Systems Diagnosis of Chronic Diseases, Explored by Metabolomics and Ultra-weak Photon Emission

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Chapter 1

General introduction and aim of the thesis

1. Chronic diseases, unmet medical needs

Treating chronic diseases such as rheumatoid arthritis (RA) and type 2 diabetes mellitus (T2DM) is a hot topic that has been discussed widely and investigated extensively, but never solved, due in part to their high complexity (e.g., dynamic disease processes, multiple pathologies, and associated complications). The onset of a chronic disease usually starts from a slowly developing, asymptomatic stage, which can last several years until clinically detectable signs of disease appear, then progresses to an irreversible stage. With respect to prevalence, approximately 1% of the global population currently has RA, and this percentage is increasing. For T2DM, epidemiology studies estimate that 285 million individuals are currently affected worldwide, and this number is projected to reach 439 million by 2030 [1]; moreover, a large number of individuals are undiagnosed due to only mild symptoms in the early stages of the disease [2][3]. This long-term undiagnosed state can directly and/or indirectly affect quality of life, serving as a major cause of morbidity, hospitalization, systematic complications, and even mortality. At the same time, the costs associated with caring for patients with diabetes are extremely high, with hospitalization and complications accounting for the largest portion of these costs. Thus, from the perspective of both patients and the economy, it is essential to develop more reasonable and efficient approaches to diagnose these diseases early, thereby increasing treatment efficacy.

2. Diagnosing chronic disease using a systems approach

Early diagnosis is an essential step in the detection of chronic disease, helping the clinician identify the appropriate target for intervention and decreasing the risk of complications, reducing mortality, and reducing economic costs. With respect to chronic diseases, subtle perturbations associated with metabolic disorders are often present for years before the appearance of clinically severe symptoms. Therefore,

the slow development of chronic disease, as well as dynamic phenotypes, make diagnosing a chronic disease more complex and challenging, as well as leading to complications if not diagnosed in an early stage. Current diagnostic approaches are based primarily on a single marker (usually the most relevant marker), which is sometimes not directly applicable and/or might not adequately reflect the chronic disease. The ability to predict disease early and to dynamically observe chronic disease remain challenging and if solved can—to a certain extent—prevent the development of irreversible lesions. Given the complexity and long-term dynamics of chronic disease, a personalized approach to phenotyping may help improve our understanding of the early stages of chronic disease. In addition, integrating disease-related information using a systems approach may help improve our knowledge of all stages of the disease, thus improving the accuracy of diagnosing chronic disease.

3. Personalized medicine: going beyond the "one-size-fits-all" approach

The definition of "health" is shifting changing from the notion of complete well-being towards a state of dynamic control (i.e., homeostasis); thus, reduced resilience of the body's systems can lead to disease [4]. This loss of resilience can occur at any time point and/or with dynamics unique to each individual. Thus, with respect to disease, it is reasonable to assume that each patient will experience a unique situation that reflects that patient's personalized disease characteristics. Given the shift in our concept of health in recent decades, the Western model for treating disease is also shifting from the "one disease-one target-one drug" approach towards a more personalized approach that focuses on the individual patient [5]. The concept of personalized medicine, which reveals unique symptoms that are related to disease, has the ultimate goal of helping improve diagnostics and

prognostics, improving healthcare by providing accurate, personalized treatment targets, and providing opportunities to minimize—or even eliminate—side effects and non-responded therapies in patients.

The reductionist approach helps improve our understanding of complex processes by dividing these processes into smaller, simpler units. Although living organisms are rather complex, with many interactions, systematic approach–based integrative analysis has the advantage of providing an overall understanding by evaluating "what the complex system looks like, how complex systems connect and interact, and why the various components function in the organism as they do." Therefore, in recent decades Western medicine has been shifting from identifying individual components to identifying interactions within intricate networks. In addition, a systems approach can be considered a guide for developing complementary approaches to healthcare [5] and may contribute to personalized diagnostics/prevention, evaluation, and intervention.

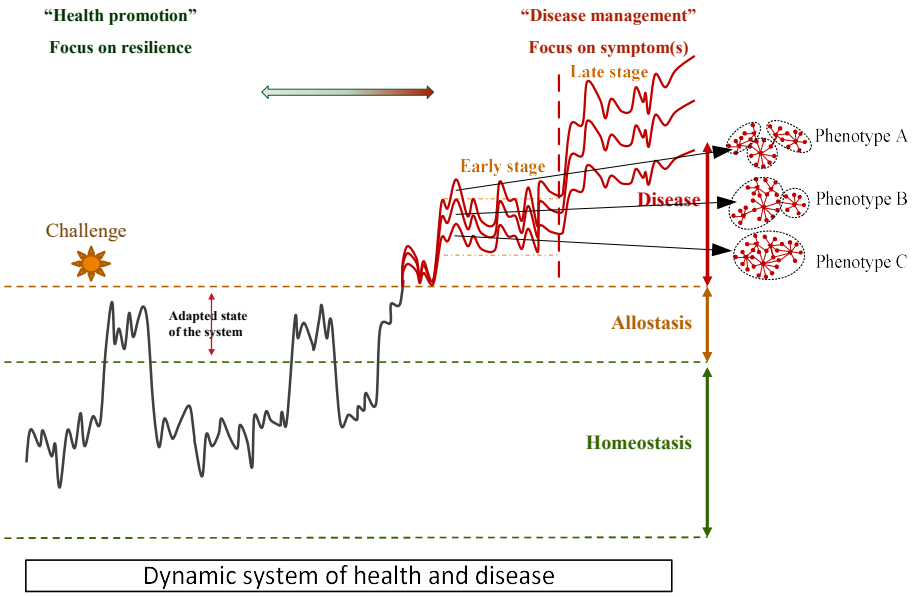

Fig. 1: Schematic diagram illustrating health (homeostasis) and the dynamic development of disease.

In the healthy state, challenges can be overcome and the body's resilience enables the system to remain healthy. The disease state develops when the body loses the ability to overcome this challenge. Subtle perturbations often occur for years (in the early stage of disease), and the disease progression can take various paths, producing various phenotypes (phenotype A, B, or C) before the appearance of serious symptoms with irreversible disease sequelae (in the late disease stage). Personalized medicine focuses on the individual patient, and a systems-based approach may help improve our understanding of phenotypes by measuring complex interactions between intricate networks, even in the early stages of disease.

Combining integrative thinking at the systems level with integrative measuring techniques and bioinformatics can help overcome challenges related to understanding living systems and disorders, and can help move towards truly personalized medicine. With respect to integrative thinking, traditional Chinese medicine (TCM)–based concepts may provide a suitable holistic model, as TCM describes disease syndromes/phenotypes as an experience-based reference from the systems level. Such descriptions may also help with the development of specific treatments based on various syndromes and phenotypes, thereby achieving personalized medicine, which is particularly applicable to chronic disease [6], [7].

With respect to systems-based approaches, metabolomics has many advantages, including linking current bodies of knowledge and providing biological interpretations of the pathophysiology of disease [8]; specifically, these approaches provide a comprehensive picture of small molecular metabolites in biological systems and can be used as a readout of an organism's physiological status [9]. These integrative tools provide a wealth of biological information beyond single molecules by simultaneously measuring a range of metabolites—including lipid metabolites, fatty acid–derived oxylipins, organic acids, sugars, amino acids and their biogenic metabolites, etc.,—in order to provide an overview of the disease state and reflect system-wide perturbations. Therefore, metabolomics is considered a suitable approach for obtaining evidence-based scientific data; moreover, in principle metabolomics is an appropriate method for studying the complexity of chronic diseases from the perspective of systems biology. In addition, combining metabolomics with TCM concept–based diagnostics may provide comprehensive data that can be used as a readout to reflect even the early stages of disease and/or

specific phenotypes, thereby facilitating early diagnosis and personalized medicine. However, because metabolomics approaches do not necessarily cover the entire metabolome, choices must be made based on available metabolomics platforms.

Recently, the rapid, highly sensitive, non-invasive measurement of ultra-weak photon emission (UPE) has been proposed for supporting TCM-based diagnostics [10]. UPE measures spontaneously emitted photons at the surface of the skin [11]; therefore, UPE has been proposed to reflect the body's physiological and pathological status and is considered to have potential in terms of clinically diagnosing and observing disease [12]–[14]. Because of the relationship between UPE and reactive oxygen species (ROS), which play an importantly role in inflammatory disease during metabolic processes, UPE may be correlated with oxidative metabolic processes, thereby reflecting the dynamics of disease [15]–[18]. In addition, UPE has potential applications for systematically characterizing TCM-based diagnostics[19], [20]. Given that both metabolomics and UPE have distinct advantages in terms of reflecting disease, combining metabolomics with TCM-based diagnostics will provide a robust model for investigating the biological processes that underlie UPE.

4. Scope and outline of this thesis

Given the challenges described above, this thesis aimed to investigate system-wide perturbations by providing *i*) a systems view of chronic disease, and *ii*) personalized phenotyping guided by TCM-based principles. By using a systems approach, the biological meaning of relevant molecules related to disease/phenotype was revealed by metabolomics, and the relationship between metabolomics and UPE was investigated, thereby providing a molecular basis for UPE and bridging different techniques.

In **Chapter 2** and **Chapter 3**, we used metabolomics in an animal model of RA to evaluate metabolic perturbations in a disease situation from various perspectives, including inflammatory- and ROS-related oxylipins (Chapter 2) and amine-related energy levels (Chapter 3). These studies revealed metabolic characteristics of RA in a commonly used animal model using two well-established platforms. To further understand and further characterize the relationship between metabolic processes and UPE, we then examined the correlations between metabolites (i.e., the integrated dataset described in Chapters 2 and 3) and UPE intensity (measured in the same group of mice) using correlation network analysis; these results are discussed in **Chapter 4**. Such a combination study provides more information and an overall look at the complex pathophysiology underlying RA from a systems perspective. Correlation networks were also created to explore the relationship between UPE and metabolomics under disease conditions and in health.

Personalized phenotyping guided by TCM-based diagnostic principles, metabolomics, and UPE provides a unique contribution to personalized medicine. An explorative study combining metabolomics and UPE with TCM-based diagnostics may further our understanding of personalized medicine from a systems perspective. Thus, information obtained from several analytic technologies can be integrated, helping generate a systems view of disease, with the ultimate goal of achieving personalized medicine. In **Chapter 5**, we provide a general overview of the applications of UPE that were guided by TCM-based diagnostic principles, and we discuss why linking metabolomics and UPE with TCM-based diagnostics may create new avenues for personalized medicine, systems diagnostics, and systems-based interventions for treating chronic disease. In **Chapter 6**, we present our explorative study based on the notions introduced in Chapter 5. We first examined the application of metabolomics for subtyping 44 early-stage T2DM subjects in an attempt to identify key metabolites that contribute to subtypes defined using TCM. We then examined the relationship between metabolites and UPE in these TCM-based subtype.

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Chapter 2

Collagen induced arthritis in DBA-1J mice associates with oxylipin changes in plasma

Min He, Eduard van Wijk, Ruud Berger, Mei Wang, Katrin Strassburg, Johannes C. Schoeman, Rob J. Vreeken, Herman van Wietmarschen, Amy C. Harms, Masaki Kobayashi, Thomas Hankemeier, and Jan van der Greef

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Abstract

Oxylipins play important roles in various biological processes and are considered as mediators of inflammation for a wide range of diseases such as rheumatoid arthritis (RA). The purpose of this research was to study differences in oxylipin levels between a widely used collagen-induced arthritis (CIA) mice model and healthy control (Ctrl) mice. DBA/1J male mice (age: 6-7 weeks) were selected and randomly divided into two groups, viz. a CIA- and a Ctrl group. The CIA mice were injected intraperitoneal (i.p.) with the joint cartilage component collagen type II (CII) and an adjuvant injection of lipopolysaccharide (LPS). Oxylipin metabolites were extracted from plasma for each individual sample using solid phase extraction (SPE) and were detected with high performance liquid chromatography/tandem mass spectrometry (HPLC-ESI-MS/MS), using dynamic multiple reaction monitoring (dMRM). Both univariate and multivariate statistical analysis was applied. The results in univariate student's *t*-test revealed 10 significantly up- or down-regulated oxylipins in CIA mice, which were supplemented by another 6 additional oxylipins, contributing to group clustering upon multivariate analysis. The dysregulation of these oxylipins revealed the presence of ROS-generated oxylipins and an increase of inflammation in CIA mice. The results also suggested that the Collagen-induced arthritis might associate with dysregulation of apoptosis, possibly inhibited by activated NF- κ B because of insufficient PPAR-γ ligands.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic, destructive auto-immune disease which involves primarily the joints in the extremities. The disease is characterized by the destruction of the cartilage in the joints and inflammation of the synovium. This local immune response is characterized by both cell –mediated and humoral immune factors. CD4+ T cells, activated B cells are present in the synovium together with cytokines such as interleukins (e.g. IL-1 and IL-6), tumor necrosis factor (TNFα) and interferon gamma (IF- γ) [1]–[3]. Recent studies have shown an important role of fibroblasts-like synovial cells in the pathophysiology of RA [4]–[6]. Upon pro-inflammatory stimuli and in combination with genetic and epigenetic/environmental factors, these cells, normally responsible for proper composition of the synovial fluid and extracellular matrix, transform into an aggressive phenotype. This phenotype is characterized by a reduced ability to undergo apoptosis [7]–[12], the production of extracellular enzymes like collagenase and metalloproteases responsible for the destruction of the joints [13], [14] and the secretion of (pro-/anti) inflammatory cytokines, chemokines, pro-angiogenic factors and oxylipins [15]–[17]. Due to local hypoxia, the formation of reactive oxygen and nitrogen species is promoted [18]–[21].

Although the role of cytokine/chemokine triggered signal transduction pathways such as MAP kinase and nuclear factor-kappa B (NF- κB) in the pathophysiology of RA has been subject of extensive research, the role of oxylipins is less well understood. Oxylipins are bioactive lipid mediators synthesized from omega-6 polyunsaturated fatty acid such as arachidonic acid (AA), linoleic acid (LA) and dihomo- γ -linolenic acid (DGLA) and omega-3 polyunsaturated fatty acid like eicosapentaenoic acid (EPA), docosahexanoic acid (DHA) and alfa-linolenic acid (ALA) upon liberation from membrane bound phospholipids by activation of phospholipase A2 and subsequent oxidation by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 expoxygenase (CYP450) systems [22]. This leads to the formation of, over at least hundred, bioactive

oxylipins such as prostaglandins (PG), leucotrienes (LT), thromboxanes (TBX), hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EpETrEs). They can act both on local and distant targets by secretion into the circulation system of body. AA is the substrate of pro-inflammatory lipid mediators while EPA and DHA derived lipid mediators are anti-inflammatory such as resolvins and protectins playing a role in the resolution of inflammation [23]. Nonenzymatic oxidation of polyunsaturated fatty acids produces the closely related bioactive lipids mediators like, for example, isoprostanes, HETEs and HDoHEs, indicators of oxidative stress [24]–[29]. Therefore, investigation of the changes of oxylipins in RA animal models will certainly contribute to the understanding of biochemical events in RA research.

Metabolomics is an important and rapidly emerging field of technology enabling the comprehensive analysis of a large number of metabolites associated with disease phenotypes. We have applied a metabolomics approach using a LC-MS based platform combined with elaborate statistical methods to analyze oxylipins in a validated model of RA that is collagen induced arthritis in mice. Our results point to a diminished anti-inflammatory response and increased oxidative stress in the RA-induced situation.

2. Materials and Method

2.1 Chemicals

Methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), ethyl-acetate (EtOAC) and purified water were purchased from Biosolve (Netherlands). All reagents used during the HPLC-MS/MS experiments were ultra-performance liquid chromatography grade (UPLC). Acetic acid was purchased from Sigma-Aldrich (St. Louis, Mo). Standards were purchased from Cayman (Netherlands).

2.2 Animal Studies

DBA/1J male mice (6–7 weeks; Charles River Laboratories) were used in this study. Twenty mice were randomly divided in two groups (10 in CIA group, 10 in Ctrl group as healthy control). In the CIA group, immunization with collagen type II will provoke chronic polyarthritis by the induced autoimmune response. Each mouse was intraperitoneally induced (i.p.) with joint cartilage component collagen type II (CII; 100µg diluted with a 100 µl volume 0.005M acetic acid) which was extracted from bovine nasal cartilage (Funakoshi Co., Tokyo, Japan) at day 0 (T=0). Thereafter, the CII injection was repeated i.p. on days 14,28,42 and 56. In the ctrl mice, 100 µL of 0.005M acetic acid alone was administered i.p. on the same days (0, 14,28,42 and 56).

Next, to all experimental mice, 5 mg of Lipopolysaccharide from *E. coli* 011:B4 (Chondrex, Redmond, USA) dissolved in 100 µL phosphate buffered saline (PBS) was given i.p. immediately after each injection of CII. In the Ctrl group, 100 µl PBS was similarly administered as a control. This protocol for arthritis induction is well established and extensively described [30]. All animals were maintained in a temperature and light controlled environment with free access to standard rodent chow and water. From day 71 to day 75, blood was taken from each animal of both groups (CIA mice (CIA1) died when sampling, leaving 9 animal blood samples in the CIA group) and collected in pre-cooled tubes containing EDTA

(Ethylenediaminetetraacetic acid) as coagulant (BD Vacutainer, Plymouth, UK). After centrifugation at 3000g for 10 minutes, the EDTA-plasma was collected and aliquots were stored at -80 ºC until further processing.

2.3 Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experiments were performed with the approval of the Tohoku Institute of Technology Research Ethics Committee, Sendai, Japan (approval date 18 January 2009).

2.4 Oxylipin HPLC-MS/MS Analysis on Study Mouse Samples

The details of extraction and analysis of oxylipins species were adapted for the analysis of mouse plasma from a previously described oxylipin profiling method [31]. Antioxidant mixture (5 µL) (0.4 mg/mL BHT and 0.4 mg/mL EDTA mixed with volume ratio 1:1) and a mixture of internal-standard mixtures (ISTDs) (5 µL, 1000nM) were added into each 50 µL aliquot of mouse plasma. Subsequently the samples were loaded on the activated SPE plates (Oasis-HLB 96-well plates, 60mg, 30µm) and eluted using ethyl acetate (1.5mL). The dried eluate was re-dissolved in 50 µL acetonitrile/methanol (50:50 v/v) and 5 µL were analyzed by HPLC (Agilent 1290, San Jose, CA,USA) on an Ascentis Express column (2.1 × 150 mm, particle size of 2.7 µm) coupled to electrospray ionization on a triple quadrupole mass spectrometer (Agilent 6490, San Jose, CA, USA). Performance characteristics for the adapted method including recovery, linearity (R^2), linear dynamic range and sensitivity (LOD/ LOQ) were evaluated in a separate validation experiment and the results were comparable to those published before for human plasma by Strassburg et al. [31]. The data is included in the

Supplementary Material (Table S1, figure S1, available online at http://dx.doi.org/10.1155/2015/543541).

2.5 Data Processing and Statistical Analysis

Peak areas were exported from Mass Hunter software (Agilent Technologies, version B.05.01) and ratios to internal standards were computed (target compounds/ ISTDs). Subsequently, an in-house developed QC tool [32], [33] was used to correct for instrument drift and batch effects. The reliability of the measurements was assessed by calculating the reproducibility of each metabolite in a QC pool which was measured after every 10 samples. Oxylipins which met the criteria RSD-QC lower than 35% were included in the final list for the further statistical analysis. Data were log transformed (Glog) and scaled by the standard deviation (autoscaling) in order to get a normal distribution [34], [35]. Univariate analysis (two-tailed unpaired Student's *t*-test) was employed to evaluate significant differences between groups for each metabolite (determined by $p< 0.05$). Principal component analysis (PCA) and partial least square discriminant analysis (PLSDA) were performed to further investigate the discrimination oxylipins between the two groups using tools provided in the metaboanalyst software package (http://www.metaboanalyst.ca) [36]. Cross validation was used in order to validate the performance of the PLS-DA model [37]. A permutation test with 100 iterations was performed to estimate the null distribution, by randomly permuting the class labels of the observations. *p* values of each pair of comparison in the permutation test were calculated to evaluate the null hypotheses. To select the potential important metabolites which contribute to group separation, Variable Importance in the Projection (VIP) scores based on PLS-DA analysis were used. The higher the VIP score of a metabolite is, the greater its contribution in the group clustering will be. VIP scores higher than 0.8 are considered as meaningful. Variables with VIP score higher or equal to 1 were considered as significant important features [38], [39].

3. Results

In this study, the relative concentrations of a panel of oxylipins were determined in control and CIA mice. When evaluating the results from the LC-MS/MS analysis, lower response of ISTDs peak areas were found in two samples, which lead to an extreme high peak area ratio compared with other study samples. Therefore, these two outliers from Ctrl group were excluded from statistical analysis. The list of detected endogenous oxylipins in mice plasma assigned by their precursors is given in Table 1 (details in supplementary table).

3.1 Univariate and Multivariate Analysis Results

From the QC corrected data, a total of 30 unique oxylipins out of a target list of 110 oxylipins included in the metabolomics platform met the criteria RSD-QC <35%. In order to generally visualize the variance of the samples, a principal components analysis (PCA) analysis, as an unsupervised multivariate analysis approach, was performed using these oxylipins. Fig.1 displays the PCA results in the form of a score plot. The first two principal components accounted for 60.1% of the total variance (PC1 35.6% and PC2 24.5% respectively), which means the model explains well the variance of the samples. The score plot showed a natural distribution of samples between the CIA group and Ctrl group (consisting of the symbols "Δ" or "+" plots). All 8 samples (100%) of Ctrl group clustered in PCA. Eight out of 9 mice (88.9%) of CIA group clustered as well, while one sample in CIA group was misclassified and clustered within the Ctrl group. This cluster indicates that there are some differences between the samples, which were mainly a reflection of the CIA/Ctrl groups.

Determining the oxylipin species responsible for the differences between the CIA and Ctrl group is key to unraveling the biological role of this class of compounds in RA. Student's *t*-test is one of the most widely used method to determine the statistical significance. In order to understand which of the detected

oxylipins showed significant differences between the two groups, an unpaired Student's *t*-test analysis was evaluated in each individual metabolite. From the *t*-test, 10 out of the 30 detected oxylipins (percentage of 33.3%) showed significant differences (p<0.05) namely 9,10-DiHOME, 9-KODE, 12,13-DiHOME, 14-HDoHE, 13-HDoHE, 12S-HEPE, 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10-EpOME and 10-HDoHE. In order to show the effect size and variance among the samples, a comparison of individual metabolite levels measured for CIA and control mice is displayed in Fig. 2, in the form of boxplots, with a "*" indicating statistical significance between groups. In the boxplot, lines extended from the boxes (whiskers) showed the variabilities outside from the upper and lower quartiles of the data.

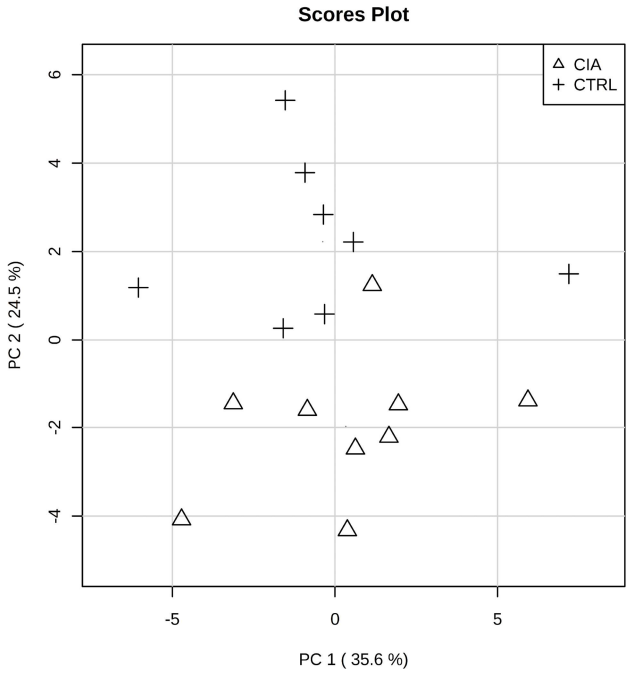

Fig. 1 PCA plot of oxylipin data in study mice plasma. PCA score plot of plasma oxylipin data from all study samples revealed general clusters in CIA mice samples and Ctrl samples. The individual samples were marked with"Δ" or "+"to show the group (CIA versus Ctrl) clustering.

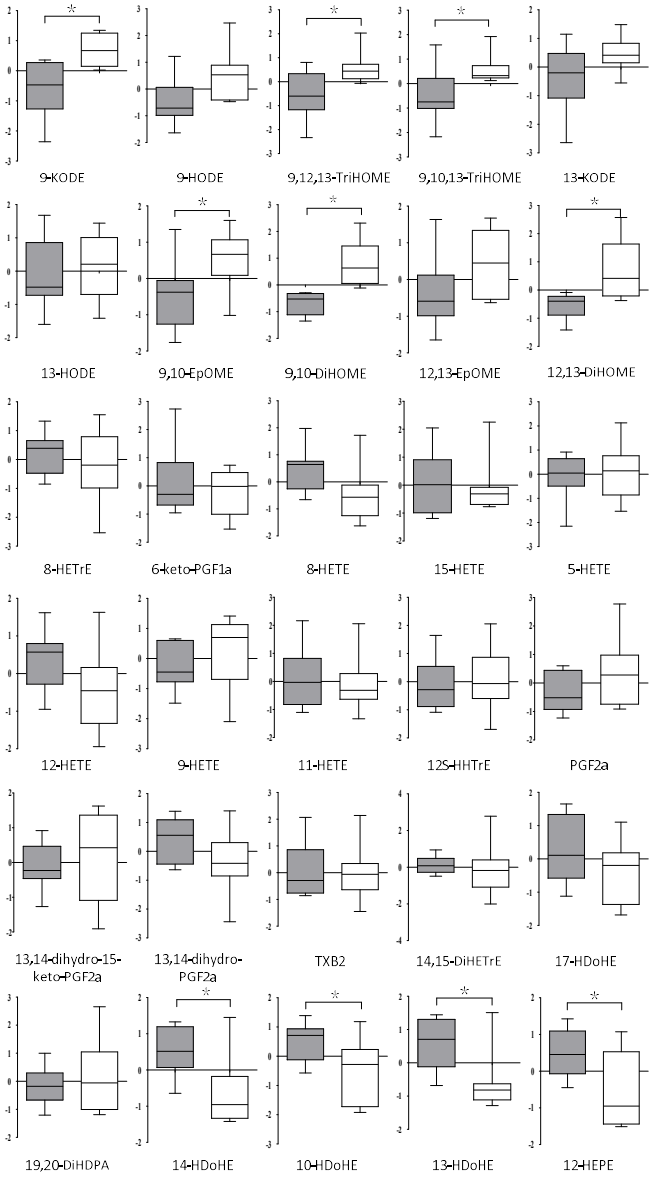

Fig. 2 Changes in metabolite levels between Ctrl and CIA mice. Individual metabolite levels for the two groups are illustrated using box-plots with the whisker drawn, after logarithmic transformation for normalization. Boxplot colored: white box: metabolites in Ctrl group; grey box: metabolites in CIA group. The metabolites which differed significantly based on Students' *t*-test ($p < 0.05$) are marked with "＊".

