Lipid signaling and inflammation: metabolomics for better diagnosis and treatment strategy

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Chapter I

General introduction and scope

Metabolomics and lipidomics

Metabolomics as a powerful tool for systems biology studies

Systems biology is an integrative biology-based approach deciphering the functions and interactions of different components of biological systems, including organisms, organelles, cells and molecules [1]. "Omics" technologies, which include analyses of genes (genomics), transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics), are powerful tools for characterizing the dynamics of a biological system. Specifically, metabolomics is the profiling of the metabolome - the complete set of metabolites which are low-molecular-weight molecules (<2000 Da) found in living organisms [2]. The metabolites are the end products of the biological processes, representing the interplay between genomic and environmental factors. In addition, even though the changes in the transcriptome and the proteome are subtle, the variations in health and disease status can be substantially reflected in the metabolome thus making the metabolome often a more sensitive readout of physiological conditions and diseased status than either the transcriptome or proteome [3]. Therefore, metabolomics plays an essential role in omics-based systems biology studies and can best reflect the phenotypic characteristics of biological systems. The acquired metabolite profiles and interconnected metabolic pathways will help to understand underlying molecular mechanisms of homeostasis maintenance and perturbations. Furthermore, identified metabolites may serve as potential biomarkers for disease diagnosis, for indicating disease progression as well as predicting clinical therapeutic outcome.

Technical strategies in metabolomics

Technological innovations are the driving force behind advances in metabolomics. The two major techniques used are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, both of which have been extensively applied in metabolomics for different research goals [4]. Specifically, NMR-based metabolomics is a high-throughput technique with strengths that allow it to be performed without the requirement of elaborate sample preparation or fractionation and thus enables a "holistic view" of the metabolites, whereas

the relative low sensitivity and less metabolite coverage are the limitations [5]. MS-based technologies use direct introduction or can be coupled with different separation technologies such as liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). The application of hyphenated MS technique has gained considerable momentum in recent years in metabolomics profiling.

Two distinct groups of technologies for metabolomics methodologies are untargeted and targeted metabolomics. Untargeted methods aim for profiling as many metabolites as possible, including unknown ones, while targeted methods are used for measuring a group of chemically characterized and biochemically annotated metabolites, often using an analytical method that is optimized to measure the selected metabolites.

Lipidomics as an emerging field in metabolomics

Lipidomics is one of the most important subfields in targeted metabolomics, studying the structure and function of lipid molecules as well as related interacting factors. Lipids represent complex classes of biomolecules with diverse characteristics in chemical structure and function, whose presence and abundance are key to metabolic homeostasis and regulation. Lipid classes like glycerolipids, phospholipids and sphingolipids are essential building blocks for cellular membranes in which the lipid composition significantly affects membrane physical properties and protein functions [6-8]. Lipids are also involved in biological processes mediating signaling cascades as individual lipid molecule/species and/or lipid (sub)classes. With the technical advancement in lipid analytics, lipidomics has been emerging and showing its potential role in metabolomics studies, offering cellular and molecular biology insight into health and disease.

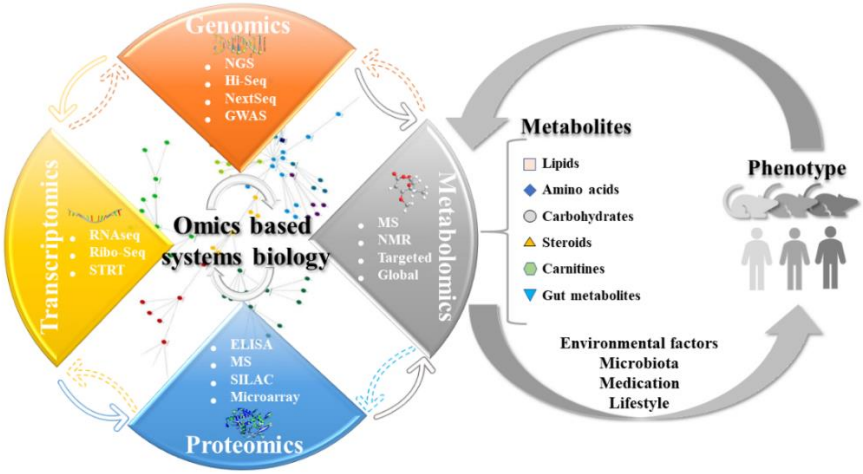

Figure 1. Omics based systems biology and phenotyping. Omics based systems biology studies include the application of genomics, transcriptomics, proteomics and metabolomics. The phenotype of an organism represents an observable trait influenced by a variety of factors: genetic, environmental/lifestyle, medication and gut microbiome. Metabolomics bridges the genotype to phenotype gap reflecting the effect of genetic factors on phenotype through the metabolome.

