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Systems diagnosis in chronic disease: prediction and evaluation

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Systems diagnosis in chronic disease: prediction and evaluation

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Knowing is owning.

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Chapter 1

General introduction and scope

General introduction

Chronic diseases are diseases of long duration (lasting longer than a year), leading to functional limitations, and/or requiring continuing medical treatment ¹. In the past decades, the number of patients with chronic diseases and the cost of treatment increased rapidly ². The World Health Organization reported that in 2012 approximately 38 million of the world's 56 million deaths were due to chronic diseases, such as rheumatoid arthritis, cardiovascular diseases, cancers, respiratory disease and diabetes ³. In low- and middle-income countries, the accumulated cost for chronic diseases is estimated to be US\$ 7 trillion in the period 2011-2025 ⁴. The growing burden of chronic diseases has focused the attention of healthcare on prevention and effective intervention. However, medications for preventing the onset of diseases are hardly effective for most chronic diseases ⁵. For millions of patients with chronic diseases, the medications they are taking may slow down the process, but not really help to improve the conditions—for example, in the case of statins, cholesterol-lowering drugs, only benefit one out of fifty patients ⁶. Patients with low-effective medications often suffer from side effects and deterioration in the condition. Along with medications, myriad factors such as lifestyle and environmental factors, will define a patient's therapeutic outcome. Therefore, a more precise intervention/treatment strategy—personalized medicine is needed to maintain or improve the condition of patients with chronic diseases. Personalized medicine is an emerging field of healthcare that uses diagnostic tests or techniques to identify groups of patients and provide optimal treatment ⁷. In other words, instead of the 'one disease—one target—one-size-fits-all' or so-called 'one-size-fits-none', individual or subtype orientated personalized medicine could provide new solutions for chronic diseases ⁸.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly affects the synovial joints and periarticular tissue, destroying cartilage and resulting in systemic inflammation. It affects 0.5% to 1% of the population in the world ⁹. At present, the aetiology of RA is still unknown, while knowledge of the pathogenesis of RA is increasing ¹⁰. The activation or infiltration of immune cells, such as Th1 cells, Th17 cells, CD20+ B cells, macrophages, mast cells and natural killer cells, have been implicated playing crucial roles in the development of RA ¹⁰.

Rheumatoid arthritis related clinical measurements

A complex interaction of genetic and environmental factors is involved in RA ¹⁰. Due to this complexity, it is important to have reliable clinical measurements to assist the decision-

making in daily clinical practice, hence multiple measurements have been developed. The most widely used instrument for monitoring disease activity is a disease activity score based on a 28 joint count (DAS28), both in clinical trials and in daily practice. It is developed based on the number of swollen joints and tender joints out of the 28 that are measured, a general health assessment score measured with a visual analogue scale (GH-VAS; score of 100, 0 = best, 100 = worst) and an inflammation factor, either erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) ¹¹. According to the European League Against Rheumatism (EULAR) response criteria, the therapeutic response is determined on the basis of changes from baseline DAS28 as well as the present DAS28 score. To be classified as responders, patients should have a significant change in DAS28 as well as a low current disease activity score. A good response is defined as an improvement in DAS28 of > 1.2 and a present DAS28 ≤ 3.2 , whereas a non-response is assigned to patients with an improvement of $0.6-1.2$ with a present DAS28 > 5.1 or patients with an improvement ≤ 0.6 . In between, an improvement > 1.2 with a present DAS28 > 3.2 or an improvement of $0.6-1.2$ with a present DAS28 < 5.1 is specified as a EULAR moderate response ¹².

Although DAS28 has been extensively validated and used in clinical trials as well as in daily practice, it should be noted that it has been claimed to be a rather unstable monitoring instrument for RA patients with a stable disease ^{13,14}. Hence two recently developed and validated instruments –the simplified disease activity index (SDAI) ¹⁵ and the clinical disease activity index (CDAI) ¹⁶, have been suggested as better alternatives to be used in clinical practice. It has been reported that DAS28, SDAI, and CDAI do not result in the same classification of patients ¹⁷.

The American College of Rheumatology (ACR) adopted another disease activity measure for RA, using the tender and swollen joint counts, global disease activity assessed by patient and physician separately, patient's assessments in terms of pain and physical function, as well as the acute-phase reactant ESR or CRP ¹⁸. To measure the effectiveness of RA treatment, the ACR criteria are established to evaluate the improvement between two measurement time points, indicated as ACR20, ACR50 or ACR70 representing 20%, 50% or 70% improvement of disease activity respectively ¹⁹.

Clinical remission is a major target for RA therapies, which is defined as 'sustained total absence of inflammation and associated manifestations of inflammation' by Pinals et al. in 1981²⁰. The most commonly used definition of remission is DAS28 < 2.6 , while new ACR/EULAR remission criteria apply either 'a Boolean definition, including tender and

swollen joint counts ≤ 1 , and CRP ≤ 1 mg/dl' or SDAI ≤ 3.3 ²¹. Both response and remission criteria are frequently employed as measurements for outcomes in clinical trials.

Moreover, clinical parameters from laboratory blood tests, such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (ACPA), can be used as diagnostic tools and for the assessment of the severity of RA. It has been reported that the (positive/negative) status, instead of concentration levels, were associated with increased mortality²². In addition, imaging tests (e.g. radiography, computed tomography, magnetic resonance imaging, and high-resolution peripheral quantitative computed tomography) are used for diagnosing and assessing the severity of joint damage^{23,24}.

The current strategy for managing rheumatoid arthritis

The management of RA rests on drug treatment, physical as well as psychological therapy. In clinical practice, anti-inflammatory drug therapy is the mainstream, which comprises non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs). The DMARDs can be divided into the conventional synthetic compounds (csDMARDs) and biological agents (bDMARDs)²⁵. For disease management, csDMARDs are the first line of medication, including methotrexate, sulfasalazine, glucocorticoids, etc. Of these csDMARDs, glucocorticoids (GCs) are the most frequently used drugs in RA²⁶. However, the use of GCs is a double-edged sword—RA patients can benefit from the anti-inflammatory effects and slowing down of radiographic progression²⁷ while suffering from adverse events, such as diabetes, osteoporosis, and cardiovascular disease (CVD)²⁸. Patients with RA have a higher CVD risk and this elevated risk is only partially explained by increased prevalence of traditional CVD risk factors such as age, gender, hypertension, etc.²⁹. It has been reported that the GC dosage was associated with increased mortality rates of patients with RA³⁰. The mechanism behind these associations still needs to be elucidated further.

For patients not responding to the csDMARDs, bDMARDs are the second-line strategy²⁵. The bDMARDs are a relatively new therapy class compared to csDMARDs. In the late 1990s, the first tumour necrosis factor inhibitor (TNFi) was introduced for treating RA, followed by other bDMARDs such as interleukin 6 (IL-6) inhibitors and T-cell co-stimulation blockers. The bDMARDs commonly used in Europe and their mechanisms of action are shown in Table 1. Biological agents are emerging therapies for reducing symptoms and slowing disease progression, highly improving the quality of life for many RA patients. However, approximately 30% of the patients fail to respond to the therapy, while they might suffer from severe side effects and joint damage^{31,32}. Additionally, compared with conventional DMARDs, the cost of biological agents is much higher—around 1000 euro per person per

month³³. Therefore, identifying the sub-group of patients that would respond favourably to biological therapy could result in a much better quality of life for the patients and at the same time reduce the current costs for treatment enormously.

Table1. Overview of biological agents used in the treatment of RA in Europe³⁴.

	Full name	Structure	Mechanism
Tumour necrosis factor inhibitor	Infliximab ^a	Chimeric human-murine monoclonal antibody	Block TNF α , prevent it from binding TNF α receptor
	Adalimumab ^b	Recombinant human monoclonal antibody	
	Golimumab ^b	Human monoclonal antibody	Block soluble and membrane-bound TNF α , lymphotoxin molecule TNF β , prevent them from binding receptors
	Certolizumab pegol ^b	Recombinant PEGylated humanized antibody	
	Etanercept ^b	Soluble TNF α receptor-Fc of human IgG1 fusion protein	
Interleukin -6 inhibitor	Tocilizumab ^a	Recombinant human monoclonal antibody	Block membrane and soluble IL-6 receptor, prevent IL-6 binding
T-cell co-stimulation blocker	Abatacept ^a	Fc human IgG1-extracellular domain CTLA-4 fusion protein	Bind CD80 and CD86 molecule of T cell, block T-cell activation
B-cell depletion therapy	Rituximab ^a	Chimeric human-murine monoclonal antibody	Bind CD20 of B cell, lead to depletion of B cells

^a Administrated by intravenous infusion

^b Administrated by subcutaneous injection

CD, cluster of differentiation; CTLA, cytotoxic T-lymphocyte-associated protein; IgG, immunoglobulin G; PGE, polyethylene glycol; TNF, tumour necrosis factor.

Personalized medicine for rheumatoid arthritis

Due to the high cost and unsuccessful response rate to biological therapies, a more personalized treatment strategy is needed. Personalized medicine, aiming to provide an optimal therapy to patients, has the potential to improve patients' quality of life as well as decrease healthcare costs⁶. In daily clinical practice, it would be ideal if a physician could tailor therapy based on the predicted therapeutic response by evaluating measureable characteristics of an RA patient.

Many studies have shown that clinical or demographic measurements could be used to predict treatment outcomes of biological therapies. Most of the studies were focusing on the TNFi-therapies using infliximab, adalimumab, or etanercept. It was reported that a low baseline health assessment questionnaire (HAQ) score and high DAS28 were associated with a good

clinical outcome and the concurrent use of methotrexate was a strong predictor of good response, especially in patients receiving etanercept ³⁵. Unsuccessful reduction in CRP has been demonstrated to be associated with non-responders to infliximab therapy and good responders to etanercept therapy after unsuccessful infliximab treatment ³⁶. Several studies have shown that positive RF and/or ACPA status were associated with positive therapeutic response ³⁷. In addition, the endogenous TNF levels were found to be positively associated with response to infliximab and adalimumab ^{38,39}. Although some of the parameters were identified to predict clinical response, the predictive value of single markers apparently is insufficient for applications in clinical practise or need further validation.

Systems approaches for personalized medicine in rheumatoid arthritis

Systems biology

The human body is a dynamic and complex system of which the condition can be determined by multiple factors, such as genes, disease status, the usage of drugs, environment, psychology, social network, and lifestyle ⁷ (Fig.1). Correspondingly, drug response is modulated by a variety of factors and might have multiple effects on system regulation. Therefore, single biomarkers might provide a too limited perspective on the diagnostic status of the human body (i.e. to evaluate disease progression) and on phenotyping patients for choosing an optimal therapy (i.e. personalized medicine).

To deal with the complexity of human health and to overcome the limitations of single biomarkers, systems biology is employed in evaluating disease progression and drug response ⁴⁰. Systems biology is a biology-based interdisciplinary field, applying a holistic approach for studying complex interactions within biological systems as well as between an organism and the outside world ⁴¹. Systems biology-based approaches offer opportunities to identify novel biomarkers and patterns of drug response from a combination of biological, psychological and social factors. Integrating this wide variety of data types could yield new insights into disease pathogenesis and provide solutions for deducing phenotypes of patients, followed by an optimal treatment strategy ⁴². The emphasis of systems biology is on building networks of relationships among variables and the robustness or resilience of the established networks, instead of examining the absolute values of individual variables ⁴². From a systems perspective, the dynamic changes in time of biomarker patterns could contribute to the further understanding of the development of diseases ⁴³. This approach may ultimately lead to the identification of new personalized markers that can predict treatment response for an individual.

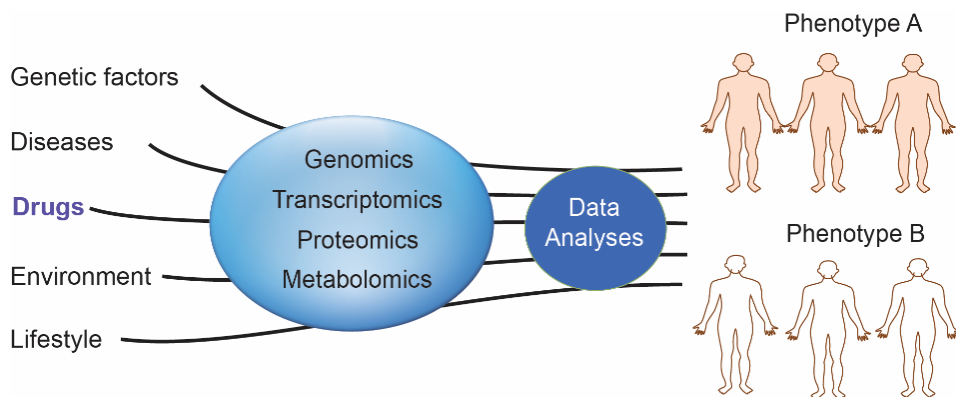


Figure 1. Systems biology and phenotyping. The condition of our body is influenced by a complex combination of genetic factors, disease status, drugs, environment, and lifestyle. With the development of systems approaches and sophisticated statistical analyses, patients can be grouped into phenotypes and ideally obtain an optimal treatment.