Given that compounds which showed nonsignificant changes from univariate approaches (such as *t*-tests) may also contribute to group clustering and provide useful information on biological interpretation, a PLS-DA model as a supervised clustering method was further applied to get a more focused view on the metabolites which contribute to group clustering. A PLS-DA scores plot using two components with total score of 43.5% (component 1 = 24.5%, component 2 = 19%) gives a reasonable group separation (figure in supplementary data). However, this model needs to be validated in order to prevent overfitting. Therefore, cross-validation and permutation test was performed. The predictive accuracy (0.88) accompanied with a goodness of fit R^2 (0.84) in cross-validation revealed a sound basis for the PLS-DA model. The permutation tests with an average of 4 misclassifications in100 iterations (p = 0.04) showed robustness of the model. Thus classification of groups based on this approach can be considered as significant based on both cross-validation and 100 permutation tests.

For this model, the Variable Importance in the Projection (VIP) score was used to summarize the relative contributions of each individual metabolite to the group separation in the PLS-DA. The VIP score shows 14 variables which contributed to the group clustering (VIP > 1), including 5 up-regulated oxylipins (14-HDoHE, 13-HDoHE, 12S-HEPE, 10-HDoHE and 8-HETE) and 9 down-regulated oxylipins (9,10-DiHOME, 9-KODE, 12,13-DiHOME, 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10-EpOME, 9-HODE,13-KODE and 12,13-EpOME). The top ten of them are also detected in univariate *t*-test results, which confirmed the importance of these oxylipins.

Given that the oxylipins 13,14-dihydro-$PGF_{2\alpha}$ and12-HETE have been implicated in inflammatory regulation in disease and also given that they showed a meaningful VIP score close to 1 (0.96, 0.95 respectively) with increasing trend in the CIA group, changes in these metabolites can provide insight in the biological interpretation for CIA and are included in further biological interpretation. The

detailed pieces of *p* value from Students' *t*-test, VIP scores from PLS-DA, and their direction of regulation are shown in Table 1.

Table 1. List of oxylipins detected in mice plasma, measured using multiple reaction monitoring (precursor ions → product ions) in LC-MS/MS analysis.

Compounds	MS transitions(m/z)	*p*-value	VIP	Regulation	Pathway
LA					
9,10-DiHOME	313.2 -> 201.1	0.0002	1.86	↓	CYP450
12,13-DiHOME	313.2 -> 183.2	0.006	1.51	↓	CYP450
9,10-EpOME	295.2 -> 171.2	0.028	1.27	↓	CYP450
12,13-EpOME	295.2 -> 195.2	0.096	1.00	↓	CYP450
9-KODE	293.2 -> 185.2	0.003	1.61	↓	5-LOX
9,12,13-TriHOME	329.2 -> 211.2	0.017	1.36	↓	5-LOX
9,10,13-TriHOME	329.2 -> 171.1	0.026	1.29	↓	5-LOX
9-HODE	295.2 -> 171.1	0.052	1.14	↓	5-LOX
13-KODE	293.2 -> 113.1	0.082	1.04	↓	12/15-LOX
13-HODE	295.2 -> 195.2	0.733	0.21	-	12/15-LOX
EPA					
12-HEPE	317.2 -> 179.1	0.016	1.37	↑	12/15-LOX
DHA					
14-HdoHE	343.2 -> 205.0	0.010	1.45	↑	ROS
13-HdoHE	343.2 -> 281.0	0.012	1.42	↑	ROS
10-HdoHE	343.2 -> 153.0	0.035	1.23	↑	ROS
17-HdoHE	343.2 -> 281.3	0.173	0.83	-	12/15 LOX
19,20-DiHDPA	361.2 -> 273.3	0.509	0.41	-	CYP450
DGLA					
6-keto-PGF1a	369.2-> 163.1	0.390	0.53	-	COX
8-HETrE	321.3 -> 303.0	0.469	0.45	-	12/15 LOX
AA					
8-HETE	319.2 -> 155.1	0.074	1.06	↑	12/15-LOX
12-HETE	319.2 -> 179.2	0.116	0.95	↑	12/15 LOX
15-HETE	319.2 -> 219.2	0.770	0.18	-	12/15-LOX
5-HETE	319.2 -> 115.1	0.713	0.23	-	5-LOX
13,14-dihydro-PGF2a	355.2 -> 275.3	0.112	0.96	↑	COX
PGF2a	353.2 -> 193.1	0.176	0.82	-	COX
13,14-dihydro-15-keto-PGF2a	353.2-> 183.1	0.618	0.31	-	COX
12S-HHTrE	279.2 -> 179.2	0.733	0.21	-	COX
TXB2	369.2 -> 169.1	0.900	0.08	-	COX
14,15-DiHETrE	337.2 -> 207.2	0.662	0.27	-	CYP450
9-HETE	319.2 -> 167.1	0.408	0.51	-	ROS
11-HETE	319.2 -> 167.1	0.820	0.14247	-	ROS

The oxylipins are grouped based on the original polyunsaturated fatty acid precursor: linoleic acid (LA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), dihomo- γ -linolenic acid (DGLA), and arachidonic acid (AA).

Their metabolic pathways include enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (P450), and nonenzymatic reactive oxygen species (ROS) pathway. The significance of changes between two groups was illustrated by *p* value from univariate test (Student's *t*-test) and VIP score from multivariate test (PLS-DA). The important regulations in the CIA group were marked with "↓"or"↑" selected based on VIP scores.

↓: downregulated in CIA group.
↑:upregulated in CIA group.

3.2 Physiological pathways of altered oxylipins

We grouped the detected oxylipins by their metabolic pathways in order to illustrate their biological roles in fig. 3. Color is used to indicate the up/down-regulation (marked in yellow/blue boxes) in the CIA group. Among these colored16 metabolites, all the 9 down-regulated oxylipins (9,10-DiHOME, 9-KODE, 12,13-DiHOME, 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10-EpOME, 9-HODE, 13-KODE and 12,13-EpOME) are derived via the LA group; 3 up-regulated oxylipins (8-HETE, 13,14-dihydro-PGF2a, 12-HETE) are derived from AA; 3 up-regulated oxylipins (14-HDoHE, 13-HDoHE and 10-HDoHE) are derived from DHA; and 1 up-regulated oxylipins (12S-HEPE) is produced from EPA.

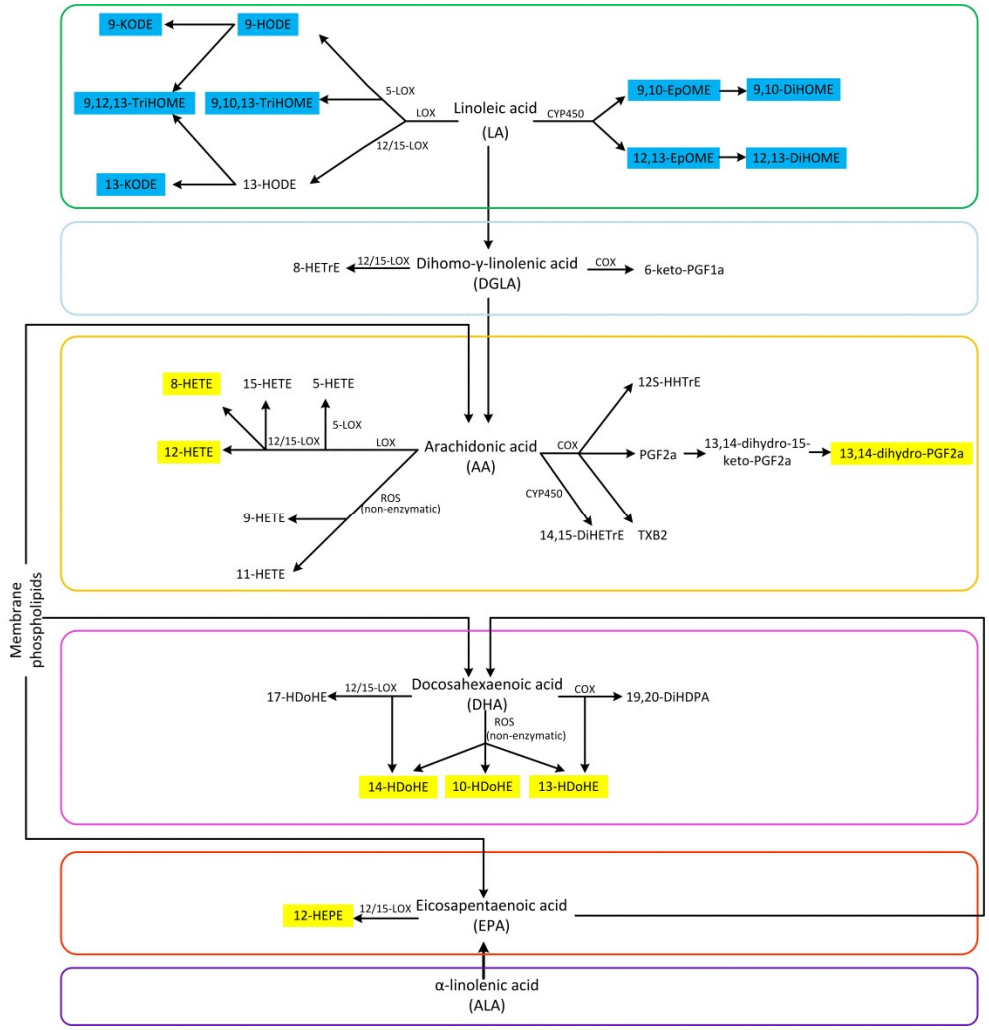

Fig. 3 Overview of regulations of oxylipins in CIA mice compared with Ctrl, including metabolic pathways. Metabolites detected in mice plasma are grouped by metabolic pathways. Important metabolites which contribute most to group clustering based on PLS-DA are colored: yellow box: up-regulated in the CIA group; blue box: down-regulated in the CIA group.

4. Discussion

Inflammation is a self-limiting innate mechanism under complex regulation with the purpose to recruit leukocytes and plasma proteins, trafficking these to the site of infection or tissue damage, supporting a robust adaptive immune response and subsequent resolution [40]. RA is the consequence of a systemic auto-immune activation/response within the synovial fluid in the joint triggering a dysregulated chronic inflammatory response, of which the exact underlying pathogenic mechanisms still remain largely unclear. RA is characterized with a strong inflamed cytokine phenotype with elevated levels of IL-1 β, IL-6, TNFα as well as increased levels of ROS [18], [41], [42], seen in fig. 4(a). Perturbations related to TNFα activation of the NF- κB pathway inhibiting apoptosis in activated antigen-presenting cells including neutrophils, macrophages, fibroblast-like cells, and B-cells, forms the general accepted pathological basis of RA [9], [10], [43], [44]. Hence we applied a comprehensive oxylipin metabolomics platform to the plasma of DBA/1J mice induced by a co-administration of type II collagen with lipopolysaccharide, to elucidate the role of these potent inflammatory mediators in RA.

We detected an increased pro-inflammatory oxylipin response, which can be attributed to the activation of NF-κB and increased ROS (Figure 4(b)). NF-κB is the transcription factor for COX-II, and its activation during RA [45], [46] can explain the increased levels of the COX derived prostaglandin $F_{2\alpha}$ measured via its downstream product 13,14-dihydro-$PGF_{2\alpha}$ in CIA mice [47], [48]. Several hydroxyl-fatty acids were also implicated as role players in the chronic inflammatory phenotype of RA. Due to two possible de novo synthesis routes for hydroxyl-fatty acids, it implicates both increased LOX activity concurrently with elevated oxidative stress within CIA mice [24]–[27]. Increased 12-LOX signaling mediators included 8-HETE and 12-HETE supporting a pro-inflammatory milieu [49], [50]. In an oral tolerance test in CIA rats, Ding et al. [51] measured elevated levels of EPA-derived 18-HEPE, while we detected increased level of a similar

metabolite 12-HEPE. Overexpression of 12-LOX in RA has been published by Liagre & Kronke [52], [53], which can further mediate the activation of NF-κ B [54]–[56], indicating the chronic nature of RA. Although 8-HETE, 12-HETE and 12-HEPE together with the docosahexaenoic acid derived HDOHEs also provide a readout for ROS induced biologically active lipid peroxidation products [24]–[27]. Oxidative stress leading to increased free radicals as well as ROS levels have been reported in RA by Ozkan et al. [18], supporting this finding.

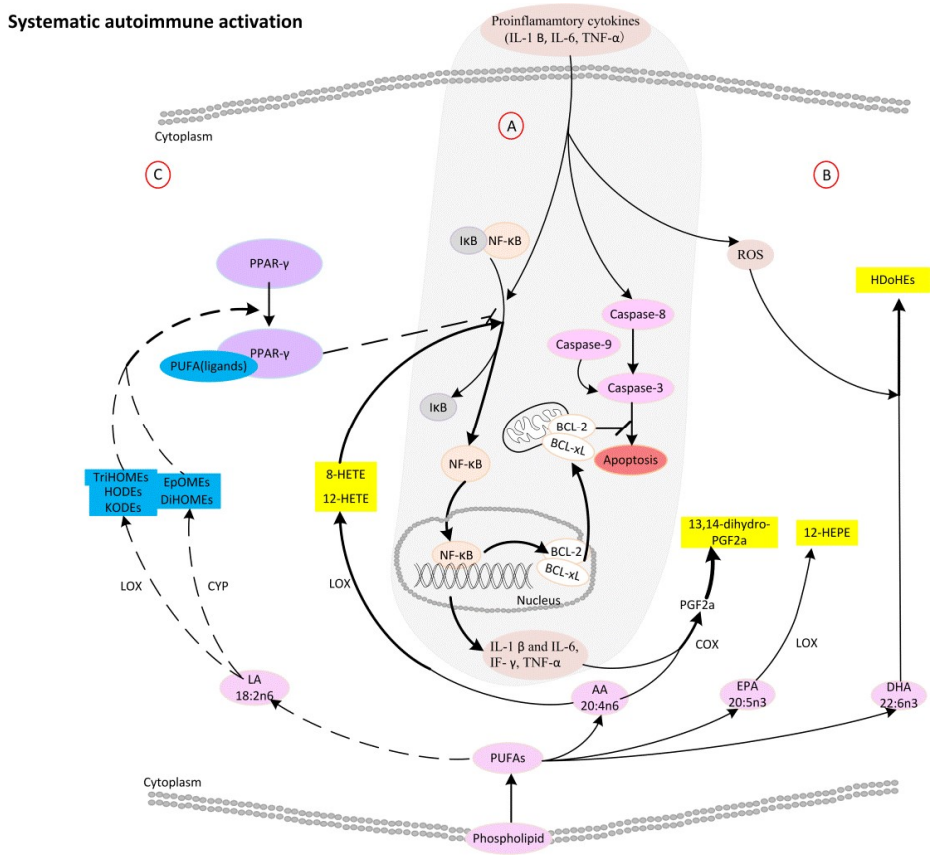

Fig. 4 A systematic auto-immune activation in RA. Appearance of pro-inflammatory cytokines (IL-1 β and IL-6, TNFα) as well as the appearance of ROS in RA. The cytokines normally induce the apoptosis via the caspase pathway, but also inhibit apoptosis through degradation IκB activating nuclear factor-κB (NF-κB), which consequently translocate to the nucleus upregulating the antiapoptotic genes (BcL2 and BcL-xL). The activated NF-κB then can also further enhance the

production of pro-inflammatory cytokines and chemokines as well as COX-II enzyme. (b) Upregulated oxylipin response. During RA increased levels of AA derived prostaglandins and HETEs are detected. 8- and 12-HETE is able to activate NF-κB exasperating RA. Due to increased levels of ROS, DHA derived peroxidation products are also found. (c) – Dysregulated anti-inflammatory response. LA derived Oxylipins including: HODEs, KODEs, TriHOMEs, DiHOMEs and EpOMEs are ligands of peroxisome proliferator-activated receptor (PPAR)-γ. Due to decreased levels of these anti-inflammatory oxylipins, the ability of PPAR-γ to inhibit the activation of NF-κB and indirectly affect apoptosis, is diminished.

Alongside the increased pro-inflammatory oxylipins, we also identified significantly decreased LA derived oxylipins in CIA mice plasma. The decreased LA cytochrome P450 products (EpOMEs, DiHOMEs) and LA LOX products (TriHOMEs) implicate a fatty acid precursor perturbation and/or a possible oxylipin enzymatic impairment in RA. AA is the ELOVL mediated elongation product of LA, and the detected increasing trend in AA derived oxylipins indicate sufficient CYP and LOX activity to rule out enzyme activity as the cause of the LA oxylipin reductions. In addition, these LA derived oxylipins as well as the decreased HODEs and KODEs are ligands for nuclear hormone receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ) activation [57]–[63], shown in Fig. 4(c). PPAR-γ are anti-inflammatory regulators of immune cells and can inhibit the activation of NF-κB [44], [46], [61], [62], [64]–[70]. Therefore, the decreased LA-derived oxylipins and PPAR-γ ligands indicate a perturbation in mechanisms related to the resolution of inflammation, unable to inhibit NF-κB activation and its downstream inhibition of apoptosis.

As discussed above, our detected oxylipins indicate insufficient PPAR-γ ligands, as well mechanisms leading to the activation of NF-κB, supporting and enhancing our understanding of the inhibition of apoptosis in CIA mice. Apoptosis plays an important role leading to the phagocytic clearances of damage cells stifling the development of chronic inflammation and autoimmunity [71]. The inhibition of apoptosis prevents the silencing of activated leukocytes, dysregulating clearance mechanisms contributing to chronic autoimmune inflammation in RA [72].

5. Conclusion

Using our comprehensive oxylipin method we were able to show that the CIA mice had an arachidonic acid dependent increased proinflammatory profile, with increased levels of oxidative stress. Several studies have been published advocating anti-inflammatory diets (the restriction of AA in the diet), leading to therapeutic benefits and ameliorating RA [73]. We also detected a significant decrease in potent anti-inflammatory oxylipins derived from linoleic acid capable of signaling via PPAR-γ to inhibit the activation of NF-κB, namely, the molecular basis for RA. Interestingly, PPAR-γ has been identified and reported as a therapeutic agent for arthritis[74]. The reduced levels of linoleic acid derived oxylipins implicated fatty acid precursor pools, shedding light on the unexplored routes of fatty acid elongation pathways in the pathogenicity of RA, and need further work. As additional metabolites have been reported to play a role in RA, a systems biology approach would complement the study of systematic auto-immune induced rheumatoid arthritis.

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7. Abbreviations

AA: arachidonic acid;
ALA: α-arachidonic acid;
CIA: Collagen induced arthritis;
CII: Collagen Type II;
COX: cyclooxygenase;
CYP 450: cytochrome P450 expoxygenases;
DGLA: dihomo- γ -linolenic acid;
DHA: docosahexaenoic acid;
DiHETrE: dihydroxyeicosatrienoic acid ;
DiHOME dihydroxyoctadeca(mono)enoic acid;
EPA: eicosapentaenoic acid;
EpETrE: epoxyeicosatrienoic acids;
EpOME: epoxyoctadecenoic acid;
HDoHE: hydroxydocosahexaenoic acid;
HEPE: hydroxyeicosapentaenoic acid;
HETE: hydroxyeicosatetraenoic acid;
HETrE: hydroxyeicosatrienoic acid;
HHTrE: hydroxyheptadecatrienoic acid;
HODE: hydroxyoctadecadienoic acid;
HOTrE: hydroxyoctadecatrienoic acid;
ISTDs: internal standards;
KETE: ketoeicosatetraenoic acid;
KODE: ketooctadecadienoic acid;
LA : linoleic acid;
LOX : lipoxygenase;
LPS : Lipopolysaccharide;
NF- κB: Nuclear factor-kappa B;
PG: prostaglandin;
PPAR: peroxisome proliferator-activated receptor;
RA: rheumatoid arthritis;
ROS: Reactive Oxygen Species;
TNF: tumor necrosis factor;
TriHOME: trihydroxyoctadecenoic acid;

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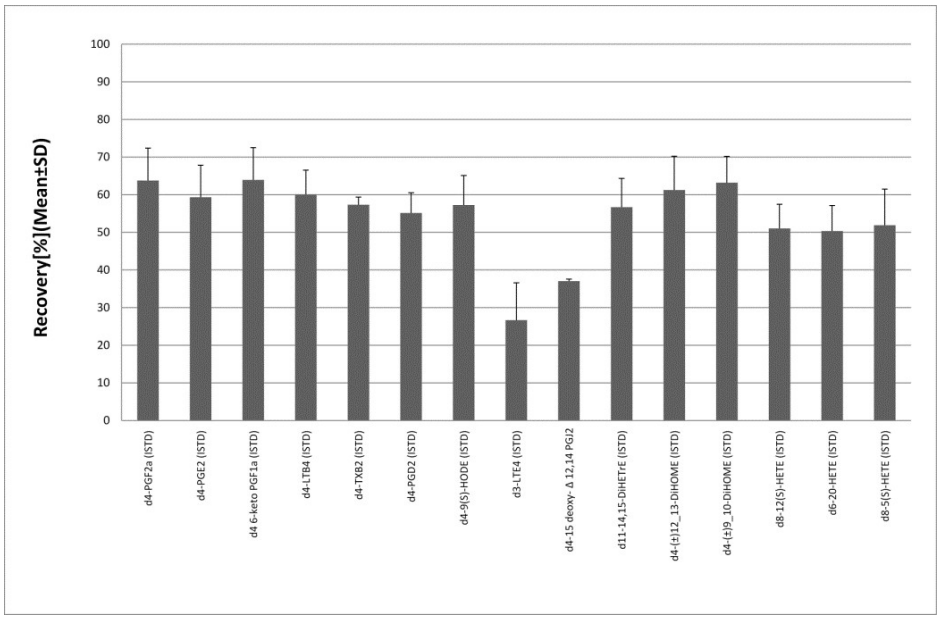
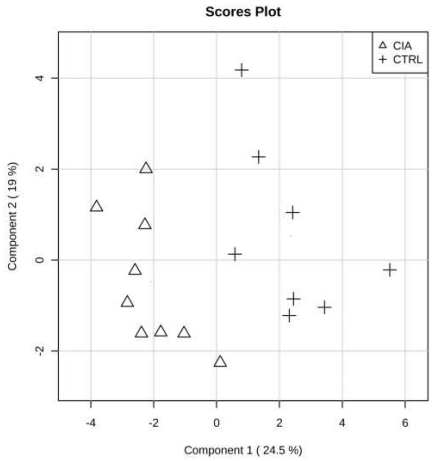
Support information

The supplementary material provides the methodology of oxylipin extraction and detection and reports performance characteristics of this method. Detailed results from supervised. PLS-DA analysis and VIP scores are also provided in order to demonstrate the important contributions of significant oxylipins to the group clusters.

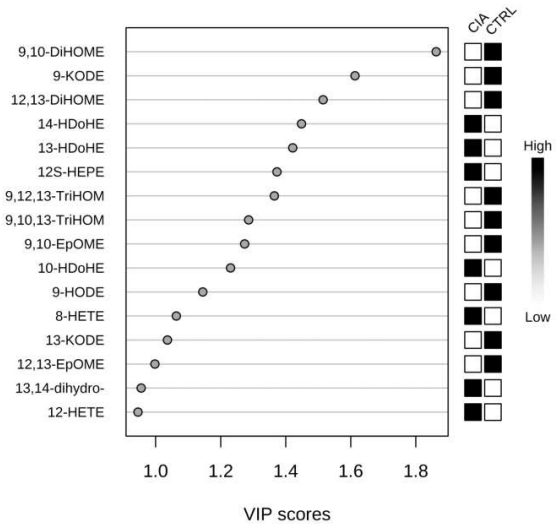
Supplementary figures



S-fig. 1: Recovery (%) of 15 deuterated oxylipins ISTDs . Oxylipins recovery for SPE extraction method was displayed in forms of percentage value.



S-Fig. 2: Score plot (component 1 vs. component 2) of PLS-DA based on the whole targeted plasma oxylipin profiling (n=30) from LC-MS in CIA model (CII+LPS induction) group and Ctrl group. Peak area ratio to relevant internal standards after Glog transformation and autoscaling was used for the PLS-DA analysis.



S-Fig. 3: Variable Importance in the Projection(VIP) scores of detected oxylipins based on PLS-DA. Fourteen oxylipins showed VIP score higher than 1, while another to are extremely closer to 1(0.96 and 0.95 respectively). The regulation information of increase (■) and decrease (□) are given in the right side of the figure.

