LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY BASED DRUG MONITORING AND METABOLOMICS FOR BIOMARKER DISCOVERY: APPLICATION IN COMPLEMENTARY TREATMENTS OF COPD

By

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ABSTRACT

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY BASED DRUG MONITORING AND METABOLOMICS FOR BIOMARKER DISCOVERY: APPLICATION IN COMPLEMENTARY TREATMENTS OF COPD

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Biomarkers serve as objective indicators of normal, diseased and therapeutic conditions to aid prediction, diagnosis and staging of diseases as well as monitoring and evaluating clinical responses to interventions. Metabolomics has emerged as a powerful holistic approach to assess physiological status of a biological system, and advances in metabolomics techniques promoted the discovery of biomarkers of wellness and disease. This dissertation presents the development of liquid chromatography-mass spectrometry (LC-MS) based metabolomics approaches to profile endogenous metabolites and monitor research subjects' consumption of drugs. The analytical approaches were applied to discover potential plasma biomarkers that can help understanding and evaluating pulmonary rehabilitation (PR) exercise and osteopathic manipulative treatment (OMT) to chronic obstructive pulmonary disease (COPD).

This dissertation is based on a pilot study, in which COPD patients were participants in a PR program where they performed exercises and either received OMT, sham OMT treatment, or no additional treatment. Prior to profiling endogenous metabolites, the first study described the development and validation of a LC-MS/MS method to screen fourteen non-steroidal anti-inflammatory drugs (NSAIDs) in plasma samples from these COPD patients, and data were compared to self-reported medication use assessed from questionnaires. The results showed that 24 of 26 subjects tested positive for at least one NSAID. However, only 3 participants self-reported NSAID drug use correctly. This work revealed the inaccuracy of self-reported medication information, suggesting that drug use

monitoring by analytical approaches should be implemented as a routine practice to support clinical trials.

Both untargeted and targeted metabolomics strategies were performed in this dissertation to assess biochemical alterations in levels of metabolites in blood plasma following PR exercise and OMT/sham/no additional treatment on COPD patients. The second study described an untargeted approach combining the power of an advanced LC-TOF-MS platform and both univariate and multivariate statistical analyses to identify potential biomarkers of the effect of exercise on the plasma metabolome. The most discriminating metabolites were identified as lipids, and most were free fatty acids, which can serve as biomarkers of exercise.

In the third study, a targeted LC-MS/MS method was developed for quantification of 57 bioactive lipid mediators in human plasma samples drawn from COPD patient participants in PR with, or without extra OMT or sham treatment. Using the targeted approach, which was intended to provide a more comprehensive assessment of lipid mediators than prior publications, the responses of circulating oxylipins, endocannabinoids and polyunsaturated fatty acids (PUFAs) to PR exercise and OMT were assessed. The results demonstrated that PR exercise significantly increased PUFAs levels in the circulatory system of COPD patients and further induced the synthesis of downstream oxylipins including epoxides, diols, monohydroxy, and ketone metabolites within one hour post-exercise, but the impact faded over 72 hour post-exercise. Extra OMT and sham treatments were associated with minor reductions in the biochemical influence of PR exercise.

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KEY TO ABBREVIATIONS

AA	Arachidonic acid
AEA	Anandamide
2-AG	2-Arachidonoylglycerol
COX	Cyclooxygenase
CYP	Cytochrome
DHA	Docosahexaenoic acid
DHET	Dihydroxyeicosatrienoic acid
DHEA	Docosahexaenoyl ethanolamide
DiHDPA	Dihydroxydocosapentaenoic acid
DiHDoHE	Dihydroxydocosahexaenoic acid
DiHETE	Dihydroxyeicosatetraenoic acid
DiHOME	Dihydroxyoctadecenoic acid
EET	Epoxyeicosatrienoic acid
sEH	soluble Epoxide hydrolase
EPA	Eicosapentaenoic acid
EpDPE	Epoxydocosapentaenoic acid
EPEA	Eicosapentaenoyl Ethanolamide
EpETE	Epoxyeicosatetraenoic acid
EpOME	Epoxyoctadecenoic acid
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
LA	Linoleic acid

LOX	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
oxoETE	oxo-Eicosatetraenoic acid
oxoODE	oxo-Octadecadienoic acid
PD	Protectin D
PG	Prostaglandin
Rv	Resolvin
TX	Thromboxane

Chapter 1 Introduction

1.1 The quest of biomarkers to assess wellness and the efficacy of treatments

Human history includes a long legacy of fighting with diseases, dating back thousands of years across numerous cultures. No one should deny that maintaining wellness and seeking effective therapies has always been one of the initial and most important goals of individuals and societies, whether in ancient or modern times. Due to their lack of mechanistic understanding and the primitive nature of early medical technologies, people have relied on limited empirical or subjective results to assess wellness and treatment effectiveness. One example is the alleviation of symptoms including fever or pain, but these medical signs were usually not reliable, quantifiable or reproducible, and relied on subjects' own experience and sense of feeling.

In the past few decades, and especially since the dawn of the 21st century, tremendous progress in chemistry, biology, and physics have improved our understanding of mechanisms of biological systems, and reshaped the ways diseases are diagnosed and treated. Advances in DNA microarrays, genomics, proteomics, and metabolomics have accelerated discovery processes, resulting in recognition of a growing number of molecular entities as therapeutic candidates. Analyzing biological parameters, *i.e.*, biomarkers, using broad analytical tools instead of subjective parameters has grown to be an efficient and objective approach for assessment of biological conditions, and has dramatically aided in understanding occurrence, progression, diagnosis, and treatments of numerous diseases (1).

The term *"biomarker"* was defined by the National Institutes of Health Biomarker Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (2). Biomarkers can be classified into different categories (3,4) including functional and physiological biomarkers, *e.g.* pulse and blood

pressure, and chemical and molecular biomarkers measured from laboratory tests. Research on the latter are dramatically growing and playing more critical roles due to the development of science and technologies as mentioned above. By sources, there are intrinsic biomarkers including measures of levels of endogenous molecules, as well as extrinsic biomarkers including measures of exposures to environmental substances. By functions, biomarkers can be subdivided further according to whether they are used for risk prediction, diagnosis, classification, staging, prognosis of diseases, as well as monitoring and evaluating clinical response to interventions (*e.g.*, drugs, vaccines, or surgery).

According to definition, biomarkers include a broad range of measurable, objective and quantifiable parameters and compounds that can play important roles in almost every stage of a disease. Hence, the reliable biomarkers can be considered as surrogates for real clinical endpoints such as survival, stroke, organ damage, myocardial infarction, which are reflecting "how a patient feels, functions, or survives" (2). There are several advantages of using biomarkers as surrogate endpoints in research or trials instead of clinical endpoints (5,6). First, it is more practical and easier. In many cases, evaluating the efficacy of a therapeutic intervention from a well-defined clinical endpoint like survival or recurrence is impractical or unethical. In contrast, recording blood pressure or quantifying a reliable protein biomarker's concentration can be performed easily and quickly. Second, biomarker measurements can provide a platform for risk assessments during clinical trials to reduce the harm that an intervention presents to subjects. Terminating a treatment early, before toxicity or overdose drug level is reached, makes trials much safer to implement. Third, since the use of biomarkers is practical and can provide interim information about intervention effectiveness, it thus can shorten clinical trial length, reduce sample sizes, and accelerate the research process.

Not all biomarkers can be used as surrogate endpoints. Good biomarkers should accurately and reproducibly correlate the measurements with mechanistically-relevant clinical endpoints (2,6). In another words, an ideal biomarker should be a validated reliable indicator that is capable to clearly distinguish normal and diseased states, with and without the context of a specific intervention. The process of determining a novel biomedical biomarker includes a series of phases: candidate biomarker discovery, quantification, verification, validation, and clinical assay development (7,8). There is an inverse relationship between the number of measurements and the number of samples in this process: in the early discovery stage, hundreds and thousands of analytes are usually profiled in an unbiased manner from a small number of samples of human materials or animal models to screen out the entities differentially expressed in different states; the selected candidate biomarkers are measured in all phases after discovery to confirm the differences. In the validation phase, a large number of human biofluid samples (thousands) are used to assess variations in populations, and candidate biomarkers that fail to show strong relationships with functional outcomes are removed from consideration in future investigations.

High-throughput technologies have boosted the process of biomarker discovery for all kinds of molecular entities ranging from DNA and mRNA to proteins and metabolites. Among them, metabolic biomarkers have begun to show more promise in disease diagnosis and therapy evaluation (9,10). The following sections will focus on metabolite biomarkers and metabolic approaches.

1.2 Metabolomics

1.2.1 From genomics to metabolomics

Since 1953 when Watson and Crick first discovered the molecular structure of DNA (11), our understanding of living organisms and the genetic basis of biological functions has dramatically increased, and half a century later in 2003 the completion of the Human

Genome Projects (12) made another landmark of biology research, and the dramatic drop in DNA sequencing costs makes genomic information widely available. However, knowledge of sequences of genomic DNA is only a starting point. Though DNA records the "code of life", genomic sequences alone are not enough to understand the complexity of a biological system since they merely hold the information of "what can happen". Whether a gene will influence the biological system functions depends on whether it is actively expressed. In fact, only a small fraction (less than 5%) of the genome in human is positively transcribed (13). The initial product of gene expression is mRNA, and the total mRNA at the global level is known as the transcriptome. Transcriptomes can be measured globally, most recently using next-generation sequencing of complementary DNA (cDNA), provide information about "what appears to be happening", and link genetic codes with the functioning of cells and organisms. However, despite that mRNA encodes for synthesis of proteins, the levels of functional protein expression in a specific cell or organ are not necessarily reflected by transcriptome profiles (14). First, alternative splicing of pre-mRNA results in synthesis of multiple proteins from a single gene (15,16). In addition, after proteins are produced, many types of post-translational modification including phosphorylation, methylation, glycosylation, and acylation change the composition and properties of proteins, which not only diversify the proteome but also play key roles in signal transduction and other cellular processes (17-19). Moreover, localization of proteins and interaction with other molecules are important to their functions (18). Hence, global measurements of proteins, termed "proteomics" became an exciting research area during the recent two decades and provides the means to answer the question of "what makes it happen" in biological systems.

Advances in genomics, transcriptomics and proteomics have provided vast information about "what might happen" as driven by the genotype; a phenotype, on the other hand, describes the resultant interaction of genotype and environment, so it directly reflects

biological status and physiological functions, telling us about "what has happened and is happening" (Figure 1) (20). Metabolites, the end products of the biological cascades, are considered the closest to phenotype (21,22). Metabolites are usually defined as small molecules with molecular weights less than 1500 Daltons. Amino acids, peptides, lipids, nucleic acids, carbohydrates, organic acids, alkaloids, inorganic compounds, etc. all fall in the category of metabolites. As is the case for the other three "omics", metabolomics involves the measurement of metabolites at the global level in biological systems or specific physiological states, representing the landscapes and "snapshots" of gene functions and enzyme activities (23).

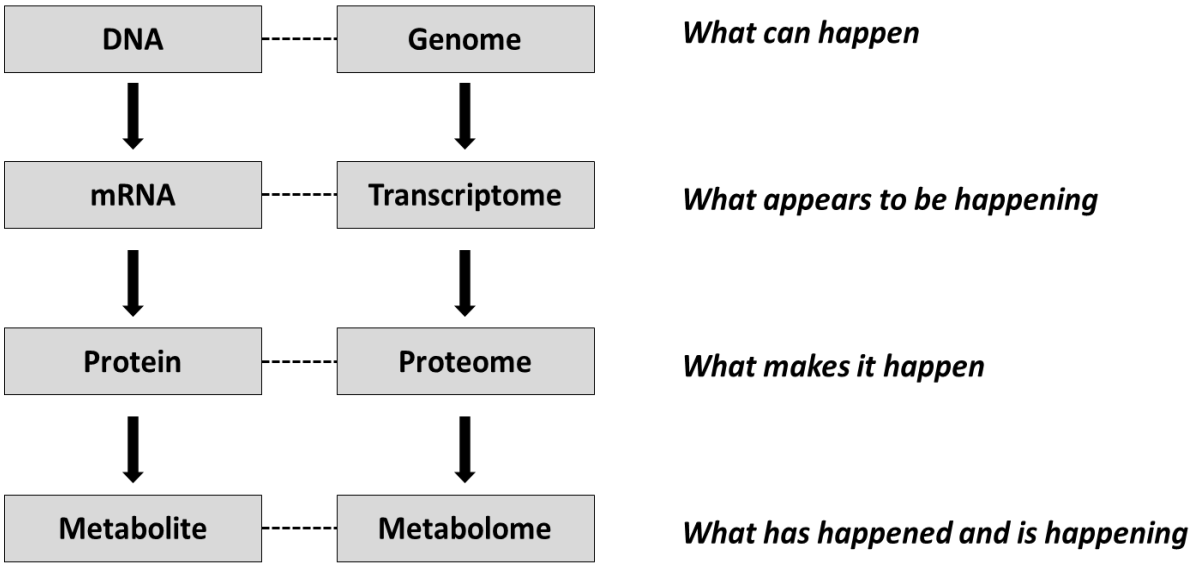

Figure 1.1. The "omics" cascade. Genomic DNA holds information about "what can happen", but the genome alone is not sufficient to understand the complexity and dynamics of a biological system. Transcriptomes provide information about "what appears to be happening", but the transcriptome profile does not necessarily reflect the level of protein expression and functions. Proteomes describe "what makes it happen", but do not reflect what functions have been performed. The final phenotype in a biological system is directly reflected by the end products of biological cascade----metabolome, so metabolomics tells "what has happened and is happening" (20).

In spite of being the youngest "omics" field, metabolomics has developed rapidly, and its applications have extended to various biological systems including plants (24-26), yeasts (27-29), bacteria (30,31), cell lines (32-35), animal models and humans (36-41). One of the

most important uses of metabolomics approaches lies in biomarker discovery and clinical research (42-45). For example, by comparative analysis of metabolite levels in diseased and healthy conditions, biomarkers of diagnosis can be screened. In addition, because metabolites represent the end products of complex networks of metabolic transformations, a biomarker that can predict diseases or monitor therapies is usually a profile of a set of molecules rather than a single compound. So the identification, quantification and metabolic fluxes of a large number of metabolites provide a powerful approach to assess gene functions, monitor environmental exposures, assist diagnosis of diseases, as well as evaluate responses and toxicological effects of treatments such as drug therapies.

1.2.2 Analytical platforms and strategies for metabolomics

Metabolomics covers the global measurements of a large number of molecules, and this requires analytical platform capable of providing simultaneous and comprehensive analysis of compounds with high chemical diversity and broad dynamic range in metabolite abundance. No single analytical method can cover all metabolic information even in a single sample, and a variety of analytical platforms have been used for metabolic biomarker discovery (43). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) including gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the major metabolomics platforms in biomarker and clinical research.

1.2.2.1 Nuclear magnetic resonance spectroscopy

NMR is the primary tool for investigation of structure-based metabolomics, and has been successfully applied for many biomedical studies, from blood biomarkers to toxicology researches (46-52). NMR owns several advantages for metabolomics profiling. First, ^{1}H-NMR offers near-universal detection. As long as the level is above detection limit, all compounds containing hydrogen, which means nearly all metabolites, can be detected in the

^{1}H NMR spectrum. Second, NMR provides highly reproducible results and quantitative detection, which especially benefits biomarker discovery. Third, no pre-treatment other than adjustment of solution pH is required for NMR analysis, so metabolites can be detected in crude extracts or whole tissues, and the structures of interesting molecules can be determined directly from the spectrum. Despite these advantages that make NMR a powerful tool for metabolomics, the approach is mainly limited by its relatively poor sensitivity and dynamic range (53) ---- usually requiring μM to mM concentrations of individual metabolites in common practice.

1.2.2.2 Mass spectrometry

Recent advances in metabolomics research have shown that mass spectrometry (MS) serves as an essential analytical tool for metabolomics analysis, and MS has become more important, even to researchers whose initial approach was based on NMR. MS is a near-universal platform for profiling, characterizing, identifying and quantifying a wide range of molecules in almost all kinds of biological samples. MS is much more sensitive than NMR (offering detection levels down to low pM concentrations) and is able to quantify metabolites over a broader concentration range. Furthermore, combinations of MS with separation techniques such as gas chromatography (GC) and liquid chromatography (LC) can greatly reduce complexity of individual mass spectra and increase the sensitivity and selectivity of the analysis. Currently, mass spectrometry-based metabolomics have been applied to biomarker studies in a wide range of diseases, for example, cancer (54-57), respiratory diseases (58-60), Alzheimer's disease (61-63), diabetes (64,65), coronary artery disease (66,67), and HIV (68,69).

1.2.2.2.1 Gas chromatography-mass spectrometry

GC-MS has been used for analyzing metabolites for many years (70). The primary advantages of GC-MS lie in its high separation efficiency, which allows resolution of most

isomeric metabolites, and reproducibility of ionization efficiency. Also, the standardized electron ionization with 70 eV of energy generates almost universal fragment ion spectra, and the availability of public databases of these spectra allows the relatively straightforward identification of metabolites providing they are known and previously characterized. Though GC-MS is primarily suited for analyzing volatile and thermally stable compounds (usually with low molecular weight), chemical derivatization is commonly employed for those non-volatile molecules to reduce their polarity, and this pre-treatment successfully expands the range of metabolites that GC-MS can detect to many classes of compounds including organic acids, amino acids, and disaccharides (71,72). However, the drawbacks of this technique also arise from the chemical derivatization, as the process often requires complete evaporation of solvent which is frequently time-consuming. In addition, many metabolites cannot form appropriate volatile derivatives, and some derivatized products are unstable and decompose during injections and separation.

1.2.2.2.2 Liquid chromatography-mass spectrometry

The development of LC-MS significantly led to a revolutionary change in analytical strategies for metabolomics. Unlike GC-MS, no derivatization is required and non-volatile polar compounds may be analyzed using LC-MS, which simplified sample preparation and dramatically broadened the range of metabolites that could be measured. Hyphenation of LC with MS was mostly due to the invention of electrospray ionization (ESI), which gently converts analytes into gas phase ions before introduction into the mass analyzer (73). ESI is considered a soft ionization technique that generates minimal fragment ions, which enables the detection of pseudomolecular ions often by proton attachment or detachment (*e.g.* $[M+H]^+$ and $[M-H]^-$) and facilitates compound annotation. In addition, ESI can be operated in either positive- and negative-ion mode, which increases the possibility of a compound to be detected.

LC-MS is a versatile technique that may integrate any of several different LC modes (including various choices of mobile phases and columns) and mass analyzers with different modes of mass analysis. Reversed-phase LC-MS (RPLC-MS) has been most widely used for metabolomic profiling with the emphasis on providing good retention and separation of relatively non-polar compounds over a large mass range (74). Reversed-phase separations do not retain polar compounds well, but alternative separation methods including hydrophilic interaction chromatography (HILIC) and aqueous normal phase chromatography (ANP) are available for the analysis of polar species (75,76). Furthermore, the appearance of ultrahigh performance LC (UHPLC) benefits the high-throughput metabolomics applications due to the improved LC resolution and peak capacity (77,78).

After LC separation, the pseudomolecular ions generated by ESI can undergo MS/MS experiments via collision-induced dissociation (CID) to produce fragment ions whose masses yield further structural information about the compound of interest. A diversity of mass analyzers are used in mass spectrometers for mass analysis: linear and 3-dimensional ion traps, single quadrupole (Q), triple quadrupole (QQQ), time-of-flight (TOF), Orbitrap, and magnetic Fourier transform ion cyclotron resonance (FT-ICR) analyzers. For example, QQQ is commonly employed for targeted analysis to monitor specific metabolites with the purpose of quantification by filtering away interfering substances that have different molecular and fragment ion masses. Modern Q-TOF, Orbitrap and FT-ICR analyzers offer high mass resolution and accuracy, which are especially useful to determine elemental composition of metabolites based on accurate mass measurements and isotopologue patterns (79,80). Together with further MS/MS or MS^n information, annotation of metabolites and identification of unknown are often achieved.

One limitation of LC-MS lies in relative difficulty to obtain consistent ion yields, and the CID process has not been standardized across laboratories, so unlike GC-MS, it is of

limited utility to compare results between laboratories. As a result, MS/MS spectrum libraries are limited in scope and utility. Another drawback of LC-MS with ESI comes from ion suppression. The ionization efficiency of one analyte can be influenced by co-eluting compounds, and the problem may become severe when analyzing complex biological samples because co-elution of analytes is almost universal, and extra efforts are required on greater separation for reliable LC-MS results.

1.2.2.3 Untargeted and targeted metabolomics for based on LC-MS

Two common LC-MS strategies are performed in metabolomics analysis: untargeted (or non-targeted) and targeted metabolomics (81).

The untargeted approach aims to achieve comprehensive analyses of all measurable metabolites, including both known and chemical unknown compounds with minimal sample preparation, often under high throughput conditions (82). In these kinds of experiments, metabolites are analyzed with minimal bias and the coverage of compounds is only limited by sample preparation and sensitivity of the analytical platform. Hence, the untargeted approach offers the opportunity to discover novel biomarkers and is primarily applied in the first stage of metabolic biomarker discovery. Due to the extensive dataset generated in untargeted analyses, chemometric methods incuding multivariate statistical analysis (*e.g.*, PCA, PLS-DA) must be coupled to compress data size and select candidate biomarkers that can differentiate "healthy vs diseased", or treated vs. control conditions. Untargeted analyses usually does not yield absolute quantification of metabolites (not it is intended to be), but ion abundances are used for relative comparison of metabolite abundances between samples. Since untargeted approaches aim to find potential biomarkers, the ability of the analytical platform to annotate or identify chemical structures is a key issue. High resolution mass analyzers including TOF, Orbitrap and FT-ICR (with mass accuracy < 5 ppm (83)), as mentioned above, are increasingly applied for the identification of biomarkers in a global metabolomics approach.

Accurate masses obtained from high resolution MS aids to distinguish metabolites with the same nominal mass (rounded to integer value) but different elemental compositions, and mass defects alone (decimal part of the mass) can also reveal information about the composition of the compound (84). Then researchers can identify metabolites via comparing the accurate molecular and fragment masses with online databases. Werner *et al*. summarized the web-databases available now for mass spectrometry metabolite identification (83), which include KEGG (http://www.kegg.com/), ChemSpider (http://www.chemspider.com/), Human Metabolome Database (HMDB) (http://www.hmdb.ca/), and LipidMaps (http://www.lipidmaps.org/). As for the novel chemical unknown biomarkers not available in databases, accurate mass and MS/MS or MS^n spectra help greatly on obtaining structure information, but NMR is usually required for complete structure identification.

Targeted metabolite profiling strategies, on the other hand, measure a small set of pre-defined metabolites using a particular analytical technique which is optimized for measurement of those compounds (85). The targeted metabolites are usually biologically or chemically related, belonging to a class of compounds or associated with a specific pathway, gene function and enzyme system (43). Targeted approaches are mainly used in the later stage of biomarker discovery to quantify the concentrations of metabolites for biomarker confirmation and validation; it is also applied in the early discovery stage for candidate biomarker selection from the pre-defined lists of analytes in the context of particular physiological states, for instance, the targeted profiling of inflammation-related metabolites in inflammatory conditions (86,87). Since absolute quantification is a primary goal, internal standards are usually employed in the experiments. Also, sample preparation methods can be optimized for the specific compounds of interest to reduce interferences from high abundance molecules in the matrix.

The most widely used MS for targeted metabolomics is QQQ owing to its low limits of detection and potential to perform precise and accurate quantitative analysis, usually involving multiple reaction monitoring (MRM) (88). In MRM mode, two stages of mass filtering are performed on a triple quadrupole mass spectrometer. In the first quadrupole, an ion of a specific mass (the precursor ion, usually a pseudomolecular ion) is selected to pass into the collision cell, and ions of all other masses are excluded. After this first mass filtering step, collision-induced dissociation (CID) results from collision of the ion with a gas, usually argon or nitrogen, and product ions are produced. Then a specific ion (product ion) is exclusively selected in the third quadrupole and passes along to the ion detector. MRM excludes all other substances that do not form ions of the same precursor and fragment masses and greatly improves ion transmission efficiency compared to scanning method, so lower detection limit is achieved (89).

1.3 COPD, pulmonary rehabilitation and OMT

1.3.1 Introduction to chronic obstructive pulmonary disease (COPD) and current state of metabolomics in biomarker discovery for COPD

Chronic obstructive pulmonary disease (COPD) presents an important cause of human morbidity and mortality throughout the world. The World Health Organization (WHO) estimated that 65 million people have COPD, and in 2005 more than 3 million people died of COPD which accounted for 5% of all global deaths (90). The Global Burden of Disease study predicted that COPD ranks as the fifth leading cause of death, but will rise to the third leading cause by 2030 (91). In economic terms, COPD and its treatments cost about $32 billion in the U.S. annually, and as the global population ages the burden of COPD will be increasing (92).

According to the definition by the Global Initiative for Chronic Obstructive Lung Disease (GOLD), COPD “is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways

and the lung to noxious particles or gases" (93). Tobacco smoking is the most primary risk factor, and occupational, indoor and outdoor air pollution are also major contributors to COPD (92,94). COPD has been described as a complex chronic inflammatory disease, associated with different types of pro-inflammatory cells (*e.g.* neutrophils, macrophages, T-lymphocytes, B- lymphocytes), chemokines (*e.g.* TNF-α, IL-8), and inflammatory lipid mediators (*e.g.* LTB_4, isoprostanes) (95,96). The inflammation of pulmonary tissue also triggers downstream systemic inflammatory responses which affect many organs remote from the lung (97). Clinical diagnosis of COPD requires spirometry. If the post-bronchodilator ratio of forced expiratory volume in 1 (FEV_1, volume that has been exhaled at the end of the first second of forced expiration) to forced vital capacity (FVC, volume of air that can be forcibly blown out after full inspiration) is less than 0.7, it is confirmed the presence of airflow limitation, i.e., COPD (93).

Currently, COPD has no curative therapy. The initial management of COPD involves smoking cessation and reduction of exposure to air pollutants. Clinical pharmacologic treatments mostly focus on improving function of the diseased gas exchanger, for example, the use of short-acting and long-acting bronchodilators, inhaled corticosteroids, anticholinergic agents alone or as a combination to prevent exacerbation and reduce symptoms by dilating the bronchi and bronchioles and regulating inflammation (98). However, the traditional inhaled agents present several health risks and side effects, and COPD, as assessed by pulmonary function tests, is relatively insensitive to the drugs (99,100). Thus, interest has emerged in developing complementary and alternative treatments, for example, pulmonary rehabilitation and osteopathic manipulative treatment which will be introduced in the next session.

Small molecule metabolites have long been known to play important roles in COPD. For example, prostanoids and leukotrienes contribute to the pulmonary inflammation, and

isoprostanes serve as biomarkers of oxidative stress which is a key feature of COPD (101). However, previous research usually focused on measuring a small number of metabolites, and the understanding of global metabolome in COPD patients has been limited. In recent years, researchers have begun to pay more attention to comprehensive metabolic profiling of biofluids from human subjects, and metabolomic strategies have emerged as promising tools for biomarker discovery of COPD. The majority of published reports answered questions about whether or how metabolic profiling was able to discriminate COPD patients from healthy individuals, distinguish COPD from other lung disorders, and describe different phenotypes or stages of COPD. Both NMR and MS-based approaches were used as the analytical platform using both targeted and untargeted strategies. Through NMR spectroscopy, Deja *et al*. discovered several metabolic biomarkers which distinguished COPD and different stages of lung cancer in human serum (102). Wang *et al*. showed that NMR-based metabolic profiles in both serum and urine discriminated COPD patients and healthy subjects, and could be used for potential diagnosis purpose (103). In another study, McClay *et al*. found that urinary levels of trigonelline, hippurate and formate were associated with lung function, but no associations were found in plasma (104). Besides blood and urine, metabolomic analyses of exhaled breath condensate were also reported to distinguish COPD patients from healthy controls (105). A few untargeted LC-MS methods were applied on COPD biomarker investigations, too. For example, Telenga *et al*. investigated lipid biomarkers using untargeted UPLC-QTof in induced sputum comparing non-smokers with smokers with, and without, COPD, and found different sphingolipids levels in different groups of subjects (106,107). Chen *et al*. also used untargeted LC-MS to profile human serum metabolites in healthy smokers and COPD smokers and found potential biomarkers for early-stage COPD including myoinositol, glycerophopshoinositol, fumarate, cysteinesulfonic acid, a modified version of fibrinogen peptide B (mFBP) (108). In more recent research,

Paige *et al*. selected 12 unknown metabolites as putative biomarkers from untargeted profiling to build a predictive model that differentiated an emphysematous COPD phenotype from other COPD phenotypes (109). On the other hand, limited research reports on COPD have described targeted metabolomics, which is usually necessary to measure low-level (nM) metabolites. Ubhi *et al*. analyzed 34 amino acids and dipeptides in serum by LC-MS/MS and showed that multiple amino acids could distinguish patients with COPD, COPD related cachexia and cancer related cachexia (110). Bowler *et al*. combined targeted and untargeted MS to measure 69 plasma sphingolipids from a cohort of 250 COPD patients. The authors reported systemic dysregulation of sphingolipids in COPD, and subphenotyping suggested strong association between sphingomyelins and emphysema, as well as glycosphingolipids and COPD exacerbations (111). In a study of a mouse model, Conlon *et al*. reported reduced levels of L-carnitine in lung to be associated with progressive emphysema by targeted MS profiling of 186 metabolites (112).

COPD is a complex inflammatory lung disease, but little information is available about metabolite biomarkers of COPD, and even less is known about the underlying mechanisms of how the metabolites are related to different phenotypes and exacerbation of COPD. Metabolomics as an emerging field has already shown potential for biomarkers discovery of COPD, but these efforts are still in their infancy and more efforts should be put in this area.

1.3.2 Introduction PR and OMT, and current state of metabolomics in biomarker discovery of PR and OMT on COPD

Compared with conventional pharmacological interventions based on drugs, pulmonary rehabilitation (PR) ---- an integrated care which includes but is not limited to exercise training, education and behavior modification, and nutritional intervention ---- encourages COPD patients to take an active role in their own health care (113). PR programs

are usually designed to last 6-12 weeks, and patients participate in 2-3 sessions per week. The common goal of PR is to improve physical and psychological conditions of patients with COPD (and other chronic respiratory diseases), and thus promote quality of life (114). Prior investigations have shown that PR programs, especially the exercise training component, reduce dyspnea, increase exercise tolerance, strengthen respiratory muscles, exert positive psychological influence, and enhance subjects reported quality of life (115,116). Due to its effectiveness and low cost, PR is currently widely recommended as part of the treatment for COPD patients.

Physical activity serves as the core of PR programs. Participants are recommended to have 60-70% of the maximum symptom-limited exercise capacity for 20-30 minutes (117). Exercise training is not known to directly improve respiratory system functions, but reduce lung COPD manifestations through the systemic body system which includes improved stamina (118). Clinical trials have evaluated the efficacy and benefits of PR exercise on COPD patients, based on outcomes mainly associated across exercise capacity, exercise tolerance, muscle strength, severity of dyspnea, supplemental oxygen use, hospitalization rates, health-related quality of life questionnaires, and psychosocial conditions (119). Although PR exercise training has been proven beneficial to COPD patients regarding of all kinds of outcomes above, the understanding of the underlying mechanisms behind the physiological and psychological response has been limited. Skeletal-muscle dysfunction is widely accepted as a major systemic manifestation often accompanying with COPD (120), and PR exercise training plays an effective role to reverse the dysfunction; however, the precise mechanism causing skeletal muscle abnormalities is still not clear, and it is much less clear how physical exercise influences subjects' biochemical functions. Though metabolomics serves as a powerful tool for investigating molecular mechanisms, it has been rarely used to study the comprehensive biochemical responses of PR exercise training on

COPD patients. One paper by Rodriguez *et al*. reported blood metabolome obtained pre- and post-exercise training from COPD patients and healthy subjects by NMR (121). Though the patients in this research were not enrolled in PR program, but the exercise training was designed following the recommendations of the American Thoracic Society guidelines which were used in PR program. The training significantly impacted metabolomics in healthy subjects with changes of levels of amino acids, creatine, and lactate in blood, but in contrast, only lactate decreased after exercise in COPD. This research was the only metabolomic investigation of COPD patents doing PR exercise, and showed that plasma metabolic profiling contributes to the phenotypic characterization of COPD patients.

Osteopathic manipulative treatment (OMT), also a drug-free intervention, was developed to treat somatic dysfunction and improve physiologic through simulating self-healing of the patients (122) . The treatment, simply speaking, is a hands-on care, and muscles and joints are manually manipulated by osteopathic physicians using a diversity of techniques including pressure, stretching, and kneading. (123). OMT as a complementary therapy has been widely used to treat many diseases including low back pain (122), chronic neck pain (124), pneumonia (125), diabetes (126), and rehabilitation from surgeries (127). It has also been used for treating respiratory disorders diseases including COPD and asthma (128,129), and specific osteopathic techniques were developed targeting the musculoskeletal and lymphatic components of the pulmonary system (130).

Although OMT has been practiced in COPD treatment for years and its practitioners have reported anecdotal evidence that OMT improved respiratory system and quality of life, few clinical trials have examined the effects of OMT on COPD patients, and the results were contrasting. One early study showed modest improvements of total lung capacity and residual volume in COPD patients treated by OMT (128). In contrast, two other trials indicated worsened pulmonary function measures after different osteopathic techniques (131,132). Yet

one additional recent investigation compared the combined effects of PR and OMT in patients with severely impaired COPD, and suggested that OMT may further improve exercise capacity and reduce residue volume with respect to PR alone (133). On the other hand, no reports have yet described metabolic profiles of COPD patients receiving OMT, though a few studies have reported the effects of OMT on a few metabolites for other diseases (134-135). In a study of patients with lower back pain, an increase from baseline in β-endorphin and Npalmitoylethanolamide and a decrease in anandamide were observed in blood (134). Another study investigated whether OMT generated cannabimimetic effects in healthy subjects, though 168% increase of anandamide in serum was observed not it did not reach statistical significance (135). Therefore, there is a large knowledge gap about whether OMT is an effective treatment to COPD and how OMT influences biochemical phenotypes, and thus more fundamental research and clinic trials are needed to answer these questions. The following chapters of this dissertation describe our efforts in this area.