Metabolomics

To map the biochemistry of biological systems, ‘-omics’ approaches, such as genomics, transcriptomics, proteomics and metabolomics, are employed (Fig.1). Of these technologies, metabolomics is a continuously developing research field, which combines sample preparation and analytical chemistry with sophisticated data analysis ⁴⁴. High-throughput techniques, such as nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), have been widely applied in metabolomics studies ⁴⁵. Especially MS-based platforms, which are capable of detecting picomolar concentrations, have been more extensively used for screening comprehensive metabolite profiles ⁴⁶. Nowadays, MS has therefore become the most widely used technique and is applied in the characterization and diagnosis of various diseases ^{47,48}.

Metabolomics platforms can be applied to measure a wide range of metabolites (molecular weight less than 1500 Daltons, e.g. amino acids, fatty acids, nucleotide, etc.) in biological fluids, tissues, and cells ⁴⁹. Metabolites are regarded as downstream products of the genome, representing the interactions between gene-regulation and the environment of a dynamic living system ⁵⁰. Metabolomic profiling could therefore provide a more direct readout of the physiological status of an individual when compared to genomics and proteomics analysis ⁴⁹. Metabolomics has been successfully used to address pathophysiological issues by studying the activity of biochemical reactions and clinically relevant pathways such as amine metabolism, oxidative stress pathways, steroids, and lipid metabolism ^{51–57}. In this sense,

metabolomics could offer a novel perspective in the search for new disease biomarkers and drug targets.

Metabolomics methodology can be divided into untargeted and targeted approaches. Untargeted metabolomics aims to measure as many analytes as possible, including unknown compounds⁵⁸. Most untargeted profiling approaches do not use internal standards (ISTDs) per metabolite with a known calibration model, and consequently results are not quantitative, thereby limiting comparisons between different studies and laboratories⁴⁶. Targeted metabolomics methods often produce quantitative results by including structural or isotope-labelled analogues as ISTDs as well as an established calibration model per metabolite⁴⁴. However, the covered quantitative range of metabolites of targeted methods is often limited by the commercially available ISTDs and of reference metabolites to establish a calibration model⁴⁶. In conclusion, each metabolomics approach has both advantages and disadvantages, so researchers should choose a method on the basis of the objective of a given study.

Metabolomics approaches have already been widely applied in studies of RA, and have contributed to the understanding of the pathogenesis and drug response⁵⁹. However, there are a limited number of studies employing metabolomic profiling to phenotype patients and to predict therapeutic response prior to biological therapies. To our knowledge, only two previous studies have used metabolomics to predict the clinical response to TNFi, both by using a ¹H-NMR-based technique. Kapoor et al.⁶⁰ screened the urine metabolome of 16 RA patients and found that histamine, glutamine, xanthurenic acid, and ethanolamine predicted TNFi response. In addition, there was a significant correlation between the baseline urine metabolic profile and the magnitude of the one-year change in disease activity. Priori et al.⁶¹ showed that the serum metabolic profiling of 27 RA patients at baseline could discriminate the response to etanercept. Additionally, higher levels of isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose and lower levels of 3-hydroxybutyrate were observed in good responders after 6 months of therapy. The potential predictors found in these ¹H-NMR based studies have thus far not been validated in other cohorts. Mass spectrometry has a much higher sensitivity and therefore requires minimal amounts of sample, offering more opportunities to identify biomarkers in studying the prediction of therapeutic responses.

Chinese medicine

Chinese medicine (CM) is a ‘holistic approach to examine the function and dysfunction of living organisms’⁶², which is similar to the principles of systems biology. Diagnosis and treatment strategies are the two most important components of CM⁴³. According to CM-based diagnostic principles, symptoms and signs are recorded from patients by practitioners

through in-depth observation, listening, questioning and pulse analyses⁶³. Based on symptom interrelationships and the dynamics of these patterns a diagnosis is made, followed by corresponding CM therapies such as acupuncture and/or herbal medicine⁶⁴. Generally, a herbal formula contains several herbs, functioning through numerous active compounds targeting on multiple pathways⁶⁵. Although quantification, qualification, and mechanisms of action of these compounds are still largely unknown at a biochemical level, in clinical practice the combination of herbs have led to improved therapeutic response and minimal side effects⁴³. Chinese medicine is capable of phenotyping patients based on captured patterns and providing a very personalized treatment strategy⁶⁶. Western medicine (WM) based diagnosis is often more reductionistic and focuses on single readouts (e.g. glucose levels in the case of type 2 diabetes), followed by a ‘one disease—one target’ treatment strategy (i.e. one-size-fits-all)⁴³. Hence, WM is effective and accurate in managing diseases, which can be monitored by single biomarkers, which is the case in later stages of diseases or acute diseases. However, the health conditions of patients with chronic diseases is typically not characterized by just a single variable. As a consequence single biomarkers have limited effectiveness in the early phases of a disease or in chronic conditions⁴³. For that reason, CM based diagnosis and therapy could provide important information to achieve treatment effectiveness in chronic diseases.

According to CM theory, RA belongs to the Bi-syndromes⁶⁷, which consist of multiple subtypes based on symptom patterns, for example ‘Cold’ and ‘Heat’ patterns. The Cold pattern can be characterised by symptoms such as loose stools and pain exacerbated with cold, while the Heat pattern shows symptoms of ‘severe pain with hot, red, swollen and inflamed joints’, pain relieved by cold, etc⁶⁸. For each pattern of RA, there is a corresponding CM treatment strategy⁶⁹. We previously reported differences in biological mechanisms between Cold and Heat RA patients as determined by metabolomic measurements of plasma and urine samples as well as gene expression analysis of CD-4 T-cells^{70,71}. A large literature mining study suggests that Cold type of diseases are related to hormone disturbances whereas immune systems disturbances are Heat type related⁷². Therefore, CM patterns may help to subtype RA patients suitable for biological therapies or to discover biomarker profiles in addition to the current practice.

Bioinformatics

One of the challenges for systems biology is dealing with the massive amounts of data generated with the multiple ‘-omics’ approaches. Large integrated datasets are produced that contain detailed information of the systems network. Typically, univariate and multivariate

data analysis techniques are then used to extract the essence of the information to answer a particular research question ⁷³.

Univariate analyses, such as t-tests, Chi-square tests, and ANOVA, are applied only to one variable at a time, to compare different sets of samples ⁷⁴. For a large dataset, multiple univariate tests are needed which could result in an increased probability of false positives and extra tests (e.g. false discovery rate tests and Bonferroni correction) are then essential to control for these false positives ⁷⁵. However, the significant differences identified by univariate analyses may not be causal, since the associations among the tested variables are not taken into consideration in univariate analyses. In order to reduce the complexity of the data and take possible relationships between variables into account, mathematical methods for multivariate analyses have been developed.

One of the reasons to use multivariate analyses is to reduce the dimensionality of the data. Common techniques are principle component analysis (PCA) and partial least squares discriminant analysis (PLSDA) ⁷⁶. Principle components analysis is most widely used as a tool to analyse correlations between variables in complex numeric datasets, identifying potential phenotypes ^{77–79}. Partial least squares discriminant analysis is applied to select biomarkers related to defined sub-groups, by analysing the additional relationships between dependent and independent variables ^{52,80}. However, overfitting of the data in the building of PLSDA models is a risk, which needs to be controlled by including permutation tests and cross validation ⁸¹. For data with different measurement levels (e.g. ordinal, categorical, binary, numeric) and non-linear associations (e.g. dataset of questionnaires), other dimensionality reduction methods are recommended, such as categorical principle components analysis (CATPCA) ^{82,83}. In addition, CATPCA with forced classification could also be used to identify relevant biomarkers for pre-defined groups of subjects, similar to PLSDA ⁸⁴.

Multivariate logistic or linear regression models are frequently applied to predict outcomes in clinical trials ^{35,85–87}. One of the problems encountered with regression models is that the number of independent variables allowed in the analysis is limited. Therefore, variable selection is needed, for example stepwise elimination. Importantly, the association between the independent variables (i.e. confounding and interacting effects) should also be taken into account. To correct for the associations between subject profiles (e.g. multiple recruitments of a subject in one analysis), generalized estimating equations (GEE) can be included in a regression analysis ⁸⁸.

In general, univariate analysis provides direct and easy-interpretable results, but it is limited by possible false positives and does not provide information on non-causal relationships of variables. In contrast, multivariate analysis handles large datasets more easily and focusses on the relationships between variables. Additionally, sophisticated multivariate statistics provides more opportunities for identifying causal relationships⁸⁹. Actually, the biochemical interpretation of the results is the ultimate goal and maybe the most challenging in systems biology studies. Having both type of statistical approaches helps to interpret the role of variables (e.g. metabolites) differing between groups in their biochemical context and provides more insights into the research question.

Scope and outline of the thesis

Nowadays, often only a limited number of patients with chronic diseases can benefit from current therapies, leading to loss of quality of life and suffering from deterioration in their health condition; additionally, the cost of healthcare is unnecessary increased. The growing burden of chronic diseases worldwide has urged healthcare systems to focus on personalized medicine—treating patients with the optimal medicine.

On the basis of systems thinking, in this thesis metabolomics, CM, as well as WM were combined to achieve a more comprehensive systems diagnosis of patients with chronic diseases. Specifically, metabolomics has been applied to characterize patients by small molecule profiles, which can be applied in phenotyping patients and matching these with optimal therapies. Chinese and Western medicine have different perspectives on diagnosis and could be highly complementary to each other, so combining both could have advantages in personalized diagnosis. Therefore, in this thesis a combination of systems approaches is used, including metabolomics, CM-based diagnosis principles as well as WM to provide systems diagnosis of patients with chronic diseases, in particular RA. With systems diagnosis, we could better identify potential biomarkers to predict the WM therapeutic response of patients with RA and monitoring disease progression.

The research described in Chapter 2-4 focuses on an observational study on RA patients, with the main aim to predict response to biological therapy. Chapter 2 aims to predict therapeutic response to TNF- α inhibitors and to elucidate the pathogenesis of RA. Established targeted platforms were used to characterise serum polar lipid, oxylinpin and amine profiles. We explored the possibility of identifying predictors of treatment outcome from metabolomic profiles, and the associations between metabolomics profiles and disease activity related parameters (CRP, ESR, and DAS28).

In addition to serum, urine is another commonly collected biological matrix in clinical studies and trials and widely used for phenotyping of patients, as urine reflects what is excreted from the human body. Chapter 3 presents the development of a method using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to analyse total oxidised lipids in human urine samples, covering prostaglandins, isoprostanes, dihydroxy-fatty acids, hydroxy-fatty acids, and the nitro-fatty acids. The method used a β -glucuronidase hydrolyses to determine the sum of free and conjugated oxidised lipids. This method was applied to urine samples of RA patients, and the associations between oxidised lipid profiles and the improvement of DAS28 and RA-related clinical parameters was investigated.

After applying serum and urinary metabolomics on RA patient samples to predict drug response and elucidate disease pathogenesis, in Chapter 4 we seek to phenotype patients for biological therapies based on CM defined symptoms. CM diagnostic principles (Cold and Heat symptoms evaluated by questionnaires), as well as WM baseline characteristics were used to classify response to biological therapy in RA patients. It is the first time that CM related diagnostic principles are used to predict RA patients' response to biological therapy are used.

As the RA patients were taken from an observational study, multiple drugs were involved and the treatment strategy for each of the patients was various. Of these concomitant drugs, glucocorticoids (GCs) have been reported to have a gender-based influence on metabolomic levels^{90,91}. Chapter 5 describes differences of the polar profiles between male and female RA patients in response to glucocorticoids treatments. We measured circulating polar lipids, such as lysophospholipids and free fatty acids, in the serum of RA patients using a targeted lipid platform. Lipid profiles between GC users and non-GC users were examined and analysed for gender differences.