Signaling lipids and their role in inflammation

Inflammation

Inflammation is part of the complex biological response of the immune system to noxious insults, such as infection, tissue injury and other detrimental invasions [9,10]. It is a protective strategy of the body, aiming for removal of harmful stimuli as well as for restoring normal homeostasis. Acute inflammation is characterized with sequentially occurring events involving molecular (*e.g.*, cytokines, chemokines, lipid mediators and free radicals), cellular (*e.g.*, leukocyte infiltration, macrophage influx) and physiological changes. If successfully resolved, the acute inflammation will be terminated. This can be followed by a post-resolution stage which involves influx of adaptive immune cells and re-assembly of tissue-resident macrophages. This suggests the complete and effective resolution will assist the shift from innate to adaptive immunity [11]. On the other hand, incomplete resolution could result in chronic inflammation, unable to fully engage an appropriate adaptive immune response.

It should be noted that not all chronic inflammatory conditions are derived from unresolved acute inflammation. Instead, some chronic inflammatory states are associated with tissue malfunction or homeostatic imbalance which is a comparatively lower magnitude response but with longer persistence than acute inflammation [12].

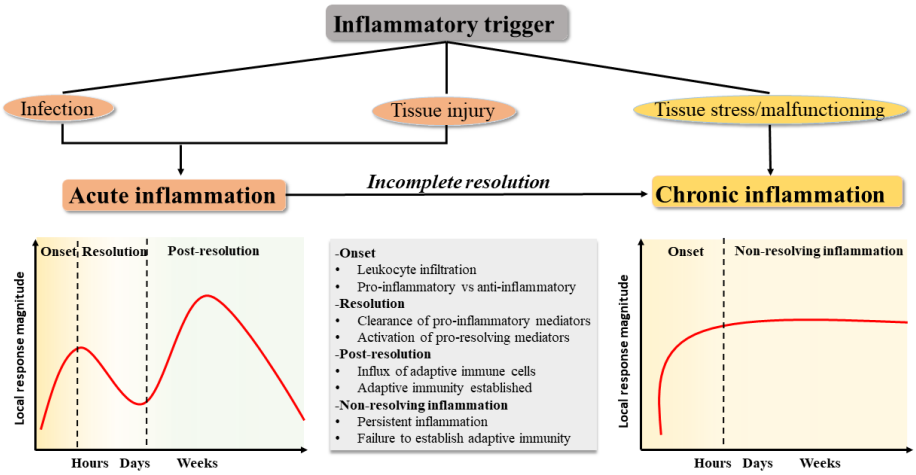

Figure 2. Causes, categories and outcomes of inflammation. A simplified illustration of inflammatory triggers for acute inflammation (*e.g.*, infection, tissue injury) or chronic inflammation (*e.g.*, tissue stress or malfunctioning) and the dynamic local response magnitude corresponding to the outset, resolved, post-resolved or non-resolved inflammation. This figure is adapted from [11,12].

Signaling lipids: introduction and inflammatory roles

Emerging evidence has highlighted the essential role of lipid signaling in inflammation/inflammatory responses [13]. Lipid signaling represents a series of biological processes involving the biosynthesis of lipid molecules, receptor-binding activities of these molecules as well as consequent cellular responses. These lipid molecules participating in signaling cascades, are so-called signaling lipids or bioactive lipids. In the following sections, typical signaling lipid classes such as oxylipins, (nitro) free fatty acids, endocannabinoids, bile acids, lysoglycerophospholipids, lysosphingolipids and platelet activating factors (PAFs) together with their reported roles in inflammation/inflammatory processes are briefly outlined.

-Oxylipins

Oxylipins (or oxylipids) are oxidized products from polyunsaturated fatty acids (PUFAs) such as linoleic acid (LA), α-linolenic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Their biosynthesis is under tight regulation through enzymatic pathways including cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP450) or non-enzymatic pathway such as via reactive oxygen species (ROS) mediation. Oxylipins are some of the most active lipid mediators in both intracellular and intercellular signaling where the specific roles across oxylipin subclasses vary significantly due to the diverse origins and formation of oxylipins.