1.4 Summary of research

The global profiling of metabolites, termed metabolomics, has emerged as a powerful tool for holistic assessment of metabolic phenotypes in biological systems. Advances in metabolomics techniques, especially the development of LC-MS, have promoted the discovery of biomarkers of physiological states. The research presented in this dissertation has aimed to develop LC-MS based analytical methods for profiling of both endogenous metabolites and exogenous drugs in order to understand molecular mechanisms of diseases and the effectiveness of therapeutic interventions from metabolite levels in clinical settings.

The various chapters of this dissertation derived from a pilot study investigating the effectiveness of osteopathic manipulative treatment (OMT) in chronic obstructive pulmonary disease (COPD) patients. The clinical trial design was as follows: COPD patients were recruited from participants in a standard 12-week pulmonary rehabilitation (PR) program at

the McLaren Ingham Regional Medical Center (IRMC) in Lansing, MI. In the pulmonary rehabilitation program, patients performed exercise training in a gym every Monday and Thursday each week. The subjects were randomly assigned into three groups: (a) OMT group receiving biweekly osteopathic structural exam (OSE) and OMT to affected areas in addition to standard PR; (b) sham group receiving standard PR as well as biweekly OSE but without OMT, thereby controlling for the effect of touch and psychological effects of being treated by a physician; (c) PR only group, subjected to PR program of exercise training only but not receiving OSE or OMT. Blood specimens were collected from subjects in the beginning of the study (Week 0), in the middle (Week 6), and at the end of the study (Week 12). At each of these weeks, blood was drawn before treatment (Monday), one hour after (Monday) and 72 hours after treatment (Thursday). Patients also were evaluated using pulmonary function and stamina tests and filled out quality-of-life questionnaires during the clinical trial. This project involved chemists, biologists, doctors, medical coordinators, hospital staff from Michigan State University and McLaren Ingham Regional Medical Center in Lansing, MI. The goal of this clinical research has been focused on correlating biochemical alterations that may shed light on the biological mechanisms underlying PR exercise and OMT. My role in this project targeted (but not limited to) development and application of analytical methods to profile metabolites from subjects' plasma samples, and discover potential metabolic biomarkers that help to understand and evaluate the effects of PR exercise and OMT on COPD.

Chapter 2 of this dissertation describes the development and validation of an assay to monitor 14 non-steroidal anti-inflammatory drugs (NSAIDs) in plasma samples of COPD patients recruited in the clinical trial. The motivation of this work was the suspicion about the accuracy of patients' self-reported drug use: only 3 out 26 subjects reported the intake of aspirin which is the most common NSAID with the function of inhibiting cyclooxygenase (COX) enzyme activities. However, the downstream lipid mediator metabolites from COX

pathway (prostaglandins, thromboxanes) were present at negligible levels for most of the patients from a preliminary test. To assess whether patient self-reporting of NSAID use was inaccurate, I developed a rapid, simple and sensitive method to screen 14 NSAIDs from only 10 μL of plasma. Results showed that among 26 human subjects, 24 (92%) tested positive for at least one NSAID, but 21 of them failed to report the drug use information correctly. These findings revealed the inaccuracy of self-reported medication information in this population, and suggest that drug use monitoring by analytical approaches should be implemented as a routine practice to support clinical trials.

Chapters 3 and 4 present the untargeted and targeted metabolomics strategies respectively for potential biomarker discovery. In Chapter 3, an untargeted approach was developed which combined the power of an advanced LC-TOF-MS platform and both univariate and multivariate statistical analyses to identify potential biomarkers of the effect of exercise on the plasma metabolome. The LC-MS method including sample preparation were high-throughput and developed for large-scale metabolic profiling in plasma. Moreover, the advanced high resolution TOF-MS technique can generate exact mass information, which facilitates the identification of interesting metabolites. The untargeted approach developed in this work can be used, with slight modification, for initial metabolic biomarker discovery of other diseases or purposes. I participated as a collaborator to modify this method for two other studies to (1) investigate the differences in metabolic profiles of plasma comparing Korean patients who had suffered acute cerebral infarction (stroke) displaying non-dampness-phlegm and dampness-phlegm phenotypes (M. H. Cha, A. D. Jones, M. M. Ko, C. Zhang, M. S. Lee. Metabolic profiles distinguish non-dampness-phlegm and dampness-phlegm patterns among Korean patients with acute cerebral infarction. *Evid. Based Complement. Alternat. Med.* **2013**. Article ID: 517018, 9 pages); and (2) investigate the variability of urinary and plasma metabolomes in a tightly controlled clinical trial with healthy volunteers and subjects

with autosomal dominant polycystic kidney disease (K. Kim, C. Mall, S. Hitchcock, C. Zhang, H. Wettersten, A. D. Jones, A. Chapman, R. H. Weiss. Mealtime, temporal, and daily variability of the human urinary and plasma metabolomes in a tightly controlled environment. *PLoS One*. **2014**. 9(1):e86223).

Chapter 4 first describes the development of a targeted LC-MS/MS method for quantification of 57 bioactive lipid mediators and polyunsaturated fatty acid precursors in human plasma samples on a triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) data acquisition. The targeted analytes include oxylipins, endocannabinoids and their fatty acid precursors. These endogenous metabolites are important signaling lipid mediators involved in many crucial biological and physiological events including inflammation, anti-inflammation and resolution, so they hold great potential as candidate biomarkers in inflammatory conditions such as COPD. The analytical method was optimized for processing of human blood samples in this study. It can also be adapted for profiling the same (or similar) set of metabolites in other biological matrices with modifications of sample preparation procedures, calibration curves, and targeted MRM transitions. Its application for characterization of the biosynthesis and diversity of oxylipins in milk and plasma during acute bovine coliform mastitis has appeared in a manuscript (V. Mavangira, J. C. Gandy, C. Zhang, Valerie E. Ryman, A. D. Jones, L. M. Sordillo. PUFAs influence differential biosynthesis of oxylipins and other lipid mediators during bovine coliform mastitis. *J. Dairy Sci*. 2014. 98: 6202-6215.). In the second part of Chapter 4, the targeted LC-MS/MS approach was employed to assess the responses of the bioactive lipids to PR exercise and OMT on COPD patients. One important finding has shown that exercise significantly increased PUFA levels in the circulatory system of COPD patients and further increased accumulation of downstream oxylipins including epoxides, diols, monohydroxy, and ketone metabolites within one hour post-exercise, but this impact faded over 72 hours.

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Chapter 2 Sensitive and Rapid UHPLC-MS/MS Measurements of Plasma NSAIDs in A Pulmonary Rehabilitation Trial of COPD Patients

2.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used around the world, and many are available as over-the-counter medications (1,2). They exhibit anti-inflammatory, analgesic, and antipyretic functions that derive from their inhibition of cyclooxygenase (COX) enzymes which catalyze formation of prostaglandins and other signaling lipid mediators (3,4). Many individuals also take NSAIDs daily to inhibit platelet aggregation, though the efficacy of such use remains controversial (5). Moreover, NSAID use has been reported to be associated with increased mortality and morbidity, for example, increased cardiovascular risk and gastrointestinal ulcers (6,7).

Because NSAIDs influence inflammatory signaling, knowledge of NSAID intake by research subjects is important for clinical studies that use biomarkers to assess treatment effectiveness. Usually, NSAID use by research subjects is collected from self-reports, commonly from questionnaires, but there are no guarantees that subject reports of medication use are correct. A few reports have compared self-reported meditation use with pharmacy records, physician reports, or electronic monitoring systems (8-12). However, over-the-counter drugs including NSAIDs may not be included in such monitoring, and record keeping is subject to errors and incomplete documentation. Furthermore, even if a patient has a prescription for a specific medication, this does not guarantee that the patient actually takes the drug. Failures of patients to fill prescriptions and adhere to medication regimens were not uncommon (13-15). Therefore, objective generation of reliable information about NSAID use is needed, particularly in clinical trials. Monitoring NSAID levels in biofluids such as blood or urine collected at relevant time points in clinical trials using validated analytical protocols provides a reliable way to obtain objective data.

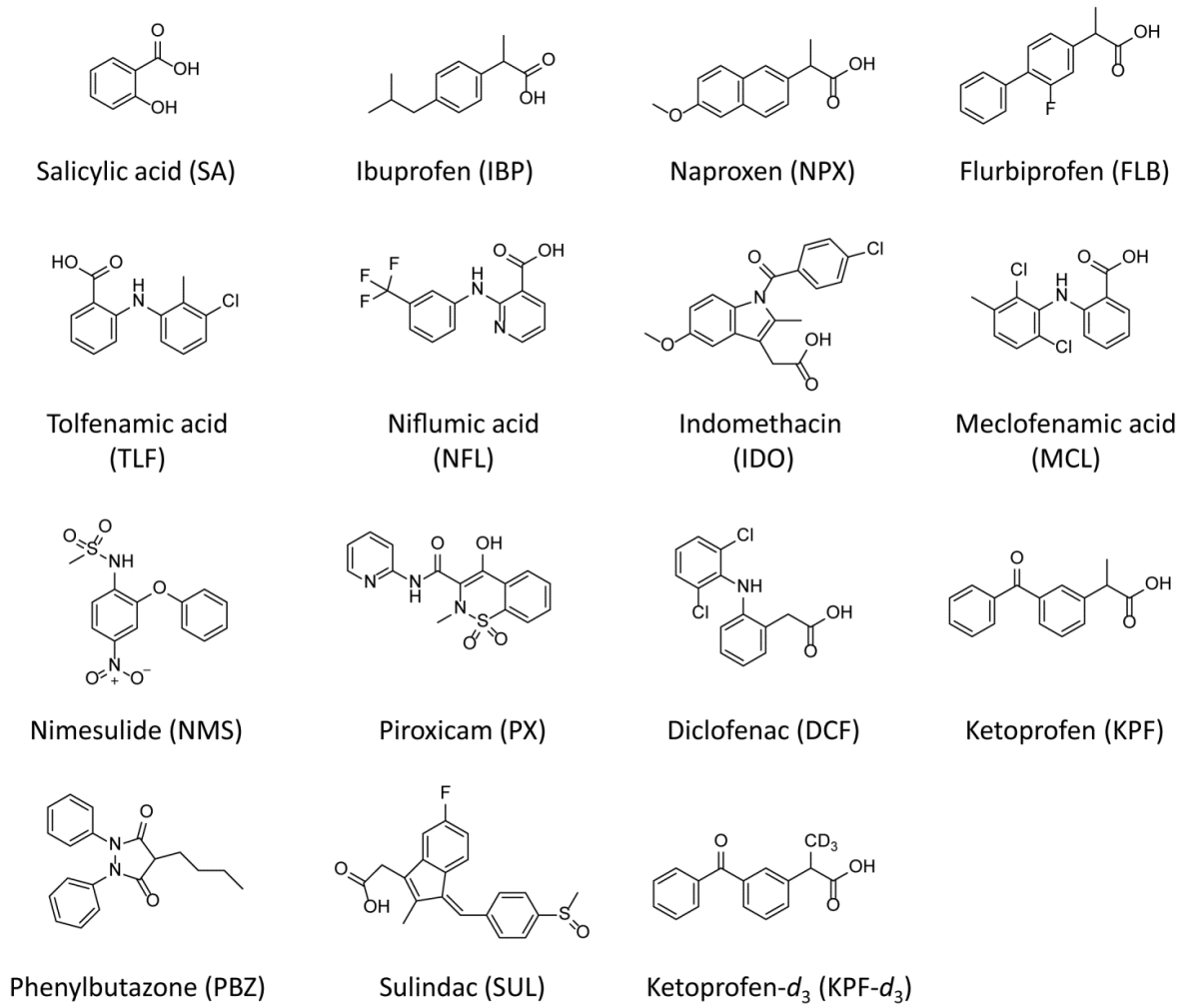

Figure 2.1. Chemical structures and abbreviations for fourteen NSAIDs and one internal standard measured in this study.

Analytical methods for the determination of multiple NSAIDs and their metabolites in matrix have been reported. For example, gas chromatography-mass spectrometry (GC-MS) has been developed for determining NSAIDs in milk, urine, blood as well as surface water (16-21). However, GC-MS analyses of NSAIDs often require derivatization that presents bottlenecks to rapid analysis needed for large-scale studies. The development of electrospray ionization opened the gate for analyses based on high performance liquid chromatography-mass spectrometry (HPLC-MS), which has been more widely used for simultaneously monitoring multiple NSAIDs in biological matrix (22-32). But previously reported methods either detected a limited range of NSAIDs (22,23,26), or processed volumes ranging from

hundreds of microliters (24,30) to as much as 5 mL of plasma (25,27). There is value in minimizing the volume of sample diverted to monitor the patient's intake of drugs inasmuch as the volume of blood drawn from patients in clinical trials is limited and a large fraction of which needs to be used for assessing biomarkers. In this study, we aimed to develop a sensitive protocol capable of monitoring the intake of 14 NSAIDs (Figure 2.1) from analysis of tiny volumes (10 μL) of blood plasma within the last 24 hours. An additional goal has been to achieve instrumental throughput of more than 10 samples per hour. By applying this fast screening, we measured plasma NSAID levels in a clinical trial of COPD patients participating in pulmonary rehabilitation and compared these levels to their self-reported NSAID use from questionnaire.

2.2 Experimental

2.2.1 Chemicals

Salicylic acid (SA), sulindac (SUL), piroxicam (PX), ketoprofen (KPF), naproxen (NPX), nimesulide (NMS), flurbiprofen (FLB), indomethacin (IDO), diclofenac (sodium salt, DCF), niflumic acid (NFL), ibuprofen (IBP), phenylbutazone (PBZ), meclofenamic acid (sodium salt, MCL), tolfenamic acid (TFL) were purchased from Cayman Chemical (Ann Arbor, MI). Internal standard ketoprofen-d_3 was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade), methanol (HPLC grade), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using the Milli-Q System from Millipore Corp (Bedford, MA).

2.2.2 UHPLC-MS/MS conditions

A Waters Quattro Premier XE mass spectrometer coupled to a Waters ACQUITY UPLC system with a binary solvent manager was used for the analyses. The autosampler was cooled to 10 ˚C, and injection volume was set to 5 μL. Chromatographic separations of NSAIDs were performed on an Ascentis Express C18 column (2.1 × 50 mm; 2.7 μm particles;

Sigma-Aldrich, St. Louis, MO), and the column temperature was held at 50 ˚C. Analytes were separated using 0.001% formic acid in water (mobile phase A) and 0.001% formic acid in acetonitrile (mobile phase B) with a flow rate of 0.35 mL/min. The mobile phase gradient began at 1% B, followed by a linear increase to 70% B at 2 min; then to 90% B at 3 min, and 99% B at 3.5 min, at which the composition was held until 4 min; then returned to 1% B until 5 min. In order to minimize contamination of the ion source from non-retained plasma constituents, column eluent was diverted to waste from 0-1.5 min and after 4 min.

Analytes were detected using electrospray ionization with polarity switching between positive- and negative-ion modes using multiple reaction monitoring (MRM). The capillary was 2.5 kV for negative mode, and 3.0 kV for positive mode. Source and desolvation temperatures were 120 ˚C and 450 ˚C, respectively. Cone gas and desolvation gas flows were 20 and 600 L/h, respectively. MRM parameters including cone voltage and collision cell potentials were individually optimized using QuanOptimize software (Waters) by injecting each drug standard. Dwell time for each transition was set to 0.03 s, and inter-scan delay was 10 ms. Data were acquired using MassLynx 4.1 software (Waters).

2.2.3 Collection of self-reported medication information and plasma samples

Ethics statement: The research protocols were approved by the Institutional Review Board at Michigan State University (IRB #12-305I). Subject files and blood samples were de-identified.

The 26 subjects in this study were patients with chronic obstructive pulmonary disease (COPD) who were participants in a 12-week pulmonary rehabilitation program at McLaren Greater Lansing Hospital (Lansing, MI), and were recruited in a clinical trial. During enrollment in the study, subjects were asked to fill out questionnaires including a survey of all medications they were taking. Blood was drawn from the antecubital fossa into a Becton-Dickinson (Franklin Lakes, NJ) sodium heparin tube, and tubes were kept on ice

during transport from the clinic to the laboratory. Each tube was then centrifuged at 1100 × *g* for 10 min at 4˚C. Each supernatant plasma was transferred to a polypropylene cryostorage tube (VWR International) and stored at -80˚C until use.

2.2.4 Sample preparation

2.2.4.1 Protein precipitation and drug extraction

Cryotubes containing frozen plasma samples were thawed on ice for approximately 30 minutes. A 10-μL aliquot of plasma was removed from each and was transferred to a polypropylene microcentrifuge tube. Then 90 μL of methanol with internal standard (KPF-d_3, 200 ng/mL; 18 ng) was added. After vortexing for 1 min, the mixtures were centrifuged at 10,000 × *g* for 10 min at 4˚C. Fifty μL supernatant was mixed with 50 μL Milli-Q water and transferred to an amber autosampler vial with 250-μL glass insert in preparation for UHPLC-MS/MS analysis.

2.2.4.2 Preparation of stock solutions, calibration solutions and quality control samples

Stock solutions (1 mg/mL) of each NSAID were prepared in methanol and stored at -20 ˚C until use. Human plasma calibration solutions were prepared at different NSAID concentration levels in plasma from 1 to 20,000 ng/mL by spiking working standard mixture solutions into blank human plasma samples, which were derived from a pooled mixture from five independent volunteers not suffering from COPD. These blank plasma samples were assessed to be free of the 14 NSAIDs by UHPLC-MS/MS. Protein precipitation and internal standard addition followed the procedures described above. Quality control (QC) samples were prepared by spiking known amounts of standards in drug-free human plasma at three plasma concentration levels: 4,000 ng/mL (high, HQC), 1,000 ng/mL (middle, MQC), and 200 ng/mL (low, LQC).

2.2.5 Method validation

Response linearity was assessed using standard spiked calibration solutions with plasma NSAID concentrations ranging from 1 to 20,000 ng/mL. The calibration curve was built by plotting the ratio of peak areas of unlabeled compounds to that of the internal standard ($A_{analyte}/A_{IS}$) against concentrations of the unlabeled analytes, fitted by a weighted (1/x) least squares linear regression using the TargetLynx component of MassLynx v. 4.1 software (Waters). The lower limit of detection (LLOD) was defined as the concentration at which the peak height was three times that of the RMS noise (S/N=3), and the lower limit of quantification (LLOQ) was the concentration with a peak height corresponding to 10 times that of the RMS noise (S/N=10). Specificity of the method was established by analyzing blank human plasma.

The intra-day precision and accuracy were determined by analyzing three QC samples at different concentration levels in five replicates within one day. This process was repeated for three consecutive days to evaluate the inter-day precision and accuracy. Calibration solutions were prepared freshly each day. Accuracy was expressed as percent of the mean calculated concentration to nominal concentration, while precision was expressed as coefficient of variation.

To evaluate method recovery for each compound, standard cocktails were spiked into three drug-free plasma samples before extraction and centrifugation (pre), giving plasma concentration levels of 4000 ng/mL (HQC), 1000 ng/mL (MQC), and 200 ng/mL (LQC). The same amount of standard cocktail was added to extracts of another three drug-free plasma samples after extraction and centrifugation (post). Each sample was analyzed in five replicates. Recovery was calculated as the mean peak area ratio of pre- and post-extraction using the following equation (33):

$$\text{Recovery} = \frac{A_{pre}/A_{IS\text{-}pre}}{A_{post}/A_{IS\text{-}post}} \times 100\%$$

To determine matrix effects of plasma constituents, the same amount of standard mixtures at three concentration levels were added in methanol: water (1:1, v/v) and drug-free plasma extract. Matrix effect was calculated by comparing the peak area ratio of in pure solvent with that in post-extraction of plasma sample, using a modified equation (33):

$$\text{Matrix Effect} = \left(\frac{A_{post}/A_{IS\text{-}post}}{A_{solvent}/A_{IS\text{-}solvent}} - 1\right) \times 100\%$$

Short-term stability of the 14 NSAIDs in plasma extracts was investigated with MQC (1000 ng/mL). The solution was stored at 10 ˚C in the autosampler, and was tested 24 and 48 hours from the time of preparation. The solution was analyzed in three replicates at each time point, and stability was determined by the concentration percentage remained in the solution.

2.3 Results and discussion

2.3.1 MS conditions

Cone and collision voltages were optimized for each compound by flow injection of 10 μL of each individual standard solution at the concentration of 10 μM using Waters QuanOptimize software, and these are listed in Table 2.1. Both positive and negative electrospray ionization modes were evaluated in the optimization process. All of the NSAIDs could be detected in both modes, but with different signal levels. For example, SA, FLB and IBP gave lower detection limits in negative mode, while PBZ and SUL had greater response in positive mode. However, detection selectivity in the plasma matrix often plays a more important role in determining limits of detection than absolute signal levels. For instance, KPF was detected well in spiked plasma in both modes, but a coeluting interfering peak in the blank human plasma extracts showed up in negative mode, while no interference was observed in positive mode. In light of this issue, positive ion mode was selected for

monitoring KPF. For the other compounds, the polarity giving higher signal was chosen as listed in Table 2.1.

2.3.2 LC conditions

Optimization of chromatographic conditions was performed using a mixed standard of NSAIDs. As shown in Figure 2.2, all compounds eluted within 3.5 min. On the other hand, MCL and DCF are isomers with similar structures, and both analytes generated two fragment ions with *m/z* of 258 and 250. The transitions for *m/z* 294>258 and 294>250 are the most sensitive for MCL and DCF respectively, so in order to maintain high sensitivity and avoid interference between transitions, the LC gradient was adjusted to separate the two compounds. Different mobile phase compositions were evaluated, and the 5 min method with flow rate of 0.350 mL/min was found to completely resolve them. In order to get good ionization efficiency and column retention for all compounds, different buffer conditions were tested. Finally, 0.001% formic acid was chosen, since IBP and NPX were observed to give about 10-fold greater response in negative mode than in more acidic conditions. Figure 2.2 shows example chromatograms of fourteen target analytes spiked into drug-free human plasma samples from healthy volunteers.

Table 2.1. Optimized UHPLC-MS/MS parameters and analytical performance for each NSAID.

Compound	ESI ion mode	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone (V)	Collision (V)	Retention time (min)	R^2	Approximate linear range in plasma (ng/mL)	LLOQ in plasma (ng/mL)	LLOD in plasma (ng/mL)
SA	-	137	93	30	15	2.35	0.996	40-20,000	51	15
IBP	-	205	161	15	7	3.02	0.997	10-20,000	15	4.6
NPX	-	229	170	15	20	2.67	0.996	20-20,000	22	6.7
FLB	-	243	199	15	7	2.87	0.996	10-20,000	18	5.4
TLF	-	260	216	20	13	3.26	0.997	2-10,000	8.0	2.4
NFL	-	281	237	20	20	3.24	0.998	2-20,000	2.7	0.8
DCF	-	294	250	15	13	2.94	0.998	4-20,000	4.2	1.2
MCL	-	294	258	20	13	3.15	0.996	20-20,000	22	6.6
NMS	-	307	229	20	20	2.76	0.999	1-20,000	2.0	0.6
PX	-	330	146	20	20	2.60	0.998	2-20,000	6.6	2.0
IDO	-	356	312	14	7	2.92	0.997	4-20,000	4.4	1.3
KPF	+	255	105	20	25	2.65	0.998	10-20,000	11	3.3
PBZ	+	309	160	26	20	3.07	0.998	4-20,000	14	4.2
SUL	+	357	233	50	43	2.46	0.996	1-20,000	2.7	0.8

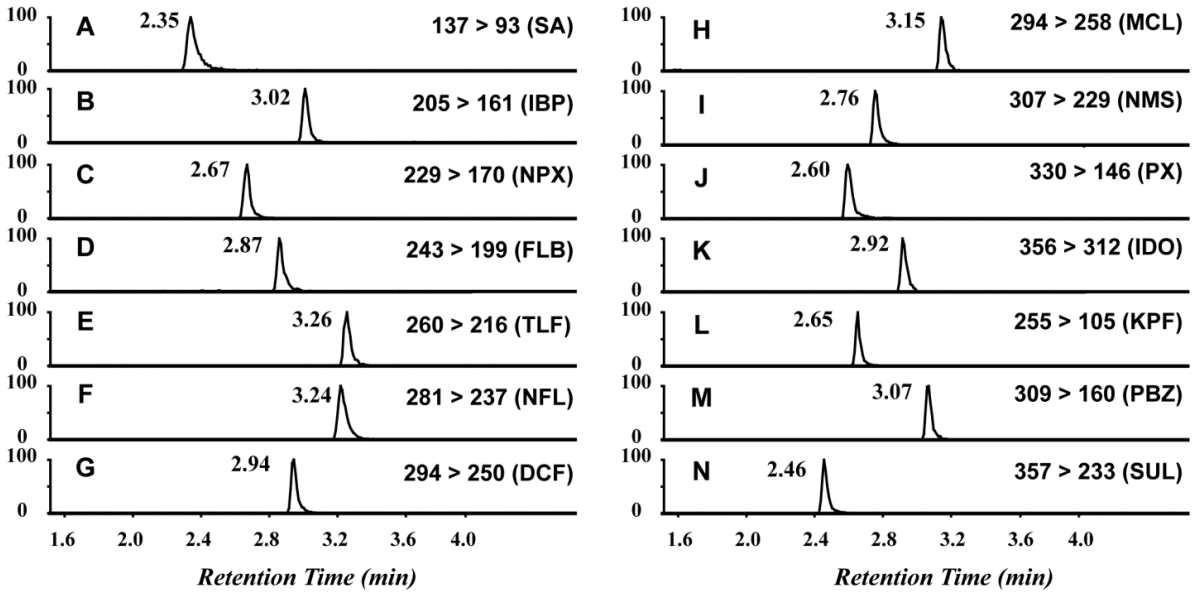

Figure 2.2. Representative multiple reaction monitoring chromatograms of fourteen target analytes. Analytes were spiked into drug-free human plasma at a concentration of 2 µg/mL. (A) salicylic acid; (B) ibuprofen; (C) naproxen; (D) flurbiprofen; (E) tolfenamic acid; (F) niflumic acid; (G) diclofenac; (H) meclofenamic acid; (I) nimesulide; (J) piroxicam; (K) indomethacin; (L) ketoprofen; (M) phenylbutazone; (N) sulindac.

2.3.3 Method validation

The range of linearity, LLOD, and LLOQ of each analyte determined in spiked drug-free plasma matrix are presented in Table 2.1 above. Calibration curves with at least six points were constructed over the plasma concentration range 1-20,000 ng/mL for all compounds using the regression of peak area ratio versus concentration. All calibration curves exhibited linear responses with correlation coefficients R^2 ranging from 0.996 to 0.999. The linear range varied for each compound, as listed in Table 2.1. LLOQ was calculated using S/N of 10, while LLOD was calculated using S/N of 3. LLOD varied from 0.6 ng/mL (NMS) to 15 ng/mL (SA). No significant endogenous interference peaks of the 14 drugs were observed in the chromatograms of blank plasma.

Chromatographic retention time reproducibility was assessed by analyzing quality control samples over three days at three concentration levels (4,000 ng/mL (high, HQC), 1,000 ng/mL (middle, MQC), and 200 ng/mL (low, LQC)). The coefficient variation of

retention time for all drugs ranged from 0.08% to 0.2% for intra-day analysis, and was between 0.08% and 0.3% for inter-day analysis (Table 2.2). These results demonstrated that the chromatographic separation was reproducible, and the drugs could be identified and measured with high confidence.

Intra-day and inter-day accuracy and precision results for each compound were summarized in Table 2.3. The intra-day accuracy was in the range from 91% to 115%, and the inter-day accuracy was between 90% and 111%. The precision, calculated as CV%, ranged from 2% to 13% for intra-day analysis, and 1% to 8.3% for inter-day assessment. This means for all the quality control analyses, calculated concentration fell between 80%-120% of the nominal concentration, and precision was less than 15%, indicating that our UHPLC-MS/MS method provided accurate and precise measurements over the range of the assay.

Table 2.4 shows that the recoveries were within the range of 86%-121%, with CV% less than 23% at all concentration levels for all compounds. By applying the simple protein precipitation procedures, we could get high recoveries with 10 µL of human plasma. Matrix effects usually become apparent in analyses of complex biological samples since co-eluting species may influence the ionization process. Matrix effects (ME) can either enhance ionization (positive ME% value) or suppress ionization (negative ME% value), and both effects were observed in the validation. The enhancement in response due to plasma matrix was less than 10%, while signal decrease was up to 29%, with all CV<18% except NPX with a CV of 24%. This indicated that the matrix effect caused by human plasma is small enough to allow for assessment of recent dosing of NSAIDs.

Table 2.5 summaries the stability of each analyte in MQC stored at 10 ˚C in the autosampler up to two days after preparation. After 24 hours and 48 hours under these conditions, the percentage concentration of all analytes in the solution were 100±8%. The

results demonstrated that the NSAIDs were stable for up to two days in the human plasma extraction matrix at 10 ˚C in the dark.

Table 2.2. Intra- and inter-day UHPLC retention times, assessed by running quality control samples over three days at three concentration levels, reported in minutes.

Compound	Intra-day(n=15)			Inter-day(n=45)		
	Mean	SD	CV(%)	Mean	SD	CV(%)
SA	2.35	0.003	0.11	2.35	0.007	0.29
IBP	3.02	0.005	0.17	3.02	0.005	0.17
NPX	2.67	0.004	0.14	2.67	0.003	0.11
FLB	2.87	0.004	0.14	2.87	0.004	0.15
TLF	3.26	0.004	0.11	3.26	0.003	0.10
NFL	3.24	0.005	0.15	3.24	0.006	0.17
DCF	2.94	0.005	0.17	2.94	0.005	0.16
MCL	3.15	0.006	0.19	3.15	0.005	0.15
NMS	2.76	0.005	0.18	2.76	0.003	0.12
PX	2.60	0.003	0.10	2.60	0.002	0.08
IDO	2.92	0.004	0.12	2.92	0.003	0.11
KPF	2.65	0.004	0.13	2.65	0.004	0.15
PBZ	3.07	0.003	0.08	3.07	0.003	0.09
SUL	2.46	0.003	0.10	2.46	0.005	0.19

Table 2.3. Intra-day (n=5) and inter-day (n=3) precision and accuracy for the fourteen NSAIDs. Precision and accuracy were determined from spiking blank human plasma samples with fourteen NSAIDs at 200 (low, LQC), 1000 (middle, MQC), 4000 ng/mL (high, MQC). (Concentrations are expressed as Mean ± SD.)

Com-pound	Nominal Conc. In plasma (ng/mL)	Intraday (n=5)			Interday (n=3)		
		Calc. conc. (ng/mL)	Preci-sion (CV,%)	Accu-racy (%)	Calc. conc. (ng/mL)	Preci-sion (CV,%)	Accu-racy (%)
SA	200	228 ± 15	6.6	114	210 ± 17	8.3	105
	1000	917 ± 58	6.3	92	902 ± 15	1.7	90
	4000	3815 ± 174	4.6	95	3804 ± 99	2.6	95
IBP	200	213 ± 26	12.0	107	218 ± 9	3.9	109
	1000	1014 ± 24	2.4	101	952 ± 55	5.8	95
	4000	4036 ± 140	3.5	101	4028 ± 104	2.6	101
NPX	200	200 ± 16	8.1	100	205 ± 12	5.7	103
	1000	987 ± 63	6.3	99	954 ± 29	3.1	95
	4000	4094 ± 113	2.8	102	4082 ± 128	3.1	102
FLB	200	212 ± 19	9.2	106	208 ± 5	2.3	104
	1000	1009 ± 46	4.6	101	1001 ± 31	3.1	100
	4000	4094 ± 155	3.8	102	3907 ± 194	5.0	98
TLF	200	197 ± 26	12.9	99	194 ± 3	1.5	97
	1000	1029 ± 46	4.5	103	974 ± 52	5.4	97
	4000	3919 ± 141	3.6	98	3780 ± 120	3.2	94
NFL	200	218 ± 7	3.1	109	200 ± 15	7.7	100
	1000	995 ± 52	5.2	100	961 ± 33	3.4	96
	4000	3767 ± 75	2.0	94	3949 ± 161	4.1	99
DCF	200	225 ± 13	5.8	112	216 ± 10	4.7	108
	1000	1006 ± 21	2.1	101	990 ± 34	3.4	99
	4000	4134 ± 113	2.7	103	4003 ± 167	4.2	100
MCL	200	229 ± 30	13.0	115	222 ± 6	2.8	111
	1000	1092 ± 57	5.2	109	1015 ± 73	7.2	102
	4000	4329 ± 210	4.9	108	4163 ± 206	4.9	104
NMS	200	204 ± 11	5.4	102	204 ± 9	4.6	102
	1000	906 ± 55	6.0	91	947 ± 40	4.3	95
	4000	3678 ± 136	3.7	92	3895 ± 196	5.0	97
PX	200	221 ± 19	8.4	111	222 ± 7	3.3	111
	1000	963 ± 44	4.6	96	949 ± 27	2.8	95
	4000	3528 ± 86	2.9	102	3741 ± 286	7.6	94
IDO	200	201 ± 20	9.8	100	203 ± 14	7.0	101
	1000	948 ± 84	8.9	95	957 ± 9	1.0	96
	4000	4257 ± 269	6.3	106	4070 ± 171	4.2	102
KPF	200	204 ± 11	5.5	102	206 ± 8	3.8	103
	1000	1003 ± 42	4.2	100	974 ± 37	3.8	97
	4000	4201 ± 148	3.5	105	4035 ± 203	5.0	101

Table 2.3 (cont'd)

PBZ	200	208 ± 15	7.1	104	198 ± 16	8.0	99
	1000	969 ± 36	3.7	97	993 ± 35	3.5	99
	4000	4101 ± 89	2.2	103	4100 ± 43	1.0	103
SUL	200	210 ± 13	6.1	105	204 ± 8	4.0	102
	1000	959 ± 52	5.4	96	991 ± 61	6.2	99
	4000	4201 ± 148	3.5	105	4003 ± 248	6.2	100

Table 2.4. Matrix effect and recovery for the fourteen NSAIDs. Matrix effect and recovery were determined from spiking blank human plasma samples (n=5) with fourteen NSAIDs at 200 (low, LQC), 1000 (middle, MQC), 4000 ng/mL (high, MQC).

