methods published, which is partly due to a lack of availability of chiral columns with low particle size (<2 μm).

In contrast to biological matrices such as urine and plasma, a particular issue with measuring oxylipin profiles in BAL-fluid and bronchial wash is the variability in fluid recovery during sampling (*i.e.* instilled volume versus recovered volume) and the estimation of the amount of mixing in the alveolar space [337]. There have been attempts to use both external (methylene blue) and internal markers (urea) in order to adjust for the recovery volume [337]. However, problems with absorption and inconsistency due to sampling procedure are still concerns that have made it difficult to address this problem. One option is to normalize the ratio of instilled/recovered volume to adjust for recovery variability.

We have observed that the coefficient of variations (CVs), tend to decrease following normalization, and routinely apply this approach.

Another obstacle in working with clinical samples is the large inter-individual differences observed within subpopulations (*e.g.* healthy, asthmatics). Coefficient of variation values of more than 100% are not uncommon. The obvious way to address this complicated issue is through appropriate study design. Given the high cost of omics analyses, it becomes increasingly important to assure selection of maximally informative experiments. Design of experiment (DOE) is a useful tool for estimation of both the systematic variance of interest, and the unwanted unsystematic noise through a limited number of screening experiments, thereby helping to select a subset of representative subjects and simultaneously maximizing the statistical power of the study [338]. An additional complication in data interpretation in working with clinical samples is that the results can be profoundly affected by environmental factors (*e.g.* diet, medication, personal habits). Accordingly, it is important to control patient inclusion criteria and perform patient phenotyping. However, caution should be used in over-interpreting study results from narrowly focused studies on discrete populations.

3.6. Data Processing and Interpretation

As with any of the omics approaches to data acquisition, the analysis of a large number of variables performed on a minimal number of individuals makes the statistical analysis challenging. This problem is a chronic issue in the preprocessing and analysis of metabolomics data, and is especially acute for clinical studies that often have relatively few individuals creating statistical issues with a lack of degrees of freedom. The majority of the univariate methods that dominate the biological sciences (*e.g.* Student's t-test) are not well-suited for metabolomics data for a number of reasons. Univariate statistical methods employ repeated testing to evaluate whether the null hypothesis for a certain variable can be rejected, in other words if it is significantly altered compared to the control group. Given the cumulative nature of type I errors in repeated testing, metabolomics analyses where a large number of variables are tested simultaneously become particularly prone to high false positive rates. Even though a range of approaches have been developed to correct for the resulting large false positive rates, most notably Bonferroni and false discovery rate (FDR) corrections [339], the use of univariate methods remains at best a compromise. Their sensitivity to missing data points further decreases the robustness of analyses based solely on traditional univariate statistical analysis of the data. As such, multivariate analysis (MVA) represents a more suitable option for the ''short and fat'' data sets typical for metabolomics studies [340, 341]. MVA aims to create a model that reduces the complexity of multi-dimensional data to a few latent variables that express the majority of the variance of the data set, as opposed to univariate methods where each variable is tested in isolation. The most utilized MVA method in metabolomics applications is principal component analysis (PCA). The model constructs a number of principal components (PC), where the first PC is oriented so that it describes the largest possible portion of the variance in the data set that can be described by a linear vector. Each subsequent PC contains a smaller portion of the variance in the data set than the previous component. Given that the MVA is based on all individual variable data points for all observations, the resulting model is robust both against false positives and missing data points. MVA is also robust against covariance, and provides information about the interrelatedness of the contributing variables. Furthermore, a confidence interval representing all of the variables is obtained, in contrast to univariate methods where each variable is analyzed as a separate unit, and consequently only provides confidence intervals for individual variables.