The COX enzyme oxidizes AA into prostaglandins, a typical pro-inflammatory subclass of oxylipins in immunomodulation and immune response through acting on nuclear factor-kappa B (NF-kB). Conversely, the pro-resolving function of oxylipins has been realized as well unveiling their multifaceted role in inflammatory response. One of the most studied prostaglandins, prostaglandin E2 (PGE2), exerts pro-inflammatory effects in pain and fever as well as altering vascular permeability and vasodilation. PGE2 was also reported to be anti-inflammatory by inhibiting cytokine generation from human lung macrophages. The effect of prostaglandin D2 (PGD2) is also context dependent. In asthma-related response, PGD2 released by mast cells can be pro-inflammatory resulting in production of chemokines and cytokines as well as airway inflammation mediated by the DP1 receptor. Contrarily, PGD2 may bind to dendritic cells (DCs) expressing DP1 and reduce cytokine generation by antigen specific T cells [14].

Leukotrienes, lipoxins, resolvins, maresins and protectins are mainly from the LOX pathway of which leukotrienes are generally regarded pro-inflammatory while the rest are putative pro-resolving or anti-inflammatory signaling molecules termed specialized pro-resolving mediators (SPMs). SPMs exert their resolving functions via acting on G protein-coupled receptors (GPCRs) on immune cells and modulating host response[15]. CYP450-derived oxylipins mainly include epoxy octadacamonoenoic acids (EpOMEs) derived from LA, epoxy eicosatrienoic acids (EpETrEs or EETs) derived from AA, eicosatetraenoic acids (EpETEs or EEQs) from EPA which are further converted to dihydroxyoctadecenoic acids (DiHOMEs), dihydroxyeicosa-trienoic acids (DHETs), dihydroxy EEQs (DHEQs)

respectively. Another CYP450-related epoxy oxylipin is 19,20-epoxydocosapentaenoic acid (EpDPE) which is derived from DHA and can be metabolized to 19,20-dihydroxydocosapentaenoic acid (DiHDPA). Inflammatory roles of CYP450-epoxy oxylipins and related compounds are understood to a lesser extent. Recent findings demonstrated their active participation (most likely EETs) in resolution phase by limiting proinflammatory monocyte lineage [16]. Collectively, due to the complexity of oxylipin generation pathways, receptor modulation as well as cellular and molecular interactions, further efforts have to be made to dig into the oxylipin family and their roles in inflammation.

-(Nitro) Free fatty acids

The key link between fatty acids and inflammation is that free fatty acids, in particular PUFAs are the precursors of oxylipin compounds as described above. The n-6 PUFAs (dominantly AA) are responsible for the generation of eicosanoids acting as inflammatory mediators and/or regulators of other mediators including inflammatory cytokines [17]. The n-3 PUFAs such as EPA and DHA are in general considered as anti-inflammatory if supplemented via nutritional products such as fish oil. The anti-inflammatory effects of n-3 PUFAs may be mediated by their incorporation into inflammatory cell membrane phospholipids at the expense of AA and this to some extent antagonizes the production of pro-inflammatory prostaglandin and leukotriene and related neutrophil chemotaxis [18,19]. In addition, anti-inflammatory effects of PUFAs are in part attributed to their inhibition of interleukin-1, tumor necrosis factor and superoxide radical production [20,21].

Eicosanoid independent mechanisms also exist regarding the roles of free fatty acids on inflammatory signaling. The n-3 PUFAs exert anti-inflammatory effects by altering expression of cell surfaced adhesion molecules related to leukocyte infiltration [22]and downregulating inflammatory gene expression via NFκB [23,24]. Saturated fatty acids are instead prone to induce inflammation via releasing TNFα and IL-6 [25] or interacting with Toll-like receptor 4 (TLR4) that triggers the innate immune response [26,27].