Compound	Nominal Conc. (ng/mL)	Recovery (%)	CV (%)	ME (%)	CV (%)
SA	200	118	8	-29	9
	1000	108	14	-23	9
	4000	97	4	-3	4
IBP	200	104	12	-3	14
	1000	86	16	-17	9
	4000	101	7	-5	8
NPX	200	109	22	-14	24
	1000	97	9	3	10
	4000	96	8	10	9
FLB	200	101	17	-13	18
	1000	107	13	-1	13
	4000	97	17	-8	17
TLF	200	121	9	1	8
	1000	104	7	-10	7
	4000	101	2	2	3
NFL	200	106	4	9	4
	1000	105	7	0	6
	4000	100	4	7	3
DCF	200	113	8	-17	8
	1000	104	4	-5	6
	4000	100	3	5	4
MCL	200	94	17	-20	12
	1000	104	10	-21	9
	4000	100	6	-4	5
NMS	200	103	6	4	6
	1000	106	6	-1	7
	4000	99	3	9	3
PX	200	109	8	-11	3
	1000	103	4	-4	4
	4000	102	3	3	2
IDO	200	112	11	-12	12
	1000	110	8	-15	6
	4000	100	4	0	5
KFP	200	107	7	-6	6
	1000	104	8	-2	7
	4000	99	5	4	6
PBZ	200	117	10	-19	11
	1000	102	5	-15	6
	4000	101	4	-6	5
SUL	200	107	8	-14	6
	1000	103	3	-12	4
	4000	100	3	-2	5

Table 2.5. Concentration variation of the fourteen NSAIDs in the stability test at 10 ˚C in the auto-sampler under dark conditions during 48 hours.

Compound	24 hour concentration percent change (%)	48 hour concentration percent change (%)
SA	-1.1	-4.3
IBP	+5.0	+4.6
NPX	-3.5	+1.3
FLB	-7.1	-4.6
TLF	+2.1	+5.3
NFL	-3.3	-2.0
DCF	+2.4	+1.9
MCL	+4.4	+6.9
NMS	-0.5	-2.2
PX	+1.3	+0.2
IDO	+0.0	-1.9
KPF	+1.3	+2.6
PBZ	+1.0	-5.2
SUL	+3.1	+0.3

2.3.4 Analysis of human plasma samples

In a clinical study involving patients with COPD, we sought to analyze bioactive lipid mediators in the plasma of the subjects. Strikingly, the downstream metabolites from COX metabolism of fatty acids (*e.g.* prostaglandins) were present at negligible levels, if detected at all. This observation led us to suspect the use of NSAIDs by most research subjects since the drugs are inhibitors of COX enzymes that produce prostaglandins and other oxylipin metabolites. Thus, we applied the UHPLC-MS/MS method to monitor the presence of the 14 NSAIDs in the plasma samples. Figure 2.3 shows the levels of the analytes observed in the 193 plasma samples collected from 26 subjects at different time points. In total, at least one NSAID was detected in plasma specimens collected from 24 of 26 (92%) subjects (153 plasma samples). Among them, the aspirin metabolite salicylic acid was detected in 18 subjects, ibuprofen was detected in plasma from 15 subjects, naproxen was detected in plasma from 6 subjects, and tolfenamic acid and sulindac were detected in 1 subject each (Appendix Table A1). This is to be contrasted with the questionnaires completed by the

subjects: aspirin was the lone self-reported medication by three subjects (salicylic acid was detected in the plasma of each).

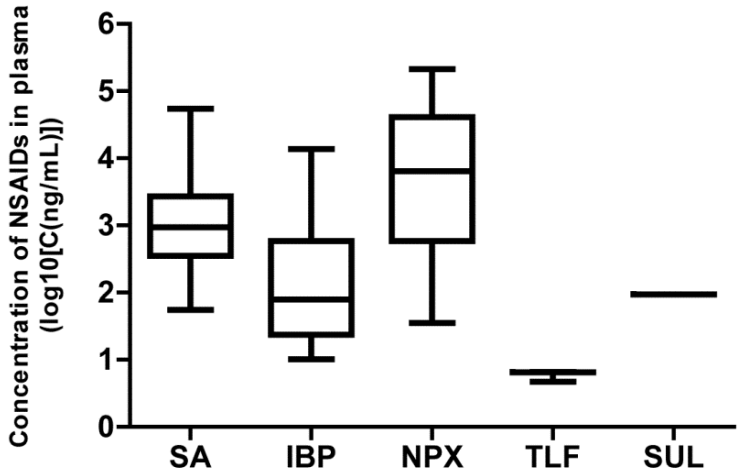

Figure 2.3. Boxplot of observed concentrations of NSAIDs in human plasma samples. NSAIDs were detected by UHPLC-MS/MS in 24 individuals (153 plasma samples) out of 26 individuals (193 plasma samples), collected over a twelve-week period at different time points for each research subject. Among them, 18 subjects tested positive for aspirin (based on measurement of its metabolite salicylic acid); 15 subjects tested positive for ibuprofen; 6 subjects tested positive for naproxen; 1 subject each was positive for tolfenamic acid and sulindac.

One notable finding is that plasma from 13 of 26 (50%) subjects had detectable levels of more than one NSAID, with 4 subjects testing positive for three different NSAIDs. Figure 2.4 shows example UHPLC-MS/MS MRM chromatograms of salicylic acid, ibuprofen, and naproxen from one plasma sample of one subject that tested positive for all three drugs at the same time. This finding suggests that researchers involved in clinical trials perhaps should be skeptical of medication self-reporting. Furthermore, the lowest concentrations detected in the plasma samples were 55, 10, 35, 5, and 94 ng/mL for salicylic acid, ibuprofen, naproxen, tolfenamic acid and sulindac, respectively. This means our method is capable for monitoring drugs with short half-lives, *e.g.* aspirin and ibuprofen, within 24 hours intake of a typical dose, as well as monitoring drugs with longer half-lives such as naproxen within 4-5 days intake of a typical dose. In separate analyses, acetaminophen and celecoxib were assayed in all plasma samples using LC-MS/MS, but neither of the drugs was detected in any plasma sample.

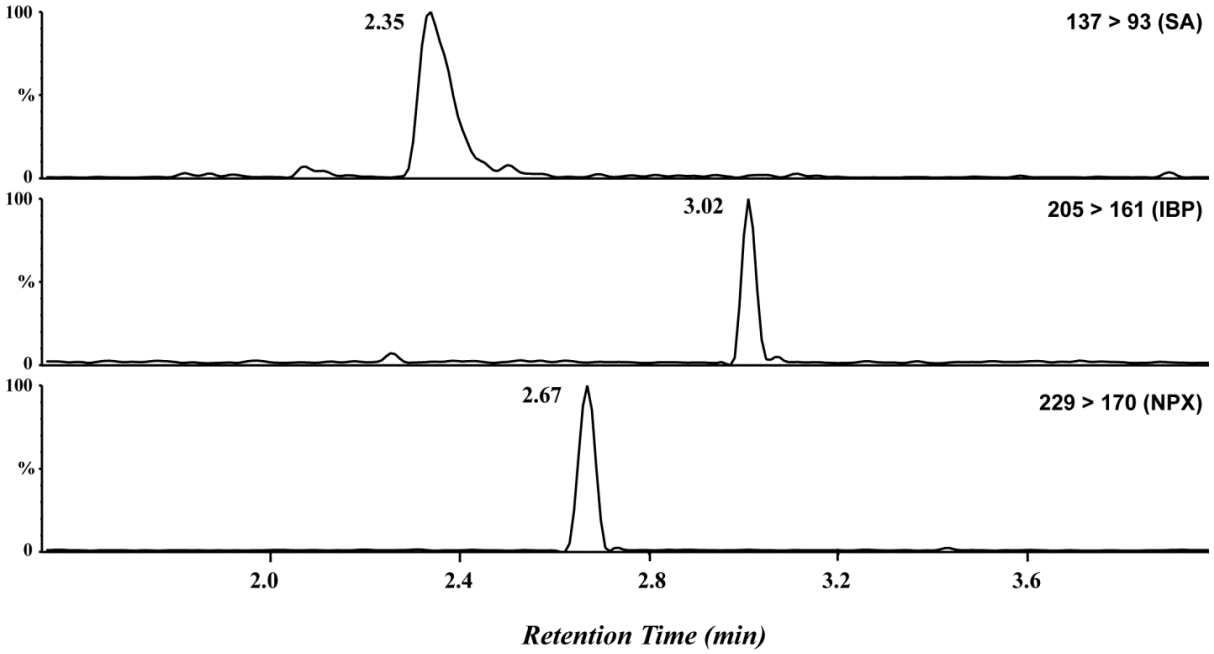

Figure 2.4. Representative multiple reaction monitoring (MRM) chromatograms of salicylic acid, ibuprofen, and naproxen from one plasma sample of one patient.

Our findings show that about 90% of our subject group of COPD patients took one or more NSAIDs, but the accuracy of self-reported NSAID use was low (3/24=12.5%). The discrepancy might be attributed to poor recollection of medication use or fatigue in filling out medication use questionnaires. Our results also suggest an important role for profiling of NSAIDs, particularly in populations likely to consume NSAIDs for various reasons. Though prior research has reported that self-reporting may be inaccurate, few comparisons of self-reported drug use and validated drug screening have been reported in the literature. In a study applying untargeted LC-MS/MS to analyze urine samples from pregnant women, the level of accordance of the questionnaire and urinary profiling was low, about 20% for all tested groups (34). One recent report used metabolite data obtained from ^{1}H NMR analysis of human urine specimens to validate self-reported ibuprofen and acetaminophen intake, and the rate of underreporting was 15-17% among 496 participants from Western populations (35). It is noticed that the participants described in this report were recruited from general

populations aged 40-59 and were considered “healthy”, and this may explain why the accuracy of self-reported drug use in this paper was much higher than our findings.

2.4 Conclusions

A rapid, simple and sensitive analytical method has been developed for determination of fourteen NSAIDs in plasma by UHPLC-MS/MS including salicylic acid (aspirin), sulindac, piroxicam, ketoprofen, naproxen, nimesulide, flurbiprofen, indomethacin, diclofenac, niflumic acid, ibuprofen, phenylbutazone, meclofenamic acid, and tolfenamic acid. The method allows the analysis of a wide variety of drugs of different NSAID sub-classes from only 10 µL of plasma using a simple sample preparation method. The method was validated, showed robust analytical performance, and was successfully applied to monitor levels of NSAIDs in COPD patients enrolled in a pulmonary rehabilitation program. Among 26 human subjects, 24 (92%) tested positive for at least one NSAID. However, only three patients had reported taking aspirin in their medicine survey. We anticipate that this approach might be used, with slight modifications, to monitor NSAIDs in plasma or serum as well as dried blood spots.

APPENDIX

APPENDIX

Table A1. Measured plasma NSAID concentration for each sample. For each patient, blood samples were drawn before, after and 72 hours after pulmonary rehabilitation exercises at week 0, 6 and 12 during the pulmonary rehabilitation program. Reported concentrations are in units of µg/mL plasma.

				NSAIDs				
Subject number	Self-reported NSAID use	Week	Time	SA	IBP	NPX	TLF	SUL
1	Aspirin	0	pre	1.48	n.d.	n.d.	n.d.	n.d.
		0	post	0.56	n.d.	n.d.	n.d.	n.d.
		6	pre	0.16	n.d.	n.d.	n.d.	n.d.
		6	post	0.17	n.d.	n.d.	n.d.	n.d.
		6	72 post	0.25	n.d.	n.d.	n.d.	n.d.
		12	pre	0.58	n.d.	n.d.	n.d.	n.d.
		12	post	0.14	n.d.	n.d.	n.d.	n.d.
		12	72 post	1.07	n.d.	n.d.	n.d.	n.d.
2	None	0	pre	1.05	0.043	n.d.	n.d.	n.d.
		0	post	0.44	0.024	n.d.	n.d.	n.d.
		6	pre	0.055	n.d.	0.35	n.d.	n.d.
		6	post	0.055	0.016	0.52	n.d.	n.d.
		6	72 post	0.51	0.021	15.9	n.d.	n.d.
		12	pre	0.99	0.024	0.042	n.d.	n.d.
		12	post	0.39	0.027	0.11	n.d.	n.d.
		12	72 post	1.29	n.d.	0.035	n.d.	n.d.
3	None	0	pre	54.6*	n.d.	n.d.	n.d.	n.d.
		0	post	14.8	n.d.	n.d.	n.d.	n.d.
		0	72 post	23.1*	n.d.	n.d.	n.d.	n.d.
		6	pre	35.3*	n.d.	n.d.	n.d.	n.d.
		6	post	21.8*	n.d.	n.d.	n.d.	n.d.
		12	pre	33.4*	n.d.	n.d.	n.d.	n.d.
		12	post	28.2*	n.d.	n.d.	n.d.	n.d.
		12	72 post	18.1	n.d.	n.d.	n.d.	n.d.
4	None	0	pre	n.d.	13.8	n.d.	n.d.	n.d.
		0	post	n.d.	2.58	n.d.	n.d.	n.d.
		0	72 post	n.d.	5.57	n.d.	n.d.	n.d.
		6	pre	n.d.	10.9	n.d.	n.d.	n.d.
		6	post	n.d.	9.28	n.d.	n.d.	n.d.
		6	72 post	n.d.	0.77	n.d.	n.d.	n.d.
		12	pre	n.d.	1.90	n.d.	n.d.	n.d.
		12	post	n.d.	0.98	n.d.	n.d.	n.d.
		12	72 post	n.d.	1.43	n.d.	n.d.	n.d.

Table A1 (cont'd)

5	None	0	pre	10.6	n.d.	n.d.	n.d.	n.d.
		0	post	0.94	n.d.	n.d.	n.d.	n.d.
		0	72 post	1.49	n.d.	n.d.	n.d.	n.d.
		6	pre	0.66	n.d.	n.d.	n.d.	n.d.
		6	72 post	0.44	n.d.	n.d.	n.d.	n.d.
		12	pre	0.17	n.d.	n.d.	n.d.	n.d.
		12	post	0.14	n.d.	n.d.	n.d.	n.d.
		12	72 post	0.29	n.d.	n.d.	n.d.	n.d.
6	None	0	pre	n.d.	0.12	n.d.	n.d.	n.d.
		0	post	n.d.	0.046	n.d.	n.d.	n.d.
		0	72 post	n.d.	0.11	n.d.	n.d.	n.d.
		6	pre	n.d.	0.027	n.d.	n.d.	n.d.
		6	post	n.d.	0.079	n.d.	n.d.	n.d.
		6	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		12	post	n.d.	0.062	n.d.	n.d.	n.d.
		12	72 post	n.d.	0.019	n.d.	n.d.	n.d.
7	None	0	pre	0.27	n.d.	n.d.	n.d.	n.d.
		0	post	n.d.	n.d.	n.d.	n.d.	n.d.
		0	72 post	1.87	n.d.	n.d.	n.d.	n.d.
		6	pre	3.69	n.d.	n.d.	n.d.	n.d.
		6	post	3.14	n.d.	n.d.	n.d.	n.d.
		12	pre	2.61	n.d.	n.d.	n.d.	n.d.
		12	post	2.22	n.d.	n.d.	n.d.	n.d.
		12	72 post	1.49	n.d.	n.d.	n.d.	n.d.
8	None	0	pre	6.38	0.087	n.d.	n.d.	n.d.
		0	post	4.34	0.089	n.d.	n.d.	n.d.
		0	72 post	4.17	0.077	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	n.d.	0.017	n.d.	n.d.	n.d.
		6	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	2.35	0.033	n.d.	n.d.	n.d.
		12	post	0.37	0.11	n.d.	n.d.	n.d.
		12	72 post	2.53	0.051	n.d.	n.d.	n.d.
9	None	0	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		0	post	n.d.	n.d.	n.d.	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		12	post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	72 post	n.d.	n.d.	n.d.	n.d.	n.d.

Table A1 (cont'd)

10	None	0	pre	n.d.	n.d.	n.d.	0.007**	n.d.
		0	post	n.d.	n.d.	n.d.	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	0.005**	n.d.
		6	pre	n.d.	n.d.	n.d.	0.006**	n.d.
		6	post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	72 post	0.077	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		12	post	0.10	n.d.	n.d.	n.d.	n.d.
		12	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
11	None	0	pre	n.d.	0.035	n.d.	n.d.	n.d.
		0	post	n.d.	0.018	n.d.	n.d.	n.d.
		0	72 post	n.d.	0.019	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	4.64	n.d.	n.d.	n.d.
		12	post	n.d.	2.28	n.d.	n.d.	n.d.
		12	72 post	n.d.	5.76	n.d.	n.d.	n.d.
12	None	0	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		0	post	n.d.	n.d.	n.d.	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	n.d.	n.d.	n.d.	n.d.	n.d.
		6	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		12	post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
13	Aspirin	0	pre	2.35	0.016	n.d.	n.d.	n.d.
		0	post	0.35	0.014**	n.d.	n.d.	n.d.
		0	72 post	1.03	n.d.	n.d.	n.d.	n.d.
		6	pre	1.30	n.d.	n.d.	n.d.	n.d.
		6	post	0.88	n.d.	n.d.	n.d.	n.d.
		6	72 post	1.23	n.d.	n.d.	n.d.	n.d.
		12	pre	0.93	n.d.	n.d.	n.d.	n.d.
		12	post	0.44	n.d.	n.d.	n.d.	n.d.
		12	72 post	1.40	0.030	n.d.	n.d.	n.d.
14	None	0	pre	1.32	n.d.	n.d.	n.d.	n.d.
		0	post	0.42	n.d.	n.d.	n.d.	n.d.
		0	72 post	1.33	n.d.	n.d.	n.d.	n.d.
		6	pre	1.92	n.d.	n.d.	n.d.	n.d.
		6	post	0.46	n.d.	n.d.	n.d.	n.d.
		6	72 post	0.49	n.d.	n.d.	n.d.	n.d.
		12	pre	0.31	n.d.	n.d.	n.d.	n.d.
		12	post	0.37	n.d.	n.d.	n.d.	n.d.

Table A1 (cont'd)

15	None	0	pre	n.d.	0.026	1.00	n.d.	n.d.
		0	post	n.d.	0.023	0.29	n.d.	n.d.
		0	72 post	n.d.	n.d.	34.9*	n.d.	n.d.
		6	pre	n.d.	n.d.	0.70	n.d.	n.d.
		6	post	n.d.	n.d.	0.58	n.d.	n.d.
		12	pre	n.d.	n.d.	105.9*	n.d.	n.d.
		12	post	n.d.	n.d.	53.1*	n.d.	n.d.
		12	72 post	n.d.	0.049	18.7	n.d.	n.d.
16	None	0	pre	1.91	0.48	n.d.	n.d.	n.d.
		0	post	0.21	0.48	n.d.	n.d.	n.d.
		0	72 post	0.38	0.28	n.d.	n.d.	n.d.
		6	pre	0.57	0.23	n.d.	n.d.	n.d.
		6	post	0.32	0.43	n.d.	n.d.	n.d.
		6	72 post	0.12	0.12	n.d.	n.d.	n.d.
		12	pre	0.68	11.2	n.d.	n.d.	n.d.
		12	post	0.33	5.13	n.d.	n.d.	n.d.
		12	72 post	0.76	0.59	n.d.	n.d.	n.d.
17	None	0	pre	n.d.	0.072	n.d.	n.d.	n.d.
		0	post	3.53	0.021	n.d.	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
18	None	0	pre	20.0	0.26	70.6*	n.d.	n.d.
		0	post	1.94	0.11	19.5	n.d.	n.d.
		0	72 post	9.6	0.21	1.03	n.d.	n.d.
		6	pre	n.d.	0.028	44.0*	n.d.	n.d.
		6	post	n.d.	0.021	47.1*	n.d.	n.d.
		6	72 post	n.d.	0.11	2.75	n.d.	n.d.
		12	pre	n.d.	0.011**	44.9*	n.d.	n.d.
		12	post	n.d.	0.053	35.1*	n.d.	n.d.
		12	72 post	n.d.	0.025	57.5*	n.d.	n.d.
19	None	0	post	5.8	0.043	n.d.	n.d.	n.d.
		0	72 post	5.0	n.d.	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	9.1	0.012**	n.d.	n.d.	n.d.
		6	72 post	8.2	n.d.	n.d.	n.d.	n.d.
20	None	0	pre	n.d.	4.3	n.d.	n.d.	n.d.
		0	post	0.17	4.4	n.d.	n.d.	n.d.
		0	72 post	n.d.	0.59	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		6	post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	pre	n.d.	12.9	n.d.	n.d.	n.d.
		12	post	n.d.	7.6	n.d.	n.d.	n.d.
		12	72 post	n.d.	0.73	n.d.	n.d.	0.09

Table A1 (cont'd)

21	None	0	pre	n.d.	n.d.	214.8*	n.d.	n.d.
		0	post	n.d.	0.010**	43.8*	n.d.	n.d.
		0	72 post	n.d.	0.017	23.0*	n.d.	n.d.
		6	pre	n.d.	0.022	0.62	n.d.	n.d.
		6	post	n.d.	n.d.	0.84	n.d.	n.d.
		6	72 post	n.d.	n.d.	0.25	n.d.	n.d.
		12	pre	n.d.	n.d.	6.3	n.d.	n.d.
		12	post	n.d.	n.d.	12.6	n.d.	n.d.
		12	72 post	n.d.	n.d.	2.8	n.d.	n.d.
22	None	0	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		0	post	n.d.	0.035	n.d.	n.d.	n.d.
		0	72 post	n.d.	0.021	n.d.	n.d.	n.d.
		6	pre	n.d.	0.012**	n.d.	n.d.	n.d.
		6	post	n.d.	0.016	n.d.	n.d.	n.d.
		12	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		12	post	n.d.	n.d.	n.d.	n.d.	n.d.
		12	72 post	n.d.	0.15	n.d.	n.d.	n.d.
23	Aspirin	0	pre	0.048	0.46	n.d.	n.d.	n.d.
		0	post	2.74	0.32	0.039	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
24	None	0	pre	n.d.	n.d.	n.d.	n.d.	n.d.
		0	post	0.22	n.d.	n.d.	n.d.	n.d.
		0	72 post	n.d.	n.d.	n.d.	n.d.	n.d.
25	None	0	pre	0.30	n.d.	n.d.	n.d.	n.d.
		0	post	0.39	n.d.	n.d.	n.d.	n.d.
		0	72 post	0.094	n.d.	n.d.	n.d.	n.d.
		6	pre	n.d.	n.d.	3.8	n.d.	n.d.
		6	post	n.d.	n.d.	6.6	n.d.	n.d.
		6	72 post	n.d.	n.d.	17.6	n.d.	n.d.
26	None	0	pre	0.39	n.d.	n.d.	n.d.	n.d.
		0	post	0.73	n.d.	n.d.	n.d.	n.d.

n.d. = Not detected

* Measured concentration higher than the highest calibration point

** Measured concentration lower than LLOQ but higher than LLOD

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Chapter 3 Untargeted Metabolomics Strategies for Biomarker Discovery: Application to Pulmonary Rehabilitation for COPD

3.1 Introduction

Chronic obstructive pulmonary disease (COPD), characterized by incompletely reversible airflow limitation, is one of the major causes of morbidity and mortality throughout the world with an overall prevalence currently at 10% in adults over 40 years old (1). It is presently the fifth leading cause of death worldwide, but is expected to become the third leading cause by 2030 (2,3). Conventional management of COPD includes pharmacologic therapy and long-term oxygen therapy, but COPD is a progressively degenerative disease insensitive to current marketed drugs and no cure on the horizon (4). Therefore, there is great value in developing complementary and alternative treatments and evaluating their efficacy. Pulmonary rehabilitation (PR) based on medical treatments, exercise training, and nutritional counseling is often prescribed (5,6). Physical activity serves as the core of PR programs. Participants are recommended to have 60-70% of the maximum symptom-limited exercise capacity for 20-30 minutes (7). Osteopathic manipulative treatment (OMT), the hands-on manual medical system, has also long been employed for patients with respiratory disorders (8). Even though increasing numbers of PR and OMT trials are being reported, research on the underlying biochemical mechanisms of these alternative medicines' effects on COPD has been limited. Metabolomics can serve as a useful tool to help understanding the underlying biochemistry.

Metabolomics is the youngest "omics" technique, following genomics, transcriptomics and proteomics, and has already shown its potential in solving many biological problems (9,10). Since metabolic state is representative of the overall physiologic status of the organism, if there is any change of a phenotype that may be caused by a physiological progression, disease development, or medical treatment and drugs, the level of

some particular endogenous metabolites, which can serve as biomarkers, will be altered compared to a control phenotype (11,12). Accordingly, analysis of these biomarkers can suggest the underlying mechanisms of diseases and facilitate evaluation of the efficacy of a specific therapeutic intervention.

A few thousands of endogenous metabolites exist in biological systems, so untargeted analyses are usually the starting point for potential biomarkers discovery. Untargeted metabolomics emphasizes on profiling all detectable metabolites, which is applied to measure as many compounds (both identified and unknown compounds) as is feasible, aiming to obtain a comprehensive picture of all metabolites in a sample (13). A powerful analytical approach for untargeted metabolomics study should achieve high-throughput profiling and comprehensive coverage. Many instrumental systems including nuclear magnetic resonance spectroscopy (NMR) (14,15), mass spectrometry (MS) combined with capillary electrophoresis (CE), gas chromatography (GC), and liquid chromatography (LC) have been applied (16-21). HPLC-MS or UHPLC-MS coupled with advanced MS has been considered as the major platform now due to its reliability and wider coverage of metabolome. Furthermore, the development in high-resolution MS techniques, *e.g.*, quadruple-time-of-flight mass spectrometry (Q-TOF-MS) and Orbitrap-MS, has promoted the untargeted metabolomics research by the power of giving exact mass, which facilitates the identification of candidate biomarkers. Another important part of untargeted metabolic approach for selecting potential biomarkers is statistical analysis. Besides univariate analysis such as *t* test and analysis of variance (ANOVA) for evaluating the change of individual compound, multivariate analysis methods including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis(OPLS-DA) are often utilized to reveal the overall pattern. PCA is an unsupervised method and is usually applied first to investigate the general trend between groups. PLS-DA

and OPLS-DA are supervised approaches and are used to maximize the difference between groups and facilitate determining the potential biomarkers. Once the candidate biomarkers are selected, the compounds are identified based on the LC-MS result and database search.

Though metabolomics serves as a powerful tool for investigating molecular mechanisms, it has been rarely used to study the comprehensive biochemical responses of PR exercise training on COPD patients. One paper by Rodriguez *et al.* reported blood metabolome obtained pre- and post-exercise training from COPD patients and healthy subjects by NMR (22). The training significantly impacted metabolomics in healthy subjects with changes of levels of amino acids, creatine, and lactate in blood, but in contrast, only lactate decreased after exercise in COPD. This research was the only metabolomic investigation of COPD patents doing PR exercise, and showed that plasma metabolic profiling contributes to the phenotypic characterization of COPD patients.

In this chapter, we demonstrate a LC-MS based untargeted strategy for profiling human plasma metabolomes to discover candidate biomarkers for evaluating the effects of PR and OMT on COPD patients. The current LC-MS method, including sample preparation, was developed for large-scale metabolic profiling in plasma. Both univariate (*t*-test) and multivariate analysis (OPLS-DA) were applied in this research to investigate the changes in metabolites and to illuminate biochemical mechanism of the complementary treatments.

3.2 Materials and methods

3.2.1 Chemicals

HPLC grade methanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using the Milli-Q-System from Millipore Corp (Bedford, MA, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2 Subjects and clinical experiment design

Ethics statement: The research protocols were approved by the Institutional Review Board at Michigan State University (IRB #12-305I). Subject files were de-identified.

In this chapter, 21 COPD patients were recruited (April, 2013-December, 2014) from participants in a standard 12-week pulmonary rehabilitation (PR) program at the McLaren Ingham Regional Medical Center (IRMC) in Lansing, MI. In the pulmonary rehabilitation program, patients were doing exercise training in fitness gymnasium (*e.g.* walking and cycling exercise) every Monday and Thursday each week. Participants are recommended to have 60-70% of the maximum symptom-limited exercise capacity for 20-30 minutes. The subjects were randomized into three groups: (a) OMT (n=5) group receiving biweekly osteopathic structural exam (OSE) and OMT to affected areas in addition to standard PR; (b) sham group (n=7) receiving standard PR as well as biweekly OSE without OMT, thereby controlling for the effect of touch; (c) PR only group (n=9) were subjected to PR program doing exercise training only but not receiving OSE or OMT.

3.2.3 Plasma sample collection and preparation

Blood specimens were collected from subjects in the beginning of the study (Week 0), in the middle (Week 6), and at the end of the study (Week 12). At each of these weeks, blood was drawn before treatment (Monday), one hour after (Monday) and 72 hours after treatment (Thursday). Approximately 5 mL of blood was drawn from the antecubital fossa into a Becton-Dickinson sodium heparin tube, and tubes were kept on ice from collection and during transport from the clinic to the laboratory. Each tube was then centrifuged at 1100 × *g* for 10 min at 4˚C. The supernatant plasma was transferred into a cryostorage tube, and was stored at -80˚C until use.

Frozen plasma samples were thawed at room temperature. Fifty μL of thawed plasma was removed to a polypropylene microcentrifuge tube containing 200 μL of methanol for

simple protein precipitation and metabolite extraction. Each mixture was vortexed well, followed by centrifugation at 10,000 × *g* for 15 min at 4˚C. A 100-µL aliquot of each supernatant was then mixed with 50 µL of Milli-Q water, and was centrifuged at 10,000 × *g* for 5 min at 4˚C to remove debris. Supernatants were then transferred into an autosampler vial with low volume (250 µL) glass insert for LC-MS analysis. Quality control (QC) samples were prepared by pooling aliquots of 20 µL from 21 plasma samples (week 0 before treatment samples from each patient).

3.2.4 Plasma metabolite profiling by LC-MS

Samples were analyzed in a randomized order using liquid chromatography-time-of-flight mass spectrometry (LC-TOF-MS) on a Waters LCT Premier mass spectrometer (Waters Corp., Milford, MA) coupled to a Shimadzu LC-20AD system and a SIL-5000 autosampler (Shimadzu, Columbia, MD). A 10 µL aliquot of each extract was injected and the separation was achieved on an Ascentis Express C18 column (2.1 mm × 10 cm; 2.7 µm particles; Sigma-Aldrich, St. Louis, MO) maintained at 45 ˚C. Water with 0.15% formic acid was used as mobile phase A and methanol/acetonitrile (1:1, v/v) as mobile phase B. A 15-min gradient at a flow rate of 0.35 mL/min was applied for the separation, and the linear gradient elution program was as follows (A/B): 0-1.0 min (99/1), 1-2 min (program to 40/60), 2-7 min (0/100), hold until 13 min, and then the gradient returned to the initial condition. Mass spectrometric detection was achieved in negative-ion mode using electrospray ionization (ESI) and data collection over *m/z* 50-1500. Multiplexed collision-induced dissociation (CID) was performed by switching four acquisition functions using W-mode ion optics with aperture 1 voltages of 10, 30, 50 and 80 volts respectively for quasi-simultaneous generation of spectra without and with fragment ions. Spectrum acquisition time was set to 0.15 seconds for each function. Before analysis, ten pooled QC samples were analyzed to equilibrate the analytical platform. During the batch, one QC sample was run after every 10 biological samples.

3.2.5 Data analysis

Peak identification, integration, and retention time alignment were performed using Waters MarkerLynx software. Parameters for Markerlynx analysis were set as follows: mass window: 0.15 amu; retention time window: 0.2 min; peak width at 5% height: 20 s; intensity threshold: 10; noise elimination level: 6; mass tolerance: 0.1 Da; deisotope data: yes. Only data acquired from collision function two were processed in MarkerLynx; the fragment ions produced at higher energy functions were used for metabolite annotation. Processed results were exported as unscaled peak areas into a Microsoft Excel spreadsheet. The retention time-ion pairs (RT-*m/z*) were used as identifiers for the detected peaks. Each RT-*m/z* pair reported by MarkerLynx software was considered as a "marker". The peak area of each marker was then normalized to the total peak area of all markers in each sample such that the total area equaled 10000.

In order to assure the reproducibility and reliability of the markers for further analysis, QC filtering was performed by assessing the relative standard deviation (RSD%) of the normalized peak area of each marker in QC samples. Those markers with RSD% higher than 30% were removed. The resulting data matrix then consisted of RT-*m/z*, normalized peak areas and de-identified subject information.

Both univariate and multivariate analyses were performed for statistical analysis. Paired Student's *t* test (α=0.05) was performed using Microsoft Excel to assess the change of marker levels due to exercises/treatment. Data were $\log_2$-transformed to achieve normal distributions prior to Student's *t* test. For multivariate analysis, the data matrix was imported into EZinfo software (Umetrics Corp., Umea, Sweden). All variables were $\log_2$-transformed and Pareto scaled. Orthogonal partial least squared discriminant analysis (OPLS-DA), a supervised clustering method, was then performed to screen out potential biomarkers which had main contributions to group and time differences. The variable importance in projection

(VIP) values from OPLS-DA, reflecting relative contribution of each variable to the model, was used to selecting biomarkers that contributed most to discrimination of sample groups.

Potential biomarkers were annotated according from their m/z values generated by Tof-MS, relative mass defect (23), fragment ions at higher energy functions and online database searching including ChemSpider (http://www.chemspider.com/), KEGG (http://www.kegg.com/), Human Metabolome Database (HMDB) (http://www.hmdb.ca/), and LipidMaps (http://www.lipidmaps.org/).

3.3 Results and discussion

3.3.1 Metabolic profiling and metabolite identification

Metabolites in extracts of plasma from COPD patients were profiled using UHPLC/MS and revealed hundreds of metabolites, several of which were of high abundance (Figure 3.1). Automated peak processing detected 1273 markers (mass-retention time pairs) after removal of ions corresponding to naturally abundant stable isotopes such as ^{13}C, and these probably represent more than 500 distinct metabolites owing to multiple ions (*e.g.* $[M-H]^-$ and fragment ions). Given the complexity of the detected metabolites, coelution was common. Among the detected "markers", 154 met the filtering criteria from multiple technical replicates of quality control (QC samples), namely that RSD% < 30%, and these were considered "stable" markers and kept for further analysis (Appendix Table A2).