Supplementary tables

S-Table 1: Linearity(R^2), reproducibility(RSD), Limitation of detection(LOD) and quantitation(LOQ) of LC/ESI-MS/MS for oxylipins detected in mice plasma

Oxylipins	Chemical class	R^2	RSD[%]	LOD[nM]	LOQ[nM]
AA					
12S-HHTrE ^	Alcohols	0.999	12	1	3.5
20-HETE	Alcohols	0.999	11	6.1	20.2
15-HETE^	Alcohols	0.904	26	2.6	8.8
11-HETE^	Alcohols	0.963	12	0.6	1.8
12-HETE ^	Alcohols	0.794	7	15.6	52.1
8-HETE^	Alcohols	0.95	30	13.1	43.5
9-HETE ^	Alcohols	0.932	19	33.9	113
5-HETE^	Alcohols	0.82	17	5.2	17.4
5S,6R-LipoxinA4	Diols	0.994	4	1.5	5.1
5S,6S-Lipoxin A4	Diols	0.996	5	4.4	14.5
6-trans-LTB4	Diols	0.99	7	8.3	27.5
LTB4	Diols	0.999	12	0.4	1.3
14,15-DiHETrE ^	Diols	1	9	0.4	1.4
11,12-DiHETrE	Diols	0.999	14	1.6	5.5
8,9-DiHETrE	Diols	0.999	17	0.7	2.4
5,6-DiHETrE	Diols	0.999	5	18.8	62.6
14,15-EpETrE	Epoxides	0.847	7	3.7	12.4
5,6-EpETrE	Epoxides	0.999	8	3.9	12.8
12S-HpETE	Hydroperoxides	0.902	10	116.7	389.1
5S-HpETE	Hydroperoxides	1	27	0.4	1.3
15-KETE	Ketones	0.998	32	4.8	16
5-KETE	Ketones	0.985	32	39.1	130.4
8-iso-PGF2a	Prostanoids/throboids	1	13	0.5	1.7
5-iPF2a-VI	Prostanoids/throboids	1	10	0.1	0.3
TXB2 ^	Prostanoids/throboids	0.999	10	1.3	4.5
PGF2a ^	Prostanoids/throboids	0.999	11	0.9	2.8
PGE2	Prostanoids/throboids	0.998	12	1.9	6.2
11beta-PGE2	Prostanoids/throboids	0.998	6	2.5	8.5
13,14-dihydro-PGF2a ^	Prostanoids/throboids	1	13	4.1	13.8
13,14-dihydro-15-keto-PGF2a^	Prostanoids/throboids	1	9	1.94	6.48
PGA2	Prostanoids/throboids	0.998	8	2.3	7.7
PGJ2	Prostanoids/throboids	1	6	0.03	0.1
d12-PGJ2	Prostanoids/throboids	0.995	10	2.3	7.6
PGD2	Prostanoids/throboids	0.996	6	2.5	8.5
HepoxilinA3	Prostanoids/throboids	0.992	7	57.7	192.3
ALA					
9-HOTrE	Alcohols	0.998	12	0.3	1
12,13-DiHODE	Diols	0.993	13	54.5	181.8
DGLA					
15S-HETrE	Alcohols	0.998	27	1.3	4.3
8-HETrE^	Alcohols	0.979	15	2.8	9.2
5-HETrE	Alcohols	0.999	23	1.6	5.4
6-keto-PGF1a^	Prostanoids/throboids	1	15	1.08	3.62

DHA					
17-HDoHE^	Alcohols	0.988	10	0.4	1.2
20-HDoHE	Alcohols	0.993	14	4.8	15.8
16-HDoHE	Alcohols	0.983	13	7.6	25.5
13-HDoHE^	Alcohols	0.977	11	113.9	379.6
14-HDoHE^	Alcohols	0.900	9	13.7	45.7
10-HDoHE ^	Alcohols	0.974	13	8.8	29.3
7-HDoHE	Alcohols	0.991	9	0.1	0.4
11-HDoHE	Alcohols	0.986	9	3.6	11.8
4-HDoHE	Alcohols	0.976	22	29.6	98.5
8-HDoHE	Alcohols	0.92	19	14	46.8
10S,17S-DiHDoHE	Diols	1	4	1.6	5.4
19,20-DiHDPA ^	Diols	1	19	3.6	11.9
19,20-EpDPE	Epoxides	0.984	16	95.6	318.7
EPA					
18-HEPE	Alcohols	0.991	19	29.9	99.7
15-HEPE	Alcohols	0.999	25	7.6	25.3
12-HEPE ^	Alcohols	0.939	11	12.3	40.8
9-HEPE	Alcohols	0.995	15	4.8	16.1
5-HEPE	Alcohols	0.999	25	1.2	4
8S,15S-DiHETE	Diols	0.832	3	40.3	134.4
5S,15S-DiHETE	Diols	0.998	4	8.2	27.2
5S,6S-DiHETE	Diols	1	9	3.0	10.1
LA					
13-HODE ^	Alcohols	0.884	9	2.7	9.2
9-HODE ^	Alcohols	0.903	8	1.6	5.3
12,13-DiHOME ^	Diols	1	3	0.9	3
9,10-DiHOME ^	Diols	0.999	5	0.6	2.1
12,13-EpOME ^	Epoxides	1	9	1.6	5.3
9,10-EpOME ^	Epoxides	0.999	26	1.1	3.7
13-KODE^	Ketones	0.996	6	1.4	4.7
9-KODE^	Ketones	0.998	9	0.6	1.9
9,12,13-TriHOME ^	Triols	0.994	8	1	3.3
9,10,13-TriHOME ^	Triols	0.996	9	2.7	9.1

^: oxylipins were also detected in the study samples.

S-Table 2: Details of detected oxylipins in mice plasma using LC-MS/MS analysis

Compounds	Lipid Maps ID	Mass (precursor ion → product ions)	Retention Time	Internal standard	Precision RSD[%]	FA group	Chemcal class
6-keto-PGF1a	LMFA03010001	369.2 → 163.1	5.21	d4 6-keto PGF1a	6.9	DGLA	Prostanoids
TXB2	LMFA03030002	369.2 → 169.1	7.05	d4-TXB2	0.4	AA	throboids
9,12,13-TriHOME	LMFA02000014	329.2 → 211.2	7.1	d4-9(S)-HODE	26.1	LA	Tiols
PGF2a	LMFA03010002	353.2 → 193.1	7.2	d4-PGF2a	7.7	AA	Prostanoids
9,10,13-TriHOME	LMFA02000168	329.2 → 171.1	7.24	d4-9(S)-HODE	19.7	LA	Tiols
13,14-dihydro-PGF2a	LMFA03010079	355.2 → 275.3	8.08	d4-PGF2a	8.7	AA	Prostanoids
13,14-dihydro-15-keto-PGF2a	LMFA03010027	353.2 → 183.1	8.67	d4-PGF2a	5.6	AA	Prostanoids
12,13-DiHOME	LMFA01050351	313.2 → 183.2	12.07	d4-(±)12_13-DiHOME	14	LA	Diols
9,10-DiHOME	LMFA01050350	313.2 → 201.1	12.46	d4-(±)9_10-DiHOME	34.5	LA	Diols
19,20-DiHDPA	LMFA04000043	361.2 → 273.3	12.87	d11-14,15-DiHETrE	23.3	DHA	Diols
14,15-DiHETrE	LMFA03050010	337.2 → 207.2	12.91	d11-14,15-DiHETrE	27.6	AA	Diols
12S-HHTrE	LMFA03050002	279.2 → 179.2	12.99	d8-12(S)-HETE	30.4	AA	Alcohols
12-HEPE	LMFA03070008	317.2 → 179.1	14.91	d8-12(S)-HETE	9.4	EPA	Alcohols
13-HODE	LMFA01050349	295.2 → 195.2	15.37	d4-9(S)-HODE	5.6	LA	Alcohols
9-HODE	LMFA01050278	295.2 → 171.1	15.5	d4-9(S)-HODE	6.5	LA	Alcohols
15-HETE	LMFA03060001	319.2 → 219.2	15.83	d8-5(S)-HETE	21.6	AA	Alcohols
13-KODE	LMFA02000016	293.2 → 113.1	15.96	d4-9(S)-HODE	4.3	LA	Ketones
17-HDoHE	LMFA04000032	343.2 → 281.3	16.1	d8-12(S)-HETE	10.7	DHA	Alcohols
11-HETE	LMFA03060028	319.2 → 167.1	16.23	d8-12(S)-HETE	10.1	AA	Alcohols
14-HDoHE	LMFA04000030	343.2 → 205.0	16.28	d8-12(S)-HETE	4.9	DHA	Alcohols
13-HDoHE	LMFA04000029	343.2 → 281.0	16.29	d8-12(S)-HETE	7.9	DHA	Alcohols
10-HDoHE	LMFA04000027	343.2 → 153.0	16.32	d8-12(S)-HETE	26.2	DHA	Alcohols
9-KODE	LMFA01060177	293.2 → 185.2	16.37	d4-9(S)-HODE	18.3	LA	Ketones
12-HETE	LMFA03060088	319.2 → 179.2	16.52	d8-12(S)-HETE	13	AA	Alcohols
8-HETE	LMFA03060006	319.2 → 155.1	16.54	d8-5(S)-HETE	6.4	AA	Alcohols
9-HETE	LMFA03060089	319.2 → 167.1	16.77	d8-12(S)-HETE	12.3	AA	Alcohols
5-HETE	LMFA03060002	319.2 → 115.1	17	d8-5(S)-HETE	18.2	AA	Alcohols
8-HETrE	LMFA03050011	321.3 → 303.0	17.06	d8-12(S)-HETE	19.6	DGLA	Alcohols
12,13-EpOME	LMFA02000038	295.2 → 195.2	17.4	d4-(±)12_13-DiHOME	24.3	LA	Epoxides
9,10-EpOME	LMFA02000037	295.2 → 171.2	17.59	d4-(±)9_10-DiHOME	5.6	LA	Epoxides

The table contains 30 oxylipins detected in study mice plasma, with their information of Mass (precursor ions accompanied with product ions) as well as the selected internal standard for ratio calculation and correction, ranked by their retention time. For oxylipins platform, RSD is acceptable if less than 35% because of the less stability and low concentration levels of oxylipins. 30 oxylipins were reliable with RSD <35%.

Chapter 3

Role of amino acids in rheumatoid arthritis studied by metabolomics

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Abstract

Background: Rheumatoid arthritis (RA) is a complex, chronic autoimmune disease characterized by various inflammatory symptoms, including joint swelling, joint pain, and both structural and functional joint damage. The most commonly used animal model for studying RA is mice with collagen-induced arthritis (CIA); the wide use of this model is due primarily to many similarities with RA in human patients. Metabolomics is used increasingly in biological studies for diagnosing disease and for predicting and evaluating drug interventions, as a large number of disease-associated metabolites can be analyzed and interpreted from a biological perspective.

Aim: To profile free amino acids and their biogenic metabolites in CIA mice plasma.

Method: Ultra-high-performance liquid chromatography/tandem mass spectrometry (UPLC-ESI-MS) coupled with multiple reaction monitoring (MRM) was used for metabolomics study.

Results: Profile of 45 amine metabolites, including free amino acids and their biogenic metabolites, in plasma was obtained from CIA mice. We found that the plasma levels of 20 amine metabolites were significantly decreased in the CIA group.

Conclusion: The results suggest that a disordered amine response is linked to RA-associated muscle wasting and energy expenditure.

Key words: Collagen-induced arthritis, mouse model, amine metabolites, systems biology.

1. Introduction

Rheumatoid arthritis (RA) is a highly prevalent chronic disease, currently affecting approximately 1% of the world's population [1]–[3]. Patients with RA typically have destruction of joint cartilage and bone accompanied by joint stiffness, hyperplasia, microvascular injury, swelling, and pain. The pathogenesis of RA is mainly associated with the secretion of cytokines such as interleukins (e.g., IL-1 and IL-6), tumor necrosis factor (TNFα), interferon gamma (IFNγ), and various pro-inflammatory mediators [4], [5]. Increased activity of the nuclear factor (NF)-κB pathway, which inhibits apoptosis in immune cells, also plays a role in RA [6]–[10]. A variety of cellular immune responses are also activated and/or dysregulated by increased cytokine levels in RA [11]–[14]. Interestingly, nearly two-thirds of patients with RA develop cachexia and sarcopenia, with a loss of skeletal muscle mass, degradation of proteins, and energy expenditure [15]–[18]. This perturbation in catabolic processes drives the body into a state of negative energy balance, leading to skeletal muscle atrophy, loss of muscle strength, and reduced physical activity [18], [19].

Considering the complex nature of RA, animal models have been useful for studying the underlying pathology and disease mechanisms. The most widely used animal model for studying chronic RA is the collagen-induced arthritis (CIA) mouse model; in addition to high reproducibility and easy induction, the physiological processes and pathogenic features of CIA mice are strikingly similar to the clinical features associated with patients with RA [20]–[23]. For example, increased levels of IL-6 , IL-1, and TNFα play a role in the development of CIA [24]. In addition, high correlation between muscle wasting and the severity of clinical arthritis has also been observed in animal models, including both monkeys and mice with CIA [25], [26].

Applying a systems biology approach using metabolomics can provide a comprehensive functional readout of the organism's physiological status [27]. Recently, van Wietmarschen and van der Greef summarized the putative

inflammatory mediators identified in RA patients using metabolomics [28]. Although some pro-inflammatory mediators have been observed in CIA mice [29], the complexity of the disease warrants a search for additional compound classes and a study of their relationship with the biochemical processes underlying RA. Free amino acids and their derivative biogenic amines play essential roles in both energy production and protein synthesis/degradation; thus, changes in the levels of these amine metabolites may reflect changes in the body's state and catabolism of proteins in RA disease. Therefore, we used a liquid chromatography mass spectrometry (LC/MS)-based amine platform to measure the levels of amine metabolites in the plasma of CIA and control mice. We observed reduced levels of amine metabolites in the plasma of CIA mice, possibly reflecting systemic changes in this model of RA. Based on these results, we speculate that decreased amine metabolite levels likely reflects muscle mass loss and protein degradation and may associates with inflammatory activity.

2. Materials and Methods

2.1 Induction of Arthritis by Co-Administration of Collagen Type II and Lipopolysaccharide

A total of 20 male DBA/1J mice (age 6–7 weeks) were obtained from Charles River Laboratories (Yokohama, Japan). The animals were randomly divided into two groups, with ten mice in the experimental (CIA) group and ten mice in the control (Ctrl) group. The protocol for inducing arthritis is well established and has been described in detail [29]–[31]. In brief, the mice were given intraperitoneal (i.p.) injections containing collagen type II (extracted from bovine nasal cartilage and dissolved in acetic acid) and lipopolysaccharide (extracted from *Escherichia. coli* 011:B4 and dissolved in phosphate-buffered saline) in order to induce chronic polyarthritis by stimulating an autoimmune response; control mice received i.p. injections of vehicle (acetic acid and phosphate-buffered saline) only. All animals were housed in a temperature- and light-controlled environment with free access to standard rodent chow and water throughout the experiments. After repeated injections (administered on days 0, 14, 28, 42, and 56), blood samples were collected from each animal on day 70 and stored in pre-cooled Vacutainer tubes (BD Vacutainer, Plymouth, UK) containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. After centrifugation, the EDTA-plasma fractions were collected and aliquots—including individual study samples and pooled quality control (QC) samples—were stored at -80ºC until further analysis. During sampling, one mouse in the CIA group died; thus, the final analysis is based on 9 CIA mice and 10 control mice.

2.2 Extraction of Amine Metabolites and Analysis using UPLC-MS/MS

The methods for extracting and analyzing amine metabolites were adapted for mouse plasma samples based on a previously described protocol [32]. For each sample, a 5-µl aliquot of plasma was used for the analysis. A mixture of internal

standards containing $^{13}C^{15}N$-labeled amine metabolites was added to each 5-µl plasma sample. After the proteins were precipitated using MeOH, the supernatant was transferred to a fresh Eppendorf tube and dried under N_2. The residue was then dissolved in borate buffer (pH 9), and 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) derivatization reagent (Waters, Etten-Leur, The Netherlands) was added. The reaction mixture was then neutralized by the addition of formic acid (20%), and the solution was transferred to injection vials for ultra-high-performance LC tandem MS (UPLC-MS/MS) analysis (injection volume: 1.0 µl) using an ACQUITY UPLC system (Waters) equipped with an AccQ-Tag Ultra column (2.1 mm × 100 mm, 1.7 µm particles, Waters) coupled to a Xevo mass spectrometer with electrospray ionization source (Waters). Multiple reaction monitoring was performed in the positive ion mode in order to monitor the analytes. A gradient elution starting with Eluent A (water containing 2% formic acid) and ramping to Eluent B (aqueous acetonitrile containing 2% formic acid) was used as the mobile phase in the UPLC system. The samples were analyzed in random order.

2.3 Data Processing and Statistical Analysis

The integrated peak areas of the target analytes were calculated using Quanlynx software (Waters) and corrected using the appropriate internal standards. The response ratio (calculated at the ratio between the target analyte and the respective internal standard) was used for further statistical analysis. The reproducibility and reliability of each metabolite measurement was determined using repeated measurements of the QC pool performed after every ten samples. By defining the acceptable relative standard deviation as <15%, 45 amine metabolites (from a starting list of 74) were considered high quality and were included in the final list for further analysis. The data were log-transformed to correct for distribution skewness and auto-scaled to achieve uniform units.

To visualize clustering of individual samples, unsupervised principal component analysis (PCA) was performed using MetaboAnalyst version 3.0 (http://www.metaboanalyst.ca) [33]. To measure the significance of differences in each individual amine metabolite between the CIA group and the Ctrl group, a two-sided unpaired Student's *t*-test was performed, assuming unequal variance; differences with a *p*-value <0.05 were considered significant (H_0: group means are equal). Fold change (FC) was then calculated in order to determine the direction ($\log_2$ of FC) and magnitude (FC ratio reflecting the CIA/Ctrl ratio) of differences between two group mean values. A positive value for the $\log_2$ of FC indicates higher levels of metabolites in the CIA group, whereas a negative value indicates lower levels of metabolites in the CIA group. In the FC analysis, a minimum threshold of 1.5 was used, meaning that the ratio of metabolites between the CIA and Ctrl groups exceeded 1.5.

3. Results

A 2D plot of the PCA scores was generated using an unsupervised pattern recognition method and was used to provide a visual overview of the natural distribution of amines detected in the plasma samples of the nine CIA and ten Ctrl mice (Fig. 1). PC1 and PC2 accounted for 56.6% and 13.9% of the variation, respectively; thus, these two principal components (i.e., PC1 and PC2) explained a total of 70.5% of the variance. From the 2D plot of the PCA scores, the CIA group (depicted with triangles symbol) and the Ctrl group (depicted with the "+" symbol) were generally distributed in distinct regions with respect to PC1, with the CIA samples clustering largely on negative side of the plot and the Ctrl samples clustering largely on the positive side of the plot, thereby reflecting group differences with respect to the composition of free amine metabolites in the plasma samples.

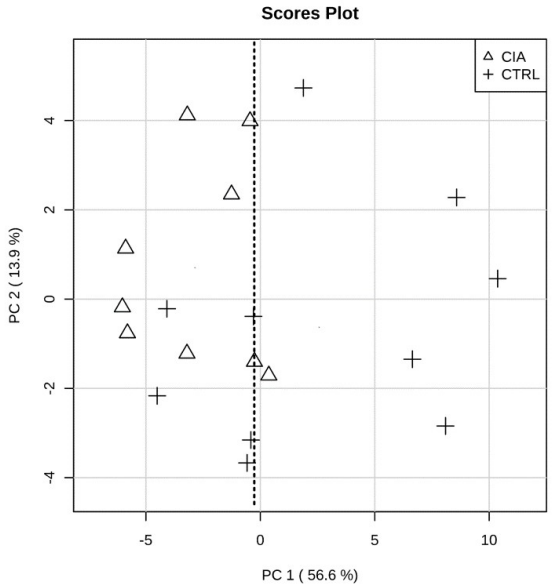

Fig. 1 2D plot of the PCA scores for the amine metabolites measured in the plasma samples from CIA (△) and control (+) mice. The plot of the PCA scores shows that the two groups form distinct clusters along the *x*-axis (corresponding to PC1), indicated by the vertical dashed line.

To measure whether the differences between the two groups were significant, we performed a Student's *t*-test for each amine metabolite. In total, 20 of the 45 detected amine metabolites differed significantly differences between the two groups ($p<0.05$); these 20 amine metabolites are shown in Fig. 2, and all 45 detected amine metabolites are summarized in Table 1. Metabolite changes were reported in the table 1 only when the *p*-values from the Student's test were lower than 0.1. Table 1 also lists the false discovery rate-adjusted *p*-values. Fold change (FC) analysis was performed to indicate the direction of change and the magnitude of change for the detected amine metabolites (FC of the CIA/Ctrl ratio). The analysis revealed that 11 amine metabolites decreased by more than one-third in the CIA group ($FC_{CIA/Ctrl}$ <0.67). The $\log_2$ value of FC indicates that 43 of the 45 amine metabolites detected (95.6%) were lower in the CIA group (i.e., a negative $\log_2$ value of FC), whereas the remaining two metabolites (methylcysteine and O-phosphoethanolamine) were higher in the CIA group.

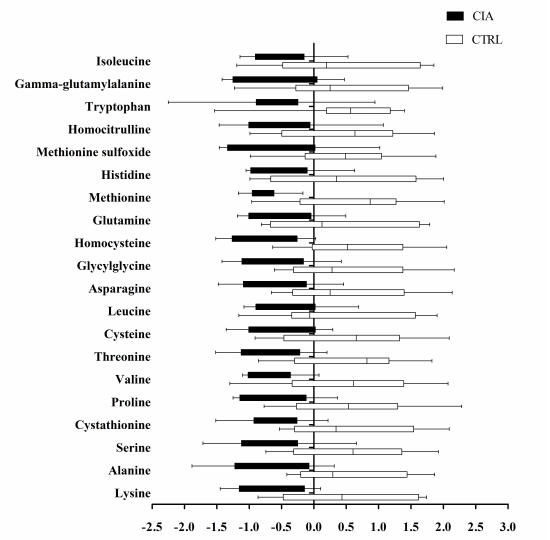

Fig. 2 Summary of the 20 amine metabolites that differed significantly between the collagen-induced arthritis (CIA) and control groups ($p<0.05$). The values are presented as the response ratio of the peak area (determined as the ratio of the target amine metabolite to its corresponding internal standard) after logarithmic transformation and auto-scaling.

Table 1. Summary of the 45 amine metabolites detected in CIA and control mice

Amine Metabolite	HMDB	*p*-value	FDR	$FC_{CIA/Ctrl}$	Direction of Change
Methionine	HMDB00696	**0.001**	0.017	0.533	↓
Homocysteine	HMDB00742	**0.001**	0.017	0.640	↓
Threonine	HMDB00167	**0.001**	0.017	0.628	↓
Proline	HMDB00162	**0.003**	0.031	0.521	↓
Alanine	HMDB00161	**0.003**	0.031	0.573	↓
Cystathionine	HMDB00099	**0.005**	0.032	0.818	↓
Valine	HMDB00883	**0.005**	0.032	0.650	↓
Glycylglycine	HMDB11733	**0.006**	0.032	0.660	↓
Lysine	HMDB00182	**0.007**	0.032	0.686	↓
Serine	HMDB00187	**0.007**	0.032	0.815	↓
Asparagine	HMDB00168	**0.009**	0.036	0.667	↓
Cysteine	HMDB00574	**0.012**	0.044	0.802	↓
Tryptophan	HMDB00929	**0.015**	0.051	0.789	↓
Homocitrulline	HMDB00679	**0.017**	0.051	0.737	↓
Methionine sulfoxide	HMDB02005	**0.017**	0.051	0.582	↓
Isoleucine	HMDB00172	**0.020**	0.056	0.682	↓
Gamma-glutamylalanine	HMDB06248	**0.021**	0.056	0.616	↓
Histidine	HMDB00177	**0.041**	0.103	0.799	↓
Glutamine	HMDB00641	**0.047**	0.107	0.775	↓
Leucine	HMDB00687	**0.048**	0.107	0.741	↓
Citrulline	HMDB00904	0.052	0.112	0.818	↓
Saccharopine	HMDB00279	0.086	0.168	0.698	↓
Ornithine	HMDB00214	0.087	0.168	0.727	↓
2-Aminoadipic acid	HMDB00510	0.093	0.168	0.732	↓
Phenylalanine	HMDB00159	0.094	0.168	0.755	↓
Homoserine	HMDB00719	0.103	0.178	0.852	-
Methylcysteine	HMDB02108	0.114	0.187	1.330	-
Sarcosine	HMDB00271	0.119	0.187	0.860	-
Arginine	HMDB00517	0.120	0.187	0.866	-
Tyrosine	HMDB00158	0.154	0.231	0.723	-
Alpha-aminobutyric acid	HMDB00452	0.165	0.239	0.765	-
Kynurenine	HMDB00684	0.270	0.379	0.861	-
Glycine	HMDB00123	0.335	0.439	0.902	-
Beta-alanine	HMDB00056	0.338	0.439	0.809	-
Putrescine	HMDB01414	0.341	0.439	0.777	-
Norepinephrine	HMDB00216	0.371	0.463	0.660	-
Glutamic acid	HMDB00148	0.399	0.485	0.850	-
5-Hydroxylysine	HMDB00450	0.417	0.494	0.630	-
Glutathione	HMDB00125	0.497	0.573	0.841	-
4-Hydroxyproline	HMDB06055	0.565	0.635	0.934	-
Aspartic acid	HMDB00191	0.713	0.781	0.960	-
Serotonin	HMDB00259	0.729	0.781	0.699	-
Spermidine	HMDB01257	0.785	0.812	0.933	-
O-Phosphoethanolamine	HMDB00224	0.794	0.812	1.015	-
Ethanolamine	HMDB00149	0.963	0.963	0.858	-

CIA, collagen-induced arthritis; Ctrl, control; HMDB, Human Metabolome Database; FC, fold change; FDR, false discovery rate

4. Discussion

RA is a chronic disease in which the immune response is dysregulated and the levels of several cytokines and factors are elevated, including TNFα, IL-1β, IL-6, IFNγ, and ROS [34], [35]; in addition, NF-κB activation is increased [36]. Changes in metabolic factors such as arachidonic acid–derived inflammatory mediators have also been reported in RA [37], suggesting that a metabolomics approach may provide insight into the biochemical processes underlying this disease.

In addition to the well-characterized inflammatory dysregulation in RA, muscle wasting and energy expenditure are also common features and are linked to the production of cytokines during the immune response [38]–[40]; muscle wasting and energy expenditure can then dysregulate the protein degradation pathway, leading to perturbed metabolic processes [15], [16], [19], [41]–[43]. Given the close relation between amine metabolites and proteins, it is therefore reasonable to speculate that changes in amine metabolites may reflect protein dysregulation which owing to muscle wasting and energy expenditure. However, few studies have focused on measuring muscle wasting in RA by measuring the plasma levels of amine metabolites.