Nitro-fatty acids (NO_2-FAs) are a special group of fatty acids formed from the nitration of unsaturated fatty acids by the radical nitrogen dioxide ($\bullet NO_2$) [28]. The formation of NO_2-FAs mediated by reactive nitrogen species (RNS) represents an adaptive response to oxidative and nitrosative stress conditions to counterbalance the potential cytotoxicity of

reactive oxygen and nitrogen, as well as to downregulate the generation of proinflammatory eicosanoids [29]. Therefore, they are considered as anti-inflammatory molecules. Their anti-inflammatory actions were reported to be associated with peroxisome proliferator-activated receptor (PPARγ) activation [30], NF-kB inhibition and superoxide radical ($O_2^{\bullet-}$) inhibition [31,32]. The immune function of NO_2-FAs is linked with metabolic regulation, such as glucose homeostasis [33]and lipid metabolism, which remains to be further elucidated [34].

-Endocannabinoids

The endocannabinoid system consists of endocannabinoids, endocannabinoid synthetic and degrading enzymes as well as cannabinoid receptors (CBRs) [35]. Endocannabinoids are endogenous lipid-based retrograde neurotransmitters and their role in inflammation was systematically reviewed in [36]. Briefly, endocannabinoids modulate inflammatory processes interacting with cannabinoid receptors CB1 and CB2 and other receptors like GPCRs, PPARγ and transient receptor potential ankyrin 1 (TRPA1). Besides, endocannabinoids indirectly interact with nonreceptor targets such as ion channels and share pathways with other lipid molecules [37,38].

The two most studied endocannabinoids are anandamide (N-arachidonoyl ethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) which belong to N-acylethanolamines (NAEs) family and monoacylglycerol (MAGs) family, respectively. They have differential affinities to CBRs and concentrations in vivo with respective major pathways involving different enzymes, thus engaged inflammatory networks are presented differently. AEA is synthesized through N-acyl-phosphatidylethanolamines (NAPE) hydrolysis by a NAPE-specific phospholipase D (NAPE-PLD) and degraded mainly by fatty acid amide hydrolase (FAAH). The synthesis of 2-AG is via enzyme diacylglycerol lipase α and β (DAGL-α/β) and the degradation is primarily mediated by monoacylglycerol lipase (MAGL) while other enzymes also involve including α,β hydrolase 6 (ABHD6), ABHD12 and FAAH. Notably, endocannabinoid signaling is intimately linked with eicosanoid pathways at both substrate and enzyme levels. This is mostly but not limited to AA and 2-AG together with the COX, LOX, CYP450 enzymes.

-Bile acids

Bile acids are cholesterol-derived steroid acids that facilitate the intestinal absorption and transport of dietary lipids. They have been recently recognized as signaling molecules that mediate through nuclear receptors farnesoid X receptor (FXR) and G protein-coupled receptor (GPCR) TGR5. TGR5-BA in immune cells inhibit inflammatory signaling cascade via targeting NF-kB, tumor necrosis factor α (TNF-α) and interleukin (IL-18,IL-1β) thus attenuating inflammation. In a more general way of regulation, BA signaling in energy expenditure, gluconeogenesis and glycogen tightly controls the systemic glycemic response which indirectly links with inflammatory factors such as obesity/leanness and insulin resistance. In addition, under chronic inflammatory conditions, BA composition can be modulated by the location and severity of inflammation [39]. More reports regarding BA signaling in inflammation have discussed the roles of bile acids in gastrointestinal disease [40,41], mucosal immunity [42] and tumorigenesis [41].

-Lysoglycerophospholipids and lysosphingolipids

Lysoglycerophospholipids and lysosphingolipids are two categories of lysophospholipids characterized based on their lipid backbones. Lysoglycerophospholipids include the following lipid classes: lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS). In particular, LPA consisting of an acyl chain esterified to a glycerol backbone, is the only type of "head group free" lysoglycerophospholipid which can be derived from phosphatidic acid (PA) via PLA1/PLA2 or from other lysoglycerophospholipids via lysophospholipase D termed autotaxin (ATX) [43]. LPA signaling is primarily mediated by binding to extracellular GPCRs LPAR1-6 and the intracellular peroxisome proliferator-activated receptor gamma (PPARγ). Its regulatory role in inflammation/inflammatory process is associated with enhancement of proinflammatory cytokines by interacting with types of immune cells in innate immunity and adaptive immunity, for example, proinflammatory cytokine secretion of M1 macrophages [44], interferon gamma (IFNγ) secretion by activated natural killer cells [45] and IL-6 production from dendritic cells [46]. Intracellular hydrolysis of LPAs is significantly attributed to the N-terminal domain of soluble epoxide hydrolase (sEH) which dephosphorylates LPAs. The C-terminal domain of sEH on the other hand is responsible

for epoxide (e.g. EETs) hydrolysis to form diols [47-49]. The regulatory role of sEH on both LPAs and epoxy oxylipins interlinks the signaling pathways of these two types of inflammatory molecules. Other classes of lysophospholipids similarly revealed their signaling biology involving inflammatory response though to a lesser extent compared to LPA [50]. LPS is regarded as an immunomodulator due to its highly expressed receptors on mast cells and in lymph node. LPI binds to G protein-coupled receptor 55 (GPR55) which is a cannabinoid receptor and is suggested to involve inflammatory pain perception [51].