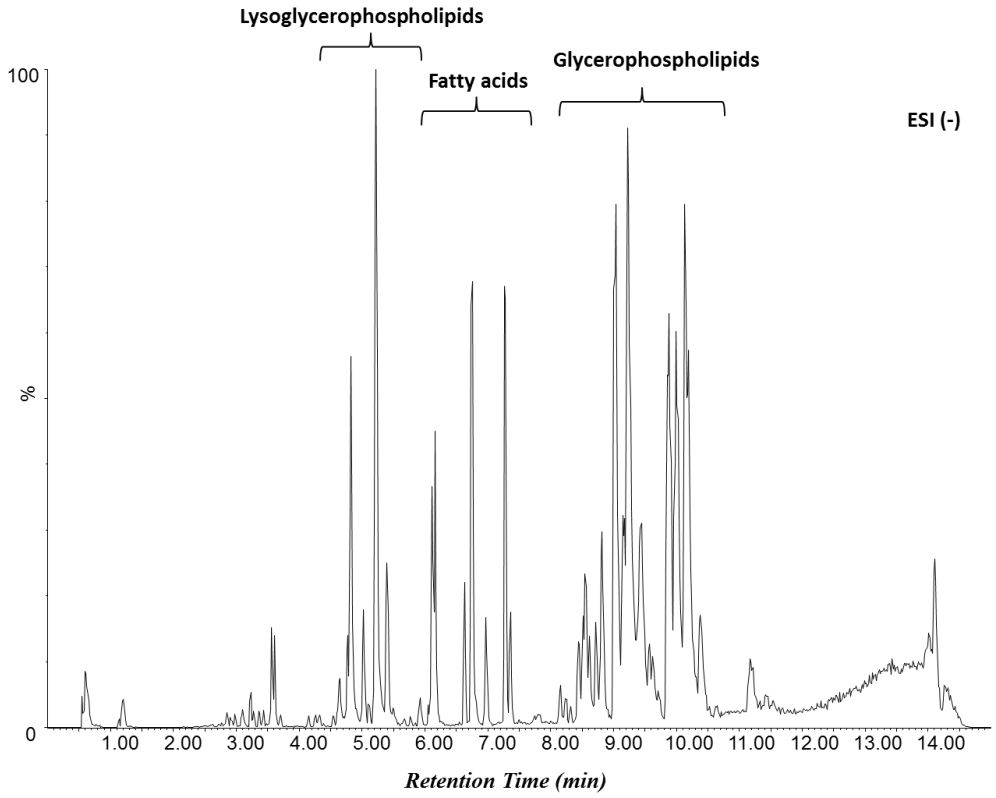


Figure 3.1. Example base peak intensity (BPI) LC/MS chromatogram of human plasma extract from a COPD patient. Mass spectra were acquired using negative mode electrospray ionization (using Aperture 1 collision voltage of 30V, function 2). Sections of the chromatogram are labeled with the most abundant metabolite categories detected. Automated peak processing by MarkerLynx software detected 1273 markers (mass-retention time pairs), and 154 met the QC filtering criteria (RSD% < 30%) and kept for further analysis.

To annotate the detected metabolites, the relative mass defect (RMD) was calculated from the ion *m/z* value for each marker. Absolute mass defect is defined as the deviation of the measured mass from its nominal mass (rounded to the nearest integer value), and RMD normalizes the mass defect to the measured mass, and is calculated as (mass defect/measured monoisotopic mass) × 10^6, expressed in parts-per-million (ppm) (23,24). Most elements heavier than nitrogen have negative mass defect (*e.g.*, oxygen -5.09 mD), and carbon has 0 mass defect. Hydrogen shows a slightly high positive mass defect (+7.83 mD) because it has no nuclear binding energy, and since most organic compounds have numerous hydrogen atoms, values of RMD reflect the fractional hydrogen content by weight (%H) (23). To illustrate, linoleate ($C_{18}H_{31}O_2^-$) has a hydrogen content of 11.2%, and the RMD of this ion is

834 ppm. The distribution of RMD values for the 154 stable markers is shown as a histogram in Figure 3.2. Most metabolites fell in the RMD range of 500-900 ppm, which corresponds to hydrogen content in the range of about 7-12% hydrogen by weight. This suggested that the majority of the markers are lipids, which are in rich of hydrogen atoms. Lipids consist of a diverse group of compounds and are the main structural components of cell membranes, and many individual lipids including fatty acids and phospholipids are abundant at µM levels in plasma. In this investigation, the extraction solvent (methanol) and chromatographic method (reverse phase) were suitable for extracting, retaining, and resolving lipid compounds, so most of the metabolites detected here were lipids.

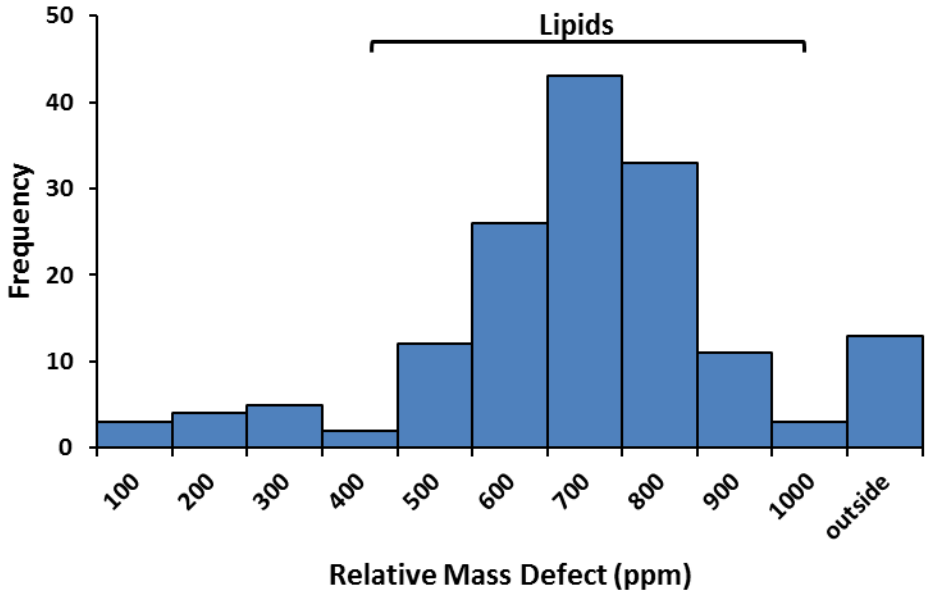


Figure 3.2. Histogram of Relative mass defect (RMD) values for 154 stable markers from human plasma extracts of COPD patients. Data were generated by LC-Tof MS in negative mode, and automated peak detection employed MarkerLynx software. RMD is calculated as (mass defect/measured monoisotopic mass) × 10^6, expressed in parts-per-million (ppm). Most metabolites fell in the RMD range of 500-900 ppm, which corresponds to hydrogen content in the range of about 7-12% hydrogen by weight. This suggested that the majority of the markers are lipids, which are in rich of hydrogen atoms.

Besides metabolite annotations based on values of RMD, high resolution (accurate mass) mass spectrometry also facilitated the identification of metabolite by providing the exact monoisotopic mass. Each exact mass was submitted for searching of online metabolite

databases including the Human Metabolome Database (HMDB) and LipidMaps for searching for matching molecular formulas. Furthermore, the characteristic fragment ions generated in higher collision energy functions aided identification. For example, the marker Rt-*m/z* 9.27_802.5557 has *m/z* of 802.5557 and was assigned to a formate adduct $[M+COOH]^-$ because in collision functions at higher energy, an ion corresponding to a neutral loss of 60 Da (*m/z* 742.5312, annotated as $[M+HCOO\text{-}CH_3OC(O)H]^-$) was detected. This neutral loss suggests a methyl substituted quaternary nitrogen, as found in phosphatidylcholine lipids, that transfers a methyl group to the formate, leading to neutral loss of methyl formate (60 Da). Ions observed at *m/z* 480.32 at higher collision energy correspond to a demethylated lysophosphatidylcholine ($C_{23}H_{47}NO_7P^-$) resulting from the further loss of a fatty acid ketene (fatty acid minus H_2O). Two carboxylate anion fragment ions at *m/z* 255.23 and 279.24 corresponded to fatty acid anions of palmitic acid (16:0) and linoleic acid (18:2) respectively. Thus, the marker Rt-*m/z* 9.27_802.5557 was identified as the phosphatidylcholine lipid PC (16:0/18:2).

3.3.2 Univariate analysis

Student's *t* test is one of the most widely used statistical methods for comparison of means between two datasets. Here, a paired *t* test (α=0.05) was performed for each treatment group (PR only, OMT and Sham) to compare differences in levels of each of the 154 markers between pre- and one hour post-exercise/treatment (short-term effect), and pre- and 72 hour post-exercises/treatment (long-term effect). Appendix Table A2 lists all 154 markers with fold changes of normalized peak area due to short-term and long-term effects, and P values from statistical analysis. The change was considered significant if $P < 0.05$. Most (~80%) of the changing metabolites exhibited slight decrease (less than 15%), and the majority of which were identified as lysophospholids and phospholipids; the metabolites with increased levels mostly exerted higher magnitude of elevation (>1.5-fold) (Appendix Table A2). In

comparisons of short-term effects, the levels of 82 metabolites altered significantly in the PR only group, while 33 and 27 metabolites had significant fold changes in the OMT and Sham groups respectively. On the other hand, for long-term effect, 32 metabolites gave statistically different levels in PR only group, while only 10 and 6 markers still had significant changes in the OMT and Sham groups. This suggested that, first, the pulmonary rehabilitation exercises alone had greater impacts on plasma metabolite profiles than additional OMT or Sham treatment for COPD patients in this clinical trial; second, the biochemical changes in plasma constituent levels due to exercises/treatment faded over 72 hours. Thus, we will focus on the analysis of short-term effects in PR only group in the following discussion.

Seventeen plasma metabolites in the PR group that exhibited more than 1.5-fold increase in the short term comparison are summarized in Table 3.1. Most (12 of 17) are free fatty acids including both saturated and unsaturated fatty acids, with palmitoleic acid (16:1) having the greatest increase (6.7-fold). Also, one cholesterol ester (cholesteryl decanoate, abbreviated as CE (10:0)) and one diacylglycerol (DG (15:0/20:4/0:0)) were identified having more than four-fold increases after exercise.

Table 3.1. Identification of plasma metabolites with more than 1.5-fold change one hour post-exercises from COPD patients (n=9) in PR only group. Blood specimens were drawn on weeks 0, 6, and 12 for each patient, so 27 pairs (n=27) in total were subjected for comparison. Data were generated using LC-MS and ESI in negative mode. Paired Student's *t* test (α=0.05) was used on $\log_2$-transformed normalized peak areas (normalized to total peak area) to compare the differences.

Marker (Rt_*m/z*)	Common name	Retention Time (min)	Formula	Measured mass (*m/z*)	Theoretical mass (*m/z*)	Short-term fold change (n=27)	
						Mean ± SEM	P Value
5.92_253.2198	Palmitoleic acid (16:1)	5.92	$C_{16}H_{29}O_2^-$	253.2198	253.2173	6.70 ± 1.85	< 0.001
6.62_255.2316	Palmitic acid (16:0)	6.62	$C_{16}H_{31}O_2^-$	255.2316	255.2330	2.68 ± 0.34	< 0.001
5.62_277.2202	*alpha*-Linolenic acid (18:3)	5.62	$C_{18}H_{29}O_2^-$	277.2202	277.2173	4.18 ± 0.93	< 0.001
6.17_279.2360	Linoleic acid (18:2)	6.17	$C_{18}H_{31}O_2^-$	279.2360	279.2330	3.31 ± 0.57	< 0.001
6.75_281.2516	Oleic acid (18:1)	6.75	$C_{18}H_{33}O_2^-$	281.2516	281.2486	4.08 ± 0.89	< 0.001
7.36_283.2613	Stearic acid (18:0)	7.36	$C_{18}H_{35}O_2^-$	283.2613	283.2643	1.60 ± 0.13	< 0.001
6.61_301.2166	Eicosapentaenoic acid (20:5)	6.61	$C_{20}H_{29}O_2^-$	301.2166	301.2173	2.58 ± 0.34	< 0.001
6.04_303.2307	Arachidonic acid (20:4)	6.04	$C_{20}H_{31}O_2^-$	303.2307	303.2330	1.69 ± 0.12	< 0.001
6.73_327.2362	Docosahexaenoic acid (22:6)	6.73	$C_{22}H_{31}O_2^-$	327.2362	327.2330	3.23 ± 0.52	< 0.001
6.19_329.2522	Docosapentaenoic acid (22:5)	7.36	$C_{22}H_{33}O_2^-$	329.2522	329.2486	1.60 ± 0.17	< 0.001
6.65_331.2674	Adrenic acid (22:4)	6.65	$C_{22}H_{35}O_2^-$	331.2674	331.2643	2.67 ± 0.44	< 0.001
6.73_365.3389	Nervonic acid (24:1)	6.73	$C_{24}H_{46}O_2^-$	365.3389	365.3425	3.17 ± 0.64	< 0.001
5.92_373.2475	unknown lipid	5.92		373.2475		2.02 ± 0.27	< 0.001
6.75_433.2072	unknown lipid	6.75		433.2072		1.95 ± 0.24	< 0.001
6.74_585.4946	CE(10:0)	6.74	$C_{37}H_{64}O_2$+$HCOO^-$	585.4946	585.4888	4.70 ± 1.22	< 0.001
6.18_597.4471	unknown lipid	6.18		597.4471		4.73 ± 1.17	< 0.001
6.74_601.4779	DG(15:0/20:4/0:0)	6.74	$C_{38}H_{65}O_5^-$	601.4779	601.4832	5.69 ± 2.26	< 0.001

3.3.3 OPLS-DA

Multivariate statistical procedures were applied to further compare metabolic profiles pre- and one hour post-exercise in PR only group. The unsupervised PCA approach was employed first to the dataset but no separation was obtained. Thus, the supervised OPLS-DA was performed, which achieved better discrimination between two blocks. Figure 3.3 displays the OPLS-DA scores plot of the first two components, which shows most of the pre-exercise samples grouped to the left of the y-axis, and most of the post-exercise samples grouped to the right of the axis.

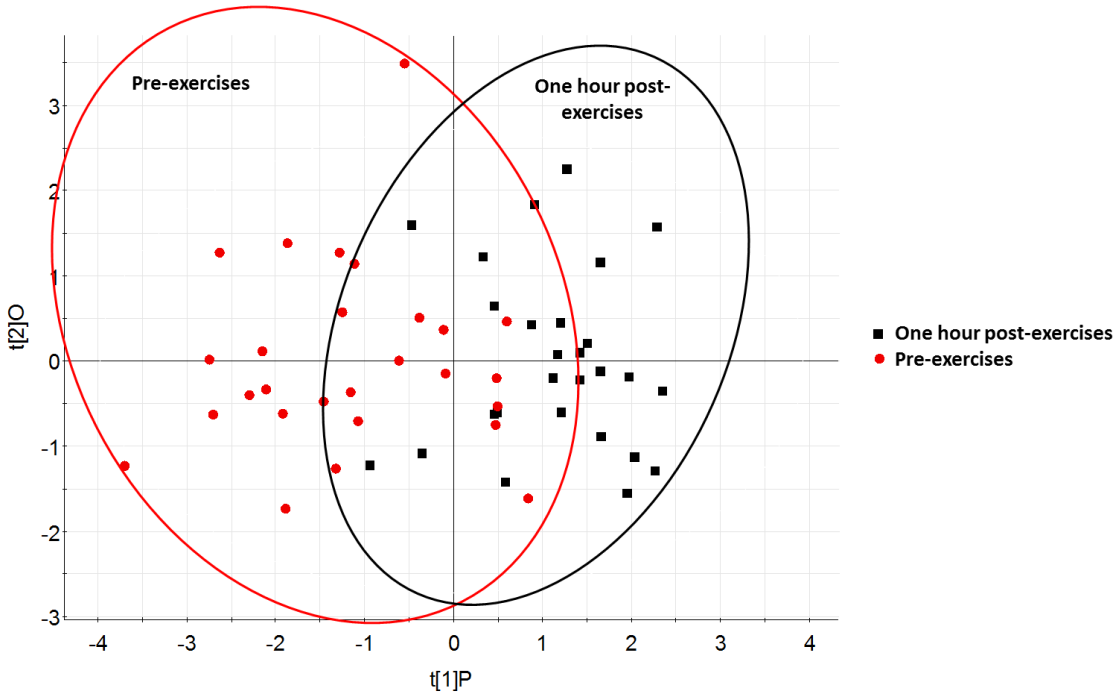
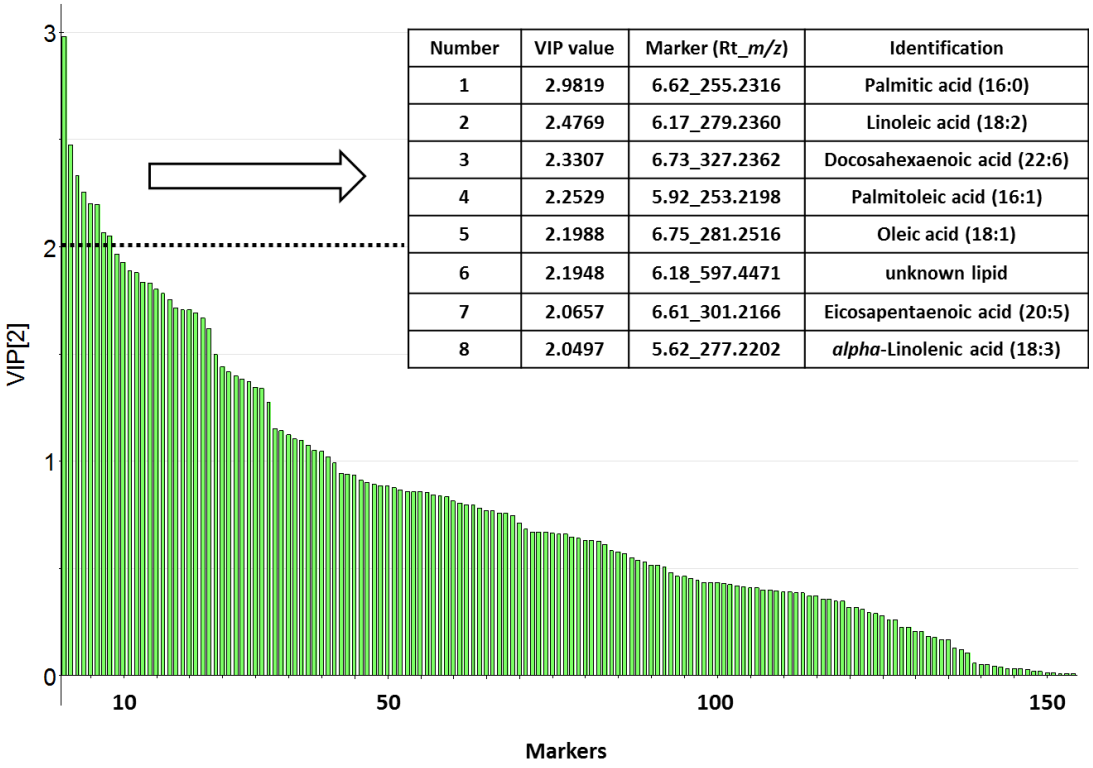


Figure 3.3. OPLSDA scores plot for COPD patients in PR only group. The red dots represent plasma samples collected pre-exercise and the black squares represent the plasma samples collected one hour post-exercise. The multivariate statistics were performed using EZinfo software on the log_2-transformed and Pareto-scaled and normalized peak area dataset of negative ions generated by LC-TOF-MS analysis.

Although the pre- and post-exercise samples were not completely distinguished in this model ($R^2Y(cum)=0.56$, $Q2(cum)=0.30$), an obvious trend of separation was observed due to exercises. The partial segregation of samples from the scores plot suggested biochemical metabolic alterations as a result of PR exercises. In addition, the OPLS-DA scores plot

suggests that some individuals did not show the biochemical changes typical of the rest of the treatment group (three samples from the post-exercise group retained negative scores on principal component 1 and clustered with the pre-exercise group). It is hypothesized that some individuals did not engage in as great an extent of exercise performance as the majority of the subjects.

In order to extract the markers responsible for the separation of pre- and post-treatment samples, analysis of variable influence on projection (VIP) values was carried out (Figure 3.4), and metabolite signals were ranked based on VIP scores. Markers with VIP values > 2.0 were considered to have greater influence on the classification and listed in the inserted table in Figure 3.4 in the order of decreasing VIP values. The metabolite contributing most (*i.e.* with the highest VIP value) to the difference between pre- and one hour post-exercises samples was palmitic acid (16:0), followed by six unsaturated fatty acids and one unknown lipid. These top 8 important features of the metabolite markers from the VIP analysis were consistent with the results from univariate analysis described above (Table 3.1), *i.e.*, they were confirmed by the paired *t* test for significant fold change (>1.5-fold increase, $P<0.001$) after exercise.



Number	VIP value	Marker (Rt_*m/z*)	Identification
1	2.9819	6.62_255.2316	Palmitic acid (16:0)
2	2.4769	6.17_279.2360	Linoleic acid (18:2)
3	2.3307	6.73_327.2362	Docosahexaenoic acid (22:6)
4	2.2529	5.92_253.2198	Palmitoleic acid (16:1)
5	2.1988	6.75_281.2516	Oleic acid (18:1)
6	2.1948	6.18_597.4471	unknown lipid
7	2.0657	6.61_301.2166	Eicosapentaenoic acid (20:5)
8	2.0497	5.62_277.2202	*alpha*-Linolenic acid (18:3)

Figure 3.4. The variable importance in projection (VIP) values generated from the OPLS-DA model for evaluating short-term effects for COPD patients in PR only group. VIP values were sorted in descending order. The metabolites with VIP value > 2.0 contributed most to the separation between pre- and post-exercise samples, and are listed in the inserted table.

3.3.4 Effects of exercise on fatty acids levels

Long chain fatty acids and their metabolites regulate many essential biochemical processes. They are important components of cell membranes, major energy storage and precursors of signaling lipid mediators (25). Effects of exercise on fatty acid have been studies for many years (26-29). During exercise, fatty acids serve as an important fuel source to provide energy for skeletal muscles. Free (or non-esterified) fatty acids are released mainly from adipose tissue to blood via hydrolysis of triglycerides, and also can be released from membrane lipids.

In the present work, the main observation was that the levels (normalized peak areas) of all free fatty acids were significantly elevated one hour after exercise (Table 3.1, Figure 3.4), which was consistent with previous reports (27,28). The relative proportions of each

individual fatty acid, however, changed after exercise as shown in Table 3.2. The percentage of the saturated stearic acid (18:0) decreased by ~ 40% one hour post-exercise ($P<0.001$). In contrast, levels of the two monounsaturated fatty acids palmitoleic acid (16:1) and oleic acid (18:1) showed significant increases in relative abundance (increased by ~ 50%, $P<0.001$, and ~15%, $P<0.01$, respectively). Our observed short-term effects of down regulation in the relative amounts of saturated fatty acids and increases in the proportion of monounsaturated fatty acids due to acute exercise was also consistent with previous reports (29). Polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (20:5) and arachidonic acid (20:4) showed significant decreases in relative abundances after exercise (decreased by ~10%, $P<0.01$, and ~40%, $P<0.001$, respectively). No consensus has emerged in in literature reports regarding the effects of exercises on PUFA levels. PUFAs, especially arachidonic acid, are known as precursors of a number of signaling lipid mediators such as various oxylipins. The decrease in the percentage distribution of the two PUFAs may result from the preferential release of monounsaturated fatty acids during exercise.

One item worthy of note is that the current work showed minimal decreases in normalized levels of other lipids, mostly glycerophospholipids and lysoglycerolipids. The levels of phospholipids in skeletal muscle was reported to be almost unaffected by exercise (26,30), but previous studies demonstrated an enhanced rate of triacylglycerol hydrolysis during exercise, which resulted in decreased levels of adipose triglycerides (31). Therefore, one limitation of the results described in this chapter is that only negative ions were detected and some classes of lipids including triglycerides do not ionize well in this condition (32). This also highlights the importance to achieve more comprehensive coverage of metabolites in untargeted metabolomics studies. Both positive and negative electrospray ionization modes will be employed to the completed set of plasma samples.

Table 3.2. Normalized levels and percentage distribution of 12 free fatty acids detected in plasma pre- and one hour post-exercise in the PR only group (n=9). Blood specimens were drawn on week 0, 6, and 12 for each patient, so 27 pairs (n=27) in total were analyzed and compared (mean ± SEM). Paired Student's *t* test (α=0.05) was used on log-transformed normalized peak areas to compare the differences between pre- and post-exercises for each fatty acid.

Fatty acid	Mean of normalized XIC peak area			Percentage of total free fatty acids (%)		
	pre	post	P value	pre	post	P value
Palmitoleic acid (16:1)	0.38 ± 0.08	1.06 ± 0.13	P < 0.001	0.65 ± 0.08	1.07 ± 0.11	P < 0.001
Palmitic acid (16:0)	4.10 ± 0.55	7.81 ± 0.55	P < 0.001	9.01 ± 0.32	8.43 ± 0.23	NS
alpha-Linolenic acid (18:3)	0.29 ± 0.04	0.66 ± 0.07	P < 0.001	0.61 ± 0.05	0.68 ± 0.05	NS
Linoleic acid (18:2)	9.13 ± 1.34	19.2 ± 1.46	P < 0.001	19.1 ± 0.50	20.4 ± 0.50	P < 0.05
Oleic acid (18:1)	18.9 ± 3.19	37.9 ± 3.12	P < 0.001	35.5 ± 1.37	39.6 ± 0.84	P < 0.01
Stearic acid (18:0)	4.18 ± 0.38	5.76 ± 0.28	P < 0.001	11.1 ± 0.91	6.90 ± 0.54	P < 0.001
Eicosapentaenoic acid (20:5)	1.76 ± 0.21	3.17 ± 0.19	P < 0.001	3.91 ± 0.13	3.50 ± 0.09	P < 0.01
Arachidonic acid (20:4)	0.83 ± 0.07	1.27 ± 0.09	P < 0.001	2.33 ± 0.26	1.40 ± 0.07	P < 0.001
Docosahexaenoic acid (22:6)	6.31 ± 0.84	12.5 ± 0.93	P < 0.001	13.1 ± 0.49	13.5 ± 0.41	NS
Docosapentaenoic acid (22:5)	0.12 ± 0.03	0.39 ± 0.06	P < 0.001	0.27 ± 0.07	0.38 ± 0.05	NS
Adrenic acid (22:4)	0.27 ± 0.03	0.50 ± 0.04	P < 0.001	0.58 ± 0.04	0.55 ± 0.03	NS
Nervonic acid (24:1)	1.85 ± 0.23	3.29 ± 0.19	P < 0.001	3.83 ± 0.13	3.63 ± 0.09	NS

NS: not significant (*i.e.* P >0.05).

3.4 Conclusions

In this chapter, an untargeted mass spectrometry-based metabolomics approach was described and applied to analyze levels of plasma metabolites in the context of a pulmonary rehabilitation program. The strategy combines the power of an advanced LC-MS platform and both univariate and multivariate statistical analysis to identify potential biomarkers of the effect of exercise on the plasma metabolome. For application in a clinical trial, the approach was used for the first time to investigate plasma metabolic alterations after pulmonary rehabilitation exercise training and OMT in a cohort of patients with COPD. The most discriminating metabolites were identified as lipids, and most of them were free long chain fatty acids, which can serve as biomarkers of exercises. During exercise, fatty acids were released and utilized for providing energy, generation of signaling mediator lipids, and biosynthesis of other lipids, but the disposition of the released fatty acids is still not clear from blood levels alone. We propose that future work should focus on the effects of exercises on fatty acids using metabolic flux analysis so that greater understanding of the dynamics of the disposition and functions of the released fatty acids can be determined.

APPENDIX

APPENDIX

Table A2. List of 154 stable markers (RSD% < 30% in QC samples) detected in plasma from COPD patients using LC-ESI-MS in negative mode. Nine, five and seven COPD patients were recruited in PR only, OMT and Sham group respectively. Blood specimens were drawn at week 0, 6, and 12 pre-, one hour post- and 72 hour post-exercises/treatments for each patient. Short-term effects (pre- vs. one hour post-treatment) and long-term effects (pre- vs 72 hour post-treatment) of exercise/treatment on COPD patients were investigated from fold change of each marker. Paired Student's *t* test (α=0.05) was used on log-transformed normalized peak areas to compare the differences. The change was considered significantly different if $P < 0.05$.

Marker (Rt_*m/z*)	Short-term (1 hour post-treatment) fold change						Long-term (72 hour post-treatment) fold change					
	PR only (n=27)		OMT (n=15)		Sham (n=21)		PR only (n=20)		OMT (n=14)		Sham (n=19)	
	Mean ± SEM	P value	Mean ± SEM	P value	Mean ± SEM	P value	Mean ± SEM	P value	Mean ± SEM	P value	Mean ± SEM	P value
0.60_336.7973	0.90 ± 0.07	0.0237	1.09 ± 0.12	0.9033	0.99 ± 0.11	0.2713	0.89 ± 0.13	0.1249	1.50 ± 0.12	0.0010	1.27 ± 0.16	0.4619
0.61_225.0682	0.86 ± 0.08	0.0068	1.15 ± 0.15	0.9047	0.77 ± 0.09	0.0035	0.83 ± 0.14	0.0211	1.36 ± 0.17	0.0782	1.21 ± 0.13	0.7261
0.62_326.7721	0.91 ± 0.07	0.0308	1.14 ± 0.15	0.9157	0.96 ± 0.07	0.2211	1.02 ± 0.14	0.2770	1.41 ± 0.18	0.0686	1.29 ± 0.13	0.1518
10.15_775.6205	0.95 ± 0.04	0.0971	1.02 ± 0.04	0.7847	1.00 ± 0.04	0.8404	0.94 ± 0.07	0.5290	1.03 ± 0.06	0.7829	1.02 ± 0.08	0.7023
10.10_830.5954	0.94 ± 0.03	0.0112	0.97 ± 0.03	0.2929	0.96 ± 0.04	0.1805	0.93 ± 0.04	0.0533	0.97 ± 0.04	0.3623	0.94 ± 0.05	0.5162
10.14_770.5978	0.91 ± 0.03	0.0076	0.99 ± 0.04	0.6605	1.01 ± 0.05	0.7508	0.92 ± 0.05	0.0730	1.00 ± 0.06	0.6663	0.97 ± 0.08	0.7469
10.16_843.6004	0.89 ± 0.06	0.0281	1.19 ± 0.12	0.2363	1.05 ± 0.12	0.9891	0.81 ± 0.11	0.0335	1.15 ± 0.11	0.5048	1.16 ± 0.10	0.6169
10.38_856.6349	1.05 ± 0.05	0.5954	1.08 ± 0.07	0.3145	1.05 ± 0.03	0.2905	1.02 ± 0.07	0.7012	1.00 ± 0.07	0.6882	1.04 ± 0.10	0.1592
14.09_368.0081	0.89 ± 0.03	0.0008	0.96 ± 0.05	0.2824	0.98 ± 0.03	0.4352	0.81 ± 0.06	0.0034	0.96 ± 0.07	0.2944	1.05 ± 0.06	0.8983
14.09_912.5547	1.00 ± 0.11	0.1925	1.09 ± 0.14	0.4622	0.92 ± 0.12	0.4226	1.05 ± 0.22	0.9837	1.13 ± 0.24	0.9237	1.06 ± 0.16	0.5194
14.09_608.7362	0.90 ± 0.09	0.0732	0.87 ± 0.10	0.0515	1.13 ± 0.10	0.5383	0.78 ± 0.14	0.7939	0.78 ± 0.10	0.0391	0.98 ± 0.12	0.7860
14.11_504.7603	0.88 ± 0.04	0.0045	0.95 ± 0.05	0.1670	1.02 ± 0.03	0.7621	0.76 ± 0.06	0.0009	0.85 ± 0.07	0.0420	1.02 ± 0.06	0.6628
14.11_472.7999	0.94 ± 0.04	0.0599	0.92 ± 0.03	0.0263	1.06 ± 0.07	0.7946	0.79 ± 0.08	0.0026	0.83 ± 0.07	0.0335	1.10 ± 0.09	0.6797
14.11_488.7837	0.94 ± 0.05	0.0584	0.99 ± 0.05	0.5413	0.98 ± 0.03	0.4162	0.83 ± 0.08	0.0101	0.84 ± 0.07	0.0275	1.00 ± 0.05	0.3035
14.10_624.7143	0.92 ± 0.05	0.0364	1.08 ± 0.09	0.6838	1.02 ± 0.04	0.9123	0.81 ± 0.08	0.0051	0.92 ± 0.11	0.1861	1.04 ± 0.07	0.7913
14.10_640.6945	0.91 ± 0.06	0.0304	1.01 ± 0.06	0.7442	1.09 ± 0.07	0.3698	0.87 ± 0.09	0.0262	0.85 ± 0.09	0.0741	1.07 ± 0.07	0.6508
14.10_776.619	0.91 ± 0.07	0.0413	1.12 ± 0.13	0.7323	1.06 ± 0.06	0.6076	0.91 ± 0.11	0.1744	0.91 ± 0.12	0.1672	1.07 ± 0.10	0.7109

Table A2 (cont'd)