Studies of the biochemical processes associated with RA revealed that activated NF-κB is linked to skeletal muscle loss [44], and this activation has been observed in animal models of RA [45], [46]. Moreover, injecting TNF and IL-1 into healthy rats causes muscle wasting [47]. Previously, we reported increased levels of inflammatory mediators and ROS-generated oxylipins in the plasma of CIA mice, and this was associated with the production of cytokines and increased NF-κB activation [29]. Increased ROS levels, which affect muscle signaling pathways, have also been measured in CIA mice [48]; similar results have been reported in tumor-bearing rats [49]. Given that increased cytokines, ROS, and NF-κB activation robustly affect muscle metabolism, we expected to identify a metabolic “signature” in the plasma of CIA mice.

Building on the previous report of increased inflammatory mediators and increased ROS-generated oxylipins in CIA mice [29], we used a targeted amine platform to evaluate the changes in plasma amine metabolites in age- and gender-matched CIA mice compared with control mice. We found that the plasma amine metabolomes were clearly distinguishable between CIA mice and control mice. Specifically, 20 amine metabolites were significantly lower in the plasma of CIA mice.

Given that certain free amino acids such as branched-chained amino acids are closely associated with protein degradation, amino acids—and their biogenic amines—might be used as a biomarker of muscle wasting [50]. In support of this notion, decreased plasma levels of some amine metabolites have been reported in other diseases (e.g., chronic obstructive pulmonary disease) and have been linked to resting energy expenditure and muscle wasting [51]. Increased excretion of nitrogen into the urine due to muscle wasting has been reported in RA patients [17], [52], and increased levels of acyl-carnitines in the urine of RA patients reflect muscle breakdown [53]. Together, these lines of evidence suggest that muscle wasting is a highly relevant phenomenon related to RA. However, to date relatively few clinical studies examined muscle wasting in RA by measuring amine metabolite levels. The large decrease in plasma amine metabolite levels (e.g., histidine, valine, leucine, phenylalanine, and tryptophan metabolites) is consistent with a previous study of CIA rats by Zhang et al. [54]. The earliest studies of amino acids regulation in RA patients date back to the mid-20th century [55], when researchers found decreased levels of several amino acids but were relatively limited with respect to the biological interpretation. Kobayashi et al. measured a similar decrease in some amine metabolites in the plasma of Japanese patients with RA [56]; although the authors used these results to demonstrate a relationship between ornithine metabolism and inflammation, they did not discuss the possible biological interpretation of non-significantly changed amine metabolites, including alanine, isoleucine, leucine, lysine, serine, and valine [56]. In addition, other clinical studies have reported inconsistent changes in the levels of amine

metabolites, and did not attribute these changes to muscle wasting [57]–[61]. The difference between our CIA mouse model and RA patients with respect to changes in amine metabolites may be due to differences in catabolic processes between mice and humans. Alternatively, the relative complexity of clinical data in patients may mask certain changes in amine metabolites, as various confounding variables are not always taken into consideration in clinical studies, including factors such as age, gender, illness stage, treatment protocol, and diet. Our findings indicate that CIA mice are a valuable tool for studying the pathological processes that underlie RA; specifically, this model is easy to induce, and researchers can easily control/exclude confounding factors that may affect the study results, including age, gender, genetic background, and drug exposure.

In summary, combining our previous oxylipin results and our current amine metabolomics results allows us to speculate upon the biological relationship between muscle wasting and the inflammatory response in RA (Fig. 3). In addition, our results indicate that muscle wasting conditions such as cachexia can be measured using a metabolomics approach (for example, by measuring amine metabolites). Lastly, our results indicate that changes in branched-chain amino acids as well as other amine metabolites may reflect muscle wasting status in RA.

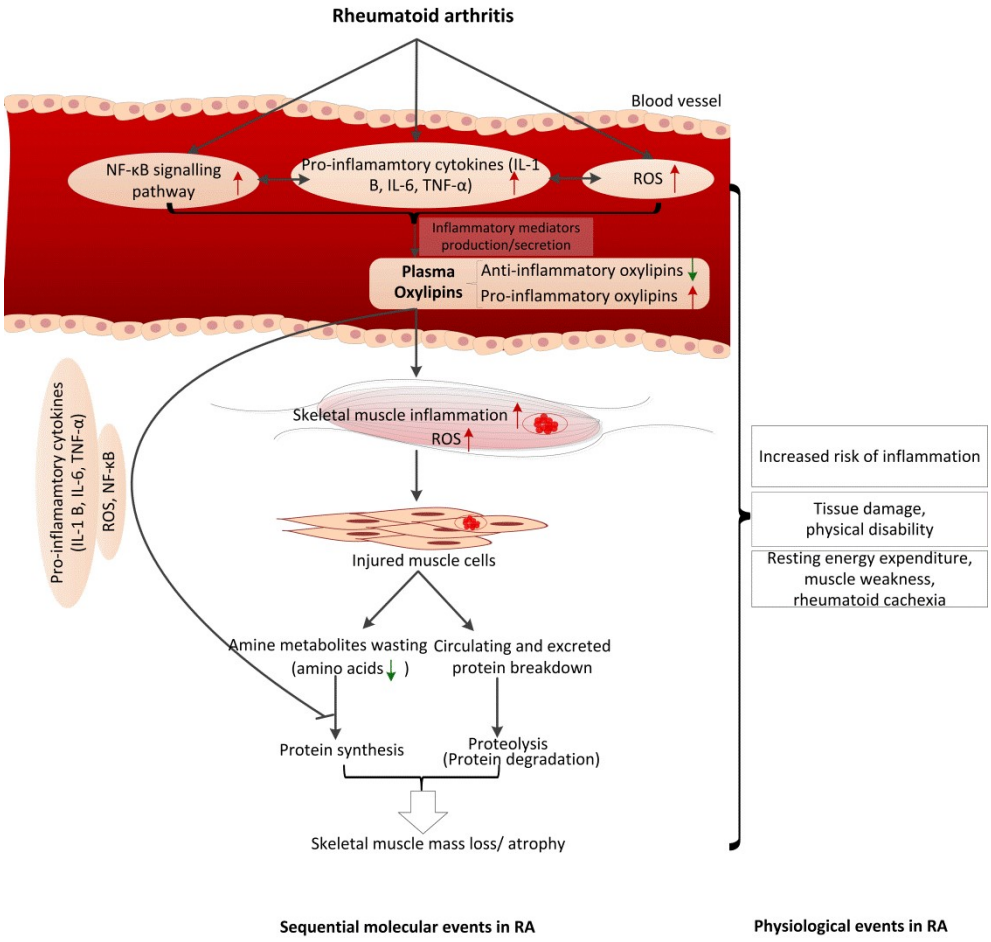

Fig. 3. Proposed biological interpretation of muscle wasting in RA. In RA, increased levels of inflammatory cytokines, ROS, and NF-κB activation play a role in the production of inflammatory oxylipins, which then trigger an inflammatory response in muscle cells. The inflammatory response then increases resting energy expenditure and thermogenesis, leading to amino acid wasting and accelerating protein breakdown. Thereafter, the accelerated protein catabolism and the subsequent reduction in amines—accompanied by the excretion of nitrogen in the urine, causes the muscle mass loss/atrophy that manifests clinically as muscle weakness / cachexia in RA patients.

5. Conclusion

In summary, using metabolomics, we found that the levels of amine metabolites are systematically decreased in the plasma of CIA mice, which is consistent with similarities between our CIA mouse model and RA patients at the metabolomics level. This result indicates that the muscle wasting and energy expenditure issues (e.g., cachexia) associated with RA—and models of RA—are highly complex.

The cachexia and sarcopenia associated with muscle atrophy, protein breakdown, and energy expenditure are not unique to RA. For example, several other chronic inflammatory diseases have been associated with catabolic wasting, including cancer [62], HIV/AIDS) [63], type 2 diabetes [64], renal failure, uremia [65], and heart failure [66]. We therefore hypothesize that systemic decreases in the levels of amine metabolites may reflect muscle mass loss and protein degradation due to inflammation.

Considering the complexity and consequences of muscle wasting in a wide variety of chronic diseases, using a metabolomics-based approach may provide a clearer understanding of the biological processes involved in these diseases.

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7. Compliance with Ethical Requirements

Conflict of interest: We have no conflict of interest to declare.

Ethical approval: The study was performed in accordance with the guidelines established in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All experiments were approved by the Tohoku Institute of Technology Research Ethics Committee, Sendai, Japan (approval date: January 18, 2009).

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Chapter 4

Spontaneous ultra-weak photon emission in correlation to inflammatory metabolism and oxidative stress in a mouse model of collagen-induced arthritis

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Abstract

The increasing prevalence of rheumatoid arthritis has driven the development of new approaches and technologies for investigating the pathophysiology of this devastating, chronic disease. From the perspective of systems biology, combining comprehensive personal data such as metabolomics profiling with ultra-weak photon emission (UPE) data may provide key information regarding the complex pathophysiology underlying rheumatoid arthritis. In this article, we integrated UPE with metabolomics-based technologies in order to investigate collagen-induced arthritis, a mouse model of rheumatoid arthritis, at the systems level, and we investigated the biological underpinnings of the complex dataset. Using correlation networks, we found that elevated inflammatory and ROS-mediated plasma metabolites are strongly correlated with a systematic reduction in amine metabolites, which is linked to muscle wasting in rheumatoid arthritis. We also found that increased UPE intensity is strongly linked to metabolic processes (with correlation co-efficiency $|r|$ value >0.7), which may be associated with lipid oxidation that related to inflammatory and/or ROS-mediated processes. Together, these results indicate that UPE is correlated with metabolomics and may serve as a valuable tool for diagnosing chronic disease by integrating inflammatory signals at the systems level. Our correlation network analysis provides important and valuable information regarding the disease process from a system-wide perspective.

1. Introduction

Rheumatoid arthritis (RA) is one of the most prevalent chronic auto-immune diseases, occurring in about approximately 1% of the population in Western countries [1], [2]. RA manifests as a complex inflammatory syndrome that typically includes joint swelling, pain, and hyperthermia, as well as synovial hyperplasia and destruction of cartilage and bones in the joints. RA is considered a systemic disease that is caused by a variety of pathophysiological processes [3]. These processes are accompanied by increased levels of cytokines such as tumor necrosis factor α (TNF-α) and interleukins (IL-1β and IL-6) in the blood and interstitial fluids, activation of NF-κB pathways (to inhibit apoptosis in various immune cells), and systemic disruptions in inflammatory metabolite synthesis [4]–[6].

Experimental studies of RA—particularly the pathophysiological mechanisms of therapeutic interventions—are often conducted using animal models. The most commonly used model for RA is the collagen-induced arthritis (CIA) mouse model, which has pathophysiological processes and features similar to patients with RA [7]–[11]. In addition, advances in metabolomics technology, which now enable researchers to measure extremely low concentrations of metabolites in several pathways simultaneously [12], has facilitated the study of RA in considerably more detail, thereby increasing our understanding of the pathological mechanisms that underlie the disease [13]. We previously studied the differences in molecular profiles between CIA mice and control mice by examining differences with respect to inflammation and reactive oxygen species (ROS), analyzed using univariate and multivariate metrics [14]. In addition to the well-characterized inflammatory phenomenon, issues related to muscle wasting and energy expenditure are also present in RA [15]–[18], and this is reflected by the presence of amine metabolites in the plasma of CIA mice [19].

Differences between CIA mice and control mice were also observed with respect to the intensity of ultra-weak photon emission (UPE), which reflects differences in the organization of the system at a biophysical level [20]. UPE is a process that occurs in all living organisms and is the spontaneous emission of light with extremely weak intensity (10^1–10^3 photons/sec/cm^2) in the UV, visible, and near-IR spectra [21]. Many studies have focused on the relationship between UPE and ROS production during metabolic processes [22]–[26]. Considering that ROS production is closely associated with inflammatory diseases and impaired metabolic processes, it is reasonable to expect that UPE is also associated with inflammatory disease and/or metabolic processes. UPE might therefore be used to help diagnose inflammation and inflammation-related diseases. UPE has been proposed for monitoring lipid peroxidation in cell membranes [27], and applications using UPE in human studies—and their potential relationship with ROS—were summarized by van Wijk [23]. Moreover, the putative relationship between UPE, physiological state, and metabolic processes has been proposed by several research groups [28]–[31]. Here, we performed an integrated analysis of the biochemical and biophysical differences between CIA mice and control mice, based on the hypothesis that a combined analysis would reveal unique insight into the biochemical and biophysical changes that occur during RA.

Network biology is an emerging field in biomedical research, and network biology tools are increasingly used to identify clusters of correlated parameters, to visualize or explore high-dimensional data, and to understand or interpret interactions that reflect part of a complex biological system [32], [33]. Correlation networks have been used in “omics” studies to combine complex data sets, for example combinations of metabolomics, genomics, and/or proteomics data sets. Correlation networks are also used to support the biological interpretation of large data profiles and to differentiate disease phenotypes [34]–[37]. Here, we expanded the systems-based approach of correlation-based analyses in order to examine the relationship between metabolomics profiling and UPE data. Using this correlation network analysis, we visualized systematic perturbations in bio-photons,

inflammatory processes, and ROS-related mediators. This approach may be used to facilitate the diagnosis of disease and/or to discriminate between disease syndromes, particularly with respect to complex chronic diseases such as RA and type 2 diabetes mellitus.

2. Materials and Methods

2.1 Animal study samples, Modelling, and ethics Statement

CIA was induced by the intraperitoneal injection of type II collagen and lipopolysaccharide in adult (6-7 weeks of age) DBA/1J male mice as described previously [38]; the CIA and control (Ctrl) groups contained 10 mice each. The injections were performed on days 0, 14, 28, 42, and 56; After 70 days' modeling, UPE intensity was measured in each paw, and blood was collected into pre-cooled EDTA tubes (BD Vacutainer, Plymouth, UK). The blood samples were centrifuged at 3000×*g* for 10 minutes, and then stored at -80ºC until metabolic measurements were performed [14]. All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). All animal care and experiments were approved by the Tohoku Institute of Technology Research Ethics Committee, Sendai, Japan.

2.2 Instruments and data acquisition

2.2.1 UPE instruments and settings

UPE was measured using a 600 series CCD camera system (Spectral Instruments, Inc., Tucson, AZ) equipped with a closed-cycle mechanical cryogenic unit (held at -120°C) as the cooling system. Prior to the UPE measurement, mice were maintained in controlled dark conditions. The detailed settings of the CCD system including figures about the measured location on mice is described in Van Wijk et al [20]. In brief, the CCD camera was mounted on the top of a dark chamber, and the animal was immobilized using isoflurane anesthesia. UPE intensity was recorded at five independent regions on each paw and used for further correlation analysis. The regions were named according to the paw measured, and numbers were added (ranging from 1 to 5, indicating the location closest to the tip of the paw through the location farthest from the tip of the paw) as follows: LFP (left front paw) 1 through LFP5; LHP (left hind paw) 1 through LHP5; RFP (right front paw) 1 through RFP5; and RHP (right hind paw) 1 through RHP5.

2.2.2 Extraction of plasma metabolites and metabolomics analysis

Plasma samples were aliquoted and extracted via different methods in order to obtain separate classes of compounds, including oxylipins, amine metabolites, and oxidative stress–related metabolites. Oxylipins (bioactive lipid mediators derived from polyunsaturated fatty acids) were extracted using solid phase extraction and analyzed using an Agilent 1290 HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer with electrospray ionization as described previously [14], [39]. Amine metabolites (including free amino acids and their biogenic metabolites) were extracted using AccQ-TagAQC derivatization and analyzed using a Waters ACQUITY UPLC coupled to a Waters Xevo mass spectrometer with electrospray ionization source as described by Noga et al. [40]. Oxidative stress–mediated metabolites—primarily PGs/IsoPGs, NO_2-FAs, lysophosphatidic acids, and sphingosine/sphingosine-related sphingolipids—were extracted using liquid–liquid extraction and analyzed using a validated method with an Agilent 1290 HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer with electrospray ionization. The peak area of each target compound was corrected using the appropriate internal standard (ISTD), leading to a ratio (target compound/ISTD) that was used for further analysis in the correlation study.

2.3 Data preprocessing and statistical analysis

The metabolomics and UPE data collected from both the CIA and Ctrl groups were included in the correlation analysis. Univariate correlations were performed using the Spearman's rank correlation method using RStudio software (version 3.0.3). Absolute values of the Spearman's rank correlation coefficient ($|r|$) >0.7 were considered to reflect a strong correlation between parameters, and this threshold was used to create highly correlated graphical networks using Cytoscape software (version 3.3.0, http://www.cytoscape.org) with the MetScape plug-in for extracting and integrating information and for visualizing the correlation networks

[41], [42]. Positive and negative correlations were indicated by positive and negative values of r, respectively.

3. Results and Discussion

3.1 Collagen-induced arthritis alters the local distribution of UPE

Differences in UPE between CIA and Ctrl mice have been reported previously [20]. A schematic figure was displayed, in order to show the CCD setup of UPE instrument as well as the locations for UPE measurements on mouse front and hind paws (Fig. 1). Here, we used correlation networks to visualize the relationship between individual UPE intensities at the locations measured in both CIA mice and in Ctrl mice (Fig. 2), as visualizing the profile of location-based UPE may provide important information regarding the disease. We then interpreted the differences and similarities between the two groups with respect to their correlation structures.

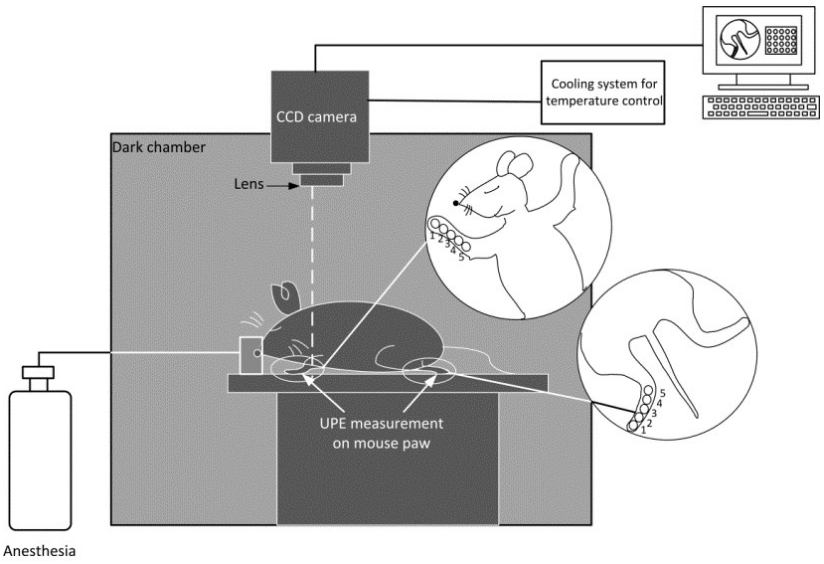

Fig. 1 Schematic figure of CCD set-up as well as locations for UPE measurements on mouse front and hind paws. Adapted from E. van Wijk et al. 2013.

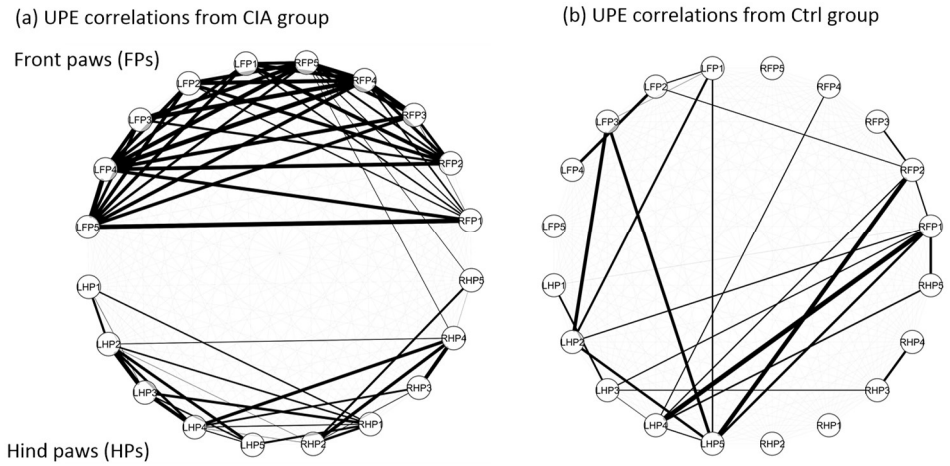

Fig. 2. Bio-photonic variance is revealed by location-based UPE-to-UPE correlation networks. The figure illustrates the differences in correlations between CIA mice (a) and Ctrl mice (b). In CIA mice, the strong correlations also indicate a strong similarity in UPE between the LFP (left front paw) and RFP (right front paw), as well as between the LHP (left hind paw) and RHP (right hind paw). The numbers (1 through 5) indicate the specific locations for the measurements (see Materials and Methods). Thus, the differences between the front paws and hind paws are clearly visible in the CIA group. The networks were established using the Spearman correlation analysis, and the lines represent Spearman correlation coefficients ($|r|$) >0.7.

The correlations were quantified using the parameters (i.e., $|r|$ values and p-values) obtained from the Spearman correlation analysis. In total, 71 and 26 strongly positive UPE-to-UPE correlations were found in the CIA and Ctrl groups, respectively; no strongly negative correlations were found. The difference in the number of strongly positive correlations between the CIA and Ctrl groups can be seen visually in Fig. 2. In the CIA group, UPE intensity was tightly correlated between the two front paws and between the two hind paws (Fig. 2a). In contrast, we found no clear correlation patterns in the Ctrl group (Fig. 2b).

3.2 Differences in metabolite correlations between CIA mice and control mice

Next, we acquired metabolic data from plasma samples using HPLC-MS/MS. The following three groups of metabolites were extracted using three validated

methods and detected using three specific instruments: amine metabolites (including free amino acids and their biogenic metabolites), oxylipins, and oxidative stress–related metabolites. A total of 110 endogenous metabolites were detected in the plasma samples, including 30 oxylipins, 45 amine metabolites, and 35 oxidative stress–related lipids. Univariate and multivariate analyses were then applied to the metabolite sets in order to characterize the differences between CIA mice and Ctrl mice at the metabolomics level. Previously, we reported the differences between CIA mice and Ctrl mice with respect to oxylipins and amine metabolites [14], [19]. Based on the oxidative stress platform, after log transformation and auto-scaling of the data, we also found a number of key metabolites that differed between the CIA the Ctrl groups (p<0.05, Student's t-test). Table 1 summarizes the key metabolites that differed significantly between the CIA and Ctrl groups.

Table 1. Summary of the key metabolites that significantly differed between the CIA and Ctrl groups

Oxylipins		Amine metabolites		Oxidative stress	
Compound	Changes	Compound	Changes	Compound	Changes
9,10-DiHOME	↓	Methionine	↓	PGE3	↓
9-KODE	↓	Homocysteine	↓	8,12-iso-iPF2a	↓
13-HDoHE	↑	Threonine	↓	cyclic-LPA C16:0	↓
14-HDoHE	↑	Proline	↓	cyclic-LPA C18:2	↓
12,13-DiHOME	↓	Alanine	↓		
9,12,13-TriHOME	↓	Valine	↓		
12-HEPE	↑	Cystathionine	↓		
9,10,13-TriHOME	↓	Lysine	↓		
9,10-EpOME	↓	Glycylglycine	↓		
10-HDoHE	↑	Serine	↓		
9-HODE*	↓	Asparagine	↓		
8-HETE*	↑	Cysteine	↓		
13-KODE*	↓	Tryptophan	↓		
12,13-EpOME*	↓	Methionine sulfoxide	↓		
13,14-dihydro-PGF2a*	↑	Homocitrulline	↓		
12-HETE*	↑	Isoleucine	↓		
		Gamma-glutamylalanine	↓		
		Histidine	↓		
		Glutamine	↓		
		Leucine	↓		

Abbreviations: DiHOME, dihydroxyoctadeca(mono)enoic acid; EpOME, epoxyoctadecamonoenoic acid; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid;
HODE, hydroxyoctadecadienoic acid; KODE, ketooctadecadienoic acid; PG, prostaglandin; TriHOME, trihydroxyoctadecenoic acid.
↓: Decreased in CIA mice; ↑: Increased in CIA mice;

*: Extra important oxylipins which contributed to the group clustering are based on multivariate analysis (VIP>1).

Differences in metabolites generally do not occur independently, but often change together with other, related metabolites, as metabolic reactions are often part of a dynamic system and have many biological processes in common [35]. Metabolic network analysis is an emerging approach used to diagnose disease, and it has the advantage of integrating "omics" datasets in order to identify links and select useful information from among chaos [34], [43]. We therefore performed a correlation network analysis in order to visualize pair-wise metabolic correlations and to extract novel information regarding dynamic alternatives. A merge between the metabolite-to-metabolite correlation networks measured in the plasma of CIA and Ctrl mice is illustrated in fig. 3a and 3b, respectively. Next, the Spearman correlation coefficient between metabolites (r_m) was calculated, and only strong correlations (either positive or negative) (i.e., with an $|r_m|$ value >0.7) were included in the resulting network. We found a total of 394 positive correlations and 91 negative correlations in the CIA group, and a total of 864 positive correlations and 117 negative correlations in the Ctrl group. In general, metabolites that are in the same chemical class or in the same biochemical pathway tended to correlate with each other; these so-called "chemical class-based" clusters and "pathway-based" clusters were more pronounced in the Ctrl group, leading a highly connected region among oxylipins and another region among amine metabolites. This network analysis revealed certain structural or pathway similarities among those highly connected metabolites with respect to significant positive correlations. Moreover, the associations between oxylipins and amine metabolites were relatively weak in the Ctrl group, possible because oxylipins and amine metabolites are generated via two separate metabolic pathways.