Sphingosine-1-phosphate (S1P) is a well-characterized lysosphingolipid with similar structure as LPA. S1P production is based on sphingosine phosphorylation catalyzed by sphingosine kinases (SphK1 and SphK2). S1P exerts its role through GPCRs (S1P 1-5) and regulates various cellular functions via S1P-S1P receptor signaling, e.g. regulation of immune cell trafficking and maintenance of vascular integrity. Specifically, the steep S1P gradient formed between lymphoid organs (low concentration of S1P) and the circulation (high concentration of S1P) provides the basis of lymphocyte egress from thymus and peripheral lymphoid organs. This S1P gradient based regulation of trafficking also applies to other immune cells which may induce a series of proinflammatory responses and relate to pro-angiogenic roles of S1P [52 53]. On the other hand, S1P-S1P receptor signaling is pivotal in regulating angiogenesis by affecting vascular maturation and strengthening the adherens junctions (AJs) between endothelial cells, thus considered to be anti-angiogenic and anti-inflammatory [52].

-Platelet activating factors

Platelet activating factors (PAFs), just as its name implies, are a group of potent mediators in platelet activation and aggregation and other inflammatory reactions [54]. PAFs are acetylated from lyso-PAFs which are released from membrane PC by PLA2 hydrolysis [55]. Thus, lyso-PAFs are in fact lysoglycerophospholipids and they are activated to exert biological functions when acetylated to PAFs.

The biosynthesis of PAF can be mediated by varieties of cell types but particularly those involved in host defense such as platelets, endothelial cells, neutrophils, monocytes, and macrophages. The generation of PAFs are under tight regulation which is in general

continuous and low abundance while external stimuli triggers will result in a larger amount of PAFs by immune cells. PAF-induced inflammatory reactions are often synergized by other pro-inflammatory molecules like prostaglandins and leukotrienes [54].

Analytical approaches targeting signaling lipids

Status quo of signaling lipid method development

The realization of biological roles and biomedical values of signaling lipids has greatly motivated the development of analytical approaches for signaling lipid profiling. Oxylipins have been most extensively studied and the methods for oxylipin analysis were reviewed by multiple papers [56-59]. Regarding the technology applied, there was an evident shift from the usage of GC-MS/MS to LC-MS/MS for oxylipin analysis [56]. Extraction strategies and separation/detection technologies for oxylipin analysis were variously reported [60-64]. To cover more metabolites, development of more comprehensive methods were undertaken. Specifically, simultaneous profiling of oxylipins and endocannabinoids were major efforts [65-67]. Methods covering a broader range of signaling lipids were also reported [68-70]. Nevertheless, only one published method covers lysophospholipids, sphingolipids while the method focuses only on a limited number of oxylipin compounds: prostaglandins and isoprostanes [71]. Hitherto, none of the analytical methods are comprehensive enough to cover all signaling lipid classes as listed above.

Improving method sensitivity is another research focus and is of paramount importance for especially low abundance compound classes (e.g. endocannabinoids, oxylipins). Nano-scale and micro-scale LC-MS/MS methods were developed for endocannabinoid profiling. These miniaturized LCMS methods enable quantitative profiling of endocannabinoids from mass-limited human CSF samples [72,73]. Yet, the robustness of nano-scale and micro-scale LC-MS/MS methods remains to be monitored during long-term instrumental running and metabolite coverage should be broadened to contain many biologically relevant lipid molecules.

A better method for signaling lipids: goal and challenges

As mentioned, a better analytical method for metabolomics profiling of signaling lipids featuring both high comprehensiveness and sensitivity with robust performance is needed to provide a bigger picture of the biochemistry of signaling lipids. There are several challenges that have to be addressed: 1) compounds are susceptible to oxidation 2) metabolite extraction is difficult to optimize due to diverse physicochemical properties and dynamic endogenous concentrations across lipid classes 3) chromatographic separation is complicated due to compound isomers 4) absolute quantification is challenging owing to potential matrix effect.