Besides patients with RA, in Chapter 6 we aim to compare the effects of two multi-herbal *Radix Rehmanniae* formulae in metabolic syndrome patients. Within the 8-week treatment period, previously described systems approaches were applied to measure response profiles at various time points, which represent the subjects' system reactions to the treatment. Both targeted and untargeted metabolomic profiling was used to characterise the changes in lipids, amino acids and sugars. We further explored the differences of therapeutic response (CM defined syndromes and WM clinical parameters) and metabolomics between these two formulae.

Finally, in Chapter 7 the conclusions and perspectives of the research of this thesis are elaborated. The advantages and challenges of the systems approaches applied are discussed and suggestions for future studies are proposed.

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Chapter 2

Exploring the inflammatory metabolomic profile to predict response to TNF- α inhibitors in rheumatoid arthritis

Based on

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Abstract

Background In clinical practice, approximately one-third of patients with rheumatoid arthritis (RA) respond insufficiently to TNF- α inhibitors (TNFis).

Objective To explore the use of a metabolomics to identify predictors for the outcome of TNFi therapy, and study the metabolomic fingerprint in active RA irrespective of patients' response.

Methods In the metabolomic profiling, lipids, oxylipins, and amines were measured in serum samples of RA patients from the observational BiOCURA cohort, before the start of biological treatment. Multivariable logistic regression models were established to identify predictors for good- and non-response in patients receiving TNFi (n = 124). The added value of metabolites over prediction using clinical parameters only was determined by comparing the area under receiver operating characteristic curve (AUC-ROC), sensitivity, specificity, positive- and negative predictive value and by the net reclassification index (NRI). The models were further validated by 10-fold cross validation and tested on the complete TNFi treatment cohort including moderate responders. Additionally, metabolites were identified that cross-sectionally associated with the RA disease activity score based on a 28-joint count (DAS28), erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP).

Results Out of 139 metabolites, the best-performing predictors were *sn1*-LPC(18:3- ω 3/ ω 6), *sn1*-LPC(15:0), ethanolamine, and lysine. The model that combined the selected metabolites with clinical parameters showed a significant larger AUC-ROC than that of the model containing only clinical parameters (p = 0.01). The combined model was able to discriminate good- and non-responders with good accuracy and to reclassify non-responders with an improvement of 30% (total NRI = 0.23) and showed a prediction error of 0.27. For the complete TNFi cohort, the NRI was 0.22. In addition, 88 metabolites were associated with DAS28, ESR or CRP (p < 0.05).

Conclusions Our study established an accurate prediction model for response to TNFi therapy, containing metabolites and clinical parameters. Associations between metabolites and disease activity may help elucidate additional pathologic mechanisms behind RA.

Introduction

Rheumatoid arthritis (RA) is a chronic, disabling disease that mainly affects the synovial joints. The disease is a multifactorial autoimmune disorder with a prevalence of 0.5-1% in industrialized countries ¹⁻³. Disease-modifying anti-rheumatic drugs (DMARDs) are the cornerstone of anti-inflammatory therapy in RA and can be divided into two categories: conventional synthetic DMARDs (csDMARDs) and biological DMARDs (bDMARDs) ⁴. The csDMARDs are relatively cheap chemical agents consisting of small active-substance molecules and are used for decades in the treatment of RA, whereas bDMARDs, predominantly antibodies such as TNF- α inhibitors (TNFis), are expensive agents that target specific inflammatory pathways and have revolutionized the treatment options for RA patients since the first trial in 1993 ⁵. Despite the success of TNFis, a substantial proportion of patients (approximately 30-40%) responds insufficiently to these biological agents ^{6,7}. At the initiation of TNFi therapy it is as yet impossible to distinguish future responders from non-responders, therefore, the only used treatment approach is by trial and error. This approach is inefficient because the clinical response can only be assessed after at least three months of treatment. Within this timeframe, non-responders might develop joint damage or may experience toxic side effects. In addition, an inefficient treatment increases healthcare costs due to intensive monitoring, more complications, higher morbidity, and medication costs. The challenge is therefore to identify responders and non-responders before initiation of TNFi treatment so that the decision can be guided and the most optimal agent can be selected for each patient. Many approaches have been explored, mostly by evaluation of clinical parameters, proteins or mRNA biomarker profiles, but none were thus far successful in such a way that they can be implemented in clinical practice⁸.

Metabolomics is a rapidly developing approach in biomarker research, involving the measurement of a large number of small-molecule metabolites in biological fluids, tissues and cells. One major advantage is that it offers a characteristic profile of each patient from minimal amounts of sample. With high-throughput techniques, such as nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography coupled to mass spectrometry (LC-MS), metabolomics provides a novel perspective on the search of new disease biomarkers and drug targets ⁹.

Metabolomic approaches in RA have already contributed to the understanding of RA and its subtypes, as well as the effect of drug treatment ¹⁰. However, there is a limited number of studies employing metabolomic profiling to predict patients' response to biological therapies. To our knowledge, only two previous studies have used metabolomics to predict the clinical

response to TNFi, both by usage of the ^1H -NMR-based technique. Kapoor et al.¹¹ screened the urine metabolome of 16 RA patients and found that histamine, glutamine, xanthurenic acid, and ethanolamine predicted TNFi response. In addition, there was a significant correlation between baseline urine metabolic profile and the magnitude of the one-year change in the disease activity. Priori et al.¹² showed that the serum metabolic profiling of 27 RA patients at baseline could discriminate the response to etanercept. Additionally, higher levels of isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose and lower levels of 3-hydroxybutyrate were observed in good responders after 6 months of therapy. The predictors found in these ^1H -NMR based studies have thus far not been validated in other cohorts. Compared with NMR, LC-MS has higher sensitivity and selectivity, thereby needing much less sample volume¹³. In the present study, LC-MS-based metabolomics was applied for measuring a large scope of metabolites consisting of lipids, oxylipins, and amines.

The baseline serum metabolome of RA patients starting biological therapy was analyzed using LC-MS. The objective was to assess the potential value of serum metabolite profiles in the prediction of response to TNFi treatment, using LC-MS. Additionally, we investigated the associations between metabolites and current disease activity, in order to gain more insight into the pathological mechanisms of RA.

Materials and Methods

Patient cohort

Patients were selected from the observational BiOCURA study (Biologicals and Outcome, Compared and predicted in Utrecht region, in Rheumatoid Arthritis) in which patients were enrolled between 2009 and 2015. In BiOCURA, RA patients eligible for bDMARD treatment in clinical practice, were followed up after start of the treatment, in one academic hospital and seven regional hospitals in the Netherlands (see Acknowledgements). The treatment included any of five TNFis, adalimumab, etanercept, infliximab, golimumab, and certolizumab pegol, or non-TNFi agents, including tocilizumab, abatacept, and rituximab. Conventional synthetic DMARDs were allowed to be used concomitantly with the bDMARD, and included methotrexate, hydroxychloroquine, leflunomide, and glucocorticoids. Apart from csDMARDs, patients continued other medication, such as statins, bisphosphonates, anti-hypertension and nonsteroidal anti-inflammatory drugs (NSAIDs), all according to regular clinical practice.

Regular visits with a treating physician and a trained research nurse were scheduled at baseline, three, six, and twelve months. Clinical parameters and blood samples were collected

from each patient before the first dose of the biological agent. Of note, blood samples were collected from fasting and non-fasting patients, as the visiting times of the patients could not be standardized in the morning. The blood was collected in a Vacutainer® SST II tube and processed immediately after clotting. Samples were centrifuged for 10 min at 1500 g at room temperature and serum was aliquoted and stored at -80°C until use for metabolomic analyses. Re-inclusion after switching to a different biological agent was possible. The study was approved by the ethics committee of the UMC Utrecht and the institutional review boards of the participating centers (see Acknowledgements). Written informed consent was obtained from each patient.

Inclusion in the present study was restricted to subjects of BiOCURA fulfilling the following criteria: at start of treatment patients should not be in clinical remission (disease activity score based on a 28-joint count, DAS28 > 2.6), after three months of therapy the DAS28 assessment needed to be available, and no (temporary) discontinuation of treatment should have occurred within the first three months of bDMARD treatment.

Clinical measurements

Demographic, clinical, and laboratory parameters of patients at baseline were obtained, including age, gender, menopausal status, body mass index (BMI), disease duration, any previously used bDMARD (biological naivety), currently used csDMARDs and non-anti-rheumatic drugs, 28 tender joint count (TJC), 28 swollen joint count (SJC), a 100mm visual analogue scale on general health (VAS-GH), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), and anti-citrullinated protein antibody (ACPA). Disease activity was assessed at baseline and at follow-up visits, using DAS28¹⁴. In clinical practice the response to biological therapy is usually measured 3-6 months after initiation¹⁵. However, in BiOCURA a substantial number of patients withdrew treatment before the 6-month time-point due to insufficient response or side effects. Using the 6-month response would thus result in (non-random) missing responses. Therefore, in this study the patients' response at 3-month was determined as the treatment response, based on the EULAR response criteria¹⁶. A EULAR good response is defined as an improvement in DAS28 of > 1.2 and a present DAS28 ≤ 3.2, whereas a EULAR non-response is assigned to patients with an improvement of 0.6-1.2 with present DAS28 > 5.1 or patients with an improvement ≤ 0.6. In between, an improvement > 1.2 with present DAS28 > 3.2 or an improvement of 0.6-1.2 with present DAS28 < 5.1 is specified as a EULAR moderate response.

Metabolomic profiling

2 Serum samples from selected subjects were measured on three targeted LC-MS platforms, which used standard operating procedures optimized from previously published methods^{17–19}, covering a broad spectrum of pre-defined metabolites. The lipid platform targets low abundance lipid species, including free fatty acids (FAs) and phospholipid derivatives, such as lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs); the oxylipins platform covers oxygenated metabolites derived from polyunsaturated fatty acids through enzymatic and non-enzymatic oxidation processes; the amine platform targets amino acids and biogenic amines. All analyses were performed by the Biomedical Metabolomics Facility Leiden (BMFL) of the Leiden University. Extra serum of the subjects was pooled and used to create internal quality control (QC) samples.

Lipids analysis

For the detection of lipids, each 20 μ L serum aliquot was spiked with internal standard (ISTD) mix and lipids were extracted by methanol. This lipid profiling was conducted using ultra performance liquid chromatography coupled to electrospray ionization-quadrupole time-of-flight (Agilent 6530 San Jose, CA, USA) with an ACQUITY UPLC™ HSS T3 column (1.8 μ m, 2.1 $\times$ 100mm)¹⁷.

Oxylipins analysis

Each 180 μ L serum aliquot was spiked with antioxidants and ISTD mix, followed by solid phase extraction. The samples were analyzed by high-performance liquid chromatography (Agilent 1260, San Jose, CA, USA) coupled to a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA), using an Ascentis® Express column (2.7 μ m, 2.1 $\times$ 150 mm)¹⁸.

Amines analysis

Each 5 μ L serum aliquot was spiked with an ISTD mix and proteins were precipitated by methanol, after which the supernatant was dried and derivatized by AQC reagent. The samples were analyzed by an ACQUITY ultra-performance liquid chromatography system coupled to Xevo Tandem quadrupole mass spectrometer (Waters, Milford, MA, USA) with an AccQ-Tag™ Ultra column (1.7 μ m, 2.1 $\times$ 100 mm)¹⁹.

Data preprocessing and correction

For the lipid and oxylipin platforms, peak determination and peak area integration were performed by Mass Hunter Quantitative Analysis (version B.05.00, Agilent technologies); for

the amine platform, TargetLynx software (version 4.1, Waters) was employed. For each metabolite, the concentration was determined by the ratio between the peak area of targeted analyte and peak area of the appropriate ISTD. These response ratios ($\text{Area}_{\text{analyte}}/\text{Area}_{\text{ISTD}}$) were used as raw metabolomic data in the subsequent analysis. For batches measured in each platform, an in-house developed method was applied to compensate and correct for instrumental drift during the measurements. The within-batch and between-batch effects of metabolomic data were corrected per metabolite using the responses of QC samples²⁰. The QC samples were measured repeatedly every 10 patient samples across the different batches per platform. The details of the data correction method are described by van der Kloet *et al*²¹. The relative standard deviation (RSD) of metabolites in the QC samples was used to assess the quality of targeted metabolites in each analytical platform.