Supervised MVA methods, where the user defines which variables belong to the X dataset (dictating variables) and which belong to the Y dataset (response variables), can also be utilized to perform regression analysis between large data sets, most commonly through partial least squares of latent structures (PLS). A recent addition to this group regression of analysis, orthogonal PLS (OPLS), greatly simplifies the interpretability by separating the variance in the data set according to the correlation to the selected Y matrix (*e.g.* disease group) [342]. The OPLS model is designed to separate the ''orthogonal'' variance, any variance not correlated to the selected Y-variables, from the variance of interest. Given that the back-drop of the method is a supervised selection of the Y-variables that determine the separation, interpretation should be performed with caution. Overinterpretation of this type of model is common, and any conclusions or decisions regarding variable selection should always be evaluated in the light of the predictability (Q^2) of the resulting method.

Another important bottleneck in the analysis of metabolomics data is the need for software capable of performing pathway mapping and network analysis to interpret the observed fluctuations in biochemical pathways [343]. However, the structural diversity of oxylipins combined with the large number of overlapping biosynthetic pathways presents a number of challenges for the informatics community. For example, the structural complexity of the arachidonic acid cascade is displayed in Fig. (**2**), which is still only a simplified overview of the full cascade. This complexity is further compounded by the fact that analogous pathways exist for all of the unsaturated fatty acids, leading to theoretically thousands of oxylipins. While the actual existence of these species has not been shown, much less any potential biological activity, it is important to have informatics structures capable of dealing with the data. Accordingly, it is vital that specific bioinformatics methods be developed to analyze the wealth of oxylipin as well as molecular lipid data being acquired. The incorporation of lipidomics information into formats integrating genome, protein, chemical and network information data structures will provide mechanistic understanding of lipid functions and interactions in the context of cellular and organism physiology. There are many software packages and web resources available, all of which are too numerous to describe in this review (see reference [344] for a comprehensive list of >150 resources). While not an exhaustive list, a few resources worth briefly mentioning here include PathVisio, [345], MetaCore [346], Cytoscape [347], VANTED [348], Ingenuity Systems, SBML applications [349] and KEGG [350]. Some of this software is designed to map the results from metabolomics experiments onto existing pathway databases such as KEGG, but few of them are capable

of processing the full range of lipidomics data [351]. These types of tools enable the visualization of the results integrated with the information provided in these databases as shown in Fig. (**7**). Other tools enable the generation of networks that are inferred from metabolomics data, such as Cytoscape (through several plugins), VANTED, some of the R/Bioconductor packages [352] and many of the commercial software packages.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a web-based resource that contains a series of databases of biological systems, consisting of genetic building blocks of genes and proteins (KEGG GENES), chemical building blocks of both endogenous and exogenous substances (KEGG LIGAND), molecular wiring diagrams of interaction and reaction networks (KEGG PATHWAY), and hierarchies and relationships of various biological objects (KEGG BRITE). For more detail, please refer to either the KEGG homepage (http://www.genome.jp/kegg/) or a comprehensive tutorial on KEGG [353]. A new and expanding function in the KEGG suite is the DISEASE functionality, which highlights biochemical pathways in several diseases including asthma. While potentially useful, a review of the KEGG asthma pathway clearly shows that additional emphasis needs to be placed on expanding the mechanistic information in this pathway in order to be of clinical utility (see KEGG Pathway hsa05310 for more detail). Accordingly, while a potentially useful initial step, these efforts demonstrate the need for increased interaction between clinical scientists and computational scientists in order to create disease models that: 1) accurately reflect the intricacies of the disease, 2) are capable of accepting standard clinical diagnostic parameters as input, and 3) can be used and applied by non-specialists.