14.10_760.6453	0.95 ± 0.06	0.1081	1.01 ± 0.07	0.7619	1.02 ± 0.07	0.7040	0.87 ± 0.08	0.0270	0.87 ± 0.11	0.0830	1.05 ± 0.08	0.6218
14.10_1034.5024	0.92 ± 0.11	0.0471	0.96 ± 0.14	0.2176	1.03 ± 0.10	0.6188	0.94 ± 0.16	0.2889	0.82 ± 0.17	0.2645	0.85 ± 0.09	0.0422
14.10_1016.5366	0.95 ± 0.09	0.1040	1.06 ± 0.13	0.6962	1.04 ± 0.10	0.6197	0.75 ± 0.16	0.0499	0.89 ± 0.21	0.8602	0.99 ± 0.13	0.9985
14.10_1032.5134	0.94 ± 0.12	0.1227	1.04 ± 0.16	0.4408	0.98 ± 0.15	0.3823	0.88 ± 0.15	0.1783	0.80 ± 0.20	0.3520	0.83 ± 0.12	0.0488
2.85_187.0107	0.85 ± 0.05	0.0084	0.97 ± 0.08	0.3328	0.86 ± 0.07	0.0535	3.14 ± 2.20	0.7769	1.03 ± 0.17	0.3994	1.44 ± 0.23	0.6012
3.15_367.1669	0.94 ± 0.07	0.2174	0.96 ± 0.12	0.6949	0.99 ± 0.10	0.8519	1.13 ± 0.25	0.6746	1.06 ± 0.11	0.0960	1.04 ± 0.11	0.7606
3.22_239.0981	0.98 ± 0.12	0.0760	1.08 ± 0.07	0.4586	0.81 ± 0.08	0.0686	0.70 ± 0.16	0.9091	1.17 ± 0.09	0.1392	1.05 ± 0.07	0.9049
3.52_267.1294	0.90 ± 0.08	0.0775	0.97 ± 0.06	0.3645	0.86 ± 0.08	0.1937	0.99 ± 0.10	0.3134	1.13 ± 0.10	0.5915	0.96 ± 0.12	0.3666
3.54_358.0854	0.79 ± 0.07	0.0492	0.99 ± 0.05	0.5924	0.71 ± 0.10	0.1200	0.96 ± 0.09	0.2810	0.99 ± 0.09	0.2605	0.94 ± 0.07	0.4943
3.55_424.0691	0.93 ± 0.03	0.0062	0.99 ± 0.04	0.5258	0.97 ± 0.03	0.2907	0.94 ± 0.07	0.5248	1.06 ± 0.04	0.2708	0.98 ± 0.04	0.3846
3.55_440.0428	0.96 ± 0.04	0.1339	0.97 ± 0.04	0.2872	0.99 ± 0.05	0.4457	1.00 ± 0.08	0.6052	1.00 ± 0.04	0.7460	1.04 ± 0.06	0.8689
3.55_312.0881	0.95 ± 0.03	0.0671	0.99 ± 0.05	0.6481	0.96 ± 0.05	0.1813	1.09 ± 0.08	0.0538	1.05 ± 0.06	0.6032	0.95 ± 0.07	0.0889
3.55_753.1216	0.96 ± 0.10	0.7015	1.08 ± 0.14	0.4850	0.79 ± 0.08	0.0507	1.24 ± 0.16	0.0824	1.07 ± 0.09	0.7464	0.87 ± 0.08	0.0704
3.55_751.1224	0.93 ± 0.04	0.0391	1.08 ± 0.06	0.3479	0.97 ± 0.04	0.2225	1.07 ± 0.10	0.3244	1.07 ± 0.07	0.5623	0.92 ± 0.09	0.1431
3.55_713.1664	0.96 ± 0.04	0.1540	1.00 ± 0.04	0.8072	0.96 ± 0.04	0.1672	0.97 ± 0.08	0.8254	1.00 ± 0.07	0.6158	1.01 ± 0.05	0.6227
3.55_735.1497	0.96 ± 0.08	0.3943	1.02 ± 0.10	0.3192	1.10 ± 0.12	0.8012	1.08 ± 0.10	0.2875	1.10 ± 0.11	0.7807	1.05 ± 0.12	0.3790
3.56_402.0853	0.97 ± 0.05	0.2077	1.00 ± 0.07	0.6760	0.95 ± 0.04	0.1406	0.73 ± 0.10	0.0041	1.04 ± 0.10	0.7956	1.04 ± 0.07	0.9481
3.60_448.3183	1.31 ± 0.26	0.8514	0.71 ± 0.08	0.0139	0.63 ± 0.13	0.0002	1.57 ± 0.39	0.9449	0.88 ± 0.21	0.2394	1.07 ± 0.28	0.8057
4.26_586.3121	0.92 ± 0.06	0.0498	0.90 ± 0.08	0.0836	1.06 ± 0.09	0.8284	0.88 ± 0.11	0.1365	0.89 ± 0.12	0.1007	1.11 ± 0.16	0.5498
4.31_512.3120	0.90 ± 0.03	0.0046	0.94 ± 0.08	0.2097	0.92 ± 0.06	0.0926	0.85 ± 0.10	0.0215	1.00 ± 0.11	0.5055	1.07 ± 0.14	0.4166
4.55_538.3168	0.90 ± 0.04	0.0098	0.92 ± 0.06	0.1131	0.91 ± 0.06	0.0466	0.80 ± 0.05	0.0020	0.88 ± 0.12	0.0791	1.00 ± 0.13	0.1533
4.60_588.3357	0.97 ± 0.05	0.1982	0.94 ± 0.06	0.2119	0.98 ± 0.05	0.3540	0.91 ± 0.08	0.0573	0.88 ± 0.09	0.0696	1.02 ± 0.08	0.4863
4.63_564.3285	0.92 ± 0.04	0.0180	0.87 ± 0.08	0.0373	0.89 ± 0.07	0.0307	0.95 ± 0.09	0.1740	0.94 ± 0.08	0.2182	1.00 ± 0.12	0.3640
4.68_612.3254	1.02 ± 0.05	0.8576	0.95 ± 0.06	0.2541	1.08 ± 0.06	0.4777	0.96 ± 0.11	0.1586	0.86 ± 0.08	0.0484	1.05 ± 0.13	0.7416
4.76_588.3358	0.95 ± 0.03	0.0493	0.91 ± 0.04	0.0317	0.97 ± 0.04	0.3163	0.94 ± 0.06	0.1181	0.91 ± 0.07	0.1152	1.03 ± 0.09	0.5233
4.77_672.308	0.94 ± 0.09	0.8173	0.79 ± 0.11	0.0295	1.01 ± 0.10	0.4097	1.18 ± 0.15	0.5093	1.04 ± 0.13	0.6295	1.03 ± 0.13	0.6478
4.80_500.293	1.04 ± 0.04	0.7264	0.95 ± 0.09	0.2436	0.97 ± 0.04	0.2390	0.98 ± 0.07	0.3747	0.86 ± 0.04	0.0060	0.98 ± 0.10	0.2628
4.81_632.3371	0.95 ± 0.04	0.1076	0.88 ± 0.06	0.0258	0.91 ± 0.06	0.0502	1.17 ± 0.11	0.2995	1.01 ± 0.08	0.6446	1.09 ± 0.11	0.8726
4.81_1083.6976	0.92 ± 0.08	0.1191	0.80 ± 0.10	0.0277	1.26 ± 0.44	0.3931	1.30 ± 0.19	0.5366	1.02 ± 0.12	0.5533	1.09 ± 0.15	0.7269

Table A2 (cont'd)

4.82_564.3367	0.91 ± 0.05	0.0171	0.85 ± 0.05	0.0091	0.92 ± 0.05	0.0560	1.00 ± 0.09	0.4615	1.00 ± 0.09	0.5846	1.04 ± 0.10	0.6775
4.82_648.3100	0.97 ± 0.05	0.2117	0.90 ± 0.05	0.0572	0.91 ± 0.06	0.0451	1.12 ± 0.09	0.4114	0.98 ± 0.07	0.4572	1.07 ± 0.09	0.9348
4.83_504.3239	0.87 ± 0.06	0.0680	0.85 ± 0.04	0.0029	0.82 ± 0.09	0.0761	1.11 ± 0.12	0.8787	0.95 ± 0.06	0.2564	0.98 ± 0.15	0.8147
4.84_476.2923	0.92 ± 0.05	0.0480	0.94 ± 0.12	0.1713	0.91 ± 0.07	0.0714	1.08 ± 0.14	0.5584	0.92 ± 0.08	0.1758	1.08 ± 0.15	0.5854
4.95_590.3432	1.08 ± 0.09	0.9492	0.94 ± 0.11	0.2049	1.03 ± 0.12	0.4192	1.11 ± 0.13	0.8717	0.98 ± 0.11	0.3855	1.13 ± 0.22	0.4069
5.01_540.3279	0.87 ± 0.03	0.0000	0.87 ± 0.04	0.0074	0.93 ± 0.04	0.0633	0.90 ± 0.04	0.0198	0.95 ± 0.05	0.2290	0.93 ± 0.06	0.0556
5.10_590.3489	0.98 ± 0.04	0.3279	0.95 ± 0.06	0.2060	1.04 ± 0.05	0.7939	0.91 ± 0.06	0.0707	0.90 ± 0.08	0.1109	1.07 ± 0.12	0.5991
5.21_608.3363	0.88 ± 0.03	0.0002	0.96 ± 0.05	0.2490	0.92 ± 0.04	0.0224	1.01 ± 0.04	0.8212	1.03 ± 0.04	0.6406	0.99 ± 0.06	0.5625
5.21_624.3097	0.90 ± 0.03	0.0010	0.95 ± 0.05	0.1871	0.97 ± 0.04	0.2917	0.99 ± 0.04	0.5862	1.01 ± 0.05	0.9690	1.07 ± 0.06	0.4297
5.21_540.3282	0.91 ± 0.03	0.0010	0.92 ± 0.04	0.0405	0.93 ± 0.04	0.0252	0.94 ± 0.04	0.0844	0.98 ± 0.04	0.4700	1.00 ± 0.07	0.2754
5.21_1035.6950	0.85 ± 0.03	0.0001	0.92 ± 0.05	0.0913	0.92 ± 0.04	0.0272	1.00 ± 0.07	0.5173	0.97 ± 0.06	0.4182	1.01 ± 0.09	0.3800
5.21_708.2759	0.91 ± 0.03	0.0107	0.95 ± 0.05	0.2297	0.91 ± 0.04	0.0135	1.13 ± 0.05	0.0372	1.02 ± 0.07	0.9684	1.09 ± 0.07	0.5591
5.21_480.3231	0.87 ± 0.03	0.0002	0.92 ± 0.05	0.0841	0.93 ± 0.04	0.0500	0.97 ± 0.05	0.3003	1.03 ± 0.04	0.6630	1.06 ± 0.06	0.9996
5.39_566.3498	0.91 ± 0.03	0.0014	0.87 ± 0.03	0.0019	0.92 ± 0.04	0.0238	0.93 ± 0.05	0.0850	0.88 ± 0.05	0.0319	0.99 ± 0.07	0.4082
5.40_650.3262	0.91 ± 0.03	0.0093	0.90 ± 0.06	0.0465	0.91 ± 0.05	0.0319	1.05 ± 0.06	0.8537	0.94 ± 0.07	0.2310	1.11 ± 0.11	0.8699
5.40_634.353	0.94 ± 0.05	0.0776	0.97 ± 0.10	0.3191	0.88 ± 0.05	0.0097	1.13 ± 0.08	0.2899	0.91 ± 0.06	0.1090	1.01 ± 0.08	0.4279
5.42_478.3056	0.94 ± 0.06	0.0625	1.05 ± 0.19	0.5203	0.91 ± 0.08	0.0821	1.15 ± 0.16	0.7350	1.01 ± 0.10	0.5798	1.15 ± 0.14	0.9020
5.53_524.3488	0.95 ± 0.05	0.1419	0.87 ± 0.04	0.0103	0.77 ± 0.10	0.1819	0.71 ± 0.09	0.0677	0.85 ± 0.11	0.7299	0.78 ± 0.14	0.8194
5.54_554.3412	0.92 ± 0.10	0.0317	0.97 ± 0.05	0.3954	1.02 ± 0.07	0.6393	0.90 ± 0.10	0.0599	1.10 ± 0.14	0.9511	1.23 ± 0.17	0.7438
5.62_277.2202	4.18 ± 0.93	0.0000	3.81 ± 1.22	0.1048	3.54 ± 0.97	0.0065	1.62 ± 0.39	0.5084	1.57 ± 0.39	0.8115	2.72 ± 1.32	0.6286
5.66_554.3498	0.93 ± 0.05	0.0619	0.94 ± 0.05	0.1734	1.02 ± 0.05	0.8668	0.95 ± 0.05	0.1809	1.03 ± 0.08	0.8120	1.03 ± 0.10	0.5949
5.78_681.3150	0.93 ± 0.05	0.0677	1.07 ± 0.08	0.7639	0.91 ± 0.10	0.0729	0.95 ± 0.12	0.1386	1.13 ± 0.09	0.3165	1.02 ± 0.12	0.6480
5.90_568.3583	0.89 ± 0.03	0.0014	0.96 ± 0.05	0.2831	1.00 ± 0.05	0.6200	0.93 ± 0.05	0.0699	0.97 ± 0.06	0.3997	1.05 ± 0.07	0.9723
5.92_253.2198	6.70 ± 1.85	0.0000	6.20 ± 1.69	0.0101	2.82 ± 0.52	0.0001	2.98 ± 1.38	0.3210	2.48 ± 0.83	0.0567	2.10 ± 0.51	0.0815
5.92_373.2475	2.02 ± 0.27	0.0009	2.49 ± 0.45	0.0117	1.89 ± 0.35	0.0669	0.92 ± 0.34	0.6883	0.99 ± 0.24	0.8389	1.42 ± 0.24	0.1154
6.04_303.2307	1.69 ± 0.12	0.0000	1.40 ± 0.14	0.0362	1.43 ± 0.11	0.0076	1.18 ± 0.17	0.8364	1.12 ± 0.23	0.6632	1.03 ± 0.08	0.8130
6.11_568.3578	0.90 ± 0.02	0.0001	0.91 ± 0.03	0.0095	0.96 ± 0.03	0.1698	0.91 ± 0.05	0.0255	0.93 ± 0.04	0.0826	1.02 ± 0.06	0.6669
6.12_636.3697	0.91 ± 0.03	0.0054	0.96 ± 0.04	0.2805	0.96 ± 0.04	0.2188	0.98 ± 0.06	0.3619	0.98 ± 0.06	0.4606	1.08 ± 0.07	0.7269
6.13_652.3431	0.88 ± 0.03	0.0002	0.96 ± 0.05	0.2787	0.97 ± 0.03	0.2084	0.93 ± 0.05	0.0712	0.97 ± 0.05	0.4244	1.05 ± 0.06	0.8119

Table A2 (cont'd)

6.17_279.2360	3.31 ± 0.57	0.0000	2.62 ± 0.53	0.0215	2.48 ± 0.38	0.0073	1.67 ± 0.41	0.9005	1.36 ± 0.37	0.7360	1.32 ± 0.19	0.8962
6.17_667.5523	1.32 ± 0.09	0.0024	1.31 ± 0.10	0.0125	1.28 ± 0.09	0.0270	1.42 ± 0.14	0.0323	1.04 ± 0.07	0.9181	1.16 ± 0.10	0.1638
6.18_375.2807	1.00 ± 0.05	0.4975	1.06 ± 0.06	0.5863	1.02 ± 0.04	0.8878	0.73 ± 0.09	0.0038	0.98 ± 0.08	0.4621	0.87 ± 0.08	0.0458
6.18_597.4471	4.73 ± 1.17	0.0001	5.20 ± 2.28	0.2275	4.56 ± 1.20	0.0108	3.48 ± 1.10	0.2265	1.76 ± 0.86	0.8897	2.07 ± 0.47	0.1606
6.19_329.2576	2.92 ± 0.42	0.0001	2.46 ± 0.58	0.0798	2.06 ± 0.46	0.0877	1.53 ± 0.50	0.6865	1.05 ± 0.34	0.9442	0.52 ± 0.14	0.4744
6.61_301.2166	2.58 ± 0.34	0.0000	2.27 ± 0.39	0.0156	2.23 ± 0.36	0.0107	1.26 ± 0.41	0.1622	1.09 ± 0.23	0.4121	1.58 ± 0.29	0.5134
6.62_255.2316	2.68 ± 0.34	0.0000	2.36 ± 0.48	0.0410	2.39 ± 0.38	0.0140	1.49 ± 0.36	0.8875	1.27 ± 0.31	0.5544	1.39 ± 0.23	0.9439
6.65_331.2674	2.67 ± 0.44	0.0000	2.06 ± 0.35	0.0338	1.88 ± 0.28	0.0469	1.53 ± 0.33	0.9665	1.21 ± 0.28	0.6179	1.10 ± 0.18	0.2505
6.68_537.4321	1.02 ± 0.05	0.8423	1.09 ± 0.10	0.8031	0.95 ± 0.10	0.9641	1.06 ± 0.08	0.9321	1.00 ± 0.13	0.9981	1.10 ± 0.09	0.5977
6.73_365.3389	3.17 ± 0.64	0.0000	3.53 ± 0.92	0.0207	2.38 ± 0.42	0.0440	1.63 ± 0.34	0.8576	1.61 ± 0.43	0.9935	1.65 ± 0.31	0.6987
6.73_327.2362	3.23 ± 0.52	0.0000	3.05 ± 0.59	0.0138	2.62 ± 0.45	0.0186	1.37 ± 0.48	0.1628	1.34 ± 0.35	0.8194	1.68 ± 0.32	0.5856
6.74_585.5010	4.70 ± 1.22	0.0003	5.28 ± 2.00	0.1471	5.00 ± 1.21	0.0323	2.74 ± 0.81	0.9010	0.99 ± 0.48	0.5800	2.68 ± 0.77	0.1591
6.74_601.4779	5.69 ± 2.26	0.0001	6.19 ± 2.81	0.1485	4.16 ± 1.16	0.0863	2.49 ± 0.69	0.5547	1.20 ± 0.47	0.2896	2.63 ± 0.98	0.4207
6.75_433.2072	1.95 ± 0.24	0.0003	1.99 ± 0.46	0.2517	1.97 ± 0.39	0.0020	1.14 ± 0.20	0.6918	1.22 ± 0.37	0.8736	1.51 ± 0.28	0.2055
6.75_281.2516	4.08 ± 0.89	0.0000	3.78 ± 0.91	0.0220	3.10 ± 0.60	0.0354	1.80 ± 0.45	0.9417	1.66 ± 0.48	0.8723	1.73 ± 0.39	0.8071
6.98_583.273	0.78 ± 0.08	0.0512	1.09 ± 0.17	0.0643	1.18 ± 0.15	0.0926	2.21 ± 1.20	0.7924	1.26 ± 0.16	0.0097	2.23 ± 0.72	0.2532
7.27_363.4933	0.94 ± 0.05	0.0852	1.04 ± 0.10	0.8999	1.03 ± 0.06	0.9318	0.78 ± 0.06	0.0022	1.08 ± 0.09	0.6869	1.05 ± 0.07	0.4915
7.27_386.4828	0.95 ± 0.05	0.0948	0.91 ± 0.09	0.0893	1.15 ± 0.09	0.2833	1.20 ± 0.10	0.0561	1.00 ± 0.09	0.5555	1.04 ± 0.05	0.6054
7.28_317.4861	0.92 ± 0.04	0.0254	0.94 ± 0.07	0.1668	1.08 ± 0.07	0.4874	1.04 ± 0.08	0.8996	0.97 ± 0.06	0.3762	0.92 ± 0.06	0.3548
7.28_364.4986	0.96 ± 0.04	0.1625	1.05 ± 0.09	0.9569	1.04 ± 0.06	0.9319	0.78 ± 0.07	0.0022	1.04 ± 0.07	0.9949	1.03 ± 0.06	0.5287
7.28_402.4568	0.92 ± 0.03	0.0108	0.96 ± 0.09	0.2831	1.07 ± 0.06	0.4676	1.14 ± 0.08	0.1381	1.01 ± 0.06	0.8372	1.01 ± 0.04	0.5068
7.36_283.2613	1.60 ± 0.13	0.0000	1.44 ± 0.15	0.0505	1.51 ± 0.15	0.0086	1.21 ± 0.17	0.9352	0.97 ± 0.18	0.2361	1.11 ± 0.12	0.9605
7.42_915.6263	0.94 ± 0.11	0.4561	0.81 ± 0.08	0.0238	0.98 ± 0.08	0.3105	1.14 ± 0.14	0.1318	0.92 ± 0.12	0.6135	1.14 ± 0.16	0.7772
7.57_595.5107	0.88 ± 0.09	0.6392	0.82 ± 0.14	0.5087	1.01 ± 0.10	0.3909	0.93 ± 0.12	0.8061	1.02 ± 0.12	0.4131	1.03 ± 0.12	0.6621
8.15_915.6256	1.02 ± 0.14	0.1681	1.33 ± 0.20	0.3613	1.22 ± 0.19	0.9861	1.26 ± 0.16	0.3649	1.47 ± 0.27	0.3749	1.37 ± 0.16	0.2112
8.43_719.5557	1.02 ± 0.04	0.9790	0.90 ± 0.06	0.0389	1.03 ± 0.08	0.4898	1.15 ± 0.07	0.0670	1.06 ± 0.07	0.6664	1.00 ± 0.08	0.9217
8.50_745.5719	1.01 ± 0.05	0.6748	0.92 ± 0.07	0.1005	1.07 ± 0.07	0.7253	1.13 ± 0.06	0.0781	1.05 ± 0.09	0.9118	0.98 ± 0.07	0.7140
8.50_797.534	0.94 ± 0.05	0.0866	0.96 ± 0.07	0.2955	1.11 ± 0.07	0.2948	1.00 ± 0.08	0.4376	1.02 ± 0.09	0.6968	1.04 ± 0.08	0.6230
8.55_824.5412	1.05 ± 0.06	0.8149	0.91 ± 0.05	0.0396	1.08 ± 0.06	0.5408	1.34 ± 0.11	0.0177	1.01 ± 0.12	0.5381	0.97 ± 0.07	0.5853

Table A2 (cont'd)

8.55_850.5567	0.98 ± 0.05	0.2640	0.92 ± 0.06	0.0925	1.07 ± 0.08	0.9319	1.12 ± 0.07	0.2889	1.09 ± 0.09	0.6617	1.03 ± 0.06	0.9933
8.70_826.5571	1.00 ± 0.06	0.3992	0.81 ± 0.04	0.0009	1.00 ± 0.09	0.3918	1.24 ± 0.11	0.0788	0.98 ± 0.07	0.5013	1.02 ± 0.11	0.8945
8.73_876.5602	0.84 ± 0.10	0.2508	0.83 ± 0.12	0.3234	1.05 ± 0.13	0.7338	1.10 ± 0.12	0.5013	0.80 ± 0.12	0.2236	1.00 ± 0.10	0.9482
8.80_850.5581	0.99 ± 0.04	0.3820	0.89 ± 0.03	0.0019	1.08 ± 0.07	0.3730	1.22 ± 0.09	0.0149	0.96 ± 0.05	0.2560	1.11 ± 0.06	0.2906
8.83_902.5488	0.93 ± 0.03	0.0301	1.08 ± 0.06	0.4176	1.03 ± 0.06	0.9809	1.12 ± 0.08	0.2405	0.97 ± 0.05	0.3932	1.06 ± 0.07	0.9880
9.00_878.5934	0.96 ± 0.04	0.1567	1.07 ± 0.08	0.7801	1.00 ± 0.06	0.5078	1.14 ± 0.11	0.3226	1.12 ± 0.09	0.4848	1.08 ± 0.08	0.8073
9.08_766.5643	0.90 ± 0.05	0.0493	0.95 ± 0.06	0.1986	1.07 ± 0.07	0.7042	1.14 ± 0.06	0.0857	1.05 ± 0.04	0.3153	1.27 ± 0.11	0.4003
9.09_910.5482	0.91 ± 0.02	0.0004	1.00 ± 0.03	0.7578	0.98 ± 0.04	0.3109	0.88 ± 0.04	0.0062	1.05 ± 0.04	0.4030	1.03 ± 0.06	0.9807
9.09_994.5140	0.92 ± 0.03	0.0045	1.05 ± 0.05	0.5126	1.03 ± 0.06	0.9887	0.87 ± 0.05	0.0103	1.06 ± 0.07	0.7588	0.98 ± 0.08	0.3835
9.09_1062.5044	0.82 ± 0.05	0.0010	1.02 ± 0.05	0.9932	1.00 ± 0.09	0.9343	0.91 ± 0.08	0.2265	1.05 ± 0.07	0.7991	1.05 ± 0.10	0.5453
9.10_826.5587	0.94 ± 0.02	0.0040	1.03 ± 0.03	0.5723	0.98 ± 0.03	0.3614	1.15 ± 0.05	0.0043	1.09 ± 0.04	0.0493	1.06 ± 0.04	0.5023
9.12_978.5396	0.91 ± 0.04	0.0075	0.97 ± 0.05	0.3696	0.93 ± 0.07	0.1136	0.84 ± 0.07	0.0337	1.00 ± 0.09	0.5921	1.02 ± 0.09	0.6029
9.12_936.5637	0.92 ± 0.08	0.4150	1.03 ± 0.09	0.8038	0.96 ± 0.07	0.1967	0.73 ± 0.12	0.3290	1.03 ± 0.11	0.3430	1.08 ± 0.08	0.5578
9.13_946.5454	0.84 ± 0.07	0.0504	0.96 ± 0.11	0.8326	1.05 ± 0.11	0.6793	1.08 ± 0.10	0.8416	0.90 ± 0.11	0.4401	1.14 ± 0.14	0.2261
9.14_894.5761	0.89 ± 0.04	0.0026	1.05 ± 0.06	0.6093	1.05 ± 0.08	0.9284	0.91 ± 0.07	0.2154	1.11 ± 0.10	0.5141	1.19 ± 0.12	0.2296
9.16_776.5674	0.93 ± 0.03	0.0085	1.01 ± 0.03	0.9951	1.00 ± 0.05	0.5987	1.02 ± 0.06	0.7722	0.97 ± 0.10	0.3621	1.07 ± 0.14	0.4774
9.19_831.5532	0.83 ± 0.09	0.3597	0.91 ± 0.05	0.0854	0.92 ± 0.09	0.8069	0.86 ± 0.07	0.0583	1.00 ± 0.05	0.7928	0.90 ± 0.09	0.6334
9.20_687.5651	0.94 ± 0.03	0.0253	1.07 ± 0.07	0.6176	1.00 ± 0.04	0.6583	1.16 ± 0.07	0.0528	1.10 ± 0.06	0.1947	0.99 ± 0.07	0.7045
9.20_899.5414	0.95 ± 0.04	0.0813	0.91 ± 0.08	0.1241	1.04 ± 0.07	0.8596	0.98 ± 0.07	0.3557	0.88 ± 0.15	0.6771	0.88 ± 0.10	0.3648
9.21_747.5853	0.94 ± 0.04	0.0465	0.99 ± 0.05	0.6380	1.01 ± 0.05	0.8338	1.09 ± 0.06	0.2649	1.05 ± 0.08	0.8173	1.03 ± 0.05	0.5552
9.21_815.5806	0.90 ± 0.06	0.0367	0.97 ± 0.07	0.3848	1.00 ± 0.05	0.7601	1.02 ± 0.05	0.9066	0.94 ± 0.13	0.4342	1.01 ± 0.06	1.0000
9.21_799.5511	0.98 ± 0.07	0.2555	1.10 ± 0.11	0.8685	1.04 ± 0.11	0.5707	1.26 ± 0.20	0.4882	1.13 ± 0.11	0.6318	1.01 ± 0.13	0.9721
9.25_854.5988	0.97 ± 0.08	0.1823	1.09 ± 0.13	0.8986	1.05 ± 0.12	0.4908	1.29 ± 0.15	0.1320	1.19 ± 0.16	0.6441	1.01 ± 0.12	0.6734
9.27_802.5557	0.93 ± 0.02	0.0018	0.95 ± 0.04	0.1680	0.97 ± 0.03	0.1564	1.07 ± 0.03	0.0789	1.04 ± 0.04	0.4778	1.02 ± 0.04	0.9949
9.27_742.5621	0.91 ± 0.03	0.0018	0.95 ± 0.03	0.1297	1.04 ± 0.03	0.3709	1.02 ± 0.04	0.9409	1.04 ± 0.04	0.3517	1.03 ± 0.05	0.4525
9.29_886.5486	0.94 ± 0.04	0.0374	0.90 ± 0.05	0.0334	1.06 ± 0.07	0.8490	0.87 ± 0.05	0.0104	0.99 ± 0.06	0.5745	0.96 ± 0.06	0.2877
9.30_828.5723	0.91 ± 0.03	0.0030	0.95 ± 0.07	0.2509	0.95 ± 0.05	0.1666	1.02 ± 0.05	0.9780	1.06 ± 0.08	0.8144	0.94 ± 0.05	0.2188
9.37_810.5910	0.92 ± 0.03	0.0068	1.03 ± 0.06	0.9529	1.09 ± 0.09	0.4763	0.95 ± 0.05	0.1422	1.13 ± 0.06	0.0831	0.99 ± 0.08	0.6343
9.39_836.6080	0.91 ± 0.03	0.0075	0.95 ± 0.19	0.4287	0.89 ± 0.07	0.2067	0.84 ± 0.08	0.2126	0.96 ± 0.13	0.3772	0.94 ± 0.11	0.3356

Table A2 (cont'd)

9.43_912.5671	0.91 ± 0.04	0.0107	1.02 ± 0.06	0.9819	1.03 ± 0.06	0.8751	0.83 ± 0.05	0.0006	1.04 ± 0.05	0.6936	1.07 ± 0.06	0.4269
9.43_980.5618	0.89 ± 0.06	0.0528	0.94 ± 0.12	0.7583	0.99 ± 0.12	0.0641	0.71 ± 0.11	0.0505	1.12 ± 0.17	0.9266	1.13 ± 0.11	0.2880
9.43_896.5942	0.83 ± 0.05	0.0018	0.96 ± 0.09	0.7159	0.94 ± 0.12	0.4956	0.81 ± 0.08	0.0637	0.94 ± 0.14	0.5741	1.24 ± 0.11	0.0441
9.43_996.5323	0.94 ± 0.06	0.0787	1.00 ± 0.06	0.6261	1.08 ± 0.08	0.8204	0.82 ± 0.07	0.0202	1.07 ± 0.07	0.7726	1.12 ± 0.07	0.2585
9.45_305.2507	0.96 ± 0.03	0.1260	1.03 ± 0.04	0.6827	1.01 ± 0.04	0.9448	0.95 ± 0.04	0.1099	1.07 ± 0.05	0.3113	0.98 ± 0.04	0.4479
9.55_878.6039	1.00 ± 0.04	0.5857	1.02 ± 0.05	0.9776	1.06 ± 0.06	0.5580	1.12 ± 0.14	0.7195	1.00 ± 0.06	0.7793	1.03 ± 0.06	0.8380
9.55_722.5355	0.99 ± 0.06	0.3360	1.03 ± 0.06	0.9237	0.97 ± 0.05	0.2654	1.22 ± 0.17	0.2587	1.27 ± 0.15	0.2945	1.00 ± 0.09	0.7170
9.57_748.5565	1.02 ± 0.08	0.3726	0.95 ± 0.12	0.5488	0.92 ± 0.05	0.0514	1.28 ± 0.17	0.4585	1.18 ± 0.17	0.7576	0.93 ± 0.10	0.9512
9.60_962.5644	0.98 ± 0.04	0.3884	0.94 ± 0.12	0.6085	0.93 ± 0.08	0.4154	0.86 ± 0.08	0.0670	0.90 ± 0.11	0.4951	1.04 ± 0.06	0.3485
9.62_786.5924	0.95 ± 0.05	0.4928	0.99 ± 0.05	0.6397	1.01 ± 0.04	0.9347	0.92 ± 0.03	0.0200	1.08 ± 0.10	0.6654	0.97 ± 0.07	0.3552
9.62_812.6059	0.94 ± 0.05	0.3070	0.98 ± 0.04	0.4212	1.06 ± 0.07	0.7431	0.97 ± 0.04	0.3066	1.11 ± 0.10	0.5124	0.97 ± 0.06	0.5168
9.62_838.619	0.98 ± 0.04	0.2660	0.95 ± 0.05	0.2046	1.09 ± 0.06	0.4444	1.00 ± 0.06	0.5494	1.03 ± 0.08	0.9024	1.02 ± 0.07	0.8772
9.8_854.5865	1.00 ± 0.03	0.8248	1.01 ± 0.05	0.9679	1.02 ± 0.04	0.9619	1.03 ± 0.06	0.9174	0.99 ± 0.07	0.5374	0.99 ± 0.06	0.8498
9.87_788.6038	0.90 ± 0.05	0.0437	0.96 ± 0.06	0.3629	0.98 ± 0.09	0.3081	0.83 ± 0.08	0.0974	1.03 ± 0.10	0.7331	0.89 ± 0.11	0.7789
9.87_1022.5484	0.96 ± 0.09	0.1208	1.14 ± 0.11	0.0095	0.98 ± 0.12	0.5357	0.88 ± 0.08	0.1137	1.23 ± 0.17	0.4558	1.11 ± 0.12	0.6849
9.88_938.5833	0.92 ± 0.04	0.0150	1.08 ± 0.09	0.7352	0.99 ± 0.06	0.4074	0.81 ± 0.05	0.0028	1.17 ± 0.12	0.2972	1.06 ± 0.05	0.4723
9.90_804.5677	0.94 ± 0.03	0.0263	0.99 ± 0.04	0.5525	1.02 ± 0.04	0.8595	0.95 ± 0.05	0.1888	0.93 ± 0.04	0.0727	0.93 ± 0.03	0.0483
9.91_880.6346	1.00 ± 0.04	0.6143	0.94 ± 0.05	0.1921	1.02 ± 0.06	0.7942	0.91 ± 0.06	0.0532	0.90 ± 0.08	0.0891	0.89 ± 0.05	0.0145
9.96_778.5881	1.00 ± 0.04	0.5921	0.97 ± 0.04	0.2894	0.98 ± 0.04	0.3606	0.97 ± 0.06	0.2908	0.97 ± 0.06	0.4421	0.96 ± 0.08	0.6813
9.99_888.5659	0.90 ± 0.05	0.0338	1.39 ± 0.29	0.2742	1.02 ± 0.13	0.3855	0.81 ± 0.06	0.0191	1.48 ± 0.36	0.3075	1.07 ± 0.12	0.9362

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Chapter 4 Quantitative Profiling of Plasma Bioactive Lipid Mediators Using LC-MS/MS: Application in Measurement of Biochemical Effects of Pulmonary Rehabilitation and OMT in COPD Patients

4.1 Introduction

Chronic obstructive pulmonary disease (COPD) presents an important cause of human morbidity and mortality throughout the world. The Global Burden of Disease study predicted that COPD ranks as the fifth leading cause of death, but will rise to the third leading cause by 2030 (1,2). COPD patients suffer from airflow limitation and are usually associated with chronic inflammatory response in the airways (3). Current clinical pharmacologic treatments mostly focus on improving function of the diseased pulmonary system, which is referred to as the "gas exchanger", to reduce symptoms by dilating the bronchi and bronchioles and regulating inflammation (4). However, traditional inhaled agents present several health risks and side effects, and COPD, as assessed by pulmonary function tests, is relatively insensitive to drug treatments (5). Thus, interest has emerged in developing complementary and alternative treatments, for example, pulmonary rehabilitation and osteopathic manipulative treatment, to improve the quality of life of COPD patients.