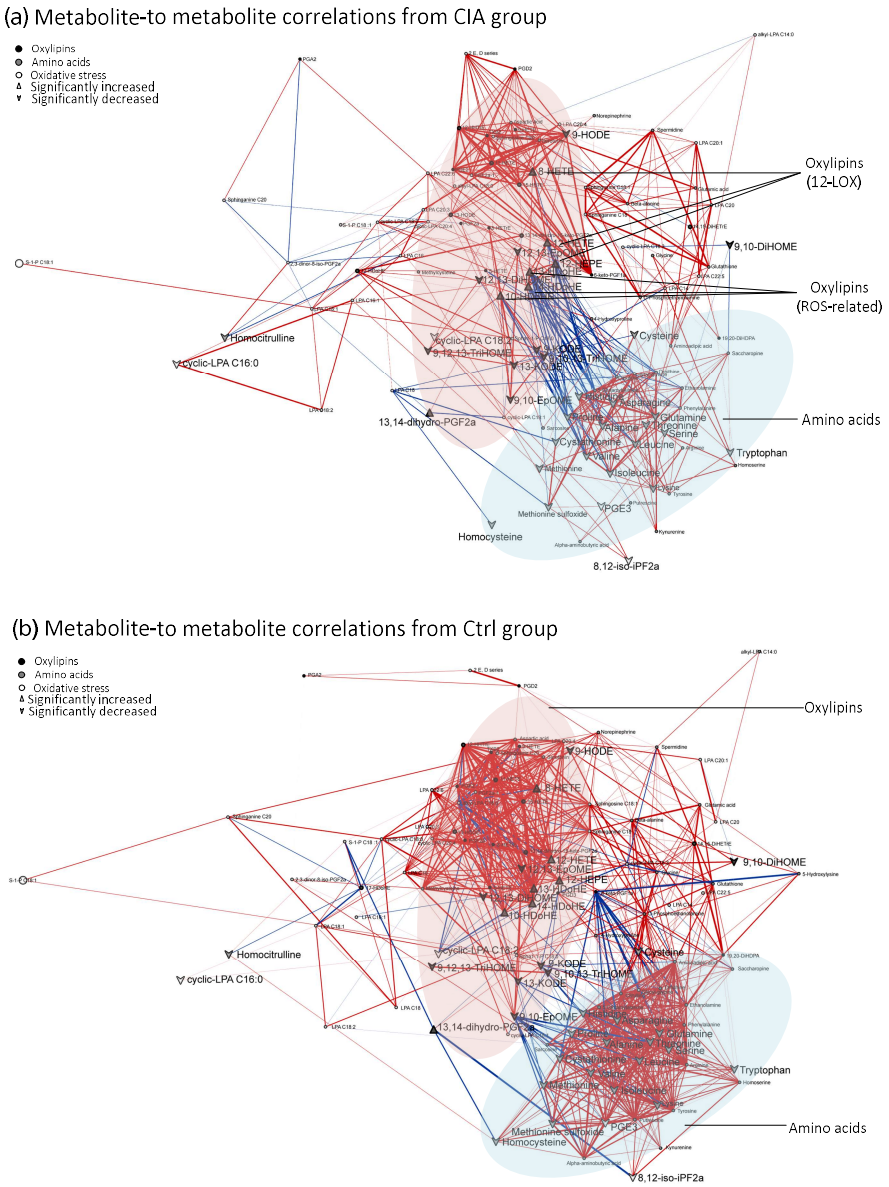

Fig. 3. Metabolic correlation networks in the CIA and Ctrl groups. Depicted are the metabolite-to-metabolite correlation networks for CIA (3a) and Ctrl (3b) mice. All of the metabolites detected in our analysis are included in the networks models.

Nodes with a positive correlation are indicated with solid red lines, and nodes with a negative correlation are indicated by solid blue lines. Shaded ellipses with a light red or light blue background indicate clusters of oxylipins or amine metabolites, respectively. Thickness of lines indicate gradient correlation strength: the thicker the line is, the stronger the correlation is (visible correlation co-efficiency: $|r|$ ranges from 0.7 to 1). (For better visualization of the detailed figures, please visit the web version of this article online: http://www.sciencedirect.com/cache/MiamiImageURL/1-s2.0-S1011134416307539-gr3_lrg.jpg/0?wchp=dGLzVlV-zSkWl&pii=S1011134416307539)

Interestingly, we found that some of the strong correlations in the Ctrl group—including both "oxylipin-to-oxylipin" and "amine-to-amine" correlations—were weaker in the CIA group. In contrast, the CIA group contained more negative oxylipin-to-amine correlations (Fig. 3a) than the Ctrl group (Fig. 3b). For example, the HETEs and HDoHEs that were elevated in CIA mice were strongly correlated with the branched chain amino acids valine, leucine, and isoleucine, as well as with cystathionine, alanine, glutamine, and asparagine. The use of HETEs and HDoHEs as inflammatory/ROS-related biomarkers has been described previously [14], and we also found that decreases in these amine metabolites may reflect muscle wasting and/or energy expenditure (cachexia) in RA [19]. Therefore, our analysis of metabolic correlation networks suggests that the increased inflammation and ROS levels reflected by oxylipins may also be associated with the onset of muscle wasting and increased energy expenditure in RA.

3.3 UPE is correlated with inflammatory signaling–related metabolites in CIA mice

As discussed in the Introduction, UPE arises as a result of metabolic reactions, particularly oxidation-reduction (redox) reactions; therefore, we hypothesized that UPE emission patterns may be correlated with metabolite patterns. To test this hypothesis, we created a correlation network to visualize potential associations between UPE intensity and peak area ratios of measured metabolites (see Materials and Methods). Therefore, we used UPE-to-metabolite correlations (i.e., between a given UPE value, u, and a given metabolite, m) in the correlation

networks, and the Spearman's correlation coefficient $|r_{um}|$ was calculated for each UPE-metabolite pair in both the CIA group and the Ctrl group.

The heat map in Fig. 4 depicts a general UPE-to-metabolite correlation profile used to compare the differences measured between the CIA group and the Ctrl group. A cluster analysis reveals clear location-based clusters in the CIA mice. The heat map also indicates a systemic change in the CIA group (i.e., the majority of positive correlations, shown in red) compared with the Ctrl group (i.e., the majority of negative correlations, shown in green). After removing relative weaker coefficient from the Spearman correlation analysis ($|r_{um}|<0.7$), networks were built to reflect the highly correlated entities and to show the most important metabolites (fig.4b). After we removed the relatively weaker correlations from the Spearman correlation analysis (i.e., $|r_{um}|$ values <0.7), we built a network to reflect the strongly correlated entities and to illustrate the most relevant metabolites (Fig. 4b). Circle-attributed networks were then used to identify the key correlations and to compare the CIA group with the Ctrl group. A total of 27 strongly positive correlations and 79 negative correlations were identified in the Ctrl group, and a total of 146 positive and 9 negative correlations were identified in the CIA group.

The correlation networks revealed that the majority of UPE-to-metabolite correlations in the Ctrl group were negative, whereas the majority of UPE-to-metabolite correlations in the CIA group were strongly positive. The major metabolites that were positively correlated with UPE in the CIA group are the monohydroxyeicosatetraenoic acids (HETEs), prostaglandins (PGs), thromboxane (TBX) synthase products, lysophosphatidic acids (LPAs), sphingolipid signaling molecules, and some amine metabolites (Fig. 4b). UPE intensity measured at various locations was correlated with various metabolites in the CIA group. For example, UPE intensity in the front paws was more strongly correlated with some LPAs, whereas UPE intensity in the hind paws was more strongly correlated with PGs (13,14-dihydro-15-keto-PGF2a, PGE2, PGD2, and 6-keto-PGF2a), TBX synthase products (TBX2 and 12-HHTrE), HETEs (8-HETE, 15-HETE, 11-HETE, and 12-HETE), and sphingolipids; see the CIA correlation networks in Fig. 4b.

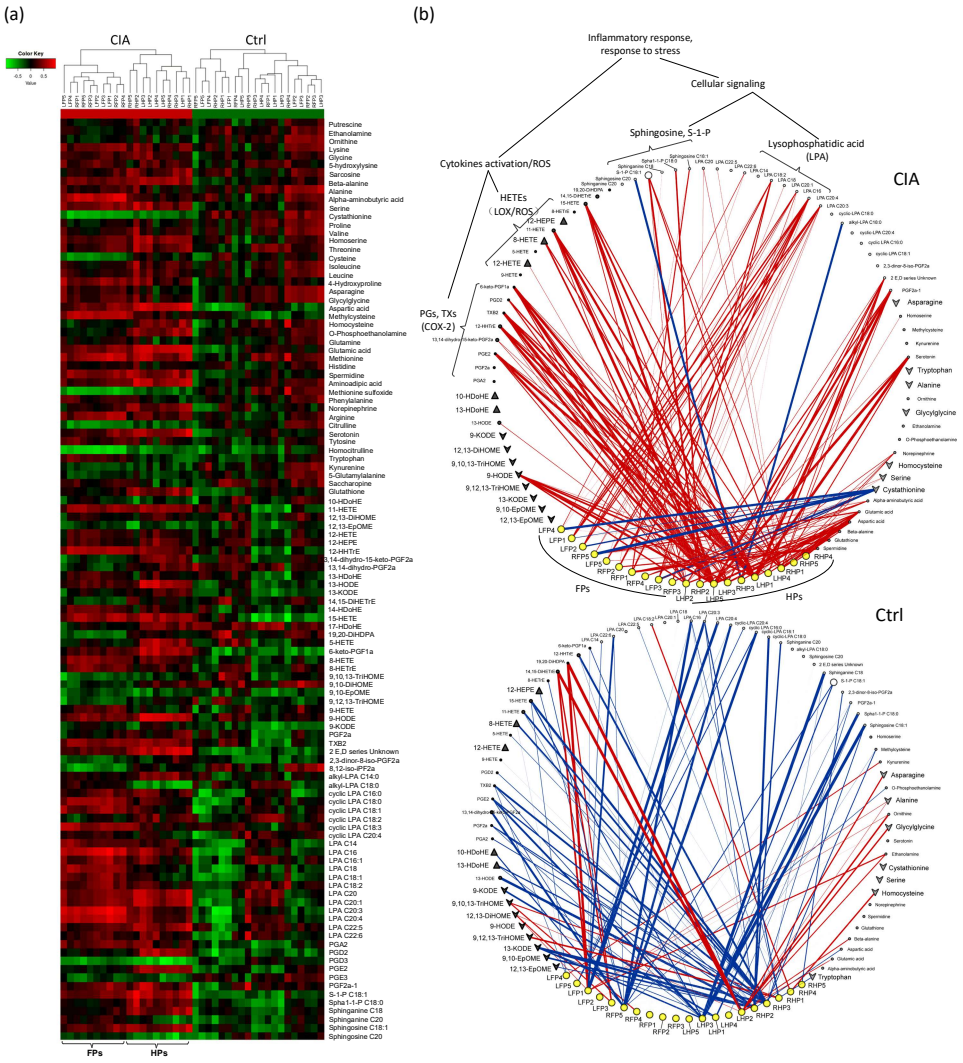

Fig. 4. Correlation-based analysis between UPE and metabolites measured in the plasma of CIA mice and Ctrl mice. a) Heat map showing the entire UPE-metabolite correlation profile, as well as the differences between the CIA and Ctrl mice. Colored blocks represent the value of the correlation coefficient, which were color-coded from 1 (strongly positive, light red) to -1 (strongly negative, light green). b) Visualized network model of the strong correlations (defined as a $|r_{um}|$ value >0.7). The red and blue lines indicate positive and negative correlations, respectively, and the thickness of the lines indicate the strength of the correlation. Several important pathway-related

networks reflect the inflammation, ROS production, and muscle wasting associated with RA. Each dot indicates an individual parameter that includes a given metabolite and UPE value: yellow dots reflect location-based UPE intensity, and black, gray, and white dots represent oxylipins, biogenic amines, and oxidative stress–related metabolites, respectively. Also shown (between the Ctrl and CIA network models) are enlarged views of the key metabolites that differed significantly based on our univariate and multivariate analyses. The up-triangles and down-triangles indicate the direction of the metabolic change in the CIA mice (i.e., up-regulation or down-regulation, respectively). (For better visualization of the detailed figures, please visit the web version of this article online: http://www.sciencedirect.com/cache/MiamiImageURL/1-s2.0-S1011134416307539-gr4_lrg.jpg/0?wchp=dGLbVBA-zSkzV&pii=S1011134416307539)

Next, the pathways related to these metabolites based on our previous study [14] and the Kyoto Encyclopedia of Genes and Genomes were organized (Fig. 5). LPAs act on G protein–coupled signaling and cellular signaling responses and function as inflammatory mediators [44], [45]. PGs and TBXs, which are synthesized from arachidonic acid via COX-II pathways, have well-established pro-inflammatory functions [46], [47]. The 12/15-LOX products (12-HETE, 15-HETE, and 8-HETE) promote the production of cytokines and activate the NF-κB pathway to inhibit cellular apoptosis [48], [49]. In addition, 8-HETE, 12-HETE, and 11-HETE can also be peroxided non-enzymatically by ROS to inhibit apoptosis [50]–[54]; therefore, these three HETEs may be important inflammatory mediators [55], [56]. The sphingomyelin-derived sphingolipids sphingosine and sphingosine-1-phosphate (S1P) are signaling molecules in immune cells that mediate neutrophil activation and apoptosis, and are therefore also considered to be inflammatory mediators [57]–[62]. Based on the correlation networks, it can be seen that these inflammatory mediators participated in the systemic perturbations (measured using both metabolomics and UPE) in the CIA mice, even though some of these mediators were not altered significantly in our univariate analysis. We also conclude that UPE intensity is correlated with systemic inflammatory mediators, ROS mediators, and cellular signaling processes; therefore, measuring UPE intensity may provide a means to diagnose inflammatory disease. In addition, UPE may also be used to monitor lipid peroxidation which relate to inflammation and ROS level in both healthy and diseased individuals (Fig. 6). Thus, a specific phenotype of a disease can be complemented by measuring both “omics” profiles

and UPE patterns, thereby providing a more detailed understanding of the disease and its underlying processes.

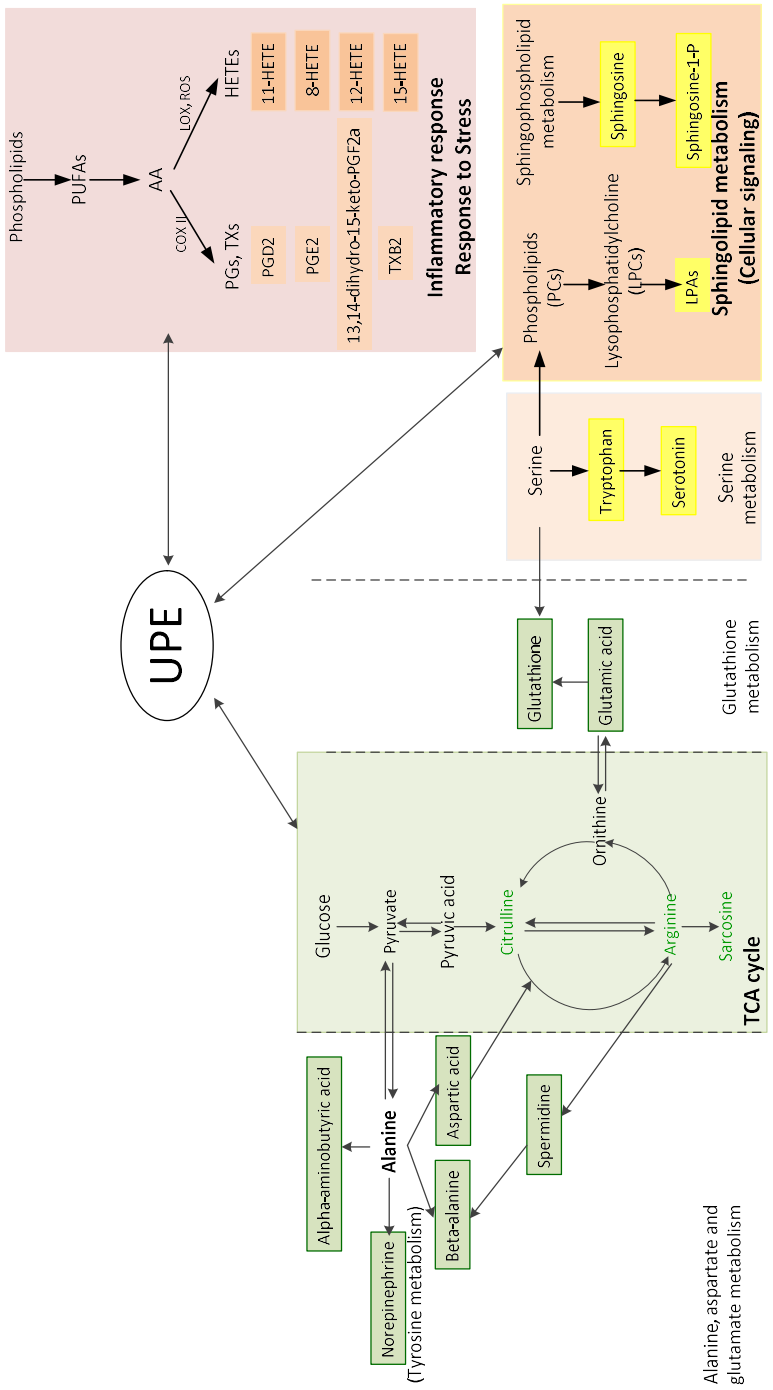

Fig. 5. Putative biochemical pathways with metabolites that can be correlated with UPE. UPE intensity can be linked to inflammatory responses and ROS-related stress in RA, which are reflected by COX-II and 12/15-LOX oxylipins derived from arachidonic acid (AA). During cell signaling, the production of LPAs and sphingosine also associates with the increased UPE intensity. Another pathway that may be linked with UPE intensity is the TCA cycle.

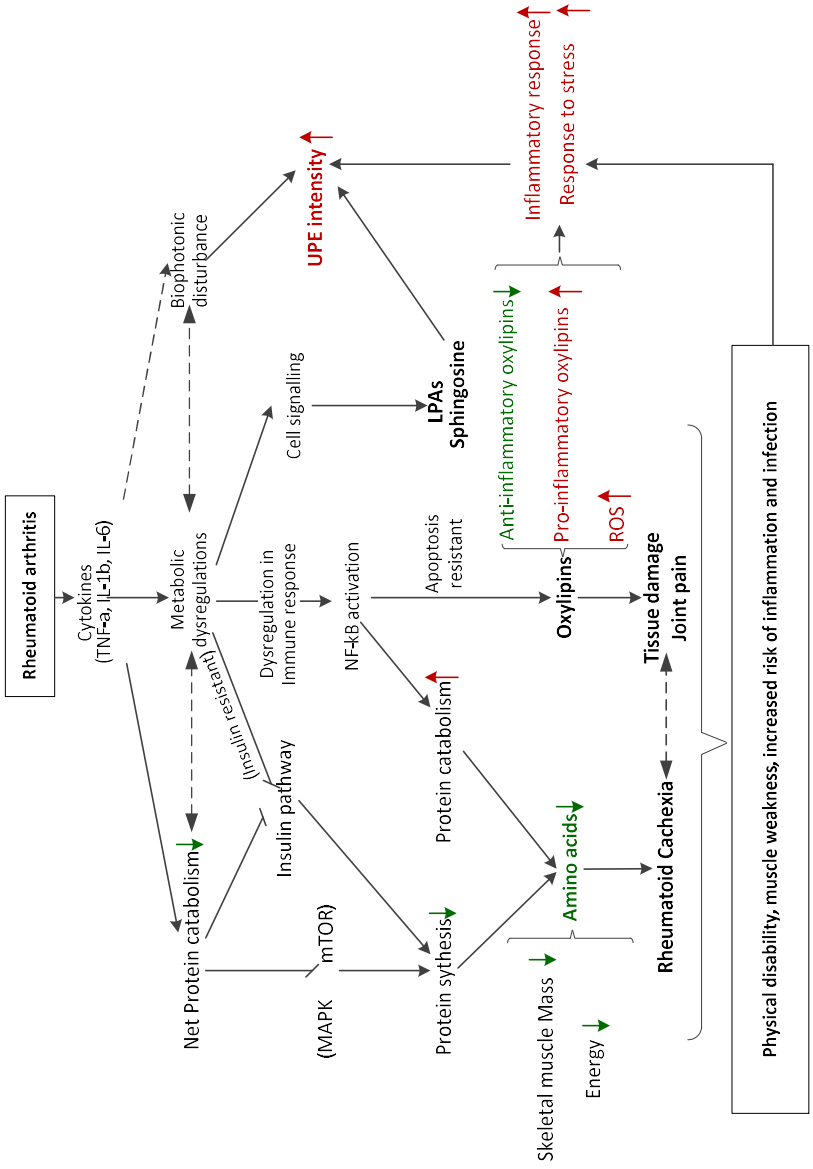

Fig. 6. Systemic and dynamic changes in RA. The production of cytokines dynamically interrupts living systems at both the biochemical level (i.e., the proteomics and metabolomics levels) and the bio-photon level. In addition, the biochemical changes are in correlation to the UPE changes. Elements marked in red/green are those increased/decreased in our CIA mice model.

In the CIA mice, a strongly negative correlation between cystathionine and UPE was measured, whereas several amine metabolites—including serotonin, tryptophan, and aspartic acid—were positively correlated with UPE. Cystathionine is a scavenger of free radicals [63]; therefore, given its significant decrease in CIA mice compared to Ctrl mice, the negative correlation between cystathionine and UPE intensity indicates that the increase in UPE intensity may be due to a decrease in antioxidants in RA. Both tryptophan metabolism and the serotonergic system have been well described as key pathways that can influence signaling in the central nervous system [64]. Thus, UPE may also be correlated with metabolic systems that are associated with neurotransmission. In addition, based upon pathways that regulate amine metabolites listed in the Kyoto Encyclopedia of Genes and Genomes, all of the other amine metabolites that were positively correlated with UPE are associated either directly or indirectly with the TCA cycle (see Fig. 5). The correlations identified between UPE intensity and these metabolites may suggest that during disease, some of the electrons that would otherwise participate in chemical reactions to produce energy (for example, with amine metabolites in the TCA cycle) actually escape and set free the energy which they carry, as photons, whereby the electrons change from high to low energy level states. Simultaneously, free radicals and/or ROS are produced, driving lipid peroxidation to produce inflammatory HETEs and PGs. While such a speculation need more rigorous validation.

The reduction in amine metabolites in the plasma of CIA mice compared to Ctrl mice may be linked to the contribution of muscle wasting in arthritis [19]. Considering that we found strong correlations between amine metabolites and UPE intensity, and given that muscle wasting is a common feature in many disease processes, including some cancers [65], HIV/AIDs [66], type 2 diabetes [67], renal failure, uremia[68], and heart failure [69] , UPE may also have potential perspective for the use of monitoring energy wasting and muscle wasting in other diseases. In this respect, future studies should examine the relationship between muscle wasting and UPE.

Interestingly, HETEs, PGs, sphingosine, and S1P—which were strongly correlated with UPE intensity in our study—are also considered to be important inflammatory biomarkers in a variety of diseases, including RA [70], cardiovascular disease and/or atherosclerosis–related inflammation [59], [61], [71], [72], congestive heart failure [60], cancers and other tumors [54], [73], some prostate diseases [55] , and nonalcoholic steatohepatitis [56]. Therefore, our finding that UPE is correlated with these inflammatory mediators may shed light on the biological mechanisms that underlie these diseases from a systems biology perspective.

4. Conclusions

Given its complex pathophysiology, RA has been studied using a variety of technologies and approaches. Indeed, integrating various data sets can provide important information regarding the disease process and possible treatment strategies. Generating correlation networks can provide valuable information, and these networks have been used recently within a wide range of “omics” studies, including proteomics, genomics, and metabolomics, thereby helping distinguish specific diseases and/or phenotypes [34], [35], [74]. Here, we performed the first study that integrates UPE with metabolomics in both diseased mice (i.e., mice with collagen-induced arthritis) and healthy control mice; this novel, powerful approach yielded meaningful information regarding RA. Moreover, we found specific correlations between metabolomics and UPE. Lastly, our correlation network analysis shows a systematic way to illustrate the complexity of RA , including dysregulation of both UPE and metabolomics.

Using our correlation networks, we also found that oxylipins were negatively correlated with certain amine metabolites in the CIA group. This may indicate a systematic perturbation under inflammation and ROS response in RA-induced situation. However, further study is needed in order to elucidate whether the inflammation and ROS are the consequence of muscle wasting, or vice versa. We also found that UPE was correlated with certain inflammatory mediators, and we expanded the biological interpretation of RA using correlation networks.

In conclusion, our correlation network analysis provides valuable information regarding the disease process from a system-wide perspective. Understanding the underlying biochemical phenomena that give rise to UPE is of great importance to learn about potential applications of UPE in early disease characterization.

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Chapter 5

A Chinese literature overview on ultra-weak photon emission as promising technology for studying system-based diagnostics

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Abstract

To present the possibilities pertaining to linking ultra-weak photon emission (UPE) with Chinese medicine–based diagnostics principles, we conducted a review of Chinese literature regarding UPE with respect to a systems view of diagnostics. Data were summarized from human clinical studies and animal models published from 1979 through 1998. The research fields can be categorized as follows: 1) human physiological states measured using UPE; 2) characteristics of human UPE in relation to various pathological states; and 3) the relationship between diagnosis (e.g., Chinese syndromes) and the dynamics of UPE in animal models. We conclude that UPE has clear potential in terms of understanding the systems view on health and disease as described using Chinese medicine–based diagnostics, particularly from a biochemistry-based regulatory perspective. Linking UPE with metabolomics can further bridge biochemistry-based Western diagnostics with the phenomenology-based Chinese diagnostics, thus opening new avenues for studying systems diagnostics in the early stage of disease, for prevention-based strategies, as well as for systems-based intervention in chronic disease.

1. Introduction

The use of ultra-weak photon emission (UPE) in living organisms was first described by Gurwitsch in 1923 [1]. At that time, the technical capabilities for measuring radiation using physical devices was rather limited. This technology became more feasible when sensitive photomultipliers were developed in the 1960s in the former Soviet Union. The early data were published primarily in Russian journals [2], [3], with only a fraction of the reports translated into English [4]. Since the 1970s, UPE has been used by research teams in Germany [5], Australia [6], Poland [7], Japan [8], the United States [9], and China [10]. UPE has been used successfully in a wide variety of organisms, including bacteria, yeast, plants, animals, and humans, as well as in cells and cellular homogenates derived from living organisms [5]–[11].