To address the need for analyzing integrated omics datasets, KEGG has recently introduced a new application called KegArray that is designed to map omics data onto the KEGG suite of databases [354]. KegArray is a Java application that provides an environment for analyzing metabolomics data (compound profiles) either alone, or in combination with transcriptomics and/or proteomics data. The KegArray tool maps omics results onto the KEGG application of choice and can thus be described as a visualization tool, but with the added advantage of a sustained interactive environment with the KEGG database suite. To provide an example of KegArray applications with oxylipin profiling data, Fig. (**7**) shows the results of a recent study involving exposure of mild asthmatics to subway air [251]. Data were formatted as a ratio of exposure to subway air/control air. The oxylipins that evidenced significant fluctuations in asthmatics following exposure to subway air were mapped to the KEGG PATHWAY database. The resulting information provided clear evidence that the 12/15-LOX pathway in the linoleic acid cascade was the site of greatest modulation. Based upon these results, further immunohistochemistry studies were performed targeting the 12/LOX pathway. Accordingly, KegArray can be a useful tool for processing metabolomics datasets, but additional work needs to be performed on generating sufficiently comprehensive lipid biochemical pathways to cover the breadth of the current analytical methods. For example, recent oxylipin profiling work focusing on the ω-3 fatty acid-derived cascades required the generation of custom pathway maps because sufficient biological detail was not available in public databases [266]. Accordingly, biochemical pathway mapping and network analysis represent both one of the most rapidly expanding fields within metabolomics and systems biology as well as one of the most significant bottlenecks in metabolomics/lipidomics data analysis.

4. FUTURE APPLICATIONS AND CONCLUSIONS

The biology of oxylipins has been extensively studied for several decades and there is a wealth of published information on their biological activity and possible role in disease processes. In addition, some of the most commonly consumed therapeutics (*e.g.* NSAIDs such as aspirin and ibuprofen) target these biological pathways. However, there is a dearth of information regarding cross-talk between the multiple biosynthetic pathways involved in oxylipin synthesis and little information on non-eicosanoid derived oxylipins. This paucity of information is demonstrated by the lack of applications of oxylipin profiling methods to clinical studies of respiratory disease. Accordingly, there is a significant need to further develop methodologies for quantification of oxylipins and apply them on samples obtained from patients with different lung diseases. With increased understanding of how these compounds are produced and their role in normal physiological and pathophysiological processes, new diagnostic tools and therapeutic strategies may be developed for patients with inflammatory respiratory conditions.

While numerous technical advances have been made, there remain multiple challenges in the field. There is a distinct need for chiral profiling methods given the important synthetic information provided by stereochemistry. In addition, some of the most interesting oxylipins, the CysLTs and eoxins, are generally unstable in oxylipin profiling methods and will therefore require special approaches (*i.e.* on-line SPE and/or polarity switching) to achieve robust recoveries. As with other metabolomics methods, another distinct challenge is interpreting the results of these profiling approaches. Given the potential cross-talk across biosynthetic pathways and biological compartments, it can be difficult to distinguish the exact function of specific compounds. This issue is further complicated by tissue heterogeneity. For example, there are a large number of different cell types in the lung, which presumably may demonstrate unique profiles for oxylipin production. This issue can be partially addressed by co-monitoring metabolite levels in a systemic matrix such as urine. However, it will require advanced statistical models in order to deconvolute the patterns of oxylipin (and other small molecule) biosynthesis on a biological compartment-specific basis. In addition, it will be necessary to have kinetic information on the production of these compounds in order to create flux models, but the short half-life of many oxylipins raises questions regarding systemic effects vs. *in situ* activity.

In this evolving era of omics technologies, systems biology, and the strive towards personalized medicine; there is a distinct drive to achieve an in-depth holistic understanding of pathophysiological processes. These efforts require the integration of data from multiple omics platforms (*e.g.* genomics, transcriptomics, proteomics, metabolomics and lipidom-