Pulmonary rehabilitation (PR) involves integrated care including, but not limited to, exercise training, education and behavior modification, and nutritional intervention (6). Prior investigations have shown that PR programs, especially the exercise training component, reduce labored breathing (dyspnea), increase exercise tolerance, strengthen respiratory muscles, exert positive psychological influence, and enhance subjects' reported quality of life (7,8). Due to its effectiveness and low cost, PR is currently widely recommended as part of

the treatment for COPD patients (9). On the other hand, osteopathic manipulative treatment (OMT), a hands-on care manipulating muscles, joints, and the movement of lymphatic fluids through these tissues, has been developed to treat somatic dysfunction and improve physiologic functions through stimulating self-healing (10). Unlike PR, fewer clinical trials have examined the effects of OMT on COPD patients, and the results were inconsistent (11-13). However, despite the long record of application of PR and OMT for treatment of COPD patients, the underlying mechanisms behind the physiological and psychological responses to these treatments have been limited. Accordingly, in this study we aimed to investigate whether PR exercise and OMT can modulate levels of bioactive lipid mediators, including oxylipins and endocannabinoids, in COPD patients' blood circulatory systems.

Oxylipins are oxidized metabolites of polyunsaturated fatty acids (PUFA) including linoleic acid (C18:2ω6, LA), arachidonic acid (C20:4 ω6, AA), eicosapentaenoic acid (C20:5 ω3, EPA), and docosahexaenoic acid (C22:6 ω3, DHA). Eicosanoids, which are derived primarily from the 20-carbon fatty acids AA and EPA, are the best known group of oxylipins (14-16). The synthesis of eicosanoids starts at the cell membrane, where for example arachidonic acid is stored in membrane phospholipids by esterification at the *sn*-2 position on the glycerol backbone. A variety of events trigger hydrolytic liberation of fatty acids from membranes by a group of enzymes called phospholipase A_2 (PLA_2), and the released fatty acids subsequently are metabolized via oxidative enzymes including cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450s (CYP), as well as other non-enzymatic pathways, as shown in Figure 4.1. Many of these oxylipins are known as

inflammatory mediators. For example, leukotrienes (LT) and prostaglandins (PG) are produced first after injury or infection, and action starts biochemical events that lead to destruction of invading pathogens and repair of damaged tissue. Leukotriene B4 (LTB4) can promote recruitment of neutrophils, and prostaglandin E2 (PGE2) is involved in vasodilation and increasing vascular permeability, which accelerates the inflammatory process (17,18). Although the inflammatory response is protective to the host, if uncontrolled, tissues would be overwhelmed by persistent inflammation, so resolution of inflammation is essential for the conservation of health. In contrast to inflammatory metabolites LT and PG, lipoxins and epoxyeicosatrienoic acids (EET), also derived from AA, have shown anti-inflammatory effects (19,20). Recent studies have indicated that AA is not the only fatty acid precursor that yields mediators involved in inflammation and anti-inflammation. The 18-carbon ω-6 linoleic acid (LA) and the two ω-3 PUFAs eicosapentaenoic (EPA, 20:5ω3) and docosahexaenoic (DHA, 22:6 ω3) acids also are converted to oxylipins through similar enzymatic and non-enzymatic pathways (Figure 4.1) (21-24).

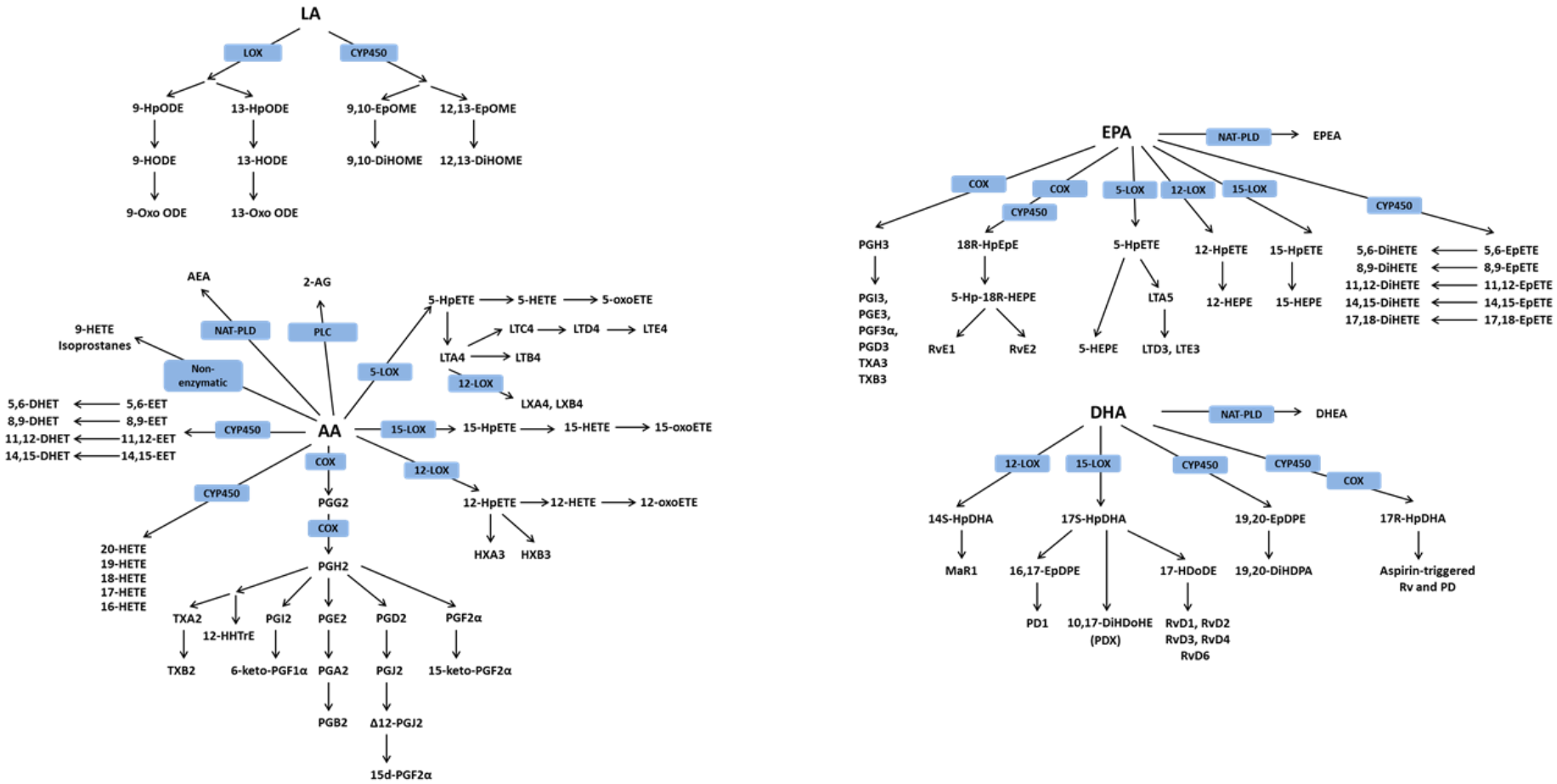

Figure 4.1. Overview of the biosynthetic pathways of oxylipins and endocannabinoids from linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

Another class of signaling lipids, endocannabinoid, *e.g.* the *N*-acylethanolamines including arachidonoyl ethanolamide (AEA), docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA), and 2-arachidonoyl glycerol (2-AG), are also derived from fatty acid release from membranes (25). Endocannabinoids have emerged as important biomarkers owing to their involvement in the regulation of many physiological processes including emotion, stress release, cognition, inflammation and reproduction (26).

An optimal method for analyzing oxylipins, endocannabinoids and their PUFA precursors needs to overcome several challenges. First, most of these lipid mediators are present at very low concentrations (in the nM to pM range) in biological fluids. Second, a great number of oxylipins have similar structures and many are isomers, which makes chromatographic separation a challenging task. Third, these bioactive lipids are produced in the same cascade and the change in levels of one oxylipin may influence the activities of all other compounds, so the method must cover a wide range of compounds. For years, enzyme-linked immuosorbent assays (ELISA) have been known as the conventional approach for quantification of oxylipins (27,28), but the similar structures of numerous oxylipins raises questions about whether ELISA measurements are specific for a single oxylipin. Gas chromatography-mass spectrometry (GC-MS) provides a platform to monitor multiple oxylipins simultaneously (29), but derivatization procedures are required, often involving three separate derivatizations to convert carboxylate, hydroxyl, and carbonyl groups to less polar derivatives. The development of electrospray ionization (ESI) opened the gate for the analysis of non-volatile molecules by mass spectrometry directly from a liquid sample

without derivatization. There is a growing body of research in the literature reporting liquid chromatography-tandem mass spectrometry (LC-MS/MS) based methods for qualitative and quantitative profiling of bioactive lipid mediators (30-41). Most approaches focused on one or two categories of bioactive lipids, for example, prostaglandins (32), COX and LOX pathway products (31,33), frequently measuring compounds derived only from arachidonic acid (30,36,37), or a limited range of endocannabinoids (35,39). However, as mentioned above, these bioactive lipids are generated through metabolic pathways organized in a complex network. Therefore, a comprehensive method that can cover multiple classes of targets is vital to promote studies related with inflammatory conditions. A few comprehensive methods for measuring lipid mediators have been reported recently for different biological matrices (34,38,40,41), but the methods either employed long analysis times or did not monitor oxylipins, endocannabinoids and PUFA precursors simultaneously.

This chapter describes the development of a high-throughput sensitive approach for quantification of 57 oxylipins and endocannabinoids derived from four major PUFAs, as well as the PUFA precursors themselves in a single analysis within 15 minutes. The method is based on solid phase extraction (SPE) sample preparation, reverse phase liquid chromatographic separation, and electrospray ionization (both positive and negative ionization modes) using multiple reaction monitoring (MRM) for quantification. The method was used to analyze bioactive lipids mediators from plasma of COPD patients involved in a clinical trial. In the pilot study, COPD patients were participating in a 12-week PR program, and some of them received additional OMT or sham OMT treatments. The aim of this clinical

trial was to determine responses of plasma bioactive lipid levels to PR exercise and OMT in patients with COPD to determine whether OMT treatments generated distinct biochemical responses.

4.2 Materials and methods

4.2.1 Clinical trial design and blood sample collection

Ethics statement: The research protocols were approved by the Institutional Review Board at Michigan State University (IRB #12-305I). Subject files were de-identified.

COPD patients were recruited from participants in a standard 12-week pulmonary rehabilitation (PR) program at the Ingham Regional Medical Center (IRMC, now McLaren Greater Lansing Hospital) in Lansing, MI. In the pulmonary rehabilitation program, patients were doing exercise training in fitness gymnasium (*e.g.* walking and cycling exercise) every Monday and Thursday each week. Participants are recommended to have 60-70% of the maximum symptom-limited exercise capacity for 20-30 minutes. Pulmonary function and stamina (6-minute walk distance) tests were performed on weeks 0, 6, and 12. In total, thirty-three patients who eventually completed the 12-week study were recruited and randomly assigned to one of the three groups: (a) OMT (n=13) group receiving biweekly osteopathic structural exam (OSE) before and after OMT in addition to PR; (b) sham group (n=7) receiving PR as well as biweekly OSE without OMT, serving as a control for the effect of touch and psychological effects of being examined by a physician; and (c) PR only group (n=13) participating in the PR program but not receiving OSE or OMT. Appendix Table A3

gives a detailed summary of the anonymized subjects including demographic information and pulmonary function test parameters.

Blood specimens were collected from subjects at week 0, 6, and 12. For each of the weeks, three blood samples were drawn: approximately 1 hour before treatment, 1 hour after treatment, and 72 hours after treatment. Approximately 5 mL of blood was drawn from the antecubital fossa into a Becton-Dickinson sodium heparin tube (BD, Franklin Lakes, NJ), and the tube was kept on ice during transport from the clinic to the laboratory. Each tube was then centrifuged at 1100 × g for 10 min at 4˚C. Each supernatant plasma was transferred to a barcoded polypropylene cryostorage tube (VWR International) and stored at -80˚C until use.

4.2.2 Chemicals

All oxylipins, endocannabinoids and PUFAs standards and deuterated internal standards were purchased from Cayman Chemical (Ann Arbor, MI). Water was purified using the Milli-Q System from Millipore Corp (Bedford, MA, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), ethyl acetate (HPLC grade) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid was purchased from Fisher Scientific (Pittsburgh, PA). Butylated hydroxytoluene (BHT) from Tokyo Kasei Kogyo Co. (Tokyo, Japan) was purchased from Fisher Scientific. Oasis HLB SPE cartridges were purchased from Waters Co. (Milford, MA).

4.2.3 Internal standards and calibration solution preparation

Seven deuterated compounds including 8,9-epoxyeicosatrienoic acid-d_{11} (8,9-EET-d_{11}), 8,9-dihydroxyeicosatrienoic acid-d_{11} (8,9-DHET-d_{11}), 5-hydroxyeicosatetraenoic acid-d_8

(5-HETE-d_8), prostaglandin E2- d_9 (PGE2-d_9), arachidonic acid-d_8 (AA-d_8), anandamide- d_8 (AEA-d_8) and 2-arachidonoylglycerol-d_8 (2-AG-d_8) were used as internal standards (IS) to adjust for instrument drift and extraction yield. By mixing the proper amount of each deuterated standard in methanol containing 0.01% BHT, the internal standard (IS) working solution was prepared at concentrations of 0.5-50 µM (8,9-EET-d_{11}: 1.0 µM; 8,9-DHET-d_{11}: 0.50 µM; 5-HETE-d_8: 0.50 µM; PGE2-d_9: 1.0 µM; AA-d_8: 50 µM; AEA-d_8: 0.5 µM; 2-AG-d_8: 4.0 µM).

A calibration-master solution was prepared by pooling the unlabeled standard stock solutions to final concentrations in the range of 1-20 µM for oxylipins and endocannabinoids, and 1000 µM for PUFAs. The calibration-master solution was further diluted serially in methanol containing 0.01% BHT to prepare calibration curves from 0.01-10,000 nM for oxylipins and endocannabinoids, and from 0.002-20 µM for PUFAs. A volume of 950 µL of each solution was mixed with 50 µL of IS working solution to make the IS containing calibration (C) solutions C1-C8 (Appendix Table A4).

4.2.4 Plasma sample preparation

Plasma samples were thawed on ice for about 30 minutes for processing. A 2.00-mL volume of methanol containing 0.01% BHT and a cocktail of deuterium-labeled internal standards were added to 500 µL of plasma (the amounts of internal standards are the same with those in calibration solutions). The mixture was vortexed and incubated at 4˚C for 20 min before centrifuging at 8000 × *g* for 15 min at 4˚C. Then each supernatant was transferred into a centrifuge tube containing 1.5 mL of Milli-Q water, resulting in a total volume of 4 mL.

Solid phase extraction (SPE) for human plasma samples followed the procedures modified from the method developed by Yang (41). Briefly, before extraction, Oasis-HLB cartridges (60 mg) (Waters, Milford, MA) were conditioned by washing successively with 2 mL of ethyl acetate, 2 mL of methanol (twice) and 2 mL water/methanol (95:5, v/v) containing 0.1% acetic acid. Each lipid extract supernatant described in the previous paragraph was loaded on the cartridge using a light partial vacuum to draw the liquid through the cartridge. Then the cartridge was washed with 2 mL of water/methanol (95:5, v/v) containing 0.1% acetic acid. Following aspiration, the targeted lipids were eluted by addition of 0.6 mL of methanol followed by 2 mL of ethyl acetate into a glass tube containing 6 µL of 30% glycerol in methanol as a trapping solution. Each eluted fraction was evaporated under vacuum at room temperature using a SpeedVac. Each residue was reconstituted in 150 µL of methanol containing 0.01% BHT, and transferred to an amber glass autosampler vial with 250-µL glass insert in preparation for UHPLC-MS/MS analysis.

4.2.5 LC-MS/MS analysis

Liquid chromatography separations were performed using an Ascentis Express C18 column (10 cm×2.1 mm; 2.7 µm particles, Sigma-Aldrich, St. Louis, MO) maintained at 50 ˚C on a Waters ACQUITY UPLC system. Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. Analytes were eluted during a 15 min gradient (Appendix Table A5) using a flow rate of 0.3 mL/min. The autosampler was cooled to 10 ˚C, and injection volume was set to 5 µL.

The column was connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Analytes were detected using electrospray ionization (ESI) with polarity switching between negative-ion mode (for oxylipins and PUFAs) and positive-ion mode (for endocannabinoids) using multiple reaction monitoring (MRM) divided into 9 data acquisition functions acquired at different times. The capillary voltage was set at 2.5 kV for negative ESI mode and 3.0 kV for positive mode. Additional source parameters in both modes were: source temperature 150 ˚C, desolvation temperature 500 ˚C, desolvation gas flow rate 600 L/h, cone gas flow rate 150 L/h. MRM parameters including cone voltage, collision voltage, precursor ion, product ion and dwell time were optimized by flow injection of pure standard for each individual compound. To avoid interference and achieve lower detection limit, more sensitive or selective transitions were chosen during method development.

4.2.6 Method validation

A series of calibration solutions was used to assess the linearity of response for each analyte (the concentrations are listed in Appendix Table A4, and the LLOD, LLOQ as well as linearity determined here were for analytes in calibration matrix (methanol containing 0.01% BHT)). For oxylipins and endocannabinoids, the ratio of analyte peak area to its internal standard peak area was plotted against the concentration, fitted by a weighted (1/x, where x represents analyte concentration) linear regression using the TargetLynx component of MassLynx 4.1 software (Waters). For the four fatty acids, the log-transformed ratio was plotted against concentration, and the fit was linear but with equal weighting applied. The LLOD was defined as the concentration at which the peak height was three times that of the

RMS noise (S/N=3), and LLOQ was the concentration with a peak height corresponding to 10 times that of the RMS noise (S/N=10).

Accuracy and precision of the method were determined by analyzing quality control (QC) samples at three concentration levels (QC1: C5; QC2: C6; QC3: C7 for oxylipins and endocannabinoids; QC1: C4; QC2: C5; QC3: C6 for PUFAs; see Appendix Table A4 for levels of calibration solutions C3-C7 for each analyte) in calibration matrix (methanol containing 0.01% BHT). All QC samples were analyzed in three technical replicates within one day with a whole set of calibration standards (intra-day), and this process was repeated for three consecutive days (inter-day). Accuracy is defined as the degree of closeness of the average calculated concentration to the nominal concentration, reported as the percent of the expected concentration. Precision is defined as the closeness of the measurements to each other, and was expressed as coefficient of variation (CV).

The analytes measured were endogenous metabolites present in all plasma samples, so there is no “true blank” plasma available. So the seven deuterated internal standards were considered as the representative for different lipid classes, and were used to determine the influence of extraction and matrix effect. The IS mixture was spiked into human plasma before extraction steps, and integrated peak areas for each deuterated standard were compared with the same amount of the corresponding internal standard spiked in the calibration solution (methanol containing 0.01% BHT). The influence of recovery and matrix effect together was calculated as $Area_{pre}/Area_{methanol}$.

The short-term stability in calibration matrix was determined by triplicate measurements of QC samples in concentration level of C6 for oxylipins and endocannabinoids, and C5 for PUFAs (see Appendix Table A4 for the levels of C6 and C5 of each analyte). The QC samples were stored at 10 ˚C in the autosampler, and were tested 48 hours from the time of preparation. The percent difference between nominal concentration and average measured concentration was calculated as a measure of analyte stability.

4.2.7 Data processing and statistical analysis

TargetLynx software was used for peak detection, integration and quantification. Plasma concentrations were calculated from the calibration curves for each compound. For those concentrations lower than LLOQ, a value of the LLOQ/2 was inserted into the spreadsheet to facilitate log-transformation. Metabolites with more than 20% of observations with concentration lower than LLOQ were excluded. All concentrations were log_2-transformed (log2) to better approximate a normal distribution. Statistical analysis was performed using JMP Pro 12 (SAS, Cary, NC, USA). Student's *t* test was used for comparing metabolites' changes within group and one way ANOVA with Tukey-Kramer post test were used to compare differences between groups. Significance was set as $\alpha<0.05$.

4.3 Results and discussion

4.3.1 Method development

4.3.1.1 LC method development

As mentioned above, the development of LC-MS/MS methods for comprehensive quantification of bioactive lipid mediators encounters several challenges. The high structural

similarity between these compounds, especially oxylipin isomers, requires sufficient chromatographic separation to resolve the isomers. Even though MRM transitions (precursor ion-product ion) employ different *m/z* values to quantify different isomers, it is common for multiple isomers to yield product ions of the same mass, albeit at different yields, so chromatographic resolution remains important. Moreover, usually a large number of samples are collected in clinical trial; therefore, high-throughput methods (*e.g.*, with relatively short gradient) would be preferable than more time consuming protocols.

The total instrumental analysis time was 15 minutes in our method (including between-sample equilibration time), which is shorter than previously published methods (33,38). Within the 15 minute run time, most critical separation pairs (compounds with identical molecular compositions, common product ions, and close elution time (41)) can be discriminated. For example, Figure 4.2 shows the extracted ion chromatogram of two prostaglandins (PG) PGD2 and PGE2, which share the same transition of *m/z* 351.2 > 271. The method achieved baseline chromatographic resolution of these two isomers even though they were measured using identical precursor and product ions. The overlapped chromatograms of all 49 oxylipins, 4 endocannabinoids, 4 PUFAs, and 7 deuterated internal standards are presented in Figure 4.3. Detailed retention times for entire group of metabolites are listed in Table 4.1.

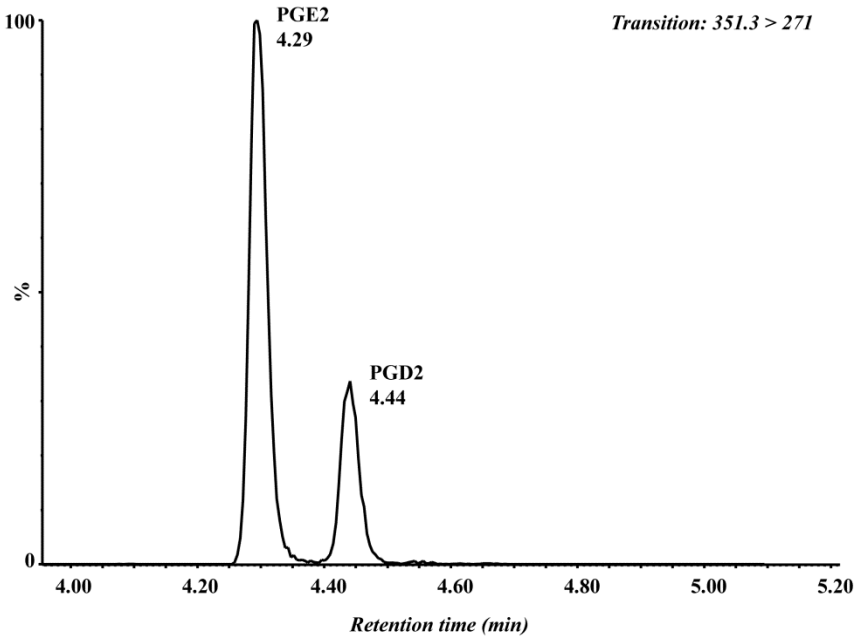

Figure 4.2. Extracted ion LC/MS/MS chromatogram of PGE2 and PGD2 with the same transition of *m/z* 351.2 > 217 using electrospray ionization in negative-ion mode. The developed LC method separated critical isomer pairs (compounds with identical molecular compositions, similar fragments, and close elution time). The two isomers, PGE2 and PGD2, were baseline separated, so even though they were assigned identical precursor and product ions, neither interfered with detection of the other.

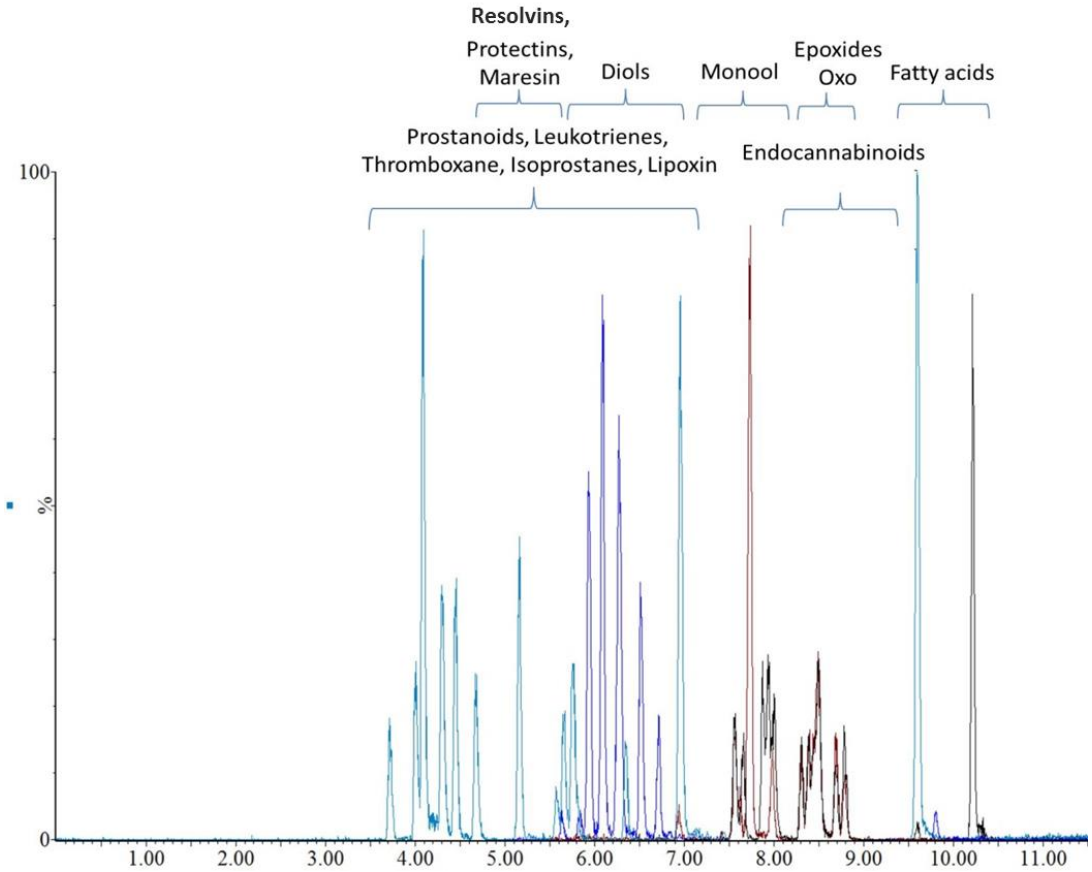

Figure 4.3. Overlapped LC-MS/MS chromatogram (MRM mode) for all analytes and internal standards. The analytes include 49 oxylipins, 4 endocannabinoids, 4 PUFAs, and 7 deuterated internal standards. Oxylipins and PUFAs were detected using electrospray ionization in negative-ion mode, and endocannabinoids were detected in positive-ion mode.

Table 4.1. Optimized MRM parameters and retention time for each analyte. The standard of each analyte (~10 μM) was flow-injected individually to optimize MS parameters including ion source cone potential, collision potential, and *m/z* of precursor ion and product ion through QuanOptimize module as part of the Waters Masslynx software package. In the whole optimization process, the most sensitive and selective transitions were chosen to the MRM method. Sometimes isomers' had identical most abundant fragment ions, but since the LC method can resolve most of the analytes, the same MRM transitions were allowed. The transition pairs were arranged into different segments to increase the time averaging for each data point and increase the signal-to-noise ratio.

Compound	Seg-ment	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone potential (V)	Collision potential (V)	Internal standard
6-keto-PGF1α	1	3.72	369.3	163	21	22	PGE2-d_9
8-isoPGF2α		4.01	353.2	193	21	22	PGE2-d_9
TXB2		4.01	369.2	169	51	16	PGE2-d_9
LTD4		4.29	495.3	177	51	22	8,9-DHET-d_{11}
PGE2-d_9		4.28	360.2	280	21	16	-
PGE2		4.30	351.2	271	55	16	PGE2-d_9
PGD2		4.44	351.2	271	55	16	PGE2-d_9
RvD2		4.45	375.2	175	21	22	8,9-DHET-d_{11}
LXA4		4.67	351.2	115	55	16	8,9-DHET-d_{11}
RvD1		4.69	375.2	141	21	10	8,9-DHET-d_{11}
8-isoPGA2	2	5.15	333.2	175	55	22	PGE2-d_9
8-isoPGA1		5.16	335.2	235	39	16	PGE2-d_9
10,17-DiHDoHE	3	5.66	359.2	153	15	16	8,9-DHET-d_{11}
7-MaR1		5.58	359.2	177	51	16	8,9-DHET-d_{11}
17,18-DiHETE		5.64	335.2	247	39	16	8,9-DHET-d_{11}
LTB4		5.76	335.2	195	21	16	8,9-DHET-d_{11}
7,17-hydroxy-DPA		5.81	361.2	143	27	16	8,9-DHET-d_{11}
14,15-DiHETE		5.84	335.2	207	27	16	8,9-DHET-d_{11}
12,13-DiHOME		5.94	313.2	183	45	22	8,9-DHET-d_{11}
9,10-DiHOME		6.09	313.2	201	27	22	8,9-DHET-d_{11}
5,6-DiHETE	4	6.26	335.2	145	21	16	8,9-DHET-d_{11}
19,20-DiHDPA		6.27	361.2	273	27	16	8,9-DHET-d_{11}
14,15-DHET		6.30	337.2	207	33	16	8,9-DHET-d_{11}
12-HHTrE		6.36	279.2	179	45	10	5-HETE-d8
11,12-DHET		6.53	337.2	167	51	22	8,9-DHET-d_{11}
8,9-DHET-d_{11}		6.68	348.3	127	55	22	-
8,9-DHET		6.73	337.2	127	15	22	8,9-DHET-d_{11}
20-HETE		6.94	319.2	245	55	16	5-HETE-d_8
15d-PGJ2		6.95	315.2	271	39	10	PGE2-d_9

Table 4.1 (cont'd)

13-HODE	5	7.37	295.2	195	30	16	5-HETE-d_8
9-HODE		7.42	295.2	171	45	16	5-HETE-d_8
15-HETE		7.59	319.2	219	40	10	5-HETE-d_8
11-HETE		7.75	319.2	167	51	16	5-HETE-d_8
17-HDoHE		7.65	343.2	281	45	10	5-HETE-d_8
17,18-EpETE		7.68	317.2	255	15	10	8,9-EET-d_{11}
13-OxoODE		7.71	293.2	113	15	22	8,9-EET-d_{11}
9-HETE	6	8.00	319.2	151	15	10	5-HETE-d_8
14,15-EpETE		7.90	317.2	207	27	10	8,9-EET-d_{11}
15-OxoETE		7.88	317.2	113	45	16	8,9-EET-d_{11}
9-OxoODE		7.90	293.2	185	27	16	8,9-EET-d_{11}
5-HETE-d_8		8.05	327.2	116	55	16	-
11,12-EpETE		7.96	317.2	167	15	10	8,9-EET-d_{11}
5-HETE		8.09	319.2	115	33	10	5-HETE-d8
8,9-EpETE		8.03	317.2	255	15	10	8,9-EET-d_{11}
19.20-EpDPE	7	8.33	343.2	299	27	10	8,9-EET-d_{11}
12,13-EpOME		8.40	295.2	195	30	16	8,9-EET-d_{11}
14,15-EET		8.47	319.2	219	40	10	8,9-EET-d_{11}
9,10-EpOME		8.50	295.2	171	45	16	8,9-EET-d_{11}
16,17-EpDPE		8.55	343.2	233	15	10	8,9-EET-d_{11}
5-OxoETE		8.64	317.2	203	45	22	8,9-EET-d_{11}
11,12-EET		8.72	319.2	179	27	10	8,9-EET-d_{11}
8,9-EET-d_{11}		8.76	330.3	268	15	10	-
8,9-EET		8.81	319.2	155	27	10	8,9-EET-d_{11}
EPEA	8	8.06	346.3	62	55	16	AEA-d_8
AEA		8.87	348.2	62	33	16	AEA-d_8
DHEA		8.79	372.3	62	15	16	AEA-d_8
AEA-d_8		8.83	356.3	63	33	10	-
2-AG		9.50	379.2	287	39	16	2-AG-d_8
2-AG-d_8		9.46	387.3	294	21	16	-
EPA	9	9.64	301.2	257	39	22	AA-d_8
DHA		10.09	327.2	283	51	22	AA-d_8
LA		10.35	279.2	59	45	22	AA-d_8
AA-d_8		10.20	311.2	267	21	20	-
AA		10.24	303.2	259	21	22	AA-d_8

4.3.1.2 Mass spectrometry optimization

All oxylipins and PUFAs were analyzed using electrospray ionization in negative ion mode, while the endocannabinoids, which lack acidic functional groups, were detected in positive mode. The ability of fast polarity switching of the Waters Xevo TQ-S instrument enabled monitoring both positive and negative ionizing compounds in a single injection.