Application studies: signaling lipids in inflammatory pathophysiology

Analytical method development enables addressing biological or clinical questions and the pursuit of a better understanding of inflammation related pathophysiology regarding the roles of signaling lipids. In following applications, we present the rationales and essentiality of studying signaling lipids in neurodegenerative diseases (Alzheimer's disease), autoimmune diseases (mucous membrane pemphigoid; rheumatoid arthritis) and exercise physiology.

Alzheimer's disease

-signaling lipids for understanding APOE related pathology

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common cause of dementia. It is recognized by the presence of senile plaques formed by amyloid β (Aβ) deposit and neurofibrillary tangles (NFTs) formed by tau protein aggregation within the patient's brain [74]. The signs of the disease include forgetting recent events or conversations followed by severe memory impairment and losing the ability to carry out daily tasks. While the cognitive changes are merely subtle, recognized in prodromal stage of AD, this is often called mild cognitive impairment (MCI) [75]. Current medications only temporarily slow progression of symptoms with modest benefit on cognition [76].

Despite the unclarified pathogenesis, evidence has indicated the association between AD and immunological mechanisms in the brain. Microglia cells are key players in maintaining the plasticity of neuronal circuits and synaptic remodeling [77]. When activated by pathological triggers, they initiate innate immune response and produce proinflammatory cytokines that induce production of amyloid β precursor protein (APP) and Aβ plaques which can be counterbalanced by anti-inflammatory cytokines [78]. Activated microglia are accompanied by increasing levels of free radicals and nitric oxide which relate to oxidative and nitrosative stress [79,80]. These collectively implicate the active and complex inflammatory processes in AD development.

One risk factor for developing AD is the APOE gene coding for the apolipoprotein E protein (apoE) which is a major lipid transporter and regulator of many signaling pathways. Three different alleles of APOE, known as ε2, ε3 and ε4, exist in humans. Possession of the APOE ε4 allele is the most prominent risk factor for sporadic, late-onset AD, possibly attributed to affecting neuropathological markers of AD: neuroinflammation, Aβ plaques and tau protein aggregations [81]. Conversely, alleles of ε2 and ε3 are regarded to play protective and neutral roles respectively in the development of AD. While the APOE genotype is a well-acknowledged primary contributor for AD, how precisely APOE may cause AD has been rarely studied and understood.

Understanding APOE related molecular characteristics from a metabolic perspective will undoubtably provide new insights into APOE related pathological mechanisms of AD presenting integral features of inflammatory lipid mediators associated with APOE genotype.

Mucous membrane pemphigoid

-signaling lipids for diagnosis and drug development

Chronic cicatrizing conjunctivitis (CCC) is an autoimmune disease including 35 immunological and neoplastic entities which lead to ocular surface scarring [82]. It often causes ocular surface dysfunction and loss of visual ability. Within the classification, mucous membrane pemphigoid (MMP) is a progressive inflammatory and scarring disease and when it is related to eye symptoms, which is the most common case, it progresses with

conjunctival cicatrization with the disease status worsening from early to late stage and may finally cause blindness [83]. In the clinic, the current gold standard for diagnosis is direct immunofluorescence (DIF) which is often with low diagnostic sensitivity, particularly in MMP with ocular manifestation alone. This prompts a better diagnostic strategy, not only for accurate diagnosis but also for directing new drug development for solving the clinical dilemma of inefficient medical intervention.

In ophthalmology, there has been emerging evidence of the success of metabolomics. In human studies, tear samples have been utilized for metabolomics profiling due to their easy accessibility while the drawback is that tear is prone to contamination when being collected. Other studies performed include in vitro cell analysis of human conjunctival epithelial cells [84]. Therefore, it is feasible to identify potential inflammatory biomarkers using metabolomics profiling and to study molecular dynamics regarding the onset and progression of MMP for better guiding drug development.

Rheumatoid arthritis

-signaling lipids for drug efficacy prediction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by joint pain, stiffness, and swelling due to synovial inflammation and effusion, ultimately leading to joint destruction and physical disability when untreated [85]. Both genetic and environmental factors play essential roles in RA development while the actual cause of RA remains largely unknown. Extensive studies have demonstrated the infiltration of various immune cells and immunomodulatory molecules secreted by them contribute actively to RA pathogenesis [86]. Two critical cellular components, monocytes/macrophages and T cells, mutually interact with each other via cell recruitment, differentiation and cytokine production, i.e., interleukins, interferon-γ and TNFα [87,88].