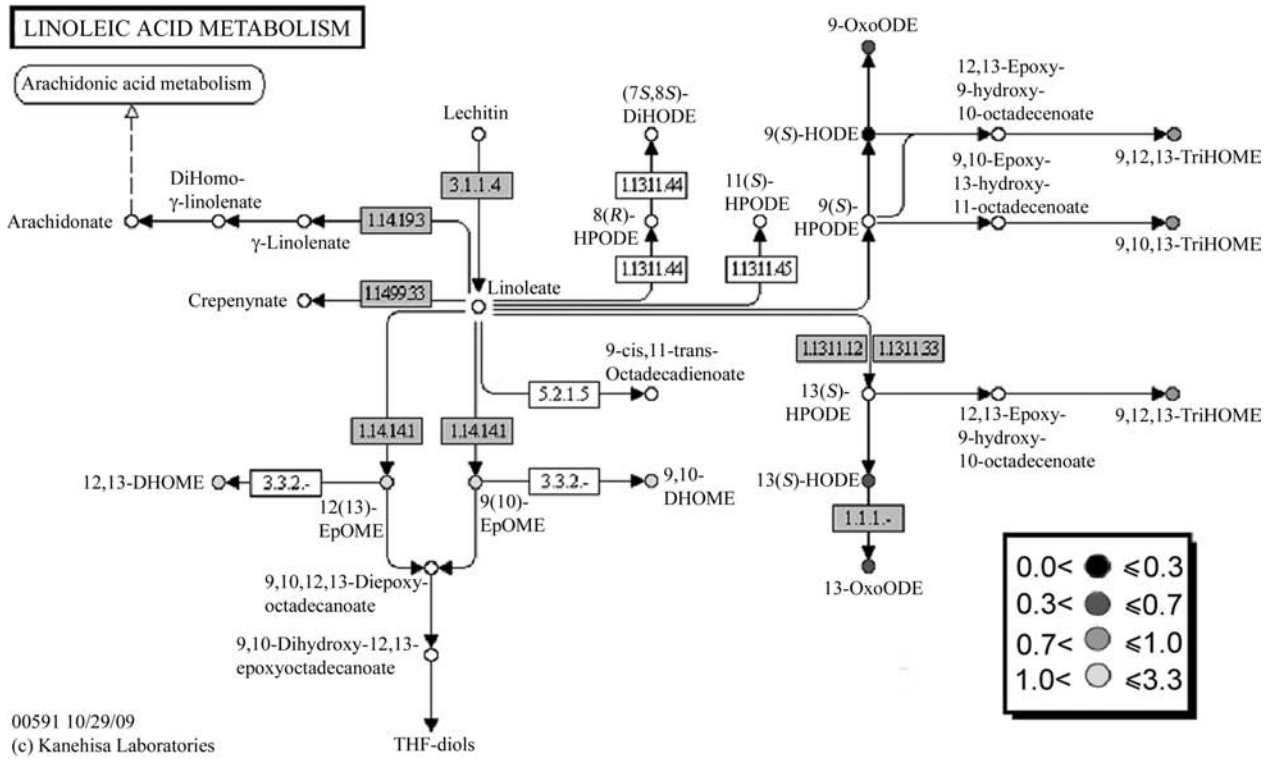

Fig. (7). Linoleic acid pathway from the KEGG database. Fluctuations in oxylipin metabolism following exposure to subway air in asthmatics relative to healthy individuals were mapped using the KegArray tool. The ratios (asthmatics/ healthy) between the averaged intra-individual oxylipin levels (Subway/Control) were used to visualize differences between the groups. As indicated in the figure, the main ratio difference observed between groups was the activity in the 12/15-LOX pathway, which for the healthy individuals indicated increased levels following subway air exposure in contrast to the asthmatics for which a decrease or no change occurred. KegArray was run with a 1.5 threshold with black, dark gray and medium gray colored compounds indicating ≤67%, 67%≤33% and 33%≤0% decreases ("down-regulations"), respectively. The light gray colored compounds indicated no change between groups ("non-regulation").

ics) with detailed clinical phenotyping and patient meta-data. This need is particularly relevant in the quest to understand heterogeneous diseases with complex etiologies, because different causes can lead to similar symptoms. This workflow involves multivariate statistics, which is capable of incorporating both quantitative and qualitative data from molecular, physiological and clinical sources [341, 355], combined with bioinformatics efforts at biochemical pathway reconstruction and network analysis [354, 356-358]. Accordingly, translational clinical metabolomics has evolved into an interdisciplinary field that involves the coordinated collaboration of multiple heterogeneous research groups; ranging from basic science involving biologists and analytical chemists to bioinformaticians and medical practitioners. This combination of research efforts can be quite powerful in elucidating disease mechanisms and identifying new biomarkers. An example of the necessary interdisciplinary team is demonstrated by the U-BIOPRED project (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes) project, which is funded under the Innovative Medicines Initiative (IMI). This 5-year project will apply a systems biology approach to integrate rigorous clinical patient phenotyping with a suite of omics platforms, including lipidomics and oxylipin profiling, to identify biomarkers of severe asthma [359]. The shear size of the project highlights one of the remaining major challenges in this field, the need for significant resources in order to acquire the clinical material as well as the diverse large-scale datasets and the necessary bioinformatics/statistical expertise to achieve the desired integrative analyses.