UPE occurs spontaneously in living organisms, without the need for external intervention [12]. The emission range of UPE is approximately 10–10^3 photons/sec/cm^2. The spectral range of the photons emitted from living systems is 300–750 nm [13]; the photons emitted from human tissue ranges from 420–570 nm [14]. The source of UPE is closely related to the electronic transport and the generation of reactive oxygen species (ROS) during oxidative metabolic processes, with UPE originating from the transition from either the singlet excited state (such as singlet oxygen $^1O_2$) or the triplet excited level of carbonyl species ($^3R{=}O^*$) to the singlet ground state [15], [16]. Biological ROS—including the reactions of superoxide radical ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$)—are produced dynamically during chemical metabolic redox reactions, including lipid peroxidation and protein/nucleic acid generation; moreover, during these metabolic processes, electrons can become excited, and energy is emitted in the form of photons [17]. Similar to the ROS theory described above, photons can also be released during the metabolism of radical nitrogen species (RNS). ROS causes the oxidation of biomolecules such as nucleic acids, proteins, and lipids, which play essential roles in many cellular processes, including cell signaling, apoptosis,

and pro/anti-inflammatory regulation [18], [19]. Therefore, UPE can be measured in order to detect the physiological state of the human body and to measure dynamic changes in health [12], [13], [20].

In humans, UPE is usually measured using a photomultiplier tube (PMT) or a charge-coupled device (CCD). Emitted photons can be measured directly through the skin in a light-tight, dark environment [21], [22]. The use of UPE as a diagnostic tool for health-related issues in humans has been reviewed recently [23]. The intensity of UPE emitted from the human body can be influenced by several physiological states, including age [24], gender [25], biological rhythms [22], [26]–[29], and conscious activities [30]–[32], thus leading to the discovery of putative diagnostic properties of photon emission. For example, hypothyroidism can be diagnosed by measuring the emission of photons from the index finger of human subjects [33]. Furthermore, differences in the intensity of photon emissions have been measured between patients with multiple sclerosis and healthy subjects [34], [35]. Moreover, patients with hemiparesis have asymmetrical UPE intensity between the left and right hands, suggesting that measuring photon emission symmetry could be used as a novel diagnostic parameter in addition to measuring UPE intensity [36], [37]. Based on the aforementioned experimental observations, UPE has been proposed as a non-invasive indicator of the integrated states and dynamic changes in human health [12], [20], [38].

In the newly emerging systems-based view of health, biology can be considered a hierarchy of various levels of organization, ranging from low levels (e.g., biochemistry and molecules) to the cellular and organ levels, all the way up to the integrated systems level [38]. In Western medicine, “omics” technologies are often utilized to study genes, proteins, and metabolites at relatively low organizational levels [39]. Recent work suggests that the dynamic distribution of UPE emissions from the human body can reflect both the health status at a large-scale organization level and the dynamics of the system [13], [20]. Similar to UPE, Chinese medicine integrates physiological and pathological information at a higher level of

organization—i.e., the phenotype level—in order to obtain a holistic description of the body's state. Two important types of descriptions are frequently used: constitution differentiation and syndrome differentiation [39]–[41]. However, Chinese medicine–based diagnostics is a descriptive, phenomenological approach based on many clinical observations, and the insights regarding molecular and mechanistic biology have been explored only recently [42]. Given that UPE may provide important insight into health at a high level of organization, measuring UPE parameters may provide novel scientific insights into Chinese medicine–based diagnostics and may help guide Western medicine towards a systems-based view of life, both from a diagnostic perspective and from an intervention perspective. Therefore, it is important to explore the history of this relationship between UPE and Chinese medicine–based diagnostics.

Applications in which UPE has been used to understand and measure systemic organization can be found in Chinese literature; these publications have generally focused on the relationship between UPE and Chinese medicine–based concepts in both human and animal studies. In this review, we summarize these studies published in Chinese scientific journals from 1979 through 1998. In studies published in 1979-1998, Chinese medicine–based concepts were used to establish UPE experimental designs. After the turn of the century, UPE research interests in China shifted from healthcare to plant and agriculture area [43], [44], and no more literature fit in the area regarding UPE and Chinese medicine–based concepts then. Because much of the clinical data was published in Chinese, UPE research is relatively unknown among scientists in non-Chinese-speaking countries. By reviewing this literature, we hope to educate scientists in terms of the possibilities regarding linking UPE with Chinese medicine–based diagnostics principles. Furthermore, because Western UPE researchers rarely study Chinese medicine–based diagnostics from a systemic regulatory perspective, this review will also provide a basis for further research in this specific area.

2. Temporal variations in UPE intensity among healthy human subjects

According to the Chinese medicine theory, one's health depends on a dynamic balance between one's physiological state and the surrounding environment. The human body can adapt in response to many environmental factors (e.g., changes in the seasons) and internal environmental changes (e.g., emotional variations). These patterns of change that result from changes in the internal and external environments are essential for obtaining a diagnosis in Chinese medicine. Therefore, Chinese physicians are taught to make a comprehensive diagnosis that includes an evaluation of how the body responds to the surrounding environment at various ages, as well as the effect of seasonal fluctuations [45]–[47].

In China, UPE measurements have been used to study temporal changes in human physiological states since the 1980s. Zheng [48] investigated the effect of gender and age on UPE measured from the fingertips of seven groups of healthy subjects; these results are summarized in Figure 1. In general, the intensity of UPE was higher among males than among females, and UPE intensity tended to increase with age. This association between age and UPE was later confirmed by Sauermann et al.[24]. In a separate study, Yan [49] examined the relationship between age and UPE by measuring the specific acupuncture point LI1 (also known as the Shangyang acupuncture point); Yan found higher UPE intensity among young subjects (17-49 years of age) compared with both older subjects (50-72 years age) and children (11-16 years of age).

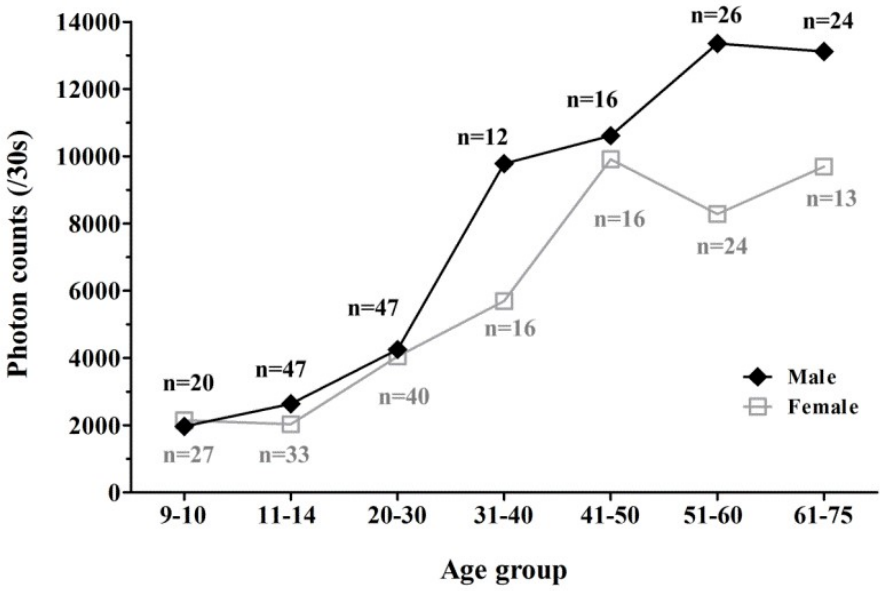

Fig.1: UPE intensity measured in male and female human subjects at the indicated ages (in years). UPE intensity was measured as the average photon counts (per 30 seconds) of the total photon emission from ten fingertips; the data are the average of five separate measurements per subject[48].

Yang measured UPE intensity at various acupuncture points located at the extremities and on the torsos of male and female children and adults [50], [51]. Consistent with the studies described above, Yang found that UPE intensity was higher in men than in women and higher in adults than in children. The association between UPE intensity and season (i.e., higher photon emission in the summer compared to the winter) that was originally reported by Zheng [52] for the fingers of healthy subjects has been later confirmed with UPE measurements of other body locations by Popp and Cohen [34], Van Wijk [53], Bieske et al. [54], and Jung et al. [55]; importantly, these authors did not refer and probably had no prior knowledge of Chinese literature regarding UPE measurements. These findings indicate that measuring UPE can provide insight into the state of harmony between the human body and the environment. Thus, deviations from these temporal rhythms in UPE intensity might be utilized further in order to study the pathological state and Chinese medicine–based diagnostic patterns.

3. The association between UPE and pathological state based on Chinese medicine–based diagnostic principles

In Chinese medicine, illness is viewed as a disruption of the body's dynamic balance. The body's dynamic balance is an abstract way to describe the flow of energy through the entire body, as well as the exchange between the body and the external environment. Measuring this flow of energy—particularly interruptions in this flow—provides important diagnostic information regarding the occurrence of specific illnesses. The aims of acupuncture are to regulate this flow of energy, remove blockages that interrupt energy flow, and help the ailing body re-establish its dynamic homeostasis [56]–[60]. In Western medicine–based terms, this might indicate a dysregulation of processes, which can be experienced as chronic disease.

The dynamic balance concept was recently correlated with symmetry—and asymmetry—in UPE intensity between the left and right sides of the human body [13], [37], [61], [62]. As far back as the early 1980s, this UPE left-right symmetry was identified by Chinese researchers as an important parameter for distinguishing between health and disease [52]. Thus, healthy subjects can be characterized by a symmetry in UPE intensity between acupuncture points on the two sides of the body [63]–[65]. Significant differences in UPE intensity at acupuncture points between the left and right sides of the body have been observed in typical "Western" diseases, including hypertension, facial nerve paralysis, and constipation [63]–[68]. Figure 2 shows an example of UPE asymmetry measured using acupuncture points on the hand. The left side of the figure shows disease states diagnosed using Western medicine. These specific diseases correspond to acupuncture point locations at which significant UPE asymmetry was measured. The right side of the figure shows the acupuncture point numbers and related meridian channels. These meridian channels always correspond with a diagnosis of the specific corresponding diseases in Chinese medicine [46], [63]–[65], [67]. Here, UPE may serve to bridge the Western medicine and Chinese medicine concepts. In other words, because UPE can be used to demonstrate potential deviations from

homeostasis in a meridian, and because these deviations can also be related to specific Western diseases, UPE provides the opportunity to connect Chinese medicine–based diagnoses with specific Western diseases [61], [69]; in this way, the long history of knowledge regarding Chinese medicine can be used to enrich Western medicine.

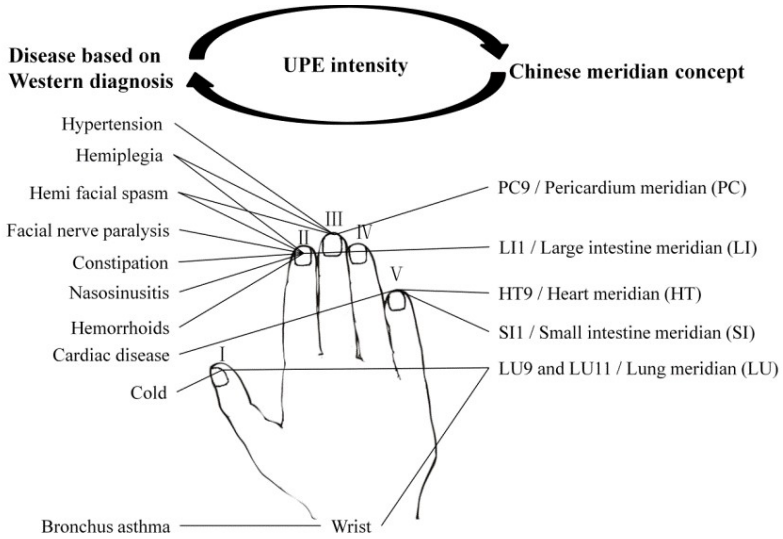

Fig.2: UPE patterns are related with both the Western medical concept of disease and Chinese medicine concepts [63]–[65], [67]**.**

The Western medicine description of diseases corresponding to Chinese acupuncture points and specific UPE intensity asymmetries.

I: Thumb; II: Index finger; III: Middle finger; IV: Ring finger; V: Pinkie

PC9: Zhongchong acupuncture point on the middle fingertip; LI1: Shangyang acupuncture point on the index fingertip; HT9: Shaochong acupuncture point on the pinkie fingertip; SI1: Shaoze acupuncture point on the pinkie fingertip; LU9: Taiyuan acupuncture point on the wrist; LU11: Shaoshang acupuncture point on the index fingertip.

Other studies have shown an uneven distribution of UPE intensity at acupuncture points at various body locations [50], [51], [70]. Higher intensity UPE has been measured at acupuncture points compared with non-acupuncture points; this difference was based on measurements of more than 150 acupuncture points together with their surrounding non-acupuncture points. Thus, the authors

suggested that acupuncture points with higher UPE intensity generally coincide with the theoretical meridians [71]–[73]. Interestingly, Guo et al. used chemical indicators to obtain fluorescence-based images of visible ROS distributions in an animal model and found that the areas with the strongest fluorescence were superimposable on human meridian lines [74]. Given that ROS content defines UPE intensity in living systems [9], [18], [19], [75], [76], the meridian-like lines of ROS activity measured in animals support—albeit indirectly—the correspondence between meridians and UPE intensity in humans.

In Chinese medicine, needles are used to stimulate acupuncture points and to trigger a dynamic interaction between the acupuncture points and the connective tissue along the meridian [77], [78]. This dynamic interaction was measured in several Chinese studies by measuring changes in UPE intensity [79], [80]. After placing needles in the acupuncture points of the forearm or calf, UPE intensity will change significantly at the acupuncture points of a finger or toe, respectively. In addition, UPE asymmetry can also be used to measure the therapeutic effect of acupuncture in patients. For example, left-right UPE asymmetry was measured at various acupuncture points on both sides of the body and was found to change following acupuncture [81]. Some studies also examined the therapeutic effect of acupuncture treatment by comparing the concentration changes in ROS-related enzymes and endogenous metabolites before and after treatment; these studies have been performed in both human subjects and animal models [82]–[86]. In addition, adiposity decreased when ROS-related anti-oxidant products (e.g., a recombinant superoxide dismutase protein) were applied to specific acupuncture points in obese subjects, and this therapeutic effect is similar to the effect of Chinese acupuncture [86]. The aforementioned studies of the therapeutic effect of acupuncture based on UPE and ROS measurements suggest that linking UPE parameters to changes in ROS may provide more opportunities to study the effect of acupuncture at the biochemical level.

4. UPE in relation to Chinese syndromes based in studies using animal models

Chinese studies have provided examples for how to study basic Chinese diagnostics concepts using UPE measurements, and this has been supported by similar UPE studies conducted in both Japan [87] and Korea [37], [88]. The pattern of UPE in the human body—and the changes in UPE intensity at specific body locations following acupuncture—appear to coincide with the meridian theory of Chinese medicine. Thus, the question arises whether UPE can also reflect the Chinese diagnostic syndrome theory.

The term “Chinese syndrome” refers to a combined pattern of physiology, psychology, and pathology in relation to a specific condition. The goal of syndrome differentiation is to understand illness as a pattern of relationships. Typically, several diagnostic procedures are used in order to identify the syndrome; these procedures include inspection, listening and smelling, inquiry, and palpation. Correctly identifying a Chinese syndrome is the basis of personalized therapies that use Chinese herbs, nutritional advice, acupuncture, physical exercise, and medication [89], [90]. To obtain a better understanding of Chinese syndromes from a modern biological perspective, several Western analytical tools—for example, omics-based approaches—have been used to study basic Chinese syndromes in patients with chronic diseases such as rheumatoid arthritis and diabetes. Using this approach, chemical biomarkers have been identified successfully for subtypes of patients with diabetes or rheumatoid arthritis [91], [92].

Given its potential for measuring overarching regulatory processes, UPE may be a useful diagnostic tool for identifying Chinese syndromes. In the Chinese literature, UPE has been used in three animal models to study deficiency syndromes [10], [93]–[95]. Marked reductions in UPE intensity at the acupuncture points located at the governor vessel (gV) and the conception vessel (cV) meridian channels were observed in Yang deficiency rats and Blood deficiency rats,

respectively; an increase in UPE intensity was measured after stimulating these acupuncture points [96]. In another study, a rabbit model of Qi deficiency was established by excessive intake of Rhubarb. In this model, a rapid decline in UPE intensity, followed by a slow rise in intensity, was measured in the rabbit's ears, reflecting the rabbit's altered dynamics as it progressed from illness to a healthy state [97]. In addition, the UPE level of the rabbit's organs (e.g., the spleen and stomach) decreased considerably, suggesting that UPE can also reveal changes in organs induced by treatment with herbs [98]. The Chinese research showed an intriguing change in UPE intensity related to the specific dynamics of deficiency syndromes. As more UPE parameters are identified in the future, they will likely provide more information regarding Chinese syndromes.

5. Perspective: UPE-guided metabolomics based on Chinese medicine–based diagnostics

In this review, we discussed the UPE research that has been performed in China within the past century with respect to physiological and pathological conditions. Importantly, our review revealed that UPE experimental observations are closely correlated with Chinese medicine–based diagnostic concepts. Some researchers have hypothesized that this correlation may be due to the concordance between the coherence theory of photon emissions in humans and the energetic properties of living organisms as developed in Chinese medicine [99], [100].

Here, we propose that a UPE-guided metabolomics approach based on Chinese diagnostic theory may improve the dialogue between Western medicine and Chinese medicine. UPE parameters and Chinese diagnostics reflect dynamic responses that arise as a result of internal and/or external disturbances in the human body at a relatively high organizational level. In addition, because its origin lies in oxidative metabolic processes, UPE has been proposed to link to metabolic

networks [20]. Various ROS-regulating metabolites have been detected in several diseases, including cardiovascular disease, hypertension, rheumatoid arthritis, and type 2 diabetes [91], [92]. Several metabolomics platforms—such as platforms based on amino acids and oxylipins—have been established, and these platforms reflect ROS/oxidative stress products, as well as their biosynthetic pathways [101]–[103]. Given that ROS play an important role in mechanisms associated with UPE and metabolic processes, they might serve as a direct biochemical bridge between UPE and metabolomics.

If UPE parameters can be linked to ROS-related metabolic pathways, the Chinese diagnostic principle, which is characterized by UPE, may be related to biochemical mechanisms. Thus, UPE might be used to detect early perturbations, even before they can be detected using metabolomics. In this way, UPE measurements could be used to indicate when metabolomics measurements would be warranted. Alternatively, depending on the UPE parameter that is changed, a specific metabolomics platform can be used for further analysis. In other words, by characterizing Chinese diagnostics using UPE parameters, and by studying the relationship between UPE and metabolomics, UPE-guided metabolomics based on Chinese diagnostics can be used to improve healthcare.

6. Conclusions

In this review, we discussed the UPE research linked to Chinese medicine that was published in the Chinese literature in the last century. Several experimental observations using UPE were found to be highly correlated with Chinese medicine–based diagnostic concepts. A UPE-based metabolomics approach guided by the Chinese medicine–based diagnostic concept may provide a biochemical bridge between Western medicine and Chinese medicine. From this perspective, three areas of UPE-based research should be explored further: *i*) the UPE-based

methodologies should be developed and optimized; *ii*) experimental work should bridge UPE with Chinese medicine–based diagnostics and metabolomics; and *iii*) dynamic UPE-based data should be integrated with other system-based diagnostic measurements.

Linking UPE, a dynamic diagnostics tool, with omics measurements in systems biology studies will increase our understanding of the diagnosis, prediction, and treatment of many diseases. Moreover, combining UPE with metabolomics based on ROS production might provide an effective approach for studying the relationship between health and disease and will help improve our understanding of the healthy state.

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Chapter 6

Traditional Chinese medicine-based subtyping of early-stage type 2 diabetes using plasma metabolomics combined with ultra-weak photon emission

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Abstract

Ethnopharmacological relevance: The prevalence of type 2 diabetes mellitus (T2DM) is increasing rapidly worldwide. Because of the limited success of generic interventions, focus has shifted toward personalized strategies, particularly in early stages of the disease. Traditional Chinese medicine (TCM) is based on a systems view combined with personalized strategies and has improved our knowledge with respect to personalized diagnostics. From a systems biology perspective, this understanding can be improved in order to yield a biochemical basis for such strategies, for example using metabolomics combined with other system-based diagnostic methods such as ultra-weak photon emission (UPE). In this respect, UPE has been used successfully to support TCM-based subtyping. Combining these technologies will further support TCM-based subtyping of diseases such as T2DM.

Aim of the study: The aim of this study was to investigate the feasibility of using plasma metabolomics to stratify the following TCM-based subtypes: Qi-Yin deficiency, Qi-Yin deficiency with dampness, and Qi-Yin deficiency with stagnation. Furthermore, we studied the relationship between plasma metabolomics and UPE with respect to TCM-based subtyping in order to obtain biochemical information for further interpreting disease subtypes.

Materials and methods: Plasma samples obtained from 44 subjects were extracted and analyzed using both liquid chromatography/tandem mass spectrometry and gas chromatography/tandem mass spectrometry. We then profiled various classes of metabolites, including amine metabolites, organic acids, sugars, and lysophosphatidic acid–derived metabolites, as well as lipids, including sphingomyelin phosphatidylcholine, phosphoethanolamine, lyso-

phosphatidylcholine, lyso-phosphoethanolamine, , cholesterol esters and triglycerides. Multivariate analysis (principal component analysis and orthogonal projections to latent structures discriminant analysis) was used to analyze the metabolomics profiles and to study TCM-based stratification. Finally, Spearman's rank correlation-based networks were used to correlate the metabolites with the UPE parameters.

Results and discussion: Principal component analysis of plasma metabolites revealed differences among the TCM-based pre-T2DM subtypes. Relatively high levels of lipids (e.g., triglycerides and cholesterol esters) were important discriminators of two of the three subtypes and may be associated with a higher risk of cardiovascular disease. Correlation networks revealed that plasma metabolomics and UPE yielded similar TCM-based subtypes. Finally, plasma metabolomics data indicate that the lipid profile is an essential component captured by UPE with respect to stratifying subtypes of T2DM.

Conclusions: Metabolic differences exist among different TCM-based subtypes of pre-T2DM, and profiling plasma metabolites can be used to discriminate among these subtypes. Plasma metabolomics provides biochemical insights into system-based UPE measurements.

Key words: Type 2 diabetes mellitus, plasma metabolites, disease subtypes, ultra-weak photon emission, correlation networks

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic, devastating complex disease. T2DM is characterized by increased fasting plasma glucose levels, impaired postprandial insulin secretion, decreased insulin sensitivity, and impaired pancreatic beta-cell function [1]. In addition, patients with T2DM have increased levels of inflammatory factors such as TNFα, IL-6, IL-8, and reactive active species [2], [3], altered levels of hormones, peptides, proteins, and enzyme activity, as well as other metabolic perturbations [4]. Striking, nearly all of these metabolic changes are often present years before the patient presents with clinical symptoms leading to a diagnosis of T2DM [5], [6].

Based on epidemiology studies, an estimated 285 million individuals are affected by diabetes worldwide, and this number continues to increase [7]. Furthermore, this number is likely an underestimate, as many individuals are not diagnosed in an early stage due to insufficient knowledge regarding the multi-symptom relationships at a systems level [8], [9]. Receiving a diagnosis only in a later stage of diabetes—together with the severe complications associated with disease progression—can lead to high costs and can reduce the efficacy of treatment [10]. For example, long-term dysglycemia increases the risk of severe complications such as hypertension, blindness, renal failure, and cardiovascular disease [11], [12]. These complications reduce quality of life and are a major cause of morbidity, hospitalization, and mortality among patients with diabetes. Current diagnostic tests are based primarily on a single screening tool such as the oral glucose tolerance test or measuring fasting plasma glucose. Understanding the symptoms that develop in an early stage of the disease and developing indicators of disease progression would likely contribute to improving both prevention and treatment strategies, including strategies based on changes in lifestyle. Moreover, treatments based on generic observations—which have led to the notion of one drug-one target-one disease (or one-size-fits-all)—are extremely limited,

particularly in early stages of the disease. Therefore, system-based approaches are needed in order to achieve personalized approaches.

Integrative holistic forms of medicine such as traditional Chinese medicine (TCM) provide descriptions of disease syndromes and subtypes at a systems level, including descriptions that can be used to diagnose early syndromes of chronic diseases. Such descriptions can be used as a guide or reference in order to achieve personalized medicine. In this respect, TCM has provided descriptions of pre-T2DM syndromes, indicating its potential for helping develop personalized medicine [13], [14]. To bridge TCM with Western medicine, evidence-based scientific data is needed at the biochemical level. Thus, modern systems biology research—including metabolomics—is a promising approach for exploring the biochemistry underlying TCM subtyping.

Metabolic disorders are often present for years before the appearance of clinical disease, and metabolomics is a widely used technique for predicting and diagnosing disease [15]. Metabolomics provides a comprehensive profile of small molecular metabolites in biological systems and can be used as a readout of the organism's physiological status [16]. In principle, this approach is well suited to studying complex TCM-based diagnostics. Metabolomics is generally performed on fluids such as blood, urine, and cerebrospinal fluid. Urine is commonly used for metabolomics, as it easily obtained, contains information regarding the excretion of products, and can reflect how metabolic processes change during the disease process. Several studies have used urine metabolomics to explore TCM-based diagnostics and T2DM syndrome subtypes [17], [18]. In addition to urine, blood also contains information regarding the body's regulatory status and dynamics. Thus, performing metabolomics on different fluids can provide complementary information, thereby improving our understanding of T2DM. An explorative study at TNO (https://clinicaltrials.gov/ct2/show /NCT00469287) was designed in which 44 pre-T2DM subjects received a diagnosis by a panel of three TCM-trained

physicians [17], and we explored these TCM-based subtypes using plasma metabolomics.

Recently, a sensitive, non-invasive technique has been proposed for supporting TCM-based diagnostics [19]. This technique, called ultra-weak photon emission (UPE), is used to measure spontaneous photon emissions from the skin's surface [20]. Because UPE reflects the body's physiological and pathological status, it represents a promising tool for use in clinical diagnostics at a systems level [21], [22]. The underlying biochemistry of UPE is related to metabolism and is correlated with reactive oxygen species in oxidative metabolic processes [23]–[26]. Although the use of UPE properties for characterizing TCM-based diagnostics has been summarized previously [19], [20], [27], further understanding of the molecular basis of UPE is needed. Therefore, combining metabolomics with TCM-based diagnostics can be used to investigate the biological meaning of UPE and to explore the added value of each technology. Importantly, UPE was used previously to subtype the same cohort of 44 subjects with pre-T2DM [27], thereby enabling us to study the correlation between UPE and plasma metabolomics.