To reach the lowest detection limit, the standard of each analyte (~10 µM) was flow-injected individually to optimize mass spectrometer parameters of ion source cone voltage and collision cell potential through QuanOptimize module as part of the Waters Masslynx software package. The analyst provides the neutral molecular mass of each analyte, and the software calculates the instrumental conditions that yield the most abundant signal, and recommends *m/z* of the most abundant product ion. In the MS/MS optimization process, the most sensitive and selective transitions of product ions to precursor ions were chosen for the MRM method. Sometimes isomers had identical most abundant fragment ions, but since the LC method can resolve most of the analytes, the same MRM transitions were allowed. For example, the MRM transition *m/z* 295.2 > 171 was used for detecting both 9-HODE (RT=7.42 min) and 9,10-EpOME (RT=8.50 min), the transition *m/z* 295.2 > 195 was used for detecting both 13-HODE (RT=7.37 min) and 12,13-EpOME (RT=8.40 min), and the transition *m/z* 351.2 > 271 for PGE2 and PGD2 as mentioned above. For co-eluting or overlapping isomers, unique fragment ions were chosen to avoid interference, *e.g.*, 14,15-EpETE and 15-OxoETE had almost the same retention time (~7.89 min) and the same precursor $[M-H]^-$ ion at *m/z* 317.2, but their unique product ions of *m/z* 207 and 113

respectively enabled resolution of the two species. The optimized mass spectrometric parameters for all compounds are given in Table 4.1 above.

To improve signal-to-noise ratios, the transition pairs (precursor/product ions) were arranged into different data acquisition functions with durations spanning retention times of the target metabolites rather than over the entire data analysis period (Table 4.1). Thus, only a fraction of the ion pairs was monitored in any single time period. This allowed for longer transition dwell times without sacrificing the number of data points across the width of chromatographic peaks to an unacceptable value. In this method, dwell times ranged from 9-41 ms with nine acquisition periods.

4.3.1.3 Method validation

Using the series of diluted calibration solutions, the linearity of the method response was determined from the calibration curves established for each compound (Appendix Table A6). The goodness of fit, R^2, exceeded >0.99 for all analytes. Values of LLOQ and LLOD were estimated as the concentrations producing S/N=10 and S/N=3 respectively. The estimated LLOQ ranged from 0.05-2.16 nM for oxylipins, 0.63-4.80 nM for endocannabinoids, and 0.01-0.02 µM for PUFAs in the calibration matrix (methanol containing 0.01% BHT; Appendix Table A6).

To evaluate the reproducibility and variability of the method, intra-day and inter-day accuracy and precision were determined for three concentration levels in calibration solution by daily triplicate injections over three days. Appendix Table A7 lists the intra-day accuracy and precision for each analyte, and Appendix Table A8 summarizes the inter-day results. The

results showed that for almost all analytes in the three concentration levels, the accuracy fell between 80% - 120% of the expected values, and precision was less than ±15%, indicating our method can be considered accurate and precise over the concentration range.

Due to the lack of a "true blank" plasma matrix, in this validation, recovery and matrix effects were evaluated together by comparing instrument responses for deuterated ISs spiked into human plasma before extraction with responses for the same ISs in calibration solutions (methanol containing 0.01% BHT). Responses for ISs in plasma ranged from 28% (PGE2-d_9) to 52% (8,9-EET-d_{11}) (Appendix Figure A1), with CV% less than 10% in all cases. PGE2-d_9 exerted slightly lower recovery and matrix effect probably due to the loss during SPE washing and more ion suppression during its elution time.

Appendix Table A9 lists the short-term stability of each analyte in the calibration solution, stored at autosampler temperature. The measured concentrations for all compounds after 48 hours were within 15% of the concentrations measured at the starting point, with CV% less than 15% (with an exception of 5,6-DiHETE whose CV% is 17%). This indicates that the analytes were stable for up to two days in the calibration matrix at 10 ˚C in the dark, which is a typical autosampler condition.

4.3.2 Results of profiling plasma bioactive lipids from COPD patients

Of the total 57 metabolites measured, 36 had more than 20% of measured concentration lower than LLOQ and were excluded from statistical analysis. Data were $\log_2$-transformed for further statistical analysis, and after the transformation all achieved normal distributions.

4.3.2.1 Basal plasma levels of bioactive lipids in COPD patients

Baseline plasma concentrations of oxylipins, endocannabinoids and fatty acid precursors for subjects from the three treatment groups were measured before the PR program started, and listed in Table 4.2. Among the four fatty acid precursors, linoleic acid (LA) was present in the highest concentration (mean plasma concentrations PR/OMT/sham: 26/23/13 μM), followed by arachidonic acid (AA) (PR/OMT/sham: 5.3/4.7/2.4 μM) and docosahexaenoic acid (DHA) (PR/OMT/sham: 4.3/5.3/2.7 μM) with similar abundance, and eicosapentaenoic acid (EPA) with lowest level (PR/OMT/sham: 0.5/0.5/0.4 μM). One way ANOVA with Tukey-Kramer post test revealed that levels of most of the measured metabolites showed no significant difference between the three randomized groups at baseline pre-treatment levels. But the levels of free LA and AA in the sham group were significantly lower (~half) than the other two treatment groups ($P<0.05$). Also levels of 9-hydroxyoctadecadienoic acid (9-HODE), 11-hydroxyeicosatetraenoic acid (11-HETE) and 20-HETE in PR and OMT groups were ~2-fold higher than sham group ($P<0.05$), and levels of 8,9- and 14,15-dihydroxyeicosatrienoic acid (DHET) in PR only group were ~1.5-2 folds higher than the other two groups ($P<0.05$). Diversity of diet, perhaps including consumption of dietary supplements, may explain the variation of these metabolites, especially the two free fatty acids (LA and AA). Alternatively, differences in free fatty acid and other oxylipin levels may reflect differences in baseline physiological states of specific individual subjects. In this pilot clinical trial, sample sizes were relatively small in each group (n=13, 13 and 7 for PR only, OMT and sham group); with the fact that plasma metabolites levels varied greatly

between individuals, it was not surprising that difference existed in several compounds. Small sample size is one major limitation of the current study, and to account for baseline metabolic differences between individuals, clinical trials designed with larger sample size should be performed. As shown in Table 4.2, epoxide metabolites levels were present in the same order as their PUFA precursors, with two epoxyoctadecenoic acids (EpOMEs) derived from LA showing greatest levels (mean plasma concentration ~ 200-400 nM), followed by AA-epoxides and DHA-epoxides with levels ~ 5-20 nM, and EPA-epoxides with lowest levels (~ 0.2-1 nM). As for vicinal diols, which are hydrolysis products of the fatty acid epoxides, two LA-diols 9,10-and 12,13-dihydroxyoctadecenoic acid (DiHOME) and one EPA-diol 17,18-dihydroxyeicosatetraenoic acid (DiHETE) presented the most abundant diols (~ 15-50 nM), followed by the other two EPA-diols (5,6-DiHETE and 14,15-DiHETE) , two AA-diols 11,12- and 14,15- dihydroxyeicosatrienoic acid (DHET) and DHA-diol 19,20-dihydroxydocosapentaenoic acid (DiHDPA) with levels ~ 1.0-7.5 nM. 8,9-DHET was the least abundant diol (~ 0.3-0.5 nM). Four monohydroxy alcohols from AA (~ 0.05-1.7 nM), two monohydroxy alcohols from LA (2.1-10 nM) and two ketones derived from LA (~1.1-2.1 nM) were detected. Prostaglandin 6-keto-PGF1α and lipoxin LXA4 derived from AA were also detected (~ 0.1-0.8 nM). Among endocannabinoids, AA- and DHA-derived metabolites anandamide (AEA) and docosahexaenoyl ethanolamide (DHEA) were detected and presented ranging from 1.5- 6.6 nM.

Table 4.2. Baseline levels (Mean±SEM) of measured oxylipins, endocannabinoids and fatty acids for COPD patients from PR only (n=13), OMT (n=13) and sham (n=7) treatment groups at week 0 before starting PR program. Concentration of oxylipins and endocannabinoids are in units of nM, and concentration of free fatty acids are in units of μM.

Category	Fatty acid Precursor	Compound	PR only Group	OMT Group	Sham Group
Epoxide	LA	9,10-EpOME	383±89	221±58	264±58
		12,13-EpOME	256±64	140±35	168±39
	AA	8,9-EET	6.2±1.0	4.7±1.0	6.0±0.7
		11,12-EET	8.0±1.4	5.8±1.1	7.8±1.1
		14,15-EET	16.7±2.55	12.2±2.4	17.7±2.4
	EPA	8,9-EpETE	0.75±0.21	0.91±0.27	1.10±0.48
		11,12-EpETE	0.50±0.13	0.54±0.16	0.73±0.34
		14,15-EpETE	0.53±0.15	0.64±0.18	0.84±0.42
		17,18-EpETE	0.20±0.06	0.38±0.11	0.27±0.22
	DHA	16,17-EpDPE	5.2±0.9	6.2±1.6	8.1±2.7
		19,20-EpDPE	7.3±1.4	7.6±2.1	9.9±4.1
Diol	LA	9,10-DiHOME	24.3±8.3	14.9±3.0	30.4±11.0
		12,13-DiHOME	31.8±9.1	18.2±2.9	48.7±14.4
	AA	8,9-DHET*	0.54±0.06	0.36±0.04	0.30±0.06
		11,12-DHET	2.2±0.6	1.0±0.1	1.3±0.2
		14,15-DHET*	3.9±0.8	1.8±0.2	2.6±0.4
	EPA	5,6-DiHETE	7.2±3.3	4.1±1.4	5.8±3.1
		14,15-DiHETE	1.7±0.5	1.1±0.2	2.8±1.6
		17,18-DiHETE	19.5±7.3	10.6±1.4	18.2±7.8
	DHA	19,20-DiHDPA	4.1±0.9	2.5±0.2	3.2±0.7
Monohydroxy	LA	9-HODE*	4.1±1.0	5.3±1.1	2.1±1.0
		13-HODE	8.4±1.78	10.4±1.6	7.1±3.5
	AA	5-HETE	1.7±0.1	1.2±0.1	1.3±0.3
		11-HETE*	0.12±0.03	0.13±0.02	0.05±0.02
		15-HETE	0.43±0.10	0.40±0.08	0.19±0.04
		20-HETE*	0.63±0.14	0.73±0.13	0.30±0.12
Ketone	LA	9-OxoODE	2.1±0.3	2.1±0.2	1.5±0.3
		13-OxoODE	1.2±0.3	1.5±0.3	1.1±0.4
	AA	6-keto-PGF1α	0.11±0.02	0.12±0.02	0.15±0.04
Trihydroxy	AA	LXA4	0.28±0.08	0.15±0.02	0.74±0.44
Endocannabinoid	AA	AEA	1.87±0.2	1.5±0.2	1.6±0.3
	DHA	DHEA	5.4±0.8	5.7±0.9	6.6±1.2

Table 4.2 (cont'd)

Fatty acid	LA	LA*	25.5±4.4	23.1±2.8	13.2±2.9
	AA	AA*	5.3±0.9	4.7±0.5	2.4±0.4
	EPA	EPA	0.46±0.13	0.54±0.11	0.43±0.14
	DHA	DHA	4.3±0.87	5.3±1.3	2.7±1.2

* One way ANOVA analysis was performed to compare baseline metabolite levels (week 0) between groups. The metabolite with p value <0.05 from Tukey-Kramer HSD post test are labeled with *.

4.3.2.2 Short term effect of exercise, OMT and sham on bioactive lipids

The first comparisons of plasma metabolite levels evaluated changes in metabolite profiles between the baseline pre-exercise and one hour post-exercise in PR only group. As shown in Table 4.3, the levels of almost all bioactive lipids were elevated significantly (~ 1.1-3.1 folds increase) one hour after the subjects finished PR program exercise only, with the exception of LXA4, 6-keto-PGF1α and the two endocannabinoids AEA and DHEA. Exercise alone (PR group) resulted in higher plasma levels of free PUFAs, with DHA exerting the greatest increase (2.9-fold, P<0.0001), followed by LA (2.2-fold, P<0.0001), AA (2.0-fold, P<0.0001) and EPA (1.8 fold, P<0.0001). The PUFA precursors' release was associated with increased levels of downstream metabolites. More specifically, of all measured metabolites, EpOMEs, the LA-derived epoxides had the greatest increase (~ 3-fold, P<0.0001). Epoxides derived from AA and DHA exhibited similar increase (1.8-1.9 folds, P<0.0001), while four EPA-derived epoxides showed a slightly greater fold increase (1.9-2.5 fold, P<0.0001). Among vicinal diols, which are direct metabolites of fatty acid epoxides, DiHOMEs from LA cascade also exhibited greatest fold-changes (1.5-fold increase, P<0.001), and DHETs, DiHETEs and DiHDPA from AA, EPA and DHA respectively were observed 1.1-1.5 fold more abundant after exercise alone (P<0.0001 to P<0.05). As for monohydroxy fatty acids

(alcohols) derived from AA, 5-HETE, 11-HETE, 15-HETE and 20-HETE exhibited increases ranging from 1.9- to 2.3-fold ($P<0.01$). LA-derived alcohols 9-HODE and 13-HODE altered by 2.4- and 2.0-fold increases ($P<0.01$), and their downstream ketones 9-OxoODE and 13-OxoODE were observed to increase with less magnitude than HODEs (2.0-fold, $P<0.0005$, and 1.8-fold, $P<0.0001$).

Most metabolites elevated in the PR only group due to exercise also showed significantly higher levels in OMT group one hour post-treatment (~ 1.2-2.9 folds increase), except 6 vicinal diols including 8,9-DHET, 14,15-DHET 5,6-DiHETE, 14,15-DiHETE, 17,18-DiHETE and 19,20-DiHDPA (Table 4.3). The increases of PUFAs induced by exercise+OMT (OMT group) are ~ 1.8 to 2.8-fold ($P<0.01$), which are parallel to those induced by exercise alone (PR group) but with about 5% less increase in OMT group. All epoxides exerted ~1.7 to 3-fold increase in OMT group, with the changing order as follows: EpOMEs (LA-epoxides, ~3-fold, $P<0.0001$) > EpETEs (EPA-epoxides, ~1.9 to 2.8-fold, $P<0.01$) > EpDPEs (DHA-epoxides, ~2-fold, $P<0.01$) > EET (AA-epoxides, ~1.8-fold, $P<0.01$), which were also parallel to the fold-change magnitude and order in PR group as mentioned above. LA-derived diols DiHOMEs exhibited the highest increase among all diols in OMT group (~1.7-fold, $P<0.001$), which were slightly higher (~ 5%) than exercise alone. However, unlike PR only group, all the other diols derived from AA, EPA, and DHA did not show statistically significant change with additional OMT, except 11,12-DHET (1.3-fold increase, $P<0.05$). As for monohydroxy metabolites, exercise+OMT induced ~1.5 to 2.3-fold increase to the four HETEs (derived from AA, $P<0.01$ at least) and ~ 2.0-fold increase to

HODEs (derived from LA, P<0.001), which were ~5-10% lesser than the increase after exercise alone. HODEs' ketone metabolites 9-OxoODE and 9-OxoODE increased by ~ 1.6-fold (P<0.01), also ~15% lesser than the change induced by PR exercise alone.

On the other hand, sham "treatment" in addition to PR exercise had significant short-term effect only on LA (1.6-fold increase, P<0.05) and one of its epoxide product 12,13-EpOME (2.3-fold increase, P<0.05), which were ~20% less increase compared with true OMT. Though differences were observed between the three groups as described above, one way ANOVA with Tukey post test showed that only the fold-change of 9,10-DiHOME was significantly higher in OMT than the other two groups (P<0.05). It is recommended that larger cohorts of subjects in each treatment group be recruited to establish whether additional changes in oxylipin profiles that are statistically significant can be assessed.

Table 4.3. Short-term effects of PR exercise, OMT and sham on plasma levels of bioactive lipids. One hour-post/pre exercise and treatment fold changes (Mean±SEM) of measured oxylipins, endocannabinoids and fatty acid precursors levels for COPD patients from PR only (n=38), OMT (n=38) and sham (n=21) treatment groups. Fold change was log_2 transformed and Student's *t* test (two-tailed) was used to compare mean fold change (log_2 transformed) of each group with 0 (α=0.05). If P<0.05, the change is considered significant and listed in the table.

Category	Fatty acid Precursor	Compound	1 hour post-/pre- fold change			P-value		
			PR only	OMT	Sham	PR only	OMT	Sham
Epoxide	LA	9,10 EpOME	3.0 ± 0.4	2.8 ± 0.5	2.2 ± 0.4	<0.0001	0.0001	
		12,13 EpOME	3.1 ± 0.4	2.9 ± 0.5	2.3 ± 0.4	<0.0001	<0.0001	0.037
	AA	8,9 EET	1.8 ± 0.1	1.7 ± 0.2	1.4 ± 0.2	<0.0001	0.0079	
		11,12 EET	1.8 ± 0.1	1.8 ± 0.2	1.5 ± 0.2	<0.0001	0.0035	
		14,15 EET	1.8 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	<0.0001	0.0066	
	EPA	8,9 EpETE	1.9 ± 0.2	1.9 ± 0.3	1.6 ± 0.3	<0.0001	0.0035	
		11,12 EpETE	2.4 ± 0.3	2.1 ± 0.3	1.6 ± 0.3	<0.0001	0.0019	
		14,15 EpETE	2.2 ± 0.3	2.1 ± 0.3	2.0 ± 0.4	<0.0001	0.004	
		17,18 EpETE	2.5 ± 0.4	2.8 ± 0.7	2.6 ± 0.9	0.0017	0.0037	
	DHA	16,17 EpDPE	1.9 ± 0.2	1.9 ± 0.3	1.6 ± 0.3	<0.0001	0.0101	
		19,20 EpDPE	1.9 ± 0.2	2.1 ± 0.4	1.7 ± 0.3	<0.0001	0.0089	
Diol	LA	9,10 DiHOME	1.5 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	0.0004	0.0079	
		12,13 DiHOME	1.6 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	0.0003	0.0164	
	AA	8,9 DHET	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	0.0152		
		11,12 DHET	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	<0.0001	0.0235	
		14,15 DHET	1.2 ± 0.0	1.3 ± 0.1	1.1 ± 0.1	0.0002		
	EPA	5,6 DiHETE	1.5 ± 0.2	1.9 ± 0.4	1.8 ± 0.4	0.0376		
		14,15 DiHETE	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.0061		
		17,18 DiHETE	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.0232		
	DHA	19,20 DiHDPA	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	0.0004		

Table 4.3 (cont'd)

Monohydroxy	LA	9-HODE	2.4 ± 0.3	2.0 ± 0.3	2.4 ± 1.0	0.0019	0.0001	
		13-HODE	2.0 ± 0.2	1.8 ± 0.2	1.8 ± 0.6	0.0018	0.0008	
	AA	5-HETE	2.4 ± 0.8	1.5 ± 0.1	1.4 ± 0.2	0.0017	0.0007	
		11-HETE	2.2 ± 0.4	2.0 ± 0.4	1.5 ± 0.3	0.0073	0.002	
		15-HETE	1.9 ± 0.2	2.0 ± 0.3	2.1 ± 0.6	0.0019	0.0002	
		20-HETE	2.3 ± 0.3	2.3 ± 0.3	2.0 ± 0.4	0.0002	<0.0001	
Ketone	LA	9-OxoODE	2.0 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	0.0003	0.0017	
		13-OxoODE	1.8 ± 0.1	1.5 ± 0.2	1.9 ± 0.5	<0.0001	0.0036	
	AA	6-keto-PGF1α	1.5 ± 0.2	1.3 ± 0.1	1.2 ± 0.2			
Trihydroxy	AA	Lipoxin A4	1.5 ± 0.3	1.5 ± 0.3	2.8 ± 1.1			
Endocannabinoid	AA	AEA	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1			
	DHA	DHEA	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.2			
Fatty acid	LA	LA	2.2 ± 0.2	2.0 ± 0.2	1.6 ± 0.2	<0.0001	0.0002	0.0485
	AA	AA	2.0 ± 0.2	1.9 ± 0.2	1.5 ± 0.2	<0.0001	0.0016	
	EPA	EPA	1.8 ± 0.2	1.8 ± 0.2	1.5 ± 0.2	<0.0001	0.0006	
	DHA	DHA	2.9 ± 0.4	2.8 ± 0.4	2.0 ± 0.3	<0.0001	0.0011	

4.3.2.3 Short term effect of exercise, OMT and sham on ratios of epoxide/diol

Epoxides can be hydrolyzed to vicinal diols by the catalytic action of soluble epoxide hydrolase (sEH) *in vivo*. Prior studies reported that accumulation of epoxide oxylipins provides anti-inflammatory effects, but the subsequent vicinal diol metabolites often induce inflammatory responses (22,42,43). To investigate the potential benefits of modulated oxylipin levels, we compared the ratios of epoxides to their downstream diol products pre- and one hour post- exercise/treatment. Table 4.4 summarizes the fold change of the ratios for 8 pairs across four fatty acid precursors. All epoxide/diol ratios were elevated (fold change >1.0) after exercise in all three treatment groups. The changes in epoxide/diol ratios for PR only and OMT groups were all significant (~1.3-2.7-fold increase, $P < 0.05$ at least), however, only the ratios of 9,10- and 12, 13- EpOME/DiHOME in the sham group were statistically significant (~2.5-fold increase, $P<0.05$). Specifically in PR only and OMT group, the ratios of two EpETE/DiHETE pairs (derived from EPA) and two EpOME/DiHOME (derived from LA) have increased similarly by ~ 1.8-2.7 folds after exercise/treatment, followed by the ratios of three EET/DHET pairs (derived from AA) and one EpDPE/DiHDPA pairs (derived from DHA) which were elevated by ~ 1.3-1.7 folds. Our results provided evidence that the accumulation, in plasma, of epoxide metabolites of released free fatty acids was greater than the hydrolysis of epoxides to diols one hour after exercise and OMT. Since PUFA epoxides exhibit a variety of functions in physiological regulation, the observation that exercise induces increases in blood levels of numerous PUFA

epoxides suggests enticing mechanisms by which PR and exercise contribute to altered physiological functions.

4.2.3.4 Long term effect of exercise, OMT and sham on bioactive lipids

After 72 hours post-treatment, the average plasma concentrations of most oxylipins and PUFAs in the three groups decreased compared to one hour post-treatment levels, but were marginally greater than the pre-exercise/treatment baseline levels (Table 4.5). However, results of *t* test indicated that only 13-HODE in PR only group (2-fold, $P<0.05$) and OMT group (1,8-fold, $P<0.01$), and 9-HODE in OMT group (1.8-fold, $P<0.01$) remained statistically significant elevated levels after 72 hours, while no metabolite in the sham group showed significant change. One way ANOVA displayed that the fold change of 9-HODE and 13-HODE was also significantly different between sham group and the other two groups (~5-10% less in sham group, $P<0.05$). These results indicated that the biochemical influence on oxylipin due to PR exercise and OMT faded over 72 hours.

4.2.3.5 Effects of exercise, OMT and sham on bioactive lipids over the 3-month program

The basal (pre-treatment) metabolite levels at week 0 were compared with the basal concentrations at week 12. The three month PR program did not significantly alter most of plasma metabolites levels, taken all subjects together regardless of group. Only 17,18-EpETE exhibited significant elevation (2.8-fold, $P<0.05$) and DHEA had significant reduction (0.9-fold, $P<0.05$). No statistically significant differences in levels of any metabolites were observed between the three groups from the ANOVA result.

Table 4.4. Short-term effects of PR exercise, OMT and sham on epoxide/diol ratio. One hour post-/one hour pre-exercise and treatment fold change (Mean±SEM) of 8 epoxide/diol ratios for COPD patients from PR only (n=38), OMT (n=38) and sham (n=21) treatment groups. Student's *t* test was performed to compare mean fold change of each group with 1 (α=0.05). Fold change was $\log_2$ transformed and Student's *t* test (two-tailed) was used to compare mean fold change ($\log_2$ transformed) of each group with 0 (α=0.05). If P<0.05, the change is considered significant and listed in the table.

Fatty acid precursor	Epoxide/Diol	1 hour post/pre-treatment fold change			P-value		
		Exercise	OMT	Sham	Exercise	OMT	Sham
LA	9,10-EpOME/9,10-DiHOME	2.0 ± 0.2	1.8 ± 0.3	2.4 ± 0.4	<0.0001	0.0232	0.0106
	12,13-EpOME/12,13-DiHOME	2.1 ± 0.2	2.0 ± 0.4	2.6 ± 0.6	<0.0001	0.0081	0.0226
AA	8,9-EET/8,9-DHET	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	<0.0001	0.0058	
	11,12-EET/11,12-DHET	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	0.0002	0.0649	
	14,15-EET/14,15-DHET	1.5 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	0.0002	0.0388	
EPA	14,15-EpETE/14,15-DiHETE	2.0 ± 0.3	1.8 ± 0.3	2.6 ± 0.8	0.001	0.0408	
	17,18-EpETE/17,18-DiHETE	2.5 ± 0.6	2.7 ± 0.8	2.6 ± 1.0	0.0252	0.0274	
DHA	19,20-EpDPE/19,20-DiHDPA	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	0.0013	0.034	

Table 4.5. Long-term effects of PR exercise, OMT and sham on bioactive lipids. Seventy-two hour-post/pre exercises and treatment fold change (Mean±SEM) of measured oxylipins, endocannabinoids and fatty acid precursors levels for COPD patients from PR only (n=38), OMT (n=38) and sham (n=21) treatment groups. Fold change was $\log_2$ transformed and Student's *t* test (two-tailed) was used to compare mean fold change ($\log_2$ transformed) of each group with 0 (α=0.05). If P<0.05, the change is considered significant and listed in the table.

Category	Fatty acid Precursor	Compound	72 hour post-/pre- fold change			P-value		
			Exercise	OMT	Sham	Exercise	OMT	Sham
Epoxide	LA	9,10 EpOME	2.0 ± 0.4	1.4 ± 0.2	1.6 ± 0.3			
		12,13 EpOME	2.1 ± 0.4	1.5 ± 0.2	1.6 ± 0.3			
	AA	8,9 EET	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1			
		11,12 EET	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.1			
		14,15 EET	1.3 ± 0.2	1.1 ± 0.1	1.2 ± 0.1			
	EPA	8,9 EpETE	1.4 ± 0.2	1.2 ± 0.1	1.2 ± 0.2			
		11,12 EpETE	1.6 ± 0.4	1.6 ± 0.2	1.2 ± 0.3			
		14,15 EpETE	1.4 ± 0.3	1.4 ± 0.2	1.7 ± 0.6			
		17,18 EpETE	1.7 ± 0.4	1.5 ± 0.2	1.4 ± 0.3			
	DHA	16,17 EpDPE	1.4 ± 0.2	1.1 ± 0.1	1.2 ± 0.2			
		19,20 EpDPE	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.4			
Diol	LA	9,10 DiHOME	1.7 ± 0.2	1.8 ± 0.3	1.1 ± 0.2			
		12,13 DiHOME	1.5 ± 0.2	1.7 ± 0.2	1.1 ± 0.2			
	AA	8,9 DHET	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1			
		11,12 DHET	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1			
		14,15 DHET	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1			
	EPA	5,6 DiHETE	1.5 ± 0.3	1.7 ± 0.4	1.4 ± 0.4			
		14,15 DiHETE	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.2			
		17,18 DiHETE	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1			
	DHA	19,20 DiHDPA	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1			

Table 4.5 (cont'd)

Monohydroxy	LA	9-HODE	2.3 ± 0.4	1.8 ± 0.2	1.7 ± 0.7		0.01
		13-HODE	2.0 ± 0.3	1.8 ± 0.2	1.5 ± 0.4	0.0369	0.0078
	AA	5-HETE	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1		
		11-HETE	2.0 ± 0.5	1.3 ± 0.1	1.7 ± 0.6		
		15-HETE	1.8 ± 0.4	1.2 ± 0.1	1.5 ± 0.4		
		20-HETE	1.5 ± 0.2	1.9 ± 0.4	1.0 ± 0.1		
Ketone	LA	9-OxoODE	1.5 ± 0.2	1.2 ± 0.1	1.6 ± 0.6		
		13-OxoODE	1.6 ± 0.2	1.3 ± 0.1	1.5 ± 0.3		
	AA	6-keto-PGF1α	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.1		
Trihydroxy	AA	Lipoxin A4	1.4 ± 0.3	1.5 ± 0.3	2.1 ± 0.7		
Endocannabinoid	AA	AEA	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1		
	DHA	DHEA	1.0 ± 0.1	1.2 ± 0.1	0.8 ± 0.1		
Fatty acid	LA	LA	1.5 ± 0.2	1.2 ± 0.1	1.2 ± 0.1		
	AA	AA	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.1		
	EPA	EPA	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1		
	DHA	DHA	2.1 ± 0.5	1.5 ± 0.2	1.2 ± 0.1		

4.3.3 Discussion

In the current work, the development and validation of a rapid, sensitive, quantitative and robust UHPLC-MS/MS method to profile oxylipins, endocannabinoids and PUFAs was presented. This targeted approach was successfully applied to profile circulating bioactive lipid mediators from COPD patients who participated in a clinical trial receiving PR and OMT. To our knowledge, this is the first study to investigate the biochemical effects of PR exercise and OMT on a broad range of oxylipins and endocannabinoids in COPD patients. The results of this investigation provide evidence that PR exercise significantly increased levels of circulating PUFAs and further increased many downstream oxylipin levels within one hour post-exercise, but this impact faded over 72 hour post-exercise. Extra OMT and sham treatments had minimal effects, and if anything, may have reduced the influence of PR exercise on these bioactive lipids.

Plasma levels of the four PUFA precursors, LA, AA, EPA, and DHA, significantly increased (~2-3 folds) in the circulation system of the COPD patients one hour after PR exercise. During exercise, fatty acids serve as an important fuel source to provide energy for skeletal muscles. Horowitz reviewed the sources of circulating fatty acids during exercise (44), and identified adipose tissue (including both upper-body and lower-body subcutaneous adipose tissues, as well as intra-abdominal adipose tissue) triglycerides as the main source. Plasma and intramuscular triglycerides are also important fat sources contributing to fatty acid oxidation during moderate-intensity exercise. The mobilization of fatty acids from their triglyceride storage tissue to systemic circulation is the first step during exercise. However, they must be transported into muscles to be oxidized to provide energy. More importantly, with regard to bioactive lipid mediators and chronic inflammation conditions, how free fatty acids are transported between different organs (including blood) and cells and become the substrates of COX, LOX and CYP enzymes for oxylipin synthesis remains unclear. It has

been broadly accepted that the PUFA precursors of oxylipins and endocannabinoids are released from membrane phospholipids upon stimulus (14); however, whether these free circulating PUFAs are used to synthesize membrane phospholipids first or can directly diffuse from the cellular bilayers to serve as precursors for signaling mediators is unknown. Further studies are required to complete the underlying mechanisms based on metabolic flux analysis of fatty acids.

In this quantitative test, oxylipins from COX metabolism were present at negligible levels in most subjects' blood samples if detected at all, and as a result are not reported here. This can be explained in the context of the NSAID monitoring described in Chapter Two, which demonstrated that most patients in this study consumed at least one NSAID even though the accuracy of their self-reported drug use was low. NSAIDs, *e.g.* aspirin, ibuprofen and naproxen, can inhibit COX enzymes activities (45), therefore, the production of downstream COX pathway metabolites (*e.g.* prostaglandins, thromboxanes) were blocked.

Epoxides, important products from CYP pathways, presented significant increases post-exercise in both PR only and OMT groups. A number of studies showed that arachidonic acid epoxides (EETs) are highly bioactive mediators that exert many beneficial functions including anti-inflammation, vascular relaxation, and promotion of angiogenesis (46,47). EETs relax vascular cells via activating smooth muscle large-conductance calcium-activated potassium channels (BK_{Ca}) (47), and reduce inflammation by inhibiting transcription factor NF-κB and IκB kinase (48). Recent evidence demonstrated that EETs are involved in regulation of renal, pulmonary, and cardiac functions, and have emerged as potential therapeutic targets (49,50). Many notable observations suggested that EETs showed the ability to limit pulmonary inflammation (51). For example, 14,15-EET was found as a potent modulator of the hyperreactivity triggered by TNF-α in human airway smooth muscle cells (52), and also protected against cigarette smoke extract-induced lung injury (53). Previous

research on human plasma lipid mediators mainly focused on AA-derived EETs, whereas ω-3 fatty acids metabolites have received limited attention. But EpETEs and EpDPEs ---- epoxy signaling lipids converted by CYP450 from ω-3 fatty acids EPA and DHA respectively ---- share similar properties and activities with EETs, and also show anti-inflammatory, anti-hyperalgesic, and anti-thrombotic effects. Compared with EETs, EpETEs and EpDPEs have been reported to be as much as 1000-fold more potent than the EETs for vasodilation and anti-inflammation (22,23,54,55). Our present work indicated that the previously unappreciated EpETEs and EpDPEs also responded to PR exercise stimulus and the fold increases of EpETEs were greater than EETs. The findings of this research suggest that exercise increases circulating levels of EpETEs and EpDPEs that have potential for contributing to the regulation and resolution of pulmonary inflammation.

The most important metabolism pathway of epoxides involves the conversion to corresponding diols by the action of soluble epoxide hydrolase (sEH) (19). As mentioned, AA-, EPA- and DHA- derived epoxides demonstrate anti-inflammatory properties, and their hydrolysis by sEH converts them to diols that differ in biological function. Thus, decreasing hydrolysis and accumulating epoxides should benefit anti-inflammation. Many studies have already shown that inhibition of sEH attenuated inflammation in various disease models (56-60). For instance, Smith *et al.* showed that tobacco smoke-induced lung inflammation in rats was significantly diminished by sEH inhibitors (57). Here we observed significant elevations of all detected diols one hour post-exercise in this study, but the epoxide/diol ratios also increased, indicating that physical activities accumulated more epoxides than diols, and suggested a mechanism for beneficial influence of exercise. Another research analyzed plasma arachidonic acid epoxides and diols in healthy volunteers who did exercise testing on a bicycle ergometer, but the authors only observed increase of 14,15-DHET during exercise, and 11,12-DHET and 14,15-EET two minutes post-exercise (61). The difference between

these findings and the current work may have resulted from a very short (2 min) blood collection time point after exercise, as epoxides and diols may not have had sufficient time for release or accumulation in the circulatory system. In our study, the endogenous epoxides and diols exhibited notably significant increases at one hour post-exercise but declined nearly to baseline levels over 72 hours. However, three collection time points do not allow complete assessment of the dynamics of oxylipin circulation after exercise. In fact, knowledge about dynamic profiles of these endogenous metabolites *in vivo* is limited, and further investigations of the fluxes of oxylipins in humans are important to understand their physiologic functions and assess their roles in exercise-modulated effects on health.