This review has described the extensive biology of oxylipins, demonstrating the need for profiling methods for elucidating their roles in respiratory diseases. While an amazing amount of work has been done in this field as evidenced by the extensive body of published literature, there remains a great deal to be done. Subsequently, the field of clinical oxylipin profiling as well as lipidomics/ metabolomics in general looks bright indeed. In addition to improving our understanding of respiratory tract pathophysiological processes, oxylipin profiling is an integral component of the "omics revolution", which can potentially provide new diagnostic fingerprints and biomarkers, thereby putting us one step closer to personalized medicine.

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ABBREVIATIONS

AA	=	Arachidonic acid
ARDS	=	Acute respiratory distress syndrome
BAL	=	Bronchoalveolar lavage
COPD	=	Chronic obstructive pulmonary disease
COX	=	Cyclooxygenase
CV	=	Coefficient of variation
CYP	=	Cytochrome P450
CysLT	=	Cysteinyl-leukotrienes
DGLA	=	Dihomo-γ-linolenic acid
DHA	=	Docosahexaenoic acid
DHET	=	Dihydroxyeicosatrienoic acid
DiHOME	=	Dihydroxyoctadecenoic acid
EAA	=	Extrinsic allergic alveolitis
EBC	=	Exhaled breath condensate
EET	=	Epoxyeicosatrienoic acid
EIA	=	Enzyme-immunoassay
EX	=	Eoxin
EpOME	=	Epoxyoctadecenoic acid
EPA	=	Eicosapentaenoic acid
FDR	=	False discovery rate
FEV_1	=	Expiratory volume in one second
GPL	=	Glycerophospholipid-liposome
HEPE	=	Hydroxyeicosapentaenoic acid
HETE	=	Hydroxyeicosatetraenoic acid
HODE	=	Hydroxyoctadecadienoic acid
HOTE	=	Hydroxyoctadecatrienoic acid
HpDoHE	=	Hydroperoxydocosahexaenoic acid
HPEPE	=	Hydroperoxyeicosapentaenoic acid
HPETE	=	Hydroperoxyeicosatetraenoic acid
HPLC	=	High performance liquid chromatography
phGPx	=	Glutathione peroxidase
PBMC	=	Peripheral blood mononuclear cells
PMN	=	Polymorphonuclear leukocytes
HPODE	=	Hydroperoxyoctadecadienoic acid
IPF	=	Idiopathic pulmonary fibrosis
KETE	=	Oxoeicosatetraenoic acid
KODE	=	Oxooctadecadienoic acid
KOTE	=	Oxooctadecatrienoic acid
LOX	=	Lipoxygenase
LT	=	Leukotriene
LX	=	Lipoxin
MAPK	=	Mitogen-activated protein kinase
MS	=	Mass spectrometry
MS/MS	=	Tandem mass spectrometry
MSN	=	Multiple stages of tandem mass spectrometry
MVA	=	Multivariate analysis
NSAID	=	Nonsteroidal anti-inflammatory drug
OPLS	=	Orthogonal projections to latent structures
PC	=	Principal component
PCA	=	Principal component analysis
PD1	=	Protectin D1
PG	=	Prostaglandin
PLA	=	Phospholipase A
PLS	=	Partial least squares
PPAR	=	Peroxisome proliferator-activated receptor
PUFA	=	Polyunsaturated fatty acid
Rv	=	Resolvin
sEH	=	Soluble epoxide hydrolase
SRM	=	Selective reaction monitoring
TNF	=	Tumor necrosis factor
TGF	=	Transforming growth factor
TriHOME	=	Trihydroxyoctadecenoic acid
TX	=	Thromboxane

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