Metabolite measurements, which were lower than the limitation of detection (LOD) of the platform, were imputed by half of the observed minimum value for the corresponding metabolite. Subsequently, log-transformation and auto-scaling of the metabolites were applied to reach or approximate a normal distribution, with a mean of 0 and SD of 1. The resulting data were used as input for all subsequent statistical analyses.

Statistical analyses

Metabolites were used to develop a model for the prediction of response to TNFi at three months, and to assess their association with disease activity in general of the total cohort. An overview of the analyses is provided in Fig 1 and will be discussed in more detail below. All analyses were performed in IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, N.Y., USA), MedCalc for Windows, version 16.2.1 (MedCalc Software, Ostend, Belgium), and R (Version 3.2.3).

Development of models for predicting TNFi response

In order to increase the possibility of picking up predictors related to response, we focused on subjects with EULAR good- versus non-response to TNFi treatment only, since EULAR moderate responders are an in-between category in which some patients continue treatment and others discontinue due to inefficacy (BiOCURA data). With TNFi good responders and non-responders as an outcome, we built two multivariable logistic regression models: a clinical model containing clinical parameters only, and one combined model with metabolites as well as the same clinical parameters. We selected the following 16 baseline clinical parameters: age, gender, menopausal status, BMI, smoking status, alcohol consumption, DAS28, (log-transformed) CRP, concomitant csDMARDs (methotrexate, hydroxychloro-

quine, leflunomide and glucocorticoids) and non-DMARDs (statins, antihypertensive drugs, bisphosphonates and NSAIDs), regardless of the predictive ability (i.e. p-value) of each single parameter.

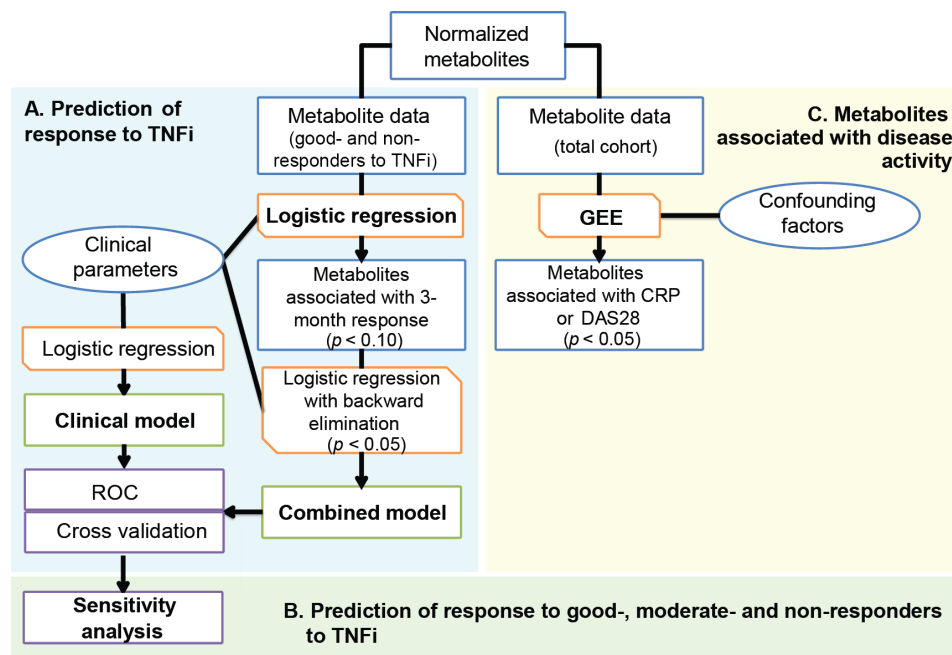


Figure 1. Flowchart of statistical analyses. (A) Prediction of response to TNFi: All steps to build a prediction model on TNFi response were performed on the TNFi subset with EULAR good-response or non-response (n=124). (B) Sensitivity analysis on the complete cohort of TNFi initiating patients. (C) Metabolites associated with disease activity. Analyses to investigate metabolites association with CRP, ESR or DAS28 were performed on the total cohort of patients using bDMARDs (n=231; including TNFi and non-TNFi treated patients). Blue boxes/circles indicate (selection of) respectively metabolites or clinical parameters, whereas orange boxes indicate the performed analyses. bDMARDs: biological disease-modifying anti-rheumatic drugs; CRP: C-reactive protein; DAS28: disease activity score based on a 28-joint count; ESR: erythrocyte sedimentation rate; GEE: generalized estimating equation, LC-MS: liquid chromatography coupled to mass spectrometry; ROC: receiver operating characteristic; TNFi: TNF- α inhibitor.

To decrease the number of metabolites in the combined model, a (non-strict) pre-selection on metabolites was performed. The predictive value of each individual metabolite to TNFi treatment outcome (good- or non-response) was investigated while combined with the clinical parameters, and all metabolites with $p < 0.10$ were pre-selected. Subsequently, the pre-selected metabolites together with the clinical parameters were added to the logistic regression model on response with backward elimination of the metabolites only, to build the

final combined model (thus keeping all clinical parameters in). The final clinical model contained the same clinical parameters without any elimination.

The receiver operating characteristic (ROC) curves of the final clinical and combined model was plotted, with the area under the curve (AUC) as an indicator of its predictive ability. Sensitivities, specificities, misclassification rates (MR), and positive- and negative predictive values (PPV/NPV) were calculated based on the optimal cutoff (Youden's index ²²). This optimal cut-off was applied to both models, so that per patient per model, either a non-response or good response was predicted, which could be compared to the observed response. Based on these predicted and observed responses, the net reclassification index (NRI) was calculated to determine if the addition of metabolites reclassifies more patients into the correct category. For example, when a future non-responder is classified at baseline to have a high probability of good response by the clinical model, but is classified into the low probability category in the combined model, the reclassification is "more correct" and the NRI for non-responders will increase, as well as the total NRI (=NRI responders + NRI non-responders). The robustness of the final models was judged by 10-fold cross validation of the model, using the `cv.glm` function of the R-package `boot`.

As an additional sensitivity analysis, the developed models were also applied to the complete cohort of TNFi initiating patients, including moderate responders (n = 49). In this step, the regression coefficients of the developed clinical and combined model were frozen and used to create a prediction rule. Subsequently, this clinical and combined prediction rule were compared for their abilities to distinguish EULAR non-responders from EULAR moderate- and good responders, using the same measurements for predictive ability as described before.

Metabolites and disease activity

In order to investigate associations between metabolites and RA disease activity (CRP, ESR and DAS28), we analyzed the complete cohort of 231 patients with TNFi and non-TNFi therapy irrespective of the EULAR response. However, in BiOCURA some RA patients were re-included after switching to a different biological agent (usually non-TNFi after a TNFi), thereby having multiple baseline visits and follow-up periods. In order to account for the effects of subjects with multiple inclusions, generalized estimating equation (GEE) was used for these analyses, as GEE is a regression-based method that allows analyses of repeated measurements within subjects ²³. Because we were interested in the association of each individual metabolite with CRP, ESR and DAS28, and clinical characteristics of patients might influence the metabolite levels (influential factors), we corrected for baseline clinical parameters except CRP, ESR and DAS28, to gain more reproducible outcomes. As such, with

the (log-transformed) CRP, (log-transformed) ESR or DAS28 as the dependent variable, each individual metabolite was added into GEE as an independent variable while corrected for the possible influential factors. Cytoscape was used to visualize the significant associations²⁴.

Results

In total, 231 RA patients from BiOCURA cohort fulfilled the selection criteria for the present study. Baseline characteristics of all patients are shown in Table S1. Of all patients, 173 (74.9%) received TNFi treatment and 58 patients (25.1%) received a non-TNFi treatment. Concomitant csDMARDs and non-DMARDs were very diverse, and frequently included methotrexate (166 patients; 72%), glucocorticoids (93 patients; 40%) and bisphosphonates (101 patients; 44%) (Table S2). Only 14 patients (6.1%) used no concomitant csDMARDs. The baseline characteristics of patients receiving TNFi and comparisons between non- and good responders are shown in Table 1. Among these patients, 64 were EULAR non-responders and 60 were good-responders. The number of RF positive patients and the SJC was significantly higher in the good responders, besides there were no significant differences in other baseline clinical variables between good responders and non-responders. Missing data was present for < 5% for each of the variables.

Table 1. Baseline characteristics of all selected TNFi initiating subjects (n=173), and split for all EULAR non-responders (n=64) and good responders (n=60).

	Subjects with TNFi (n=173)	Non-responders (n=64)	Good responders (n=60)	p- value
Female, n (%)	130 (75.1)	50 (78.1)	43 (71.1)	0.41
Menopausal status of females, n (%)				0.84
Pre-menopause	40 (30.8)	16 (32.0)	16 (37.2)	
Post-menopause	82 (63.1)	28 (56.0)	26 (60.5)	
Unknown	8 (6.1)	6 (12.0)	1 (2.3)	
Age, years, mean (SD)	54.6 (12.4)	53.7 (13.2)	53.9 (11.7)	0.95
Disease duration, years, median (IQR)	6.0 (2.0-12.0)	5.0 (2.0-11.5)	5.5 (2.0-11.0)	0.95
Smoking, currently, n (%)	42 (24.3)	17 (26.6)	15 (25.0)	0.84
Alcohol, >7 units/week, n (%)	31 (17.9)	8 (12.5)	12 (20.0)	0.24
BMI, kg/m², mean (SD)	26.8 (5.0)	27.3 (5.2)	26.5 (5.2)	0.42
Positive RF, n(%)^a	114 (65.9)	39 (60.9)	47 (78.3)	0.04
Positive ACPA, n(%)^a	123 (71.1)	41 (64.1)	46 (76.7)	0.13
CRP, mg/dL, median (IQR)	6.0 (3.0-13.0)	5.0 (2.8-10.0)	8.0 (3.8-15.0)	0.12
Baseline DAS28, mean (SD)	4.5 (1.1)	4.3 (1.2)	4.6 (0.9)	0.14

TJC, median (IQR)	7.0 (2.0-13.0)	6.5 (1.3-13.8)	6.5 (3.0-11.0)	0.73
SJC, median (IQR)	2.0 (0.0-4.0)	1.0 (0.0-3.0)	2.0 (1.0-4.0)	0.02
ESR, mm/h, median (IQR)	20.5 (11.0-34.8)	18.5 (5.3-34.0)	18.0 (10.3-33.0)	0.65
VAS-GH, mean (SD)	56.5 (23.1)	56.3 (23.4)	56.0 (23.2)	0.94

^a The positivity for RF and ACPA was determined by the hospitals using different measurement methods and cut-offs, according to their own laboratory standards. Therefore we were not able to show an exact titer.

Descriptive statistics are expressed as number (%) for dichotomized variables, and mean $\pm$ standard deviation (SD) and median and interquartile range (IQR) for respectively normally and non-normally distributed variables. The p-value is calculated for the difference between responders and non-responders. ACPA, anti-citrullinated protein antibody; BMI, body mass index; DAS28, disease activity score based on a 28-joint count; ESR, erythrocyte sedimentation rate; IQR: interquartile range; RF: rheumatoid factor; SJC, 28 swollen joint count; TJC, 28 tender joint count; VAS-GH, 100mm visual analogue scale on general health.

The metabolomic profiling of 231 serum samples was measured using established lipid, oxylipin, and amine platforms, thereby providing a broad view on the serum metabolites. Due to inadequate serum volume, 25 samples (of which 10 good-, 5 moderate-, and 10 non-responders) could not be analyzed in oxylipins platform. After analysis and data pre-processing, one sample in lipid platform and one sample in amine platform were excluded due to abnormal ISTD areas. For each platform, RSDs of detected metabolites in QC samples were applied as quality indicators. In total, 139 metabolites were measured: 40 amines with QC RSD <0.15 , 68 lipids (lysophospholipids and fatty acids) with QC RSD <0.30 , and 31 oxylipins both with QC RSD <0.30 (Table S3). The details of targeted compounds are shown in Tables S4-S6.

Metabolites and TNFi response

In the TNFi cohort, the clinical and combined model were built for the prediction of response containing all selected clinical baseline parameters (as listed in the Materials and Methods section “Development of models for predicting TNFi response”). In the model building part, we did not exclude any clinical parameters. Firstly because the comparison of the clinical and combined model containing the same (complete) set of clinical parameters, allows us to compare the sole added value of metabolites over clinical parameters alone. Moreover, clinical parameters might serve as partially influential factors (i.e. confounders) on metabolism as well, thus incorporation of all clinical parameter in the model would result in more reliable estimates of the true predictive ability of the metabolites.