2. Materials and Methods

2.1 Inclusion criteria for the selection of pre-diabetic subjects and the diagnosis of syndrome subtypes based on TCM

The recruitment of subjects and the diagnosis of pre-T2DM subtypes by TCM-trained physicians were described previously [17]. In brief, clinical parameters were obtained from 44 male Dutch subjects who met the following inclusion criteria: 30-70 years of age, body mass index of 26-35 kg/m^2, and a fasting glucose level of 6.1-6.9 mmol/L. No other clinical abnormalities or evidence of diabetic complications were detected. The subjects were then diagnosed separately in a blinded study by three TCM-certified physicians with at least five years of training in TCM and at least ten years of clinical experience. Three categories were based on TCM-based diagnostic terms, and 85% consensus was reached among the three CM physicians with respect to diagnosing the subjects. These three categories are defined as follows: QYD (Qi-Yin deficiency, n=15 subjects), QYD_Damp (Qi-Yin deficiency with dampness, n=20 subjects), and QYD_Stag (Qi-Yin deficiency with stagnation, n=9 subjects). Blood samples were collected after overnight fasting and used for the metabolomics study. In addition, UPE was measured from the palmar and dorsal surfaces of both hands.

2.2 Ethics statement

This explorative study was designed and conducted by TNO (Zeist, the Netherlands; https://clinicaltrials.gov/ct2/show/NCT00469287) and was approved by the Medical Ethics Committee of Tilburg (METOPP).

2.3 Data acquisition

2.3.1 Plasma metabolomics profiling

Metabolic profiles were measured by the Netherlands Organization for Applied Scientific Research (TNO, Zeist, the Netherlands). Heparinized blood samples were collected, and plasma was obtained by centrifugation (2000×*g* at 4ºC for 15 min). The plasma samples were aliquoted and stored at -20ºC prior to metabolite extraction and mass spectrometry.

Using a gas chromatography/mass spectrometry (GC-MS) platform, a large variety of metabolic classes were measured, including amine metabolites, organic acids, sugars, and lysophosphatidic acid (LPA)-derived metabolites. The details of the extraction and the GC-MS analysis protocol have been published previously [28]. In brief, 100-µl aliquots of plasma were spiked with a mixture of internal standards (ISTDs) and deproteinized with methanol. After centrifugation, the supernatant was transferred to a new sample vial for evaporation and two-step derivatization. The derivatized extracts were then analyzed using an Agilent 6890 gas chromatograph on a DB5-MS capillary column (30 m × 250 µm i.d., 0.25-µm film thickness; J&W Scientific, Folsom, CA) coupled to an Agilent 5973 mass selective detector; helium was used as the carrier gas at a flow rate of 1.7 ml/min for temperature-programmed gradient chromatographic separation. The raw data were pre-processed and exported using ChemStation G1701CA software (version D.01.02, Agilent), providing response ratios to the appropriate internal ISTD for each metabolite; these ratios were used for further statistical analysis.

For liquid chromatography/tandem mass spectrometry (LC-MS) lipid measurements, seven classes of lipids, including both polar lipids—such as phosphatidylcholine, phosphoethanolamine, lyso-phosphatidylcholine, lyso-phosphoethanolamine, and sphingomyelin —and non-polar lipids—such as cholesterol esters and triglycerides—were investigated using targeted analysis as reported previously by van Wietmarschen et al. [29] and Draisma et al. [30]. In brief, 10-µl aliquots of plasma were deproteinized by the addition of isopropanol containing a mixture of ISTDs. The lipids were separated and analyzed using a TSQ Quantum Discovery Triple Quad mass spectrometer coupled to a Surveyor

MS HPLC system on an Alltech Prosphere C4 300Å column (150 x 3.2 mm, particle size of 5 μm; Alltech, Lexington, KY) in combination with a Symmetry 300 C4 guard column (2.1 × 10 mm, particle size of 3.5 μm; Waters, Milford, MA) in positive ionization mode. The peak areas of the target lipids were integrated, and raw data were exported using LCQuan software (version 2; Thermo Fisher Scientific, Waltham, MA), yielding response ratios to the appropriate internal ISTD for each metabolite; these ratios were used for further statistical analysis.

During the GC-MS and LC-MS experiments, quality control (QC) samples were prepared by pooling equal amounts of plasma from each sample, then dividing the pooled samples into aliquots; these QC samples were used to check the performance of the LC-MS platform as well as to identify temporal trends in the acquired data. The relative standard deviation (RSD) of each target peak in the QC samples was used to confirm the quality of the data acquired from each analytical platform.

2.3.2 UPE measurements

UPE signals were measured from the same cohort of 44 subjects. A photomultiplier system (provided by Meluna Research B.V., Geldermalsen, the Netherland) with two detecting heads located at the top of a dark chamber was used to measure UPE. Each detecting head contains a 9558QB photomultiplier tube within a spectral sensitivity range of 190-650 nm (Electron Tubes Enterprises Ltd., Ruislip, UK) and an electronically controlled shutter. The dark chamber was maintained at 20±1.0°C. The settings used to measure UPE have been described previously [31], [32]. All measurements were controlled automatically via computer-driven software. UPE signals were measured at the following four hand surfaces: left dorsal (LD), right dorsal (RD), left palm (LP), and right palm (RP).

2.4 Data preprocessing and statistical analysis

2.4.1 Metabolomics data processing and analysis

Before performing a statistical analysis on the metabolomics data, the log-transformed dataset was processed using various scaling options (i.e., autoscaling, range scaling, and pareto-scaling) using the online software package MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) [33]. The pareto-scaling approach (mean-centered and scaling by the square root of the standard deviation of each variable) was chosen because it provided the best grouping performance, consistently explaining the largest variabilities when considering the same number of principal components (both 2D and 3D) [34]–[36]. Preliminary selection of variables prior to multivariate analysis is needed in order to: *i*) limit the dataset of variables for reliably separating the sample groups; *ii*) remove irrelevant and/or confounding variables; and *iii*) decide which variables to retain for the multivariate analysis; however, this selection is not needed in order to identify potential biomarkers, which has been applied in metabolic profiling studies [37], using *p*-values obtained from a one-way analysis of variance (ANOVA) ($p<0.1$) in GC-MS and LC-MS. Multiple comparisons, including principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA), were conducted using MetaboAnalyst 3.0, which provides standard validation information, including cross-validation and a permutation test to prevent over-fit of the models to the data [33].

2.4.2 Acquisition of UPE data and derived parameters

From a 50-ms bin, the following ten UPE properties were calculated from all four hand surfaces: strength, FF0, FF1, FF2, alpha, gamma, theta, phi, SSI, and SSR [31], [32], [38]. Thus, a total of 40 UPE parameters were obtained from each subject.

2.4.3 Correlation analysis

The statistics software package R (version 3.0.3) was used to calculate Spearman's rank correlation coefficient in order to examine the relationship between the

metabolites and UPE parameters. A graphical overview of the correlation networks was created using CytoScape version 3.3.0 (http://www.cytoscape.org) with the MetScape plugin [39], [40]. Positive and negative correlations are indicated by positive and negative values of r, respectively.

3. Results and Discussion

3.1 Subtyping based on plasma metabolomics

TCM-based diagnostics is based on several standard diagnostic steps, including inspection, listening and smelling, inquiry and question, and palpation. The outcomes from these steps are combined to create an individual profile, which is used to establish a diagnosis. In this study, 26 variables were determined using TCM-based diagnostics [17]. From this exploratory study, plasma samples were used to obtain evidence-based information that was used to help subtype the pre-T2DM subjects.

We used two validated metabolomics methods based on GC-MS and LC-MS. GC-MS yielded 147 untargeted metabolites, and LC-MS yielded 110 targeted metabolites; all of these metabolites were included in the total metabolomics profile. The metabolites detected by GC-MS included various metabolic classes, but primarily included amine metabolites, organic acids, sugars, and fatty acids such as LPA and LPA-derived metabolites. The metabolites detected by LC-MS included seven classes of lipids, including both polar lipids such as phosphatidylcholine, phosphoethanolamine, lyso-phosphatidylcholine, lyso-phosphoethanolamine, and sphingomyelin and non-polar lipids such as cholesterol esters (ChEs) and triglycerides (TGs). Given the relatively small number of subjects (44) compared to the large number of total variables (257), a first step in selecting variables was required before proceeding with a multivariate analysis; this step allowed us to optimize the variable/object ratio for discriminant type approaches, and it allowed us to remove potential irrelevant and/or confounding variables [37]. A total of 32 preliminary variables were selected based on an ANOVA analysis ($p<0.1$); these variables included 14 plasma metabolites identified by GC-MS and 17 plasma lipids identified by LC-MS. These variables were then used for subsequent multivariate analyses, including PCA, Partial least squares discriminant analysis (PLSDA), and OPLS-DA (see S-table 1 and S-fig. 1).

The first step in our analysis focused on investigating whether plasma metabolomics could be used to discriminate between the three TCM-based syndrome subtypes of pre-T2DM (i.e., QYD vs. QYD_Damp, QYD vs. QYD_Stag, and QYD_Damp vs. QYD_Stag). A 3D PCA plot was used to visualize the natural distribution of the three groups in 3-dimensional space [37], [41]. The first three principal components analyzed described 66.5% of the total variance in the plasma metabolome (Fig. 1). We found no large distance between the three subtypes reflected by PCA, which is not surprising given that their TCM-based diagnostic patterns are all-linked (interrelated) and TCM-based syndromes subtypes are not independent but with dynamic changes towards different direction [13], [14]. However, we did observe tendency of clusters within the subtypes, with minor overlap in the PCA analysis.

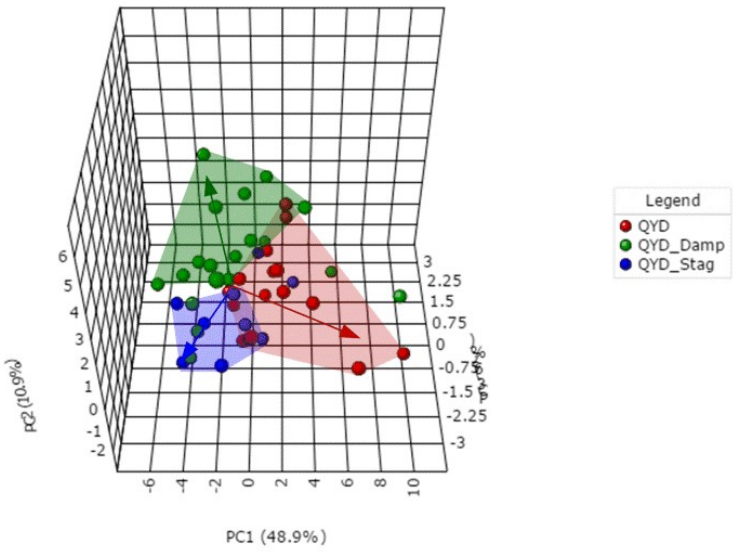

Fig. 1: 3D PCA score plot based on plasma metabolite profiling, acquired and integrated from GC-MS and LC-MS, for visualizing clusters of the three pre-T2DM subtypes (QYD, QYD_Damp and QYD_Stag).

Next, we used supervised models, including LDA, PLSDA, and OPLS-DA, in order to identify relevant plasma metabolites (S-fig. 2). The OPLS-DA model provided the highest R^2 and Q^2 values and was therefore used to identify the most relevant variables based on score plots [42][43]. Furthermore, permutation tests with 1000 iterations (p<0.05) showed a good performance of the model. Fig. 2 shows the OPLS-DA score plots for the first two principal components between each pair of subtypes (see also S-fig. 3).

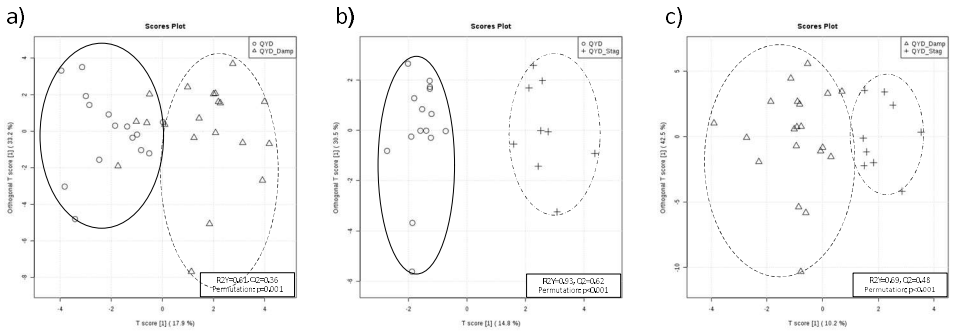

Fig. 2: OPLS-DA score plots of plasma metabolite profilling (integrated from LC-MS and GC-MS) for comparing differences between each pair of subtypes. a) QYD vs. QYD_Damp; b) QYD vs. QYD_Stag; and c) QYD_Damp vs. QYD_Stag.

Table 1 summarizes the relevant metabolites (defined as the combination of covariance |p[1]|>0.7 and correlation coefficient |p(corr)|>0.3[43]) for each pair of groups, together with their contribution between each pair of subtypes (QYD vs. QYD_Damp, QYD vs. QYD_Stag, and QYD_Stag vs. QYD_Damp). As shown in Table 1, 15 of the 18 metabolites that contributed to the differentiation between QYD and QYD_Damp are long-chain non-polar lipids (11 TGs and 4 ChEs); these metabolites were higher in the QYD_Damp group than in the QYD group. Fourteen of these same metabolites (10 TGs and 4 ChEs) were also higher in the QYD_Stag group than in the QYD group. Thus, we conclude that an increase in long-chain non-polar lipids is associated with the QYD_Damp and QYD_Stag groups.

The physiological mechanisms that underlie the early phases of T2DM have been linked to lifestyle issues such as the consumption of a diet high in fat and calories [44]–[47], which is similar to chronic fatigue syndrome and/or mild inflammatory status [17]. Triglycerides are the precursors of phospholipids, which are the building blocks of cell membranes and play an important role in energy homeostasis. Cholesterol esters are a stored form of cholesterol that is normally exported as a high-density lipoprotein (HDL) and returned to the liver. High levels of cholesterol and triglycerides (hypercholesterolemia and hypertriglyceridemia, respectively) are associated with fat accumulation, atherosclerosis, and cardiovascular disease [48], [49]. Therefore, patients in the pre-T2DM subgroups QYD_Damp and QYD_Stag may have an increased risk of developing atherosclerosis and/or cardiovascular disease in a later disease stage.

Table 1: List of relevant metabolites identified by OPLS-DA

QYD_Damp. vs. QYD		**QYD_Stag. vs. QYD**		**QYD_Stag. vs. QYD_Damp.**	
Metabolite	Change	Metabolite	Change	Metabolite	Change
C52_5_TG	↑	C22_5_ChE	↑	Beta-Alanine	↓
C54_6_TG	↑	C54_7_TG	↑	6926ukx10*	↓
C54_5_TG	↑	C54_6_TG	↑	1-Methylhistidine % 10227\01.03 uk x 45*	↓
C54_7_TG	↑	C58_10_TG	↑	31944uk05*	↓
C56_8_TG	↑	C52_6_TG	↑		
C56_7_TG	↑	C56_8_TG	↑		
C56_9_TG	↑	C18_3_ChE	↑		
C58_8_TG	↑	C52_5_TG	↑		
1-Methylhistidine % 10227\01.03 uk x 45*	↑	C22_6_ChE	↑		
C58_9_TG	↑	C56_7_TG	↑		
C52_6_TG	↑	C56_9_TG	↑		
C18_3_ChE	↑	C20_3_ChE	↑		
C16_0_ChE	↑	C54_5_TG	↑		
C22_6_ChE	↑	C58_9_TG	↑		
C20_3_ChE	↑	31944uk05	↓		
Creatinine	↑				
1-Palmitoyl-L-alpha-lysophosphatidic acid	↓				
1-Stearoyl-sn-glycero-3-phosphocholine	↓				

↑, increase; ↓, decrease.
*, Structural unidentified metabolites in GC-MS untargeted measurement.

Although TGs and ChEs were increased in both the QYD_Damp and QYD_Stag groups relative to the QYD group, these two groups had several metabolic differences (Table 1). The relatively lower levels of amine metabolites in the QYD_Stag group (and/or the relatively higher levels in the QYD_Damp group) may suggest that the difference between the QYD_Stag and QYD_Damp subtypes is based primarily in differences in the TCA cycle and/or muscle catabolism processes [50]. In summary, 23 metabolites contribute to the stratification of pre-T2DM subtypes. Thus, different subtypes of pre-T2DM may be discriminated based on differences in plasma metabolomics, including plasma lipids and amine metabolites.

Previously, Wei, et al. reported that urine metabolomics can be used to reflect changes in carbohydrate metabolism and renal function in patients with QYD_Stag syndrome; specifically, two of the three TCM-based subtypes could be stratified [17]. In contrast, plasma metabolomics provides stratification among the three subgroups, which is likely due to the use of a lipidomics platform, which measures a class of compounds that cannot be measured using urine metabolomics. This finding suggests that measuring lipid metabolomics is important for accurately subtyping pre-T2DM.

3.2 Correlation between metabolomics and UPE

We also measured UPE in our cohort of subjects with pre-T2DM. Stratification of the three TCM-based syndrome subtypes using 16 UPE parameters has been studied previously [27]. Given that both plasma metabolomics and UPE can stratify subjects into pre-T2DM subgroups, plasma metabolomics data may be used to obtain biochemical insight into UPE [51]–[53]. To explore the relationship between these two approaches, we used Spearman's rank correlation coefficient to establish a correlation-based metabolite-to-UPE network. Such a correlation

network may provide additional information that may further stratify disease subtypes and may provide a biochemical interpretation of UPE parameters.

We generated correlation networks between the 23 metabolites and 16 UPE parameters that contributed to the stratification of subtypes in order to visualize the most relevant correlations related to the three subtypes (Figure 3). These networks revealed clearly distinct distributions of UPE-to-metabolite correlations between the three subtypes. Specifically, the QYD_Damp subtype contained relatively few correlations, whereas the QYD and QYD_Stag subtypes contained relatively more positive and negative correlations, respectively. Moreover, although clear links are visible between UPE parameters and specific classes of metabolites (e.g., TGs and ChEs), the correlations differ among the subtypes. The differences between the three networks provide a clear distinction between the subgroups and might serve as an additional diagnostic tool.

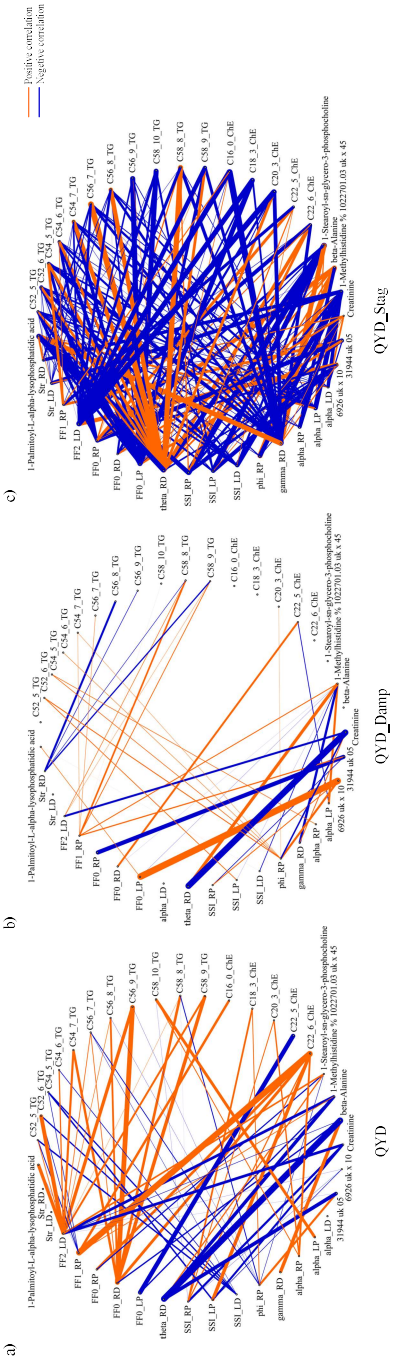

Fig. 3: Correlation networks between metabolic variables and UPE parameters for the indicated pre-T2DM subtypes. Only the correlations between metabolites and UPE parameters with $|r|>0.3$ are plotted。

4. Conclusions and perspectives

Here, we report that plasma metabolomics can be used to stratify the three TCM-based subtypes of early-stage type 2 diabetes, providing better stratification than urine metabolomics. Specifically, increased levels of plasma lipids such as TGs and ChEs may indicate a relatively higher risk of developing cardiovascular disease among patients with specific subtypes. In addition, we used UPE as a non-invasive method for subtyping pre-T2DM, and the UPE parameters were correlated with specific plasma metabolites—primarily lipid metabolites—and these correlations differed among the three subtypes. Thus, combining UPE and plasma metabolomics provides additional insight into the diagnosis of disease and the underlying biochemistry of UPE from a systems biology perspective.

The ability to identify the pre-T2DM syndrome subtype based on TCM is essential for achieving a personalized treatment plan, thereby significantly improving patient care. These results provide a window of opportunity for combining metabolomics with UPE in order to achieve personalized medicine and improve the early diagnosis of disease. Nevertheless, metabolomics platforms do not necessarily cover the entire metabolome, and choices must be made based on the metabolomics platforms that are currently available. Given the difficulties associated with obtaining comprehensive information regarding the dynamic changes reflected by measuring metabolomics, linking metabolomics to UPE under the guidance of TCM-based diagnostics is particularly attractive, promoting the early diagnosis of T2DM. Additional research is needed in order to expand the correlation networks between metabolites and UPE parameters. In addition, current approaches for stratifying T2DM are based on various criteria, which must be consistent for further clinical diagnosis. Therefore, additional research is needed in order to understand TCM-based concepts such as disease syndromes and subtypes.

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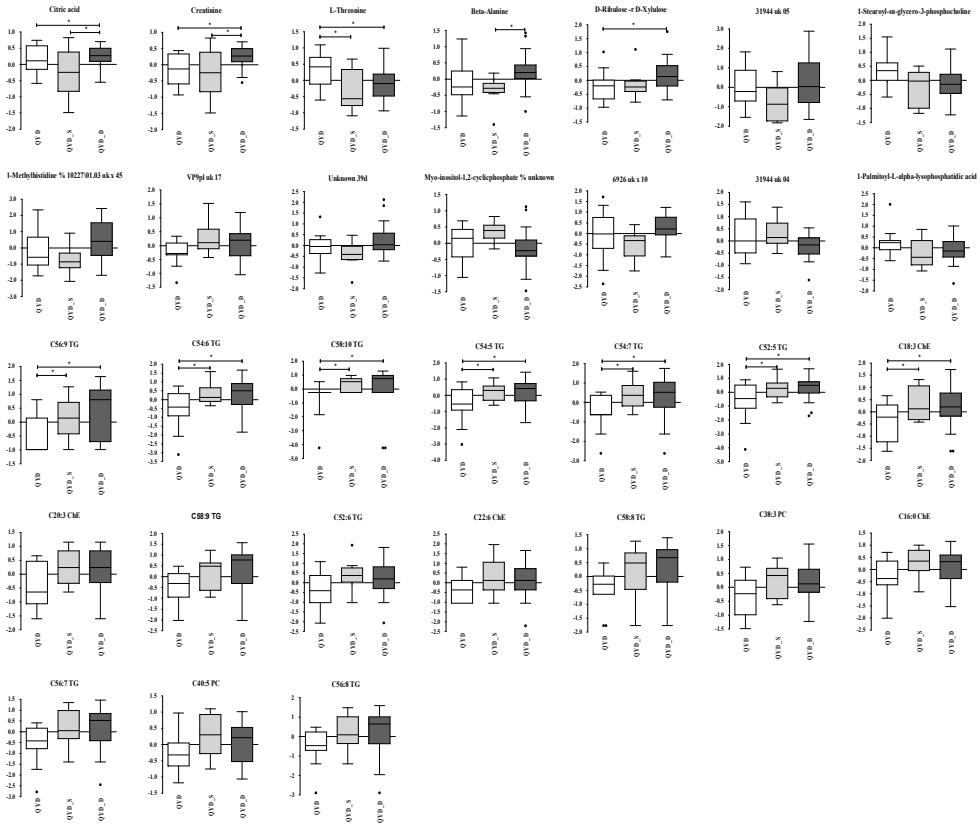
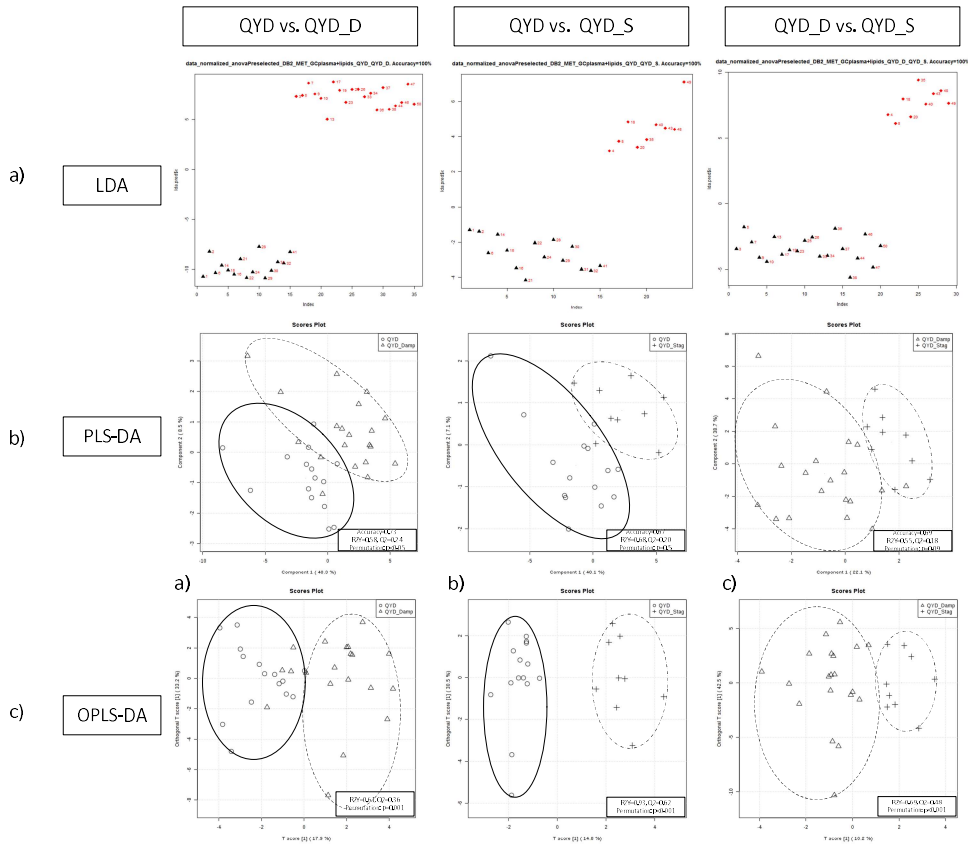
Supplementary information