In this regard, pharmacological treatment of RA has been always aiming for immune-system suppression to block the effects from inflammatory molecules. Specifically, current therapies include non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDS). The latter consists of conventional synthetic ones (csDMARDs) and biological ones (bDMARDs). In clinical practice guided by international

recommendations [89,90], the gold standard is to use csDMARDs as first line drug and then should be added with bDMARDs if csDMARDs fail to achieve remission. The adjustment of agent with another mode of action is commonly seen due to intolerance or inadequate response to csDMARDs. Considering the foremost goal in RA treatment is to achieve as early sustained drug-free remission as possible, switching treatment upon initial treatment failure may result in belated therapeutic benefits. However, it is still inconclusive on how to choose the class of DMARDs to initiate patient-tailored treatment. It would be therefore beneficial if measurable characteristics of an RA patient could be applied to assist a personalized clinical plan.

In the context of signaling lipids with biologically interlinked natures, unravelling the metabolic dynamics among RA patients prior to treatment will help discover metabolic biomarkers and relevant pathways indicative of inflammatory status and predictive of drug response of RA patients. This will drive the innovation of standardized management of RA and ultimately benefit each individual RA patient by personalizing the therapeutic regimen.

Exercise physiology

-signaling lipids in response to chronic exercise training modalities

Physical exercise is widely acknowledged as an efficient drug-free strategy to improve overall health and wellness. The profound effects of physical exercise involve the modulation of the immune system where corresponding immune responses are exercise type, intensity and frequency dependent [91,92]. Acute exercises of endurance exercise (EE) and resistance exercise (RE) both provoked alterations in increasing immune cell populations but stronger effect was seen from EE [93]. In terms of exercise period, a single and acute exercise bout induces a transient immune response which is presumably modulated by post-exercise immune surveillance [94], while chronic exercise plays a more important role in promoting immunological adaptations towards an anti-inflammatory status and reducing incidence of infection over time, if proper training is performed [95,96]. On the other hand, exercise-induced inflammation has arisen the awareness of multifaceted roles of exercise methods [97,98].

Metabolites, especially lipids, are essential parts of molecular orchestration of exercise related immune and inflammatory regulation. In a system-wide study exploring multi-omic changes in response to acute exercise, a large proportion of metabolites/lipids were altered remarkably across all time points compared to transcripts and proteins [99]. Exercise associated metabolic regulation of signaling lipids is extensive, including fatty acid oxidation [100,101], sphingolipids [102], endocannabinoids [92,103]and oxylipins [104]. Notwithstanding, current knowledge about regulatory effects of exercise training on lipid metabolism and signaling primarily derives from acute exercise studies. Therefore, further research of chronic exercise training activities characterized with different training type, frequency and intensity will provide a comparative overview of their regulatory patterns on lipid metabolites related to immune and signaling pathways. Performing such studies will expand the metabolic landscape of exercise biology and provide instructive knowledge of exercise scheme in practice.

Scope and Outline of the Thesis

Lipid signaling is an essential biological event/process in a plethora of pathophysiological conditions. The underlying idea of this thesis is that many of the roles and the complex interplay of the individual signaling lipids in inflammatory processes and related conditions in health and disease is not well known, and therefore has to be studied integrally as a complex network. In order to study this complex interplay, an improved broad analytical method is necessary to analyze a wide range of different signaling lipid classes such as oxylipins, (nitro) free fatty acids, endocannabinoids, bile acids and different subclasses of lysophospholipids. Therefore, the aim of this thesis is to develop a better method to study signaling lipids, and to apply it to study the role of these molecules in several relevant biological questions for a better understanding of inflammation related pathophysiology including autoimmune diseases, neurodegeneration and regulatory effect of exercise training.