Next, each metabolite was tested for their association with good and non-response to TNFi. *sn1*-LPC (18:3- ω 3/ ω 6), *sn1*-LPC (16:1), *sn1*-LPC (15:0), *sn2*-LPC (18:1), LPE (20:3- ω 3/ ω 6), LPE (18:1), *sn2*-LPC (18:0), LPE (18:0), *sn1*-LPC (18:0), 9,10-DiHOME, 9-HODE, 11-

HDoHE, 8-HETE, 9,10-EpOME, ethanolamine, and lysine were preselected based on $p < 0.10$. Multivariable logistic regression with backward selection was carried out on preselected metabolites to establish the final combined model. As shown in Table 2, four metabolites significantly added to the prediction of clinical response to TNFi therapy, specifically good responders showed higher levels of *sn1*-LPC (15:0) and lysine, as well as lower levels of *sn1*-LPC (18:3- ω 3/ ω 6) and ethanolamine at baseline.

Table 2. Remaining metabolites and their estimated contribution in the prediction of response to TNFi in the final prediction model.

	Coefficient	Standard error	p-value	aOR (95%-CI)
<i>sn1</i>-LPC (18:3-ω3/ω6)	-1.54	0.53	0.004	0.21 (0.08-0.61)
<i>sn1</i>-LPC (15:0)	1.67	0.56	0.003	5.32 (1.76-16.07)
Ethanolamine	-1.61	0.53	0.002	0.20 (0.07-0.57)
Lysine	1.02	0.40	0.010	2.78 (1.27-6.09)

aOR: adjusted odds ratio; CI: confidence interval.

The clinical model containing the selected 16 clinical baseline parameters (Cox & Snell R-square = 0.147, see Fig S1) showed reasonable predictive abilities to differentiate good responders and non-responders, with an AUC-ROC of 0.720 (0.622-0.818) (Fig 2) and a sensitivity of 78.2%, specificity of 60.0%, PPV of 68.3%, NPV of 71.4 % and MR of 30.5% (Table S7). In contrast, the combined model (Cox & Snell R-square = 0.433, see Fig S2) showed a good overall discriminative ability with an AUC-ROC of 0.841 (0.765-0.917) (Fig 2), and its performance is significantly better than the clinical model (difference between areas = 0.121, $p = 0.01$). After determining the optimal cut-off, sensitivity was 70.9%, specificity 90.0%, PPV 88.6%, NPV 73.8% and MR 20.0% (Table S7). The high specificity and PPV suggest that the patients classified as good responders are frequently correctly classified (low false positive rate).

Additionally an NRI was calculated to investigate whether the higher accuracy also results in a better reclassification of individual patients. The reclassification of non-responders by the combined model was 30% better and of responders 7% worse, resulting in a total NRI of 0.23 (Table 3). The clinical and combined models showed prediction errors of 0.303 and 0.269 respectively, which can be considered moderately robust. In the sensitivity analysis on all responders to TNFi, the clinical model performed weakly with an AUC-ROC of 0.641 (0.548-0.734) (Fig S3), as compared to the combined model with 0.760 (0.682-0.837) (Fig S4). The reclassification of non-responders by the combined model was 30% better and for responders 8% worse (total NRI = 0.22, Table S8).

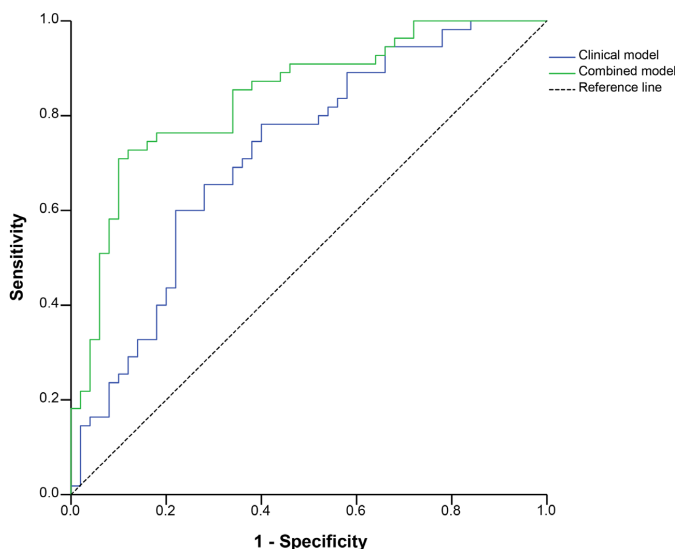


Figure 2. Receiver operating characteristic curves of clinical and combined model between good- and non-responder to TNFi. The clinical model containing the 16 selected baseline clinical parameters; the combined model included four metabolites with $p < 0.05$ in multivariable logistic regression with backward selection in addition to the clinical model.

Table 3. Net reclassification index of prediction models for good- and non-responders to TNFi..

Observed response (n=105) ^a	Predicted by clinical model	Predicted by combined model	
		Non-response	Good response
Non-responders (n=50)	Non-response	30 (equal)	0 (worsening)
	Good response	15 (improvement)	5 (equal)
Good responders (n=55)	Non-response	6 (equal)	6 (improvement)
	Good response	10 (worsening)	33 (equal)

An optimal cut-off for the clinical and combined model was chosen based on the Youden's index, after which the predicted response of each patient per model was compared to the observed response. Shown are the number of patients, split for future non-responders and good responders (observed response), that were allocated at baseline to a predicted category (non-response/good-response) by both the clinical and combined model. These allocations could be correct or wrong, based on the observed response. There are four possibilities of allocations that represent an equally good or bad performance of both models (e.g. '30' represents 30 non-responders that were correctly classified as non-responders by both the clinical and combined model). Two categories denote an improvement in the prediction by the combined model: either a future non-responder that switches from response in the clinical model to non-response in the combined model ($n=15$), or a future responder switching from non-response to response ($n=6$). The two remaining discordant categories denote a worsening of prediction by the combined model. The NRI for non-responders was $15/50 - 0/50 = 30\%$ improvement, while the NRI for

responders was $6/55 - 10/55 = -7\%$ due to a net worsening in prediction by the combined model. The total NRI was $0.30 + (-0.07) = 0.23$.

^a Due to the missing data of the clinical parameters, 19 out of 124 patients initiating TNFi therapy were excluded from the multivariable logistic regression models (clinical and combined model). Thus, 105 patients remained in the analyses.

Metabolites and disease activity

Metabolites were investigated for their cross-sectionally association with disease activity. In total, 88 metabolites out of 139 were significantly associated with baseline DAS28, ESR or CRP ($p < 0.05$) (Table S9). In Fig 3, the associations between metabolites and the three clinical parameters are visualized. With respect to the amines, glutathione was positively associated with all the three parameters; sarcosine was negatively associated with both DAS28 and ESR, whereas serotonin was positively associated with CRP and ESR. Besides from the overlapping amines, five amines were negatively associated with baseline DAS28 (histidine, threonine, glycylglycine, asparagine, and α -aminobutyric acid), while ten amines showed a positive association with CRP (valine, leucine, tryptophan, γ -glutamylalanine, isoleucine, ornithine, arginine, 4-hydroxyproline, kynurenine, and proline) and two amines a positive association with ESR (taurine, cysteine). Fatty acids with aliphatic tails of 14 to 24 carbons were found positively associated with CRP, ESR and/or DAS28, while lysophosphatidylethanolamines (LPEs) and lysophosphatidylcholines (LPCs) overall showed negative correlations with these parameters. Oxylipins were divided into different classes based on relevant pathways—the auto-oxidation pathway with reactive oxygen species (ROS) and the enzymatic pathways with cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450). Specifically oxylipins synthesized by ROS (10-HDoHE, 11-HDoHE, 11-HETE, 14-HDoHE, 8-HETE, 13-HDoHE, 9-HODE), COX (PGE₂, TXB₁, TXB₂, TXB₃) and LOX (12-HETE, 15S-HETrE, 5-HETE, 9-HOTrE) were positively associated with CRP, ESR or DAS28, whereas only two downstream products in COX and LOX pathways, 13,14-dihydro-PGF_{2a} and 9,12,13-TriHOME respectively, were found to be negatively associated with the clinical parameters. CYP450 synthesized oxylipins (11,12-DiHETrE, 14,15-DiHETrE, 19,20-DiHDPa, 5,6-DiHETrE, 8,9-DiHETrE) were all negatively associated with CRP.

Discussion

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LPC (15:0), *snl*-LPC (18:3- ω 3/ ω 6), ethanolamine, and lysine, and the high NRI for non-responders indicates that these metabolites are especially useful to identify non-responders in advance. Additionally, our sensitivity analysis confirmed the added effect of metabolites in the prediction of (non-)response in all patients initiating TNFi. When the prediction rule is further validated, non-responders can be identified and offered more suitable treatments, which prevents joint damage, potentially toxic side-effects and saves healthcare costs.

Two of the identified predictors were also found to predict response in the two previous studies ^{11,12}. A lower level of ethanolamine was also associated with response to TNFi in the study by Kapoor et al. ¹¹, however, opposite results for a higher level of lysine were found, which predicted good response in our study and non-response in the study by Priori et al. ¹². In addition, both of these previous studies reported high values of glutamine as predictors of response to TNFi, though glutamine did not predict response in our study with- or without correction for multiple clinical parameters ($p = 0.312$ and $p = 0.555$ respectively). Several differences in study design compared to our study might explain these apparent discrepancies, such as different analytical platforms (technique and targeted panels) and statistical method applied, on top of a smaller sample size as well as the use of urine instead of serum in one of the two studies ¹². Therefore, *snl*-LPC (15:0), *snl*-LPC (18:3- ω 3/ ω 6), ethanolamine and lysine hold potential in predicting response to TNFi therapy when measured with LC-MS and on top of prediction by clinical parameters.

Two amino acids, ethanolamine and lysine, predicted good response to TNFi in our study. Ethanolamine is a primary amino acid and down-regulated levels were found to be predictive for good response to TNFi in our study. It has been reported that abundance of ethanolamine was lower in synovial fluid of RA patients than in non-RA patients ¹². The derivatives of ethanolamine play important roles in many pathways. For example, cytidine diphosphate-ethanolamine can be used as a substrate for *de novo* synthesizing phospholipids phosphatidylethanolamines (PE), which are structural components of biological membranes ¹². Up-regulation of lysine was found to be predictive for good response to TNFi in the present study. Lysine is known to be related to RA treatment. A previous study that measured metabolites in 20 patients before and after three months of TNFi treatment, reported that the concentration of lysine was elevated after three months of TNFi therapy ²⁵. What its exact role in inflammation is remained speculative, however in animal studies, in which acute inflammatory processes were induced, it was indicated that amino acids are redirected from muscle to the liver for acute-phase protein synthesis and gluconeogenesis ^{26,27}. Although absolute levels of ethanolamine and lysine were not directly found associated with DAS28 or

CRP, it is possible that the relative abundance of these metabolites is to some extent informative for the inflammatory status in RA.

Interestingly, a down-regulation of *sn1*-LPC (18:3- ω 3/ ω 6) was found in good responders, while a down-regulated *sn1*-LPC (15:0) was found in non-responders. The most common structure of LPCs is with an even-chain fatty acid on *sn1* or *sn2* position, such as *sn1*-LPC (18:3- ω 3/ ω 6) with an 18-carbon fatty acid. However, *sn1*-LPC (15:0) carries an odd-saturated fatty acid (OCS-FA) chain. As part of lipid metabolism, LPCs and FAs can interconvert²⁸. It is reported that the serum levels of OCS-FAs are associated with the consumption of dairy products and the reduced disease risks of coronary heart disease and type 2 diabetes²⁹, but no study has specified the function of OSC-FA chain LPCs in the human body, and the associations between LPCs and RA have not been explored. Although in the prediction models we adjusted the metabolites for multiple clinical parameters, we were unable to correct for the dietary influences or fasted/non-fasted status. We assumed that dietary variability would be equally distributed across response groups, however, the observed levels of OCS-FAs as predictors of response might indicate that this is not the case for dairy products.