In contrast to the epoxides of AA, EPA, and DHA, metabolites of the 18-carbon fatty acid LA were also measured pre- and post-exercise in the current investigation. The epoxide metabolites of linoleic acid 9,10-EpOME and 12,13-EpOME, referred to "leukotoxin/iso-leukotoxin", are generated by leukocytes and have exhibited pro-inflammatory activities (62). Elevated levels of EpOMEs are associated with acute respiratory distress syndrome and the pathology following severe burn injury (63). Compared to EpOMEs, their soluble epoxide hydrolase (sEH) metabolites 9,10-DiHOME and 12,13-DiHOME have been reported to be more toxic than the epoxides (64,65), suggesting that EpOMEs serve as protoxicants but their corresponding diols actively were responsible for EpOME-associated toxicity (66). In our results, though both EpOMEs and DiHOMEs levels elevated one hour after exercise, the ratios of the two EpOME/DiHOME pairs also showed ~ 2-fold increases, indicating the greater accumulation of less toxic EpOME due to exercise on COPD patients.

Epoxides of PUFA were not the only metabolites to exhibit increases in plasma concentrations after exercise, as numerous hydroxyfatty acids also rose in abundance. One of these, 13-HODE, forms in the 15-LOX pathway as a stable oxidation product of linoleic acid, and it exerts contrasting effects in different conditions and stages (67). 13-HODE induces

pro-inflammatory responses by stimulating production of NF-κB, generates inflammatory cytokines (*e.g.* IL-8, IL-1β) as well as exhibits chemotactic activities (68), and its levels rise when oxidative stress is increased (69). On the other hand, 13-HODE also regulates the activity of the anti-inflammatory peroxisomal proliferator-activating receptor-γ (PPARγ) depending on its concentration (68). PPARγ was reported to be involved in airway inflammation and remodeling in asthma (70), and agonists of PPARγ showed anti-inflammatory activities in different inflammatory cells and reduced inflammation of asthma and COPD (71). Besides, 9-HODE and the two corresponding keto-dienes 13-OxoODE and 9-OxoODE are also endogenous PPARγ ligands and exert anti-inflammatory effects (72,73). A study of lung cancer demonstrated that 13-HODE and 15-HETE (also a 15-LOX product, in this case of AA) levels were significantly reduced in human lung cancer tissue compared with non-tumor lung tissue (74). In another investigation, after subway air exposure, healthy individuals had greater increases of 13-HODE, 9-HODE, 13-OxoODE and 9-OxoODE than asthmatic subjects in bronchoalveolar lavage fluid (BALF), suggesting a possible reduced anti-inflammatory response in asthmatics (75). Regarding the response of HODEs during exercise, only limited research has been performed. Nieman . assessed several plasma metabolite responses in healthy subjects to 75-km cycling, and showed elevated levels of 13-HODE and 9-HODE immediately and 1.5 hour but not 21 hours post-exercise (76); the elevated HODE concentrations were not correlated with levels of inflammatory cytokines but related with oxidative stress biomarkers (F2-isoprostanes). Our results showed that plasma levels of 9-HODE and 13-HODE increased ~2-fold one hour after PR exercise in COPD patients, and 13-HODE levels still remained elevated 72 hours after exercise which may suggest transformation of 13-HODE to its keto-dienes is probably slower in COPD context than normal conditions. Due to HODEs' contrasting effects and the knowledge gaps about their functions and dynamics, whether the observed moderate exercise-induced accumulation

of HODEs provides benefits to COPD patients is not clear. Further questions including how much exercise yields the threshold of generating beneficial amount of HODEs and OxoODEs should be addressed in future studies.

We also observed the elevation of plasma levels of all the detected HETEs one hour after PR exercise in COPD patients. HETEs are monohydroxy metabolites of 20-carbon fatty acids (*e.g.* AA) produced via different pathways, and exhibit different functions in airway diseases. As mentioned above, 15-HETE also forms via the 15-LOX pathway and has been associated with pro-inflammatory responses in pulmonary diseases (77,78). For example, severe asthmatics with persistent elevated airway eosinophil levels manifested high levels of 15-HETE in bronchoalveolar lavage fluid (BALF). But similar to HODEs, 15-HETE also has binding affinity to PPARγ and modulates anti-inflammatory activities (79). Another HETE isomer, 5-HETE, forms via the 5-LOX pathway upon inflammatory stimulus and can mediate neutrophil chemotaxis (80). A targeted eicosanoid lipidomics report showed that 5-HETE was elevated in exhaled breath condensate of tobacco smokers compared to healthy individuals (16). 20-HETE is synthesized by CYP450 via hydroxylation at the terminal carbon, and regulates vascular activities (81). A study showed that 20-HETE induced hyperpolarization and controlled relaxation of airway smooth muscle in human bronchi (82). The isomer 11-HETE is a non-enzymatic monohydroxy fatty acid and little is yet known about its functions. One study reported higher serum 11-HETE levels in patients with lung adenocarcinoma than those without cancer (83).

Endocannabinoids make up another class of fatty acid metabolites that are synthesized from membrane phospholipids on demand in many physiological and pathophysiological processes, especially involved in regulating central nervous system functions (84), but research on the roles of these metabolites in human airway diseases are limited. One study reported increased level of the ethanolamide of AA (AEA) in BALF of patients with allergic

asthma, suggesting endocannabinoids may play roles in pulmonary inflammatory response (85). As for the effects of exercise on plasma endocannabinoids, two studies showed increased AEA in healthy subjects after 45 min and 1.5 hour of cycling, but 2-AG levels remained the same (86,87). Our study targeted endocannabinoids to assess whether improvements in patient-reported quality of life might be associated with treatment-related changes in their plasma levels. We did not observe significant changes of any monitored endocannabinoids in COPD patients following PR exercise and OMT; probably the moderate intensity exercise performed by subjects with chronic inflammation hardly altered endocannabinoid levels in circulating system.

In this clinical trial, extra OMT and sham treatments before PR exercise were associated with alterations in levels of fewer metabolites, often of lesser magnitude than observed for PR alone. The mechanistic reasons for such differences were not clear so far, but future research might consider psychological influences (*e.g.* seeing physicians and receiving extra treatments may have convinced subjects, perhaps subconsciously, to exercise at lower intensity than subjects not receiving either treatment). On the other hand, the effects of OMT on COPD are still controversial (11-13), particularly among physicians not trained in osteopathic manipulation.

4.4 Conclusions

In this chapter, a targeted mass spectrometry-based approach was developed and applied to quantify bioactive lipid mediators including 57 oxylipins, endocannabinoids and PUFAs in plasma specimens from COPD patients. As far as we know, it is the first LC-MS/MS approach that simultaneously detects oxylipins, endocannnabinoids, and PUFAs in a single analysis with the aid of fast ESI polarity switching between negative and positive modes. Additional metabolites can be readily added to the protocol if standards are available. This method offers a comprehensive platform for monitoring bioactive lipids derived from

different fatty acid precursors with pro-inflammatory, anti-inflammatory and resolving functions. Our targeted profiling platform was optimized and validated for analyzing human plasma samples to achieve good chromatographic separation of isomers as well as MRM discrimination of co-eluting compounds, and also to guarantee high sensitivity (*i.e.* down to low nM levels) over 4 orders of magnitude dynamic range (sub nM-μM).

We successfully applied the targeted metabolite profiling method to analyzing circulating lipid mediators of COPD patients who participated in a PR program receiving PR, OMT and sham treatment. Our results demonstrated that PR exercise significantly increased PUFAs levels in the circulatory system of COPD patients and also induced the increased accumulation of downstream oxylipins including epoxides, diols, monohydroxy, and ketone metabolites within one hour post-exercise. This impact faded by 72 hours post-exercise. In addition, the increased ratios of epoxides to their corresponding diols were observed as a short-lived effect. Since PUFA epoxides are well-known endogenous metabolites with anti-inflammatory effects, our results suggest that PR exercise may contribute to the recovery from tissue inflammation and damage. Extra OMT treatment was associated with slight decreases in the influence of PR exercise on blood levels of PUFAs and metabolites. In summary, our targeted bioactive lipids analysis described above revealed that PR exercise and to a lesser extent, extra OMT can alter biochemical profiles of blood constituents in COPD patients. To learn more about how these alternations may be beneficial to COPD patients will require further clinical and fundamental biochemistry studies using larger numbers of research subjects.

APPENDIX

APPENDIX

Table A3. Demographics of subjects and pulmonary function test parameters measured upon subject enrollment in the study (week 0). Reported values represent mean ± SD, with minimum and maximum values in brackets.

	PR only Group	OMT Group	Sham Group
Number of subjects	13	13	7
Age (year)	67±8 [50,81]	66±7 [52,75]	66±7 [56,79]
Gender (Female/male)	3/10	7/6	4/3
Race (Caucasian/African American)	11/2	11/2	6/1
Smoker (Y/N)	11/2	12/1	6/1
For smokers, self-reported years of smoking	35±17 [13,65]	39±8 [25,50]	35±20 [5,56]
For smokers, package per day	0.95±0.48 [0.3,2.0]	1.3±0.71 [0.8,3.0]	1.08±0.38 [0.5,1.5]
Body mass index (BMI)	33.6±8.9 [19.6, 52.3]	26.9±6.5 [17.9, 37.5]	31.7±8.7 [20.0, 45.6]
Pre-bronch SVC (L), % pred	78±15 [42,97]	78±20 [54,123]	65±21 [43,108]
Pre-bronch IC (L), % pred	89±19 [55,111]	79±24 [57,125]	67±13 [50,82]
Pre-bronch ERV (L), % pred	34±31 [5,95]	78±48 [19,171]	58±64 [11,167]
Pre-bronch MEP (cm H_2O),%pred	57±21 [29,88]	50±18 [21,84]	38±10 [23,56]
Pre-bronch MIP (cm H_2O),% pred	84±31 [40,147]	68±45 [29,201]	61±9 [49,76]
FEV1 (L), actual	1.46±0.49 [0.52,2.27]	1.12±0.39 [0.61,1.81]	1.53±0.82 [0.81,3.12]
FEV1 (L), %pred	60±17 [32,79]	45±18 [20,82]	51±15 [34,77]
post bronchodilator FEV1/FVC	0.61±0.12 [0.37,0.73]	0.47±0.11 [0.34,0.67]	0.62±0.11 [0.44,0.73]
6-min walk distance (meter)	892±220 [457,1275]	931±234 [502,1209]	874±343 [340,1327]

Table A4. The calibration (C) concentration levels for each oxylipin, endocannabinoid and poly-unsaturated fatty acid (calibration solutions: C1-C8).

Compound	C0	C1	C2	C3	C4	C5	C6	C7	C8
Oxylipins and endocannabinoids (nM)									
14,15-EET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
11,12-EET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
8,9-EET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
9,10-EpOME	0	0.19	0.95	3.8	15.2	76	380	1900	9500
12,13-EpOME	0	0.19	0.95	3.8	15.2	76	380	1900	9500
11,12-EpETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
14,15-EpETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
17,18-EpETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
16,17-EpDPE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
19,20-EpDPE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
10,17-DiHDoHE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
7,17-hydroxy-DPA	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
17-HDoHE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
12 HHTrE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
15d-PGJ2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
8-iso PGA2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
8-iso PGA1	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
6-keto-PGF1α	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
8-iso PGF2α	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
TXB2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
PGE2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
PGD2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
17,18-DiHETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
14,15-DiHETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
5,6-DiHETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
12,13-DiHOME	0	0.095	0.475	1.9	7.6	38	190	950	4750
9,10-DiHOME	0	0.095	0.475	1.9	7.6	38	190	950	4750
8,9-DHET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
11,12-DHET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
14,15-DHET	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
19,20-DiHDPA	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
8,9-EpETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
15-HETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
11-HETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
9-HETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
20-HETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
7-MaR1	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
RvD2	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
LXA4	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
RvD1	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
LTB4	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
LTD4	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475

Table A4 (cont'd)

13-OxoODE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
9-OxoODE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
15-OxoETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
5-OxoETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
9-HODE	0	0.0475	0.2375	0.95	3.8	19	95	475	2375
13-HODE	0	0.0475	0.2375	0.95	3.8	19	95	475	2375
5-HETE	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
AEA	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
2-AG	0	0.0475	0.2375	0.95	3.8	19	95	475	2375
EPEA	0	0.0095	0.0475	0.19	0.76	3.8	19	95	475
DHEA	0	0.0475	0.2375	0.95	3.8	19	95	475	2375
Poly-unsaturated fatty acids (μM)									
LA	0	0.0019	0.0095	0.0475	0.19	0.76	3.8	19	
EPA	0	0.0019	0.0095	0.0475	0.19	0.76	3.8	19	
AA	0	0.0019	0.0095	0.0475	0.19	0.76	3.8	19	
DHA	0	0.0019	0.0095	0.0475	0.19	0.76	3.8	19	

Table A5. LC gradient developed in the current method for targeted profiling of oxylipins, endocannabinoids and poly-unsaturated fatty acids (mobile phase A: 0.1% formic acid in water; mobile phase B: acetonitrile).

Time (min)	Flow rate (mL/min)	A%	B%	Curve
0.00	0.3	99%	1%	
0.50	0.3	99%	1%	6
2.00	0.3	60%	40%	6
8.00	0.3	20%	80%	6
9.00	0.3	1%	99%	6
13.00	0.3	1%	99%	6
13.01	0.3	99%	1%	6
15.00	0.3	99%	1%	6

Table A6. LLOQ, LLOD, and linearity range in calibration solution and human plasma for 57 oxylipins, endocannabinoids and poly-unsaturated fatty acids.

Compound	R^2	Linear range	LLOQ in calibration solution	LLOD in calibration solution
Oxylipins and endocannabinoids (nM)				
14,15-EET	0.9988	0.19-475	0.43	0.13
11,12-EET	0.9995	0.19-475	0.48	0.14
8,9-EET	0.9995	0.0475-475	0.20	0.06
9,10-EpOME	0.9984	0.19-1900	0.26	0.08
12,13-EpOME	0.9929	0.19-1900	0.35	0.10
11,12-EpETE	0.9992	0.19-475	0.27	0.08
14,15-EpETE	0.9994	0.19-475	0.27	0.08
17,18-EpETE	0.9996	0.19-475	0.19	0.06
16,17-EpDPE	0.9992	0.0475-475	0.11	0.03
19,20-EpDPE	0.9992	0.76-475	0.82	0.25
10,17-DiHDoHE	0.9984	0.0475-475	0.08	0.02
7,17-hydroxy-DPA	0.9997	0.095-475	0.22	0.07
17-HDoHE	0.9986	0.76-475	0.97	0.29
12-HHTrE	0.9989	0.19-475	0.11	0.03
15d-PGJ2	0.9999	0.19-475	0.19	0.06
8-isoPGA2	0.9989	0.76-95	0.28	0.09
8-isoPGA1	0.9992	0.0475-95	0.14	0.04
6-keto-PGF1α	0.9998	0.095-475	0.09	0.03
8-isoPGF2α	0.9998	0.19-475	0.32	0.10
TXB2	0.9998	0.76-475	0.76	0.23
PGE2	0.9986	0.19-475	0.28	0.08
PGD2	0.9998	0.19-475	0.31	0.09
17,18-DiHETE	0.9991	0.76-475	0.86	0.26
14,15-DiHETE	0.9992	0.19-475	0.35	0.11
5,6-DiHETE	0.9980	3.8-475	2.16	0.65
12,13-DiHOME	0.9998	0.095-950	0.05	0.01
9,10-DiHOME	0.9997	0.095-950	0.10	0.03
8,9-DHET	0.9982	0.0475-475	0.06	0.02
11,12-DHET	0.9992	0.0475-475	0.06	0.02
14,15-DHET	0.9982	0.095-475	0.11	0.03
19,20-DiHDPA	0.9991	0.095-95	0.10	0.03
8,9-EpETE	0.9991	0.38-475	0.28	0.08
15-HETE	0.9998	0.095-475	0.10	0.03
11-HETE	0.9996	0.0475-475	0.08	0.02
9-HETE	0.9987	0.0475-475	0.57	0.17
20-HETE	0.9997	0.38-475	0.41	0.12
7-MaR1	0.9983	0.19-475	0.19	0.06
RvD2	0.9993	0.38-475	0.34	0.10
LXA4	0.9942	0.095-95	0.11	0.03
RvD1	0.9984	0.095-475	0.06	0.02

Table A6 (cont'd)

LTB4	0.9992	0.0475-95	0.08	0.02
LTD4	0.9981	0.0475-95	0.05	0.01
13-OxoODE	0.9996	0.38-475	0.39	0.12
9-OxoODE	0.9993	0.76-475	0.71	0.21
15-OxoETE	0.9998	0.38-475	0.66	0.20
5-OxoETE	0.9997	0.76-475	0.73	0.22
9-HODE	0.9998	0.95-475	0.53	0.16
13-HODE	0.9996	0.95-475	0.69	0.21
5-HETE	0.9996	0.095-475	0.17	0.05
AEA	0.9957	0.76-475	0.63	0.19
2-AG	0.9988	0.95-2375	1.71	0.51
EPEA	0.9981	0.76-475	0.86	0.26
DHEA	0.9920	3.8-2375	4.80	1.44
Polyunsaturated fatty acids (μM)				
LA	0.9985	0.0475-19	0.020	0.006
EPA	0.9998	0.0095-19	0.014	0.004
AA	0.9941	0.0095-19	0.011	0.003
DHA	0.9959	0.0095-19	0.010	0.003

Table A7. Intra-day (n=3) accuracy and precision (CV%) in calibration solution for each oxylipin, endocannabinoid and PUFA. (QC1: C5; QC2: C6; QC3: C7 for oxylipins and endocannabinoids; QC1: C4; QC2: C5; QC3: C6 for PUFAs; see Table A4 for the levels of C4-C7 of each analyte).

Compound	QC1		QC2		QC3	
	Accuracy	CV%	Accuracy	CV%	Accuracy	CV%
14,15-EET	104%	3%	95%	6%	100%	6%
11,12-EET	105%	14%	93%	5%	98%	12%
8,9-EET	101%	8%	98%	2%	100%	7%
9,10-EpOME	103%	6%	107%	3%	99%	6%
12,13-EpOME	106%	5%	110%	3%	98%	5%
11,12-EpETE	90%	13%	95%	5%	99%	8%
14,15-EpETE	97%	4%	94%	7%	101%	7%
17,18-EpETE	95%	10%	94%	3%	99%	8%
16,17-EpDPE	102%	12%	94%	5%	101%	6%
19,20-EpDPE	93%	3%	102%	0.4%	100%	8%
10,17-DiHDoHE	93%	8%	100%	3%	100%	5%
7,17-hydroxy-DPA	93%	14%	101%	6%	101%	9%
17-HDoHE	87%	15%	92%	12%	101%	5%
12-HHTrE	91%	9%	95%	5%	100%	6%
15d-PGJ2	95%	0.4%	101%	1%	100%	2%
8-isoPGA2	98%	5%	101%	6%	99%	3%
8-isoPGA1	101%	3%	105%	1%	100%	2%
6-keto-PGF1α	92%	5%	94%	5%	101%	3%
8-isoPGF2α	91%	6%	93%	8%	102%	2%
TXB2	99%	1%	107%	1%	98%	0.1%
PGE2	101%	3%	100%	1%	100%	2%
PGD2	92%	7%	101%	1%	101%	3%
17,18-DiHETE	94%	14%	96%	14%	101%	4%
14,15-DiHETE	92%	11%	95%	10%	101%	2%
5,6-DiHETE	89%	11%	84%	17%	113%	12%
12,13-DiHOME	100%	3%	104%	2%	100%	2%
9,10-DiHOME	100%	5%	106%	6%	100%	2%
8,9-DHET	98%	6%	94%	7%	101%	3%
11,12-DHET	94%	6%	98%	7%	101%	4%
14,15-DHET	101%	8%	98%	5%	101%	2%
19,20-DiHDPA	102%	9%	93%	4%	101%	6%
8,9-EpETE	96%	5%	93%	10%	108%	10%
15-HETE	94%	11%	97%	1%	102%	3%
11-HETE	95%	5%	98%	3%	101%	2%
9-HETE	96%	13%	85%	10%	104%	7%
20-HETE	83%	18%	90%	7%	102%	0.4%
5-HETE	99%	17%	100%	0.4%	102%	5%
9-HODE	102%	4%	103%	3%	100%	3%
13-HODE	106%	8%	103%	4%	100%	3%
7-MaR1	87%	8%	102%	2%	101%	5%

Table A7 (cont'd)

RvD1	102%	6%	103%	2%	101%	2%
RvD2	93%	12%	99%	2%	100%	0.7%
LXA4	93%	2%	96%	9%	102%	4%
LTB4	101%	10%	102%	4%	101%	4%
LTD4	94%	3%	92%	4%	102%	3%
13-OxoODE	103%	11%	103%	2%	101%	6%
9-OxoODE	106%	13%	96%	2%	104%	10%
15-OxoETE	97%	14%	102%	0.9%	103%	3%
5-OxoETE	102%	11%	104%	4%	102%	4%
AEA	98%	3%	99%	1%	101%	3%
2-AG	97%	3%	97%	5%	100%	4%
EPEA	99%	13%	98%	5%	100%	3%
DHEA	96%	10%	96%	3%	100%	4%
LA	106%	12%	98%	5%	105%	6%
EPA	102%	4%	102%	1%	105%	2%
AA	97%	4%	107%	3%	98%	1%
DHA	106%	1%	103%	0.1%	95%	0.9%

Table A8. Inter-day (n=3) accuracy and precision (CV%) in calibration solution for each oxylipin, endocannabinoid and PUFA. (QC1: C5; QC2: C6; QC3: C7 for oxylipins and endocannabinoids; QC1: C4; QC2: C5; QC3: C6 for PUFAs; see Table A4 for the levels of C4-C7 of each analyte).

Compound	QC1		QC2		QC3	
	Accuracy	CV%	Accuracy	CV%	Accuracy	CV%
14,15-EET	94%	10%	98%	7%	101%	1%
11,12-EET	95%	11%	98%	9%	101%	2%
8,9-EET	95%	14%	100%	6%	101%	0.8%
9,10-EpOME	111%	7%	108%	4%	97%	2%
12,13-EpOME	115%	8%	113%	4%	95%	6%
11,12-EpETE	88%	2%	101%	6%	102%	4%
14,15-EpETE	83%	14%	92%	6%	103%	2%
17,18-EpETE	88%	7%	100%	6%	101%	1%
16,17-EpDPE	93%	10%	97%	3%	105%	4%
19,20-EpDPE	89%	5%	103%	6%	101%	2%
10,17-DiHDoHE	95%	4%	101%	0.3%	100%	0.2%
7,17-hydroxy-DPA	86%	7%	97%	3%	102%	2%
17-HDoHE	83%	4%	96%	8%	103%	3%
12-HHTrE	86%	5%	98%	3%	101%	0.6%
15d-PGJ2	94%	2%	101%	1%	100%	0.2%
8-iso PGA2	96%	2%	104%	2%	100%	0.5%
8-iso PGA1	99%	2%	103%	2%	100%	0.5%
6-keto-PGF1α	90%	2%	97%	2%	101%	0.2%
8-isoPGF2α	86%	5%	93%	2%	102%	0.5%
TXB2	98%	6%	101%	6%	97%	2%
PGE2	99%	2%	100%	0.5%	100%	0.1%
PGD2	87%	4%	100%	4%	101%	0.6%
17,18-DiHETE	97%	11%	95%	3%	101%	0.4%
14,15-DiHETE	88%	5%	96%	2%	103%	3%
5,6-DiHETE	83%	19%	83%	5%	111%	8%
12,13-DiHOME	103%	3%	102%	1%	99%	0.5%
9,10-DiHOME	102%	1%	102%	3%	100%	0.2%
8,9-DHET	90%	9%	95%	2%	103%	2%
11,12-DHET	92%	3%	97%	2%	101%	0.4%
14,15-DHET	98%	2%	101%	5%	102%	2%
19,20-DiHDPA	96%	9%	92%	4%	101%	0.3%
8,9-EpETE	89%	9%	96%	2%	106%	4%
15-HETE	87%	11%	93%	5%	102%	0.3%
11-HETE	89%	6%	96%	2%	101%	0.6%
9-HETE	82%	15%	85%	8%	103%	0.9%
20-HETE	86%	8%	88%	4%	103%	0.2%
5-HETE	88%	11%	92%	8%	103%	1%
9-HODE	99%	6%	98%	4%	100%	0.6%
13-HODE	102%	7%	98%	5%	100%	1%
7-MaR1	85%	3%	97%	4%	101%	0.8%

Table A8 (cont'd)

RvD1	98%	6%	100%	5%	103%	2%
RvD2	89%	4%	98%	4%	103%	4%
LXA4	89%	4%	96%	0.9%	101%	0.6%
LTB4	98%	4%	99%	3%	102%	3%
LTD4	91%	3%	93%	3%	103%	2%
13-OxoODE	96%	10%	96%	7%	103%	4%
9-OxoODE	92%	15%	92%	4%	101%	2%
15-OxoETE	96%	3%	93%	8%	102%	3%
5-OxoETE	99%	9%	94%	9%	102%	2%
AEA	95%	7%	97%	2%	101%	0.5%
2-AG	97%	4%	99%	2%	100%	0.3%
EPEA	98%	3%	99%	3%	100%	0.5%
DHEA	92%	5%	98%	2%	101%	0.4%
LA	101%	5%	95%	6%	102%	2%
EPA	102%	2%	101%	0.9%	104%	3%
AA	99%	4%	104%	2%	98%	1%
DHA	104%	5%	102%	0.5%	97%	2%

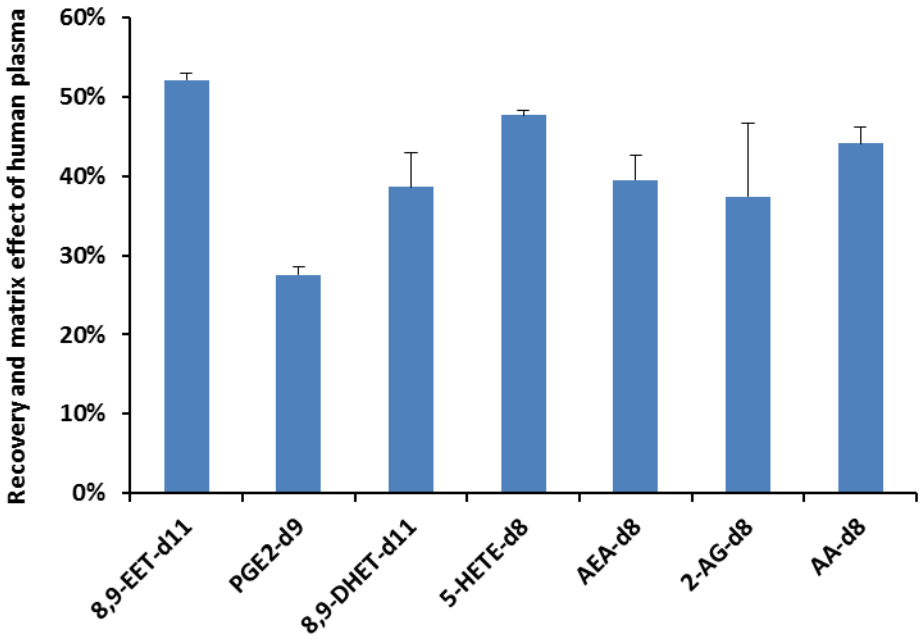

Figure A1. Recovery and matrix effect for deuterated lipid standards in human plasma (n=3). (Data are expressed by Mean±SEM).

Table A9. Percent of analyte concentration remaining after 48 hours in the stability test for each oxylipin, endocannabinoid and PUFA.

Compound	48 hours concentration remaining	CV%
14,15-EET	90.7%	5.9%
11,12-EET	85.0%	4.6%
8,9-EET	91.8%	1.7%
9,10-EpOME	94.5%	2.6%
12,13-EpOME	93.2%	2.8%
11,12-EpETE	89.0%	5.4%
14,15-EpETE	98.0%	6.8%
17,18-EpETE	88.5%	3.1%
16,17-EpDPE	94.1%	5.3%
19,20-EpDPE	93.4%	0.4%
10,17-DiHDoHE	99.5%	3.3%
7,17-hydroxy-DPA	103.6%	6.5%
17-HDoHE	100.8%	12.0%
12-HHTrE	93.5%	5.4%
15d-PGJ2	101.4%	1.1%
8-iso PGA2	96.6%	6.4%
8-iso PGA1	103.4%	1.1%
6-keto-PGF1α	96.5%	5.3%
8-isoPGF2α	103.2%	7.9%
TXB2	106.7%	1.4%
PGE2	99.7%	1.3%
PGD2	105.2%	1.3%
17,18-DiHETE	97.9%	14.0%
14,15-DiHETE	96.5%	10.4%
5,6-DiHETE	107.0%	17.2%
12,13-DiHOME	102.2%	1.8%
9,10-DiHOME	104.7%	5.9%
8,9-DHET	96.9%	7.1%
11,12-DHET	101.7%	6.9%
14,15-DHET	99.8%	4.7%
19,20-DiHDPA	98.8%	4.0%
8,9-EpETE	95.2%	10.3%
15-HETE	105.4%	1.5%
11-HETE	103.2%	3.5%
9-HETE	102.9%	9.9%
20-HETE	107.2%	6.9%
5-HETE	110.6%	0.4%
9-HODE	105.5%	3.0%
13-HODE	107.6%	4.3%
7-MaR1	106.6%	2.0%
RvD1	109.8%	2.1%
RvD2	98.1%	1.6%

Table A9 (cont'd)

LXA4	100.0%	9.1%
LTB4	105.0%	3.6%
LTD4	100.8%	3.7%
13-OxoODE	110.1%	1.9%
9-OxoODE	103.6%	1.9%
15-OxoETE	114.4%	0.9%
5-OxoETE	114.8%	4.5%
AEA	101.9%	1.2%
2-AG	96.7%	5.0%
EPEA	95.8%	4.9%
DHEA	96.2%	3.2%
LA	111.4%	4.6%
EPA	100.7%	1.0%
AA	104.5%	3.0%
DHA	100.9%	0.1%

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Chapter 5 Concluding Remarks

Maintaining wellness and seeking more effective therapies has long been one of the key goals of human societies across cultures and eras. The past few decades have witnessed tremendous progress in science and technology that have dramatically improved our understanding of mechanisms of biological systems and have reshaped the ways of diagnosing and treating diseases. Compared to the more established genomics, transcriptomics and proteomics approaches, metabolomics, which is the most closely related to organismal phenotype, is growing rapidly in application, especially in the field of discovering therapeutic targets and clinically useful biomarkers. Biomarkers serve as objective indicators of normal, diseased and therapeutic conditions to aid prediction, diagnosis and staging of diseases as well as monitoring and evaluating clinical responses to interventions. This dissertation has presented advances in the development and application of LC-MS based approaches to monitor drug use and discover potential biomarkers for evaluating therapeutic interventions and understanding fundamental mechanisms of diseases and recovery. The LC-MS protocols including sample preparation described in Chapters 2-4 were optimized for analyses of drugs and metabolites in blood plasma, but with proper modifications, all of them can be adapted for analysis of other biofluids to investigate various diseases and treatments.

Biomarker discovery is usually performed in clinical settings with human subjects because of biochemical differences between humans and model systems. In addition, the design and execution of a clinical trial always plays a key role in the research. One challenge

of conducting clinical trials is that each subject has his/her personal metabolomic phenotype, which is not only determined by genotype but also greatly influenced by the environment he/she is exposed to, including lifestyle, medications, diet, location, emotion, and gut microbiome. Information about patients is usually collected from their self-reporting. However, the findings in Chapter 2 showed that the level of accordance of the self-reporting questionnaire and objective NSAIDs screening was low. This suggested a need for analytical approaches as a routine practice to support clinical trials rather than only relying on research subjects' memories. If environmental exposures including diets and medication use can be tightly controlled (*e.g.,* providing standard meals for all subjects, as in a metabolic ward) during the clinical research period, the metabolome variation can be limited. However, this is not always practical and often expensive. For example, the subjects recruited in the research described in this dissertation were outpatients and the clinical trial lasted three months, and standardization of meals and drug controls for each individual was not feasible. Increased number of participants and objective monitoring of exposures should aid to lower variability and achieve reliable clinical results.

Mass spectrometry based metabolomics profiling is usually carried out in two ways: untargeted and targeted. Both strategies were applied in this dissertation for potential biomarker discovery which were described in Chapters 3 and 4 respectively. These measurements provide the first to investigation of plasma metabolic alterations after pulmonary rehabilitation exercise training and OMT in a cohort of patients with COPD. Increased release of long chain (C16-22) fatty acids and downstream oxylipins to the

circulatory system after PR exercise was observed. This work has improved our understanding of the influence of exercise on biochemical processes in the context of COPD. However, more questions remain including what locations and substances serve as the source of the observed lipids, and what factors govern transport, synthesis, and metabolism of the lipids. Adipose tissue triglycerides have been proposed as the main source of circulatory fatty acid during exercise to provide energy, but under chronic inflammatory conditions such as COPD, is the mechanism of fatty acids release still the same as for normal physiological conditions? After release to the circulatory system, how are fatty acids transported into organs and cells to become the precursors of oxylipins and endocannabinoids? What is the *in vivo* dynamic profile of each endogenous lipid mediator, and how is it different in a chronic inflammatory condition? More fundamental research, particularly metabolic flux analysis needs to be performed to drive improved understanding of these underlying signaling mechanisms in the context of COPD. A second set of questions addresses therapeutic effects. What duration and intensity of PR exercise can induce an effective amount of beneficial endogenous metabolites (*e.g*. EETs)? Which patients respond best to such interventions? These questions lead to the concept of personalized medicine, which aims to tailor medical practices and interventions based on the individual, often driven by the integration of genomic, transcriptomic, proteomic and metabolomics information.