S-table 1. Preliminary variables in the MS data identified by ANOVA (p<0.1)

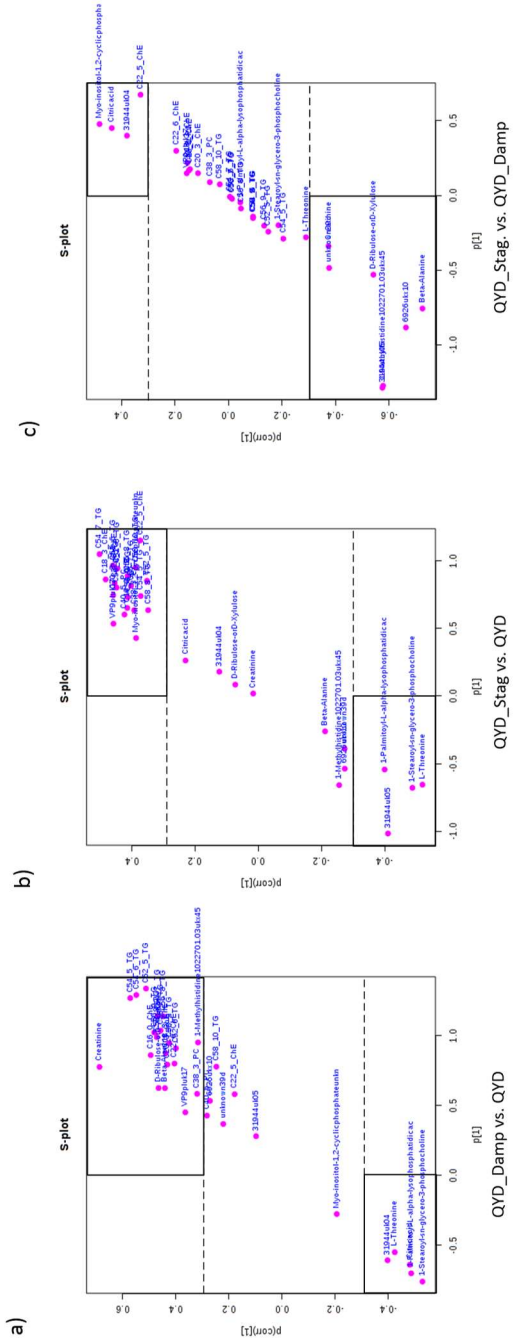
Compounds	*p*-value	Post-hoc test(Fisher's LSD) between groups
Metabolites in GC-MS		
Citric acid	0.018	QYD - QYD_Damp; QYD_Damp - QYD_Stag
Creatinine	0.019	QYD – QYD_Damp; QYD_Damp - QYD_Stag
L-Threonine	0.028	QYD - QYD_Damp; QYD - QYD_Stag
Beta-Alanine	0.039	QYD_Damp - QYD_Stag
D-Ribulose or D-Xylulose	0.049	QYD – QYD_Damp
31944 uk 05	0.052	QYD - QYD_Stag; QYD_Damp - QYD_Stag
1-Stearoyl-sn-glycero-3-phosphocholine	0.054	QYD - QYD_Damp; QYD - QYD_Stag
1-Methylhistidine % 10227\01.03 uk x 45	0.054	QYD_Damp - QYD_Stag
VP9pl uk17	0.054	QYD – QYD_Damp; QYD – QYD_Stag
unknown 39d	0.06	QYD_Damp - QYD_Stag
Myo-inositol-1,2-cyclicphosphate % unknown	0.065	QYD_Damp- QYD_Stag
6926 uk x 10	0.068	QYD_Damp - QYD_Stag
31944 uk 04	0.073	QYD_Damp- QYD_Stag
1-Palmitoyl-L-alpha-lysophosphatidic acid	0.091	QYD - QYD_Damp; QYD - QYD_Stag
Metabolites in LC-MS		
C56_9_TG	0.005	QYD - QYD_Damp; QYD - QYD_Stag
C54_6_TG	0.024	QYD - QYD_Damp; QYD - QYD_Stag
C58_10_TG	0.027	QYD - QYD_Damp; QYD - QYD_Stag
C54_5_TG	0.031	QYD - QYD_Damp; QYD - QYD_Stag
C54_7_TG	0.035	QYD - QYD_Damp; QYD - QYD_Stag
C52_5_TG	0.044	QYD - QYD_Damp; QYD - QYD_Stag
C18_3_ChE	0.050	QYD - QYD_Damp; QYD - QYD_Stag
C20_3_ChE	0.056	QYD - QYD_Damp; QYD - QYD_Stag
C58_9_TG	0.056	QYD - QYD_Damp
C52_6_TG	0.061	QYD - QYD_Damp; QYD - QYD_Stag
C22_6_ChE	0.070	QYD - QYD_Damp; QYD - QYD_Stag
C58_8_TG	0.081	QYD - QYD_Damp
C38_3_PC	0.085	QYD - QYD_Damp; QYD - QYD_Stag
C16_0_ChE	0.085	QYD - QYD_Damp; QYD - QYD_Stag
C56_7_TG	0.086	QYD - QYD_Damp; QYD - QYD_Stag
C40_5_PC	0.091	QYD - QYD_Stag
C56_8_TG	0.092	QYD - QYD_Damp



S-fig. 1: Box plots summarizing the 32 preliminary variables (plasma metabolites detected by LC-MS and GC-MS, identified by ANOVA (p<0.1)) in pre-T2DM subjects. Individual metabolite (peak area ratio between target metabolites and relevant internal standard) for the three groups are illustrated using boxplots after logarithmic transformation and pareto-scaling for data normal distribution. The metabolites which differed significantly based on ANOVA (p<0.05) were then followed by a post-hoc analysis (Fisher's least significant difference method) to show between which two groups the differences are significant (*).



S-fig. 2: Performance comparison between three supervised multivariate analysis models (LDA, PLSDA, and OPLS-DA), based on metabolite profiling in plasma of pre-T2DM subjects detected and integrated by LC-MS and GC-MS. A Permutation test with 1000 iterations (p<0.05) as well as the R2 and Q2 showed that the OPLS-DA model performed best.



S-fig. 3: Loading plots from the OPLS-DA analysis based on metabolite profiling in plasma of pre-T2DM subjects detected and integrated by LC-MS and GC-MS. Magnitude score (covariance) is combined with the reliability (correlation) results, for indicating variable influence that contributed to separation between each pair of subtypes. a) The QYD_Damp subtype versus the QYD subtype; b) the QYD_Stag subtype versus the QYD subtype; c) the QYD_Stag subtype versus the QYD_Damp subtype.

Chapter 7

Summary, Conclusions, and Perspectives

1. Summary and Conclusions

1. 1 Systems-based evaluation of chronic disease

Chronic diseases such as rheumatoid arthritis (RA) and type 2 diabetes mellitus (T2DM) involve complex processes and pathologies that result in multiple interactions within the body, including inflammatory symptoms, complications, an increased risk of morbidity, loss of mobility, and mortality. Under the chronic disease state, complex responses often lead to unpredictable, subtle perturbations and dynamic changes. A systems biology–based analytical approach that integrates comprehensive data may provide unique insight into the underlying pathophysiological mechanisms. Rather than looking for a single target to characterize chronic disease, the studies in this thesis investigated systematic processes relevant to chronic disease using systems level analyses. Specifically, metabolomics was used to measure a large set of small molecule metabolites in combination with measuring spontaneous ultra-weak photon emission (UPE).

In research, animal models of chronic disease are widely used due to their many similarities with human patients. In **Chapter 2** and **Chapter 3**, metabolomics, which provides a comprehensive measure of small molecule metabolites as a readout of physiological status, was applied to mice with collagen-induced arthritis (CIA)—a commonly used mouse model of RA—to evaluate interactions at the metabolic level under chronic disease conditions. Oxylipins are bioactive lipid mediators synthesized from polyunsaturated fatty acids. Because of their important role in inflammatory processes, we measured plasma oxylipin levels in CIA mice in order to gain insight into inflammation- and ROS-related metabolites (**Chapter 2**). Compared to control mice, we found dysregulated oxylipins in CIA mice, reflecting inflammation and increased ROS levels. In addition, we found that collagen-induced arthritis may be associated with a dysregulation of apoptosis, perhaps due to activated NF-κB as a result of reduced levels of PPAR-γ ligands. Given that free amino acids—and their derivative biogenic amines—play essential

roles in both energy production and protein synthesis/degradation, we measured plasma levels of amino acid–based metabolites in CIA mice in order to gain a different perspective regarding the levels of energetic metabolites (**Chapter 3**). Our finding of reduced levels of free amino acids together with their biogenic metabolites suggests a link between arthritis and muscle wasting/energy expenditure.

From these studies, we found that both oxylipins and amine metabolites reflecting arthritis but from different perspectives with respect to interpreting putative pathophysiological mechanisms. Systems biology–based metabolomics can provide new ways of improving the diagnosis of chronic disease and can provide insight into of the underlying pathophysiological mechanisms.

Correlation network–based analyses provide the opportunity to integrate data obtained from different technical platforms, thereby providing a correlation-based understanding of systemic interactions and regulation[1]. Interaction networks based on correlation analyses can be visualized and analyzed using software such as Cytoscape[2]. UPE is a non-invasive method for measuring photons emitted from the surface of body and may be correlated with oxidative metabolic processes[3], [4]. UPE intensity was increased in CIA mice[5]. In **Chapter 4**, we performed a correlation networks–based study to explore the relationship between metabolic processes and UPE by integrating the metabolic data described in **Chapter 2** and **Chapter 3** with UPE data measured in the same group of mice. This combination study yielded valuable information and provided insight into the disease process from a systems perspective. Our results revealed that the increase in UPE with arthritis is associated with a specific metabolites processes (primarily lipid oxidation, inflammatory metabolites and/or ROS-mediated metabolic processes). These results provide a window of knowledge into in our attempt to integrate different datasets and analyze complex interactions in RA, and these results provide further evidence to support the relationship between metabolic processes and UPE.

1.2 Personalized medicine-based phenotyping using TCM-based principles

Epidemiology studies have shown a rapid increase in the prevalence of clinic diseases, as well as a large undiagnosed patient population due primarily to mild clinical symptoms. Indeed, mild physiological perturbations can be present for years before the appearance of severe symptoms. The ability to predict disease early and to dynamically observe chronic disease remain challenging and if solved can—to a certain extent—prevent the development of irreversible lesions. In addition, if left undiagnosed long-term, chronic disease can develop in different directions, producing a wide range of phenotypes. Moreover, treatments based on generic observations (i.e., the "one drug-one target-one disease" or "one-size-fits-all" approach) are extremely limited, particularly in the early phases of a disease in which a personalized, systems-based approach is needed. Developing a personalized approach based on systems biology will reveal the unique clinical characteristics in individual patients and may shed light on the complexity and variability of chronic disease.

Traditional Chinese medicine (TCM) is based on a systems view combined with personalized strategies to provide descriptions of disease syndromes and subtypes as a guide to diagnose early syndromes of chronic diseases, an approach that has been shown to improve knowledge regarding personalized diagnostics. Based on TCM-based diagnostics, metabolomics may provide evidence-based biological mechanisms, thereby leading to personalized medicine and establishing a bridge between TCM and Western medicine. UPE reflects both the physiological and pathological status and is a potential tool for clinical diagnostics at the systems level[6] [7]; moreover, TCM-based diagnostics, metabolomics, and UPE each contributes to personalized medicine for treating chronic diseases. Integrating UPE with metabolomics under the guide of TCM-based diagnostics may create new opportunities for personalized medicine, systems-based diagnostics, and systems-based interventions for treating chronic disease (reviewed in **Chapter 5**). Based on the ideas described in **Chapter 5**, we performed an explorative study by combining

metabolomics and UPE with TCM-based diagnostics (**Chapter 6**). We examined the relationship between metabolomics and TCM-guided subtypes of early-stage T2DM ("pre-T2DM"), and we identified key metabolites—primarily plasma lipids—that contribute to phenotypic subtypes. In addition, these key plasma lipids were correlated with the UPE parameters that were used to stratify the same cohort of pre-T2DM diabetic subjects, and these correlations differed among subtypes. These differences between subgroups may be used to establish correlation networks for improved diagnostics.

2. Perspectives

Analyses at the systems biology level offer many opportunities for understanding chronic disease from various perspectives by integrating various sets of information. This systems approach requires collaboration among scientists from various fields, including medicine, analytical biology, chemical biology, and bioinformatics. Metabolomics is a systems-based approach for studying comprehensive pathophysiological mechanisms in chronic disease. However, before conducting a metabolomics study, one must select the most suitable metabolomics platform. Future studies require additional metabolomics platforms in order to supplement biochemical information and to provide a link to other techniques, including UPE.

In this thesis, UPE was measured at specific positions on the body. Measuring a larger number of anatomical positions may provide additional information regarding disease, thus helping improve our understanding of personalized medicine. Here, we visualized the relationship between metabolomics and UPE using the statistic network tool Cytoscape (www.cytoscape.org) and based on our Spearman's rank correlation analysis presents in **Chapter 4** and **Chapter 6**. In the

future, correlation-based networks may provide more comprehensive data for exploring interactions under a variety of disease conditions.

Animal models have many advantages for studying chronic disease. Specifically, the researcher can easily control and/or exclude potential confounding factors that may affect the results, including age, gender, genetic background, duration of disease, and drug exposure. However, although animal models are qualitatively similar to chronic disease in patients, metabolic differences clearly exist among patients, due to genetic differences and other factors; therefore, any biological mechanisms identified in animal models must be carefully compared to patients and validated in clinical studies involving patients.

Our study in human subjects (**Chapter 6**) illustrates the feasibility of stratifying patients using metabolomics guided by TCM-based diagnostics and provides a molecular correlate to UPE, thus illustrating that both metabolomics and UPE can be used to identify patient subtypes of pre-T2DM. Importantly, combining metabolomics and UPE measurements provides evidence-based data to support TCM-based diagnostics. Nevertheless, additional study is clearly needed in order to expand our knowledge and to achieve a systems view-based approach to personalized diagnostics.

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SAMENVATTING

Chronische ziekten, zoals reumatoïde artritis (RA) en type 2 Diabetes (T2DM), zijn gerelateerd aan complexe pathologische processen en met mogelijk ernstige consequenties zoals inflammatoire symptomen, een verhoogd risico op ziekte, verlies van mobiliteit en sterfte. In een chronische ziektetoestand ontstaan vaak complexe biologische responsen, die leiden tot onvoorspelbare subtiele veranderingen in dynamische processen. Een systeembiologische analytische benadering, die omvangrijke datasets integreert, kan wellicht een uniek inzicht geven in de onderliggende pathofysiologische processen. In plaats van te zoeken naar één enkele variabele voor de beschrijving van een ziekteproces, richt de research in dit proefschrift zich op systemische beschrijving van chronische ziekten door gebruik te maken van methoden die systemische relevante gegevens opleveren. In het bijzonder wordt metabolomics gebruikt om grote datasets te genereren rond de concentratie van kleine metabolieten in combinatie met ultra-lage fotonemissie (Ultra-low Photon Emission, UPE) om een systeemperspectief te verkrijgen.

Gezien de relatief grote overeenkomsten met de humane situatie is het gebruikelijk om in research diermodellen voor chronische ziekten te gebruiken. In **Hoofdstuk 2 en Hoofdstuk 3** is metabolomics als methodiek gebruikt om op uitgebreide schaal de concentraties van kleine metabolieten te meten als uitlezing van de fysiologische status. Dit is toegepast op een collageen-geïnduceerd artritis muizenmodel (CIA) , dat veel gebruikt wordt in RA research, om relaties op het metabolietenniveau op te sporen in chronische situaties. Oxylipiden zijn bioactieve lipidenmediatoren, die gesynthetiseerd worden uit meervoudig onverzadigde vetzuren en die een belangrijke rol spelen in ontstekingsprocessen. De plasma concentraties van oxylipiden in CIA-muizen werden gemeten om inzicht te verkrijgen in ontstekingsprocessen en metabolieten, gerelateerd aan reactieve zuurstofverbindingen **(Hoofdstuk 2)**. In vergelijking met controle muizen, werden ontregelde oxylipiden aangetroffen in de CIA-muizen, die een reflectie kunnen zijn

van ontsteking en toegenomen reactieve zuurstofverbindingen. Bovendien, werd gevonden dat de collageen geïnduceerde artritis geassocieerd kan zijn met een ontregeling van de apoptose, wellicht door activatie van NF-κB ontstaan door gereduceerde niveaus van PPAR-γ liganden. Omdat vrije aminozuren – en de daarvan afgeleide biogene aminen – een essentiële rol spelen in zowel de energieproductie als de eiwit-synthese/degradatie, zijn de plasmaconcentraties van aminozuur-gerelateerde metabolieten in CIA-muizen ook gemeten, zodat er een beter inzicht verkregen werd rond de metabolieten, betrokken bij energieregulatie (**Hoofdstuk 3**). Er werden verlaagde niveaus van zowel vrije aminozuren als biogene metabolieten gevonden hetgeen suggereert dat er een link is tussen CIA en spierafbraak/energieverbruik. Uit deze studies werd gevonden dat oxylipiden en amine-metabolieten een weerspiegeling geven van CIA, maar beiden vanuit een ander perspectief op de vermeende pathofysiologische processen. Systeembiologie gebaseerd op metabolomics, kan derhalve nieuwe inzichten verschaffen in de pathofysiologie van onderliggende processen.

De op correlatie-netwerk gebaseerde analyses bieden de mogelijkheid om gegevens te integreren, die verkregen zijn uit verschillende technische platforms, zodat een correlatie-gebaseerd begrip verkregen kan worden van systemische interacties en regulatie. UPE is een niet-invasieve methode om fotonen te meten, waarbij het belangrijk is dat de UPE-emissie gecorreleerd kan worden met oxidatieve metabole processen. In **Hoofdstuk 4** is correlatie-netwerk analyse toegepast om de relatie tussen metabole processen en UPE te verkennen, door de data gemeten aan dezelfde muizen van **Hoofdstuk 2 en Hoofdstuk 3** te integreren. Deze studie leverde belangrijke informatie op en gaf inzicht in het ziekteproces vanuit een systeemvisie. Zo blijkt uit deze studie dat de toename van UPE bij de voortgang van artritis geassocieerd is met specifieke metabole processen, in het bijzonder lipidenoxidatie, ontsteking-gerelateerde metabolieten en/of ROS-gemedieerde processen. Deze resultaten betekenen een belangrijke stap voorwaarts in de wijze waarop datasets geïntegreerd kunnen worden om complexe processen

in RA te bestuderen, maar leveren ook een verdere onderbouwing over de relatie tussen metabole processen en UPE.

Diermodellen hebben specifieke voordelen voor de bestudering van chronische processen. In het bijzonder is het voor de researcher eenvoudiger om potentieel verstorende factoren van de resultaten en de interpretatie daarvan uit te sluiten of te controleren. Onder dergelijk factoren kunnen leeftijd, geslacht, genetische achtergrond, duur van de ziekte, blootstelling aan medicatie etc. gerekend worden. Echter ondanks het feit dat diermodellen kwalitatieve overeenkomsten met de chronische ziekteprocessen bij de mens hebben, zijn er ook duidelijke verschillen door genetische variatie en andere factoren. Derhalve moeten biologische mechanismen die relevant gevonden worden in diermodellen, zorgvuldig geëvalueerd worden in patiënten en gevalideerd worden in humane klinische studies.

Epidemiologische studies laten een snelle toename zien in het voorkomen van chronische ziekten, waarbij een groot aantal patiënten met lichte klinische symptomen niet gediagnosticeerd worden. Zo kunnen milde fysiologische verstoringen over vele jaren plaatsvinden voordat zich ernstige symptomen voordoen. Niet gediagnosticeerde patiënten kunnen over langere tijd, op een verschillende wijze, chronische ziekten ontwikkelen met een uiteenlopende schaal van fenotypen. Behandelingen met een generieke aanpak (one drug – one target ; one-size-fits-all benadering) zijn uiterst beperkt vooral in de vroege fasen van ziekten, waarbij een geïndividualiseerde systeemaanpak noodzakelijk is. Ontwikkeling van een geïndividualiseerde benadering, gebaseerd op systeembiologie, kan de unieke klinische karakteristieken opleveren in individuele patiënten, zodat er meer inzicht wordt verkregen over de complexiteit en diversiteit van chronische ziekten.

Traditionele Chinese geneeskunde (TCM) is gebaseerd op een systeemvisie gecombineerd met geïndividualiseerde interventie-strategieën. Dit kan gebruikt

worden, om een betere beschrijving van ziektesyndromen of subtypen te verkregen, om vroege stadia van syndromen of chronische ziekten op te sporen. Deze beschrijving kan dan als diagnosegids dienen, in een vroeg stadium van syndromen van chronische ziekten en verrijkt tevens onze kennis voor geïndividualiseerde diagnose. Metabolomics kan meer wetenschappelijk bewijs leveren van op TCM-gebaseerde diagnostiek door opsporing van de onderliggende biologische mechanismen en geïndividualiseerde diagnose, daarbij een brugvormend tussen TCM en Westerse geneeskunde. UPE reflecteert zowel de fysiologische als de pathologische toestand en heeft potentie als klinisch systeemdiagnostische methode. De combinatie van op TCM gebaseerde diagnostische concepten, metabolomics en UPE, kunnen elk met een unieke inbreng bijdragen aan de ontwikkeling en behandeling van chronische ziekten. Op basis van deze geïntegreerde aanpak kunnen er nieuwe mogelijkheden worden gecreëerd voor geïndividualiseerde geneeskunde onderbouwd met systeemdiagnose en systeembehandeling voor chronische ziekten, zoals beschreven in **Hoofdstuk 5**. Op basis van het gedachtegoed in Hoofdstuk 5 is er een verkennende studie uitgevoerd door de combinatie van metabolomics, UPE en TCM-diagnose, zie **Hoofdstuk 6**. In het bijzonder is de vermeende relatie tussen metabolomics en op TCM gebaseerde subtypering van vroeg stadium diabetes (pre-T2DM) bestudeerd en zijn de sleutelmetabolieten – voornamelijk plasmalipiden – die bijdragen aan de subtypering geïdentificeerd. Daarnaast werden deze plasma sleutelmetabolieten gecorreleerd met de UPE parameters, die eveneens gebruikt konden worden om dezelfde subtypen te identificeren in het cohort van pre-T2DM patiënten. Deze correlaties bleken verschillend te zijn voor verschillende subtypen. Deze verschillen zouden gebruikt kunnen worden voor het differentiëren tussen subtypen. Op basis van deze verschillen tussen subtypen kunnen mogelijk correlatienetwerken ontworpen worden om verbeterde diagnostiek te verkrijgen.

In de humane studie, beschreven in **Hoofdstuk 6**, is de haalbaarheid voor subtypering van patiënten met metabolomics, TCM-diagnose en UPE geïllustreerd. Deze studie liet zien dat zowel metabolomics als UPE toegepast kunnen worden

voor de subtypering van pre-T2DM patiënten. Daarenboven geeft de combinatie van metabolomics met UPE biomedisch bewijs voor de onderbouwing van TCM-gebaseerde diagnostiek. Ondanks deze veelbelovende resultaten, zijn er aanvullende studies nodig om de kennis verder te verrijken tot een systeemvisie gebaseerd op geïndividualiseerde diagnostiek.

Samenvattend, analyse uitgevoerd op een systeembiologisch niveau, verrijkt de kennis van chronische ziekten vanuit verschillende perspectieven door de integratie van verschillende bronnen van informatie. Deze systeembenadering vereist samenwerking tussen wetenschappers uit verschillende velden waaronder geneeskunde, analytische chemie , biologie en bioinformatica. In dit proefschrift is metabolomics gebruikt in een systeembenadering voor de bestudering van pathofysiologische mechanismen in chronische ziekten door de meting van een omvangrijk aantal kleine metabolieten en door het combineren daarvan met spontane UPE. Deze resultaten bevestigen de mogelijkheid om verschillende datasets te kunnen integreren en om complexe interacties te bestuderen in chronische ziekten. Bovendien verschaffen deze resultaten aanvullend bewijs voor het aantonen van een relatie tussen metabole processen en UPE.

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(About the author)

Min He was born on September 8,1984 in Qiqihar, Heilongjiang Province, P.R. China. In 2000, after attending Qiqihar No. 1 High School, which is a key high school in Heilongjiang Province, she completed her 3-year secondary school education in 2003 and obtained her high school diploma. In September of the same year, she started her 4-year Bachelor's study (major in Pharmacy) at Jiamusi University in Heilongjiang Province, where her interests in pharmacy grew; she received her BSc degree in 2007. Next, she started her Master's study at the Changchun University of Chinese Medicine, in which she specialized in Pharmaceutical Chemistry, with a focus on bioactivity studies of anti-bacterial compounds purified from herbal materials.

After obtaining her MSc degree, her enthusiasm for scientific research deepened, and she applied successfully for the Chinese Scholarship Council (CSC) scholarship "Chinese Government Graduate Student Overseas Study Program" to study as a PhD student (scholarship no. 20108220166). She therefore began her scientific training abroad in September 2012, under the supervision of Prof. dr. Jan van der Greef, Dr. Eduard van Wijk, and Dr. Mei Wang in the Department of Analytical Biosciences at Leiden University in Leiden, the Netherlands.

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Your sincerely,

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