Secondary aim of the present study was to explore the relation between metabolite profiles and RA disease activity in general while correcting for most important clinical parameters. We found 88 individual metabolites related to CRP, ESR or DAS28, among which LPCs and FAs were abundant. We found that LPCs were negatively associated with all three parameters, which is a consistent finding across several studies in humans^{30,31}. LPCs are lysophospholipids that play important roles in pathological processes as signaling molecules^{32,33}. The *sn1*-LPC is generated by the hydrolysis of phosphatidylcholine (PC) by phospholipase A2 (PLA2). These LPCs have been studied extensively and their pro- or anti-inflammatory role and magnitude of effect are dependent on the length and (un)saturation of the fatty acyl group^{34–36}. As the precursor of LPC, PC is present in the cell membrane and can bind to CRP to initiate host defense³⁷. Long chain FAs are precursors of pro- and anti-inflammatory molecules^{18,38}, which were found positively associated with CRP, ESR and DAS28 in this study. These results indicate that the FA metabolism was more active in the RA patients with higher disease activity and inflammation. Oxylipins generated by COX, LOX, and ROS pathway have potent pro-inflammatory effects and were positively associated with CRP or DAS28 while oxylipins derived from the CYP450 pathway were negatively associated with CRP, which can be explained by their anti-inflammatory effects³⁹.

In our study, several amines were found to be significantly associated with DAS28 (6 out of 7 negatively), CRP (all 12 positively) and ESR (4 out of 5 positively). Of those amines negatively related with DAS28, histidine, asparagine, and threonine were reported to be downregulated in RA patients compared to healthy controls^{10,28}. If these markers are RA-specific, it is not surprising they also signify disease severity to a certain extent, which is indeed what can be concluded from the (negative) relation to DAS28 observed in our study. Amino acids in arginine metabolism (arginine and ornithine), tryptophan metabolism (serotonin and tryptophan) and branched-chain amino acids (isoleucine, leucine, and valine) were found to be positively associated with CRP, which may indicate as was suggested previously, that muscle proteins are degraded to amino acids and are redirected to the liver for acute-phase protein synthesis and gluconeogenesis^{26,27,40,41}. Serotonin and taurine, which were positively associated with CRP or ESR, were reported associated with oxidative stress and could therefore be linked to the inflammatory processes in RA^{42,43}. In conclusion, most of the metabolites we found to associate with disease inflammation have been described before, either in vivo or in vitro, and in most cases have been shown to be related to inflammation in RA in this study.

Although DAS28 has been extensively validated and is the most widely used instrument in clinical trials as well as in daily practice, it should also be noted that it has been claimed to be a rather instable monitoring instrument in RA patients with stable disease^{44,45}. Two recently developed and validated instruments—the simplified disease activity index (SDAI)¹⁵ and the clinical disease activity index (CDAI)⁴⁶, have been suggested to be used instead in clinical practice. Especially SDAI recently gained more relevance due to its inclusion in the American College of Rheumatism/EULAR remission criteria⁴⁷. It has been reported that DAS28, SDAI, and CDAI do not result in the same classification of patients⁴⁸. These newer instruments may reflect disease activity to a better extent, however SDAI and CDAI could not be used in our analyses because the Evaluator Global Assessment, a component included in both SDAI and CDAI, was not systemically collected in the BiOCURA study.

In this study, only metabolites at baseline were used to predict response and cross-sectionally investigate biomarkers for disease activity. As for the latter aim, investigating the change over time of metabolites might gain more insight in the most relevant metabolites regarding disease activity than any cross-sectional study, as they reflect prospective changes in RA patients.

Conclusions

Metabolomic profiling is a powerful technique, which can be applied to analyze a wide range of metabolites from small sample volumes. Therefore, it has the potential of identification of biomarkers and increasing the understanding of the metabolic pathogenic pathways involved in a disease, such as RA. In the present study, we employed metabolomic profiling to identify candidate metabolites to predict clinical response and assessed associations between metabolites and disease activity of RA. Because subjects were selected from an observational study, the heterogeneity needed to be adjusted for possible influential factors in all analyses. Yet, regardless of the possible absence of important influential factors, we showed that the predictive ability of a model can be quite high ($AUC > 0.8$) in a heterogeneous setting like clinical practice. As for its potential use, metabolites allow a better identification of non-responders on top of clinical parameters, which is of added value in determining the most fitting treatment for each individual patient. Further external validation is needed to assess the robustness of these findings and its potential value for clinical application.

This is the first time that serum metabolomic profiles analyzed by LC-MS have been demonstrated to predict therapeutic response to biological treatment in RA. It would be worth studying how these metabolites could be used for predicting patients' response with external validation in a more homogenous cohort and thereby potentially optimize the treatment strategy for patients with RA.

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Supplementary information

Tables

Table S1. Baseline characteristics of all selected subjects (n=231), and split for all EULAR good-responders and non-responders (n=80 each).

	All (n=231)	Non-responders (n=80)	Good responders (n=80)	p-value
Gender, female, n (%)	172 (74.5)	64 (80.0)	57 (71.3)	0.28
Menopausal status of females, n(%)				0.82
Pre-menopause	55 (23.8)	22 (34.4)	22 (38.6)	
Post-menopause	107 (46.3)	36 (56.3)	33 (57.9)	
Unknown	10 (4.3)	6 (9.4)	2 (3.5)	
Age, years, mean (SD)	54.5 (13.1)	52.8 (13.5)	52.8 (13.0)	1.00
Disease duration, years, mean (SD)	7.0 (3.0-14.0)	6.0 (3.0-12.0)	7.0 (3.0-12.8)	0.79
Smoking, currently	53 (22.9)	18 (22.5)	18 (22.5)	1.00
Alcohol, >7 units/week	39 (17.0)	9 (11.3)	16 (20.3)	0.13
BMI, kg/m ² , mean (SD)	26.6 (5.1)	27.1 (5.1)	26.5 (4.9)	0.47
Positive RF, n(%)	151 (65.4)	47 (58.8)	59 (73.8)	0.07
Positive ACPA, n(%)	161 (69.7)	50 (62.5)	59 (73.8)	0.17
CRP, mg/dL, median (IQR)	6.0 (3.0-16.5)	5.0 (2.0-10.5)	8.0 (3.0-20.3)	0.05
Baseline DAS28, mean (SD)	4.7 (1.1)	4.4 (1.2)	4.6 (0.9)	0.18
TJC, median (IQR)	7.0 (2.0-14.0)	7.0 (2.0-14.0)	7.0 (3.0-12.8)	0.71
SJC, median (IQR)	2.0 (0.0-4.0)	1.0 (0.0-3.0)	2.0 (0.0-4.0)	0.06
ESR, mm/h, median IQR	21.0 (21.0-39.0)	18.0 (6.0-34.0)	18.0 (10.0-36.5)	0.53
VAS-GH, mean (SD)	58.2 (22.9)	58.2 (23.1)	55.8 (22.6)	0.50

Descriptive statistics are expressed as number (%) for dichotomized variables, and mean $\pm$ standard deviation (SD) and median and interquartile range (IQR) for respectively normally and non-normally distributed variables. The p-value is calculated for the difference between responders and non-responders. ACPA, anti-citrullinated protein antibody; BMI, body mass index; DAS28, disease activity score based on 28 joint count; ESR, erythrocyte sedimentation rate; IQR: interquartile range; RF: rheumatoid factor; SJC, 28 swollen joint count; TJC, 28 tender joint count; VAS-GH, 100mm visual analogue scale on general health.

Table S2. Previously and currently used treatments of all selected subjects (n = 231) and split for good responders and non-responders (n = 80 each). P-values were calculated based on chi-square/fisher exact.

Treatment	All (n=231)	Non-responder (n=80)	Good responders (n=80)	p value
No. of previously used bDMARDs, n (%)				0.16
0 (naïve)	116 (50.2)	37 (46.3)	49 (61.3)	
1	56 (24.2)	24 (30.0)	17 (21.3)	
>1	59 (25.5)	17 (23.8)	14 (17.5)	
Initiated treatment, n (%)				0.10
Adalimumab	70 (30.3)	25 (31.3)	27 (33.8)	
Etanercept	60 (26.0)	22 (27.5)	23 (28.8)	
Golimumab	23 (10.0)	7 (8.8)	5 (6.3)	
Infliximab	10 (4.3)	6 (7.5)	3 (3.8)	
Certolizumab	10 (4.3)	4 (5.0)	2 (2.5)	
Abatacept	19 (8.2)	8 (10.0)	3 (3.8)	
Tocilizumab	25 (10.8)	3 (3.8)	14 (17.5)	
Rituximab	14 (6.1)	5 (6.3)	3 (3.8)	
Concomitant DMARDs, n (%)				1.00
Methotrexate	166 (71.9)	55 (68.8)	64 (80)	0.15
Hydroxychloroquine	50 (21.6)	18 (22.5)	19 (23.8)	1.00
Leflunomide	28 (12.1)	11 (13.8)	5 (6.3)	0.19
Glucocorticoids	93 (40.3)	36 (45.0)	26 (32.5)	0.14
Concomitant non-DMARDs, n (%)				
Statins	34 (14.7)	14 (17.5)	6 (7.5)	0.09
Anti-osteoporosis	101 (43.7)	37 (46.3)	32 (40.0)	0.52
Anti-hypertention	68 (29.4)	25 (31.2)	17 (21.3)	0.21
NSAIDs	142 (61.5)	45 (56.3)	49 (61.3)	0.63

P-values were calculated based on chi-square/fisher exact.

DMARDs: biological DMARDs, DMARDs: disease-modifying antirheumatic drug

Table S3. List of relative standard deviations (RSD) for all 139 measured metabolites

Phospholipids*	RSD	Fatty acids*	RSD	Oxylipins	RSD	Amines	RSD
LPE (16:0)	5%	FA (14:0)	8%	9,10-DiHOME	6%	Ethanolamine	6%
LPE (18:0)	3%	FA (14:1)	26%	12,13-DiHOME	6%	Ornithine	3%
LPE (18:1)	5%	FA (15:0)	23%	TXB2	7%	Lysine	5%
LPE (18:2)	5%	FA (16:0)	7%	13-HODE	7%	Glycine	4%
LPE (20:3- ω 3/ ω 6)	8%	FA (16:1)	5%	14-HDoHE	8%	Sarcosine	10%
LPE (20:4)	5%	FA (17:0)	12%	13-HDoHE	8%	Alanine	3%
LPE (20:5)	10%	FA (17:1)	11%	14,15-DiHETrE	10%	3-Aminoisobutyric acid	6%
LPE (22:5- ω 3)	9%	FA(18:0)	9%	13,14-dihydro-PGF _{2a}	11%	α -aminobutyric acid	3%
LPE (22:5- ω 6)	22%	FA (18:1)	3%	9-HODE	11%	Serine	4%
LPE (22:6)	4%	FA (18:2)	3%	19,20-DiHDPA	12%	Cystathionine	7%
<i>sn</i> 2-LPC (14:0)	11%	FA (18:3- ω 3/ ω 6)	3%	11-HETE	13%	Proline	14%
<i>sn</i> 1-LPC (14:0)	6%	FA (20:0)	18%	15S-HETrE	14%	Valine	4%
<i>sn</i> 1-LPC (15:0)	7%	FA (20:1)	8%	10-HDoHE	15%	Threonine	4%
<i>sn</i> 2-LPC (16:0)	7%	FA (20:2)	13%	5,6-DiHETrE	16%	Cysteine	6%
<i>sn</i> 1-LPC (16:0)	2%	FA (20:3- ω 3/ ω 6)	6%	11,12-DiHETrE	16%	Taurine	4%
<i>sn</i> 2-LPC (16:1)	8%	FA (20:3- ω 9)	20%	LTB4	17%	Pipecolic acid	6%
<i>sn</i> 1-LPC (16:1)	6%	FA (20:4- ω 6)	4%	8,9-DiHETrE	17%	Isoleucine	3%
<i>sn</i> 2-LPC (18:0)	3%	FA (20:5- ω 3)	6%	9,10-EpOME	17%	Leucine	4%
<i>sn</i> 1-LPC (18:0)	2%	FA (22:4)	6%	5-HETE	18%	4-Hydroxyproline	3%
<i>sn</i> 2-LPC (18:1)	4%	FA (22:5- ω 3)	3%	PGE2	18%	Asparagine	3%
<i>sn</i> 1-LPC (18:1)	6%	FA (22:5- ω 6)	4%	17,18-DiHETE	18%	Glycylglycine	4%
<i>sn</i> 2-LPC (18:2)	8%	FA (22:6)	6%	12-HETE	18%	Aspartic acid	4%
<i>sn</i> 1-LPC (18:2)	7%	FA (24:0)	15%	20-carboxy-LTB4	18%	s-Methylcysteine	5%
<i>sn</i> 2-LPC (18:3- ω 3/ ω 6)	20%	FA (24:1)	9%	TXB1	19%	Homocysteine	7%
<i>sn</i> 1-LPC (18:3- ω 3/ ω 6)	7%			12,13-EpOME	19%	O-Phosphoethanolamine	14%
<i>sn</i> 1-LPC (19:0)	24%			9-HOTrE	20%	Glutamine	4%
<i>sn</i> 1-LPC (20:1)	13%			11-HDoHE	20%	Glutamic acid	4%
<i>sn</i> 1-LPC (20:2)	7%			9,12,13-TriHOME	20%	Methionine	4%
<i>sn</i> 2-LPC (20:3- ω 3/ ω 6)	9%			TXB3	21%	Histidine	5%
<i>sn</i> 1-LPC (20:3- ω 3/ ω 6)	9%			8-HETE	22%	Methionine sulfoxide	9%
<i>sn</i> 1-LPC (20:3- ω 9)	17%			12S-HHTrE	30%	Phenylalanine	3%
<i>sn</i> 2-LPC (20:4)	6%					1-Methylhistidine	15%
<i>sn</i> 1-LPC (20:4)	8%					Arginine	8%
<i>sn</i> 2-LPC (20:5)	9%					Citrulline	3%
<i>sn</i> 1-LPC (20:5)	8%					Serotonin	11%
<i>sn</i> 1-LPC (22:4)	9%					Tyrosine	4%
<i>sn</i> 1-LPC (22:5- ω 3)	8%					Tryptophan	4%
<i>sn</i> 1-LPC (22:5- ω 6)	9%					Kynurenine	4%
<i>sn</i> 2-LPC (22:6)	8%					γ -glutamylalanine	12%