In **Chapter II** the first aim is the development of a sensitive and robust metabolomics method for signaling lipids, and the aim is to cover a wide range of signaling lipid classes

of oxylipins, (nitro) free fatty acids, endocannabinoids, bile acids and different subclasses of lysophospholipids (lysoglycerophospholipids, PAF, lyso-sphingolipids). With an optimized one-step liquid-liquid extraction protocol, the method will allow the simultaneous and efficient extraction of abovementioned lipid classes and to detect in total 261 metabolites in biological samples such as blood and cells. The method is fully characterized and validated with regards to linearity, limit of detection, extraction recovery, matrix effect and precision. Accuracy is characterized with a plasma reference sample (NIST 1950) and results are comparable to other reported data.

The developed method is applied in the following chapters to study the role of signaling lipids with regard to different pathophysiological situations involving inflammation, namely neurodegeneration (**Chapter III**) autoimmunity (**Chapter IV**, **Chapter V**), and exercise physiology (**Chapter VI**).

Inflammatory involvement in neurodegenerative diseases has been increasingly reported with oxidative stress and neuroinflammation but it has not been fully investigated. Therefore, **Chapter III** aims to study the association of markers of oxidative stress and inflammation with established biomarkers of Alzheimer's disease (AD) Aβ-42, p-tau, and t-tau (ATN biomarker system: amyloid beta, pathologic tau and neurodegeneration). From two study cohorts, 142 paired plasma-CSF samples and 40 CSF samples from mild cognitive impairment (MCI) patients have been collected for signaling lipid profiling. The isoprostane and prostaglandin data, which are readouts of oxidative stress and inflammation, are used to evaluate their associations with ATN biomarkers in CSF, in overall analyses and in APOE stratified analyses respectively. The results reveal that CSF levels of two isoprostanes (5-iPF2α VI; 8,12-iso-iPF2α VI) and one prostaglandin (PGF2α) are significantly and positively associated with CSF levels of p-tau and t-tau. Our findings demonstrate the potency of these metabolites as oxidative stress and inflammatory biomarkers in the prodromal stage of AD.

The aim of **Chapter IV** is to study the roles of signaling lipids as potential diagnostic and disease progression biomarkers in ocular mucous membrane pemphigoid (MMP), an autoimmune chronic inflammatory mucous disease. To achieve this, conjunctival cytology samples from healthy controls and patients have been collected and measured. Signaling

lipid profiles are compared between controls and patients to identify specific molecules present in only patient samples. Furthermore, to discover promising biomarkers indicating disease activity, metabolite levels in patients are compared to present dynamic metabolic patterns with disease progression from early to late stage of MMP. Our results demonstrate active roles of oxylipins, lysophospholipids, fatty acids, and endocannabinoids in disease onset and progression. In addition, the study proves the feasibility of metabolomics analysis of non-invasive conjunctive biopsy samples, thus expanding the applicability of the signaling lipid platform from blood profiling to volume-limited impression cytology sample profiling.

In **Chapter V**, the aim is to identify promising metabolic biomarkers at baseline predicting therapeutic response of achieving sustained drug-free remission (sDFR) for the treatment of early rheumatoid arthritis (RA). For this, drug-naïve RA patients were initially treated with methotrexate (MTX), tocilizumab (TCZ), or the combination. Different methods are used to find metabolic markers to predict sDFR. Next, in a systems biology approach, transcriptomics-proteomics and proteomics-metabolomics biomarker correlation networks in each group are studied and interpreted to present high potential predicting biomarkers. Overall, this study reveals the roles of predisposing inflammatory metabolites for achieving sDFR in early RA and provides new insights into personalizing treatment of early RA patients.

In **Chapter VI**, the aim is to study how exercise training could possibly alter the profiles of signaling lipids. On the basis of two-sided effects of exercise training - immune boosting or inflammation promoting - dependent on the type, intensity and time of duration of the exercise, this chapter positions the research question on investigating the impact of chronic exercise intervention on circulating levels of signaling lipids. Sixty-five middle-aged sedentary adults were recruited for a 12-week randomized controlled trial and were randomly assigned to four experimental groups (one group as no exercise control group, the other three with different training modalities). Signaling lipids have been measured in plasma samples obtained prior to and after the 12-week exercise training program. As a result, our study shows modified fasting levels of oxylipins and endocannabinoids upon exercise training comparing pre and post metabolite profiles within the group. However, no statistically significant changes are observed between exercise groups compared to control

group. Our findings suggest a minor impact of types of chronic exercise training modalities on plasma levels of inflammatory metabolites.

The thesis concludes in **Chapter VII** summarizing the work of the thesis, discussing the results as well as future perspectives of studying the role of signaling lipid in health and disease.

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