<i>sn1</i> -LPC (22:6)	7%	Glutathione	10%
LPC (O-16:0) ^c	6%		
LPC (O-18:0)	12%		
LPC (O-18:1)	6%		
LPC (O-18:2)	19%		

The RSDs were calculated on the metabolites in the QC samples. The "sn1-" or "sn2-" prefix is used to indicate the position of the fatty acid chain esterified to the glycerol backbone of a lysophospholipid. The 'O-' prefix of LPC is used to identify a plasmalogen lysophospholipid, where the fatty acid chain is attached via a vinyl ether linkage to the glycerol backbone. The 'ω3', 'ω6', 'ω9' are used to indicate double bond (C=C) at the third, sixth or ninth carbon atom from the end of the fatty acid chain.

*The number in brackets represent the long-chain fatty acid attached in the lysophospholipids (e.g. FA(14:0) represents a fatty acid with 14 carbons and 0 double bonds).

Table S4 List of detected metabolites in lipids analysis

Lipid Class	Lipid Maps	Metabolite species	Amount (n)
Free fatty acids (FA)	FA01	14:0, 14:1, 15:0, 16:0, 16:1, 17:0, 17:1, 18:0, 18:1, 18:2, 18:3-ω3/ω6, 20:0, 20:1, 20:2, 20:3-ω3/ω6, 20:3-ω9, 20:4-ω6, 20:5-ω3, 22:4, 22:5-ω3, 22:5-ω6, 22:6, 24:0, 24:1	24
Lysophosphatidylcholine (LPC)	GP0105	<i>sn1</i> : 14:0, 15:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3-ω3/ω6, 19:0, 20:1, 20:2, 20:3-ω3/ω6, 20:3-ω9, 20:4, 20:5, 22:4, 22:5-ω3, 22:5-ω6, 22:6 <i>sn2</i> : 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3-ω3/ω6, 20:3-ω3/ω6, 20:4, 20:5, 22:6	30
Lysophosphatidylethanolamine (LPE)	GP0205	16:0, 18:0, 18:1, 18:2, 20:3-ω3/ω6, 20:4, 20:5, 22:5-ω3, 22:5-ω6, 22:6	10
Plasmalogen			
Lysophosphatidylcholine (LPC-O)	GP0106	16:0, 18:0, 18:1, 18:2	4

Table S5 List of detected metabolites in oxylipins analysis.

Metabolite	Systematic name	Formula	Lipid Maps ID
10-HDoHE	(+/-)-10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-docosahexaenoic acid	C22H32O3	LMFA04000027
11,12-DiHETrE	(±)11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	C20H34O4	LMFA03050008
11-HDoHE	(+/-)-11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid	C22H32O3	LMFA04000028
11-HETE	11R-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	C20H32O3	LMFA03060028
12,13-DiHOME	(+/-)-12,13-dihydroxy-9Z,15Z-octadecadienoic acid	C18H32O4	LMFA02000046
12,13-EpOME	(+/-)-12(13)-epoxy-9Z-octadecenoic acid	C18H32O3	LMFA02000038
12-HETE	12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	C20H32O3	LMFA03060088
12S-HHTrE	12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid	C17H28O3	LMFA03050002
13,14-dihydro-PGF _{2a}	9S,11R,15S-trihydroxy-5Z-prostenoic acid	C20H36O5	LMFA03010079
13-HDoHE	(+/-)-13-hydroxy-4Z,7Z,10Z,14E,16Z,19Z-docosahexaenoic acid	C22H32O3	LMFA04000029

13-HODE	13S-hydroxy-9Z,11E-octadecadienoic acid	C18H32O3	LMFA01050349
14,15-DiHETrE	14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid	C20H34O4	LMFA03050010
14-HDoHE	(+/-)-14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid	C22H32O3	LMFA04000030
15S-HETrE	15S-hydroxy-8Z,11Z,13E-eicosatrienoic acid	C20H34O3	LMFA03050007
17,18-DiHETE	(+/-)-17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	C20H32O4	LMFA03060078
19,20-DiHDPA	(±)19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	C22H34O4	LMFA04000043
20-carboxy-LTB4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraene-1,20-dioic acid	C20H30O6	LMFA03020016
5,6-DiHETrE	5S,6S-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid	C20H32O4	LMFA03060018
5-HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	C20H32O3	LMFA03060002
8,9-DiHETrE	8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid	C20H34O4	LMFA03050006
8-HETE	(±)8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	C20H32O3	LMFA03060086
9,10-DiHOME	9,10-dihydroxy-12Z-octadecenoic acid	C18H34O4	LMFA01050350
9,10-EpOME	(+/-)-9(10)-epoxy-12Z-octadecenoic acid	C18H32O3	LMFA02000037
9,12,13-TriHOME	9S,12S,13S-trihydroxy-10E-octadecenoic acid	C18H34O5	LMFA02000014
9-HODE	9S-hydroxy-10E,12Z-octadecadienoic acid	C18H32O3	LMFA01050278
9-HOTrE	9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid	C18H30O3	LMFA02000024
LTB4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	C20H32O4	LMFA03020001
PGE2	9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid; Prostin E2	C20H32O5	LMFA03010003
TXB1	9S,11,15S-trihydroxy-thrombox-13E-enoic acid	C20H36O6	LMFA03030008
TXB2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid	C20H34O6	LMFA03030002
TXB3	9S,11,15S-trihydroxy-thromboxa-5Z,13E,17Z-trien-1-oic acid	C20H32O6	LMFA03030006

Table S6 List of detected metabolites in amines analysis

Metabolite	Chemical formula	HMDB ID	InChI Key
1-Methylhistidine	C7H11N3O2	HMDB00001	BRMWTNUJHUMWMS-LURJTMIESA-N
3-Aminoisobutyric acid	C4H9NO2	HMDB00452	QWCKQJZIFLGMSD-VKHMHEASA-N
4-Hydroxyproline	C5H9NO3	HMDB00725	PMMYEEVYMWASQN-DMTCNVIQSA-N
Alanine	C3H7NO2	HMDB00161	QNAYBMKLOCPYGI-REOHCLBHSA-N
α -aminobutyric acid	C4H9NO2	HMDB03911	QCHPKSFMHPSNR-UHFFFAOYSA-N
Arginine	C6H14N4O2	HMDB00517	ODKSFYDXXFQ-N-BYPYZUCNSA-N
Asparagine	C4H8N2O3	HMDB00168	DCXYFEDJOCNDF-REOHCLBHSA-N
Aspartic acid	C4H7NO4	HMDB00191	CKLJMWZTIZZHC-REOHCLBHSA-N
Citrulline	C6H13N3O3	HMDB00904	RHGKLRLOHJDR-BYPYZUCNSA-N
Cystathionine	C7H14N2O4S	HMDB00099	ILRYLPWNYFXEMH-WHFBIKZSA-N
Cysteine	C3H7NO2S	HMDB00574	XUJNEKJLAYXESH-REOHCLBHSA-N
Ethanolamine	C2H7NO	HMDB00149	HZAXFHJVJLSVMW-UHFFFAOYSA-N
Glutamic acid	C5H9NO4	HMDB00148	WHUUTDBJXJRKMK-VKHMHEASA-N
Glutamine	C5H10N2O3	HMDB00641	ZDXPYRJPNDRMX-VKHMHEASA-N
Glutathione	C10H17N3O6S	HMDB00125	RWSXRVCMGQZWBV-WDSKDSINSA-N
Glycine	C2H5NO2	HMDB00123	DHMQDGOQFOQNFH-UHFFFAOYSA-N
Glycylglycine	C4H8N2O3	HMDB11733	YMAWOPBAYDPSLA-UHFFFAOYSA-N
Histidine	C5H9N3	HMDB00870	NTYJJOPFIAHURM-UHFFFAOYSA-N
Homocysteine	C4H9NO2S	HMDB00742	FFHYZYDWPBMWHY-UHFFFAOYSA-N
Isoleucine	C6H13NO2	HMDB00172	AGPKZVBTJNPAG-WHFBIAKZSA-N
Kynurenine	C10H12N2O3	HMDB00684	YGPSJZOEDVAXAB-QMMMGOBSA-N
Leucine	C6H13NO2	HMDB00687	ROHFNLRQFUQHCH-YFKPBYRVSA-N
Lysine	C6H14N2O2	HMDB00182	KDXKERNBIXSRK-YFKPBYRVSA-N
Methionine	C5H11NO2S	HMDB00696	FFEARJCKVFRZRR-BYPYZUCNSA-N
Methionine sulfoxide	C5H11NO3S	HMDB02005	QEFRNWWLZKMPFJ-UHFFFAOYSA-N
O-Phosphoethanolamine	C2H8NO4P	HMDB00224	SUHOOTKUPISOBE-UHFFFAOYSA-N
Ornithine	C5H12N2O2	HMDB00214	AHLPHDHHMVZTML-BYPYZUCNSA-N
Phenylalanine	C9H11NO2	HMDB00159	COLNVLDHVKWLRT-QMMMGOBSA-N
Pipecolic acid	C6H11NO2	HMDB00716	HXEACLLIILLPRG-YFKPBYRVSA-N
Proline	C5H9NO2	HMDB00162	ONIBWKKTOPOVIA-BYPYZUCNSA-N
Sarcosine	C3H7NO2	HMDB00271	FSYKKLYZXSNPZ-UHFFFAOYSA-N
Serine	C3H7NO3	HMDB00187	MTCFGRXMIJLQNBG-REOHCLBHSA-N
Serotonin	C10H12N2O	HMDB00259	QZAYGJVTTNCVMB-UHFFFAOYSA-N
s-Methylcysteine	C4H9NO2S	HMDB02108	IDIDJDIHTAOVLG-VKHMHEASA-N
Taurine	C2H7NO3S	HMDB00251	XOAAWQZATWQOTB-UHFFFAOYSA-N
Threonine	C4H9NO3	HMDB00167	AYFVYJQAPQTCCC-GBXJSLDSA-N
Tryptophan	C11H12N2O2	HMDB00929	QIVBCDIJAJQPS-VIFPVBQESA-N
Tyrosine	C9H11NO3	HMDB00158	OUYCCASQSFEME-QMMMGOBSA-N
Valine	C5H11NO2	HMDB00883	KZSNJWFQEVHDMF-BYPYZUCNSA-N
γ -glutamylalanine	C10H17N3O6	HMDB11738	JBIFYLXEJFQWMU-WDSKDSINSA-N

Table S7. Classification table of predicted good- and non-responders and observed good- and non-responders.

	Observed Predicted	Good responder	Non-responders	Sensitivity	Specificity	PPV	NPV	MR
Clinical model	Good response	43	20	0.782	0.600	0.683	0.714	0.305
	Non-response	12	30					
Combined model	Good response	39	5	0.709	0.900	0.887	0.738	0.200
	Non-response	16	45					

PPV: positive predictive values; NPV: negative predictive values; MR: misclassification rate.

Table S8. Net reclassification index of prediction models for sensitivity analysis.

Observed response	Predicted by clinical model	Predicted by combined model	
		Non-response	Response
Non-responders (n=50)	Non-response	30 (equal)	0 (worsening)
	Response	15 (improvement)	5 (equal)
Responders (n=100)	Non-response	23 (equal)	12 (improvement)
	Response	20 (worsening)	45 (equal)

Table S9. Metabolites cross-sectionally associated with either baseline DAS28, ESR or CRP ($p < 0.05$) based on the complete cohort of bDMARD users ($n=231$).

Metabolite	LnCRP		DAS28		lnESR	
	Coefficients	p-value	Coefficients	p-value	Coefficients	p-value
<i>sn2</i> -LPC (14:0)	-0.666	0.004	-0.066	0.323	-0.604	0.002
<i>sn1</i> -LPC (14:0)	-0.713	<0.001	-0.080	0.255	-0.606	< 0.001
<i>sn2</i> -LPC (16:1)	-0.525	0.194	-0.122	0.098	-0.759	< 0.001
<i>sn1</i> -LPC (18:3- ω 3/ ω 6)	-0.223	0.487	0.048	0.564	-0.646	< 0.001
<i>sn1</i> -LPC (20:5)	-1.023	0.045	-0.007	0.944	-0.556	0.138
<i>sn1</i> -LPC (16:1)	-0.633	0.099	-0.086	0.213	-0.744	< 0.001
<i>sn2</i> -LPC (18:2)	-0.396	0.078	-0.785	0.001	-0.468	0.022
<i>sn1</i> -LPC (15:0)	-0.960	0.002	-10.176	0.243	-0.578	0.002
<i>sn1</i> -LPC (18:2)	-0.397	0.134	-0.753	0.001	-0.437	0.031
<i>sn1</i> -LPC (20:4)	-0.418	0.049	-0.056	0.475	-0.357	0.029
<i>sn2</i> -LPC (20:3)- ω 3/ ω 6	-0.093	0.510	-0.071	0.280	-0.442	0.028
LPE (22:5- ω 3)	-0.561	0.038	-0.071	0.450	-0.300	0.058
<i>sn1</i> -LPC (22:5- ω 3)	-0.710	0.009	-0.109	0.167	-0.573	0.003
<i>sn1</i> -LPC (20:3- ω 3/ ω 6)	-0.268	0.081	-0.090	0.200	-0.513	0.006
LPE (22:5- ω 6)	-0.437	0.223	-0.730	0.012	-0.602	0.002
<i>sn1</i> -LPC (22:5- ω 6)	-0.379	0.004	-0.725	< 0.001	-0.596	< 0.001
<i>sn1</i> -LPC (18:1)	-0.355	0.182	-0.081	0.314	-0.439	0.032
<i>sn1</i> -LPC (20:3- ω 9)	< 0.001	0.999	-0.132	0.059	-0.482	0.004
<i>sn1</i> -LPC (22:4)	-0.488	0.005	-0.049	0.517	-0.677	< 0.001
LPC (O-16:0)	-0.289	0.015	-0.729	< 0.001	-0.289	0.059
LPC (O-18:2)	-0.441	0.326	-0.103	0.155	-0.778	0.005
<i>sn1</i> -LPC (20:2)	-0.351	0.077	-0.011	0.875	-0.506	0.010
<i>sn1</i> -LPC (19:0)	-0.297	0.517	-0.662	0.004	-0.276	0.147
LPC (O-18:0)	-0.537	0.006	-0.057	0.520	-0.212	0.177
FA (14:1)	0.627	<0.001	0.607	< 0.001	0.444	< 0.001
FA (14:0)	0.278	0.037	0.059	0.424	0.407	< 0.001
FA (20:5- ω 3)	0.025	0.872	0.009	0.918	0.496	< 0.001
FA (18:3- ω 3/ ω 6)	0.402	0.007	0.090	0.263	0.371	0.001
FA (16:1)	0.441	0.006	0.640	< 0.001	0.477	< 0.001
FA (15:0)	0.232	0.034	0.036	0.633	0.290	0.003
FA (22:6)	0.434	0.169	-0.012	0.879	0.726	< 0.001
FA (20:4- ω 6)	0.095	0.574	0.013	0.846	0.482	< 0.001
FA (18:2)	0.443	0.039	0.067	0.314	0.459	< 0.001
FA (22:5- ω 3)	0.363	0.111	0.590	< 0.001	0.489	< 0.001
FA (17:1)	0.475	0.003	0.052	0.540	0.450	< 0.001
FA (22:5- ω 6)	0.380	0.198	-0.008	0.917	0.571	< 0.001
FA (20:3- ω 3/ ω 6)	0.194	0.206	-0.012	0.869	0.502	< 0.001
FA (16:0)	0.244	0.023	0.421	< 0.001	0.331	< 0.001

FA (20:3-ω9)	0.226	0.013	0.075	0.814	-0.045	0.749
FA (18:1)	0.547	0.009	0.583	< 0.001	0.471	< 0.001
FA (22:4)	0.438	0.060	0.597	< 0.001	0.460	< 0.001
FA (17:0)	0.220	0.001	0.353	0.001	0.215	0.013
FA (20:2)	1.032	0.064	0.002	0.989	0.406	0.002
FA (18:0)	0.175	0.015	0.351	< 0.001	0.223	0.016
FA (20:1)	0.451	0.039	0.093	0.220	0.427	< 0.001
FA (24:1)	0.349	0.151	0.529	< 0.001	0.369	0.004
TXB3	0.486	< 0.001	0.396	0.183	0.095	0.436
TXB1	0.404	0.002	0.937	< 0.001	0.596	0.001
TXB2	0.467	0.002	0.502	0.213	0.014	0.931
9,12,13-TriHOME	-0.081	0.714	-0.646	0.073	-0.505	0.008
PGE2	0.392	0.015	0.736	0.021	0.362	0.017
13,14-dihydro-PGF _{2α}	-0.352	0.008	-0.769	< 0.001	-0.411	0.060
19,20-DiHDP A	-0.683	0.020	-0.036	0.924	0.006	0.984
14,15-DiHETrE	-0.746	<0.001	0.368	0.190	0.234	0.457
11,12-DiHETrE	-0.586	0.006	0.475	0.051	0.316	0.205
9-HOTrE	0.068	0.686	0.359	0.020	0.292	0.019
8,9-DiHETrE	-0.611	0.005	-0.216	0.427	0.070	0.669
5,6-DiHETrE	-0.997	0.005	0.255	0.644	0.054	0.900
9-HODE	0.012	0.933	0.255	0.183	0.260	0.010
14-HDoHE	0.427	0.136	0.749	0.041	0.548	0.003
10-HDoHE	0.238	0.295	0.675	0.009	0.645	< 0.001
13-HDoHE	0.270	0.362	0.648	0.086	0.580	0.003
11-HETE	0.396	0.001	0.895	< 0.001	0.420	0.005
11-HDoHE	0.242	0.063	0.860	< 0.001	0.526	< 0.001
12-HETE	0.463	0.003	0.708	0.048	0.412	0.006
8-HETE	0.243	0.020	0.654	< 0.001	0.413	0.001
15S-HETrE	0.281	0.013	0.730	< 0.001	0.392	0.012
5-HETE	0.273	0.010	0.729	< 0.001	0.498	< 0.001
Ornithine	0.483	0.003	0.061	0.832	0.024	0.905
Sarcosine	-0.386	0.065	-0.664	0.003	-0.558	< 0.001
Alpha-aminobutyric acid	0.181	0.446	-0.433	0.035	-0.166	0.479
Proline	0.284	0.028	-0.051	0.573	0.103	0.373
Valine	0.840	<0.001	-0.017	0.829	0.268	0.134
Threonine	0.106	0.678	-0.205	0.006	-0.102	0.655
Cysteine	0.232	0.107	0.085	0.392	0.280	0.017
Taurine	0.626	0.279	0.124	0.086	0.461	0.005
Isoleucine	0.638	0.001	0.041	0.606	0.197	0.218
Leucine	0.642	<0.001	0.323	0.160	0.190	0.284
4-Hydroxyproline	0.285	0.024	-0.007	0.952	-0.078	0.425
Asparagine	-0.033	0.938	-0.150	0.024	-0.146	0.553
Glycylglycine	-0.030	0.944	-0.164	0.015	-0.248	0.324

Histidine	0.051	0.904	-0.284	< 0.001	-0.113	0.576
Arginine	0.434	0.012	-0.059	0.389	0.211	0.170
Serotonin	0.977	<0.001	-0.044	0.655	0.684	< 0.001
Tryptophan	0.791	<0.001	-0.113	0.216	-0.108	0.770
Kynurenine	0.529	0.026	0.082	0.338	-0.004	0.983
Gamma-glutamylalanine	0.941	< 0.001	-0.111	0.099	0.209	0.413
Glutathione	0.388	0.016	0.433	0.006	0.472	< 0.001
<i>sn</i> 2-LPC (20:5)	-0.728	0.136	-0.042	0.671	-0.435	0.274
<i>sn</i> 2-LPC (18:3- ω 3/ ω 6)	0.138	0.521	0.147	0.622	-0.314	0.073
LPE (20:5)	-0.237	0.409	0.226	0.459	0.015	0.947
<i>sn</i> 2-LPC (20:4)	-0.259	0.127	-0.064	0.413	-0.321	0.058
<i>sn</i> 2-LPC (22:6)	-0.444	0.085	-0.081	0.300	0.088	0.677
<i>sn</i> 1-LPC (22:6)	-0.456	0.095	-0.069	0.369	0.147	0.561
LPE (18:2)	-0.412	0.338	-0.026	0.788	-0.075	0.770
LPE (22:6)	-0.163	0.429	0.359	0.135	0.346	0.070
LPE (20:4)	-0.484	0.177	-0.008	0.919	0.070	0.755
<i>sn</i> 2-LPC (16:0)	-0.124	0.592	-0.015	0.852	-0.118	0.428
<i>sn</i> 2-LPC (18:1)	-0.110	0.561	-0.057	0.451	-0.298	0.126
<i>sn</i> 1-LPC (16:0)	-0.191	0.260	-0.037	0.626	-0.180	0.195
LPE (16:0)	-0.283	0.174	0.021	0.814	-0.109	0.401
LPE (20:3- ω 3/ ω 6)	-0.359	0.336	-0.052	0.550	-0.023	0.900
LPE (18:1)	-0.316	0.394	-0.037	0.747	0.178	0.439
<i>sn</i> 2-LPC (17:0)	0.133	0.449	0.203	0.492	-0.194	0.199
<i>sn</i> 2-LPC (18:0)	-0.085	0.550	-0.048	0.504	-0.086	0.464
LPC (O-18:1)	-0.179	0.174	-0.022	0.757	-0.142	0.198
LPE (18:0)	-0.130	0.396	0.056	0.547	0.247	0.125
<i>sn</i> 1-LPC (18:0)	-0.077	0.542	-0.051	0.476	-0.064	0.556
<i>sn</i> 1-LPC (20:1)	-0.164	0.356	0.021	0.713	-0.036	0.704
FA (20:0)	0.301	0.110	0.035	0.630	0.191	0.151
FA (24:0)	0.184	0.334	0.076	0.404	0.144	0.347
20-carboxy-LTB4	0.102	0.549	0.262	0.212	0.130	0.313
17,18-DiHETE	-0.405	0.053	0.549	0.081	0.153	0.624
LTB4	-0.056	0.691	0.185	0.550	0.228	0.188
12,13-DiHOME	0.048	0.823	0.244	0.205	0.092	0.479
9,10-DiHOME	-0.108	0.623	0.133	0.543	0.089	0.529
12S-HHTrE	0.209	0.592	0.528	0.189	-0.172	0.414
13-HODE	-0.111	0.446	0.065	0.774	0.160	0.141
12,13-EpOME	-0.066	0.665	0.124	0.476	0.169	0.065
9,10-EpOME	-0.090	0.554	0.111	0.536	0.199	0.059
Ethanolamine	0.091	0.878	0.039	0.620	0.400	0.102
Lysine	0.191	0.291	-0.072	0.246	0.021	0.915
Glycine	0.009	0.987	-0.037	0.673	-0.230	0.453
Alanine	0.138	0.602	-0.097	0.144	-0.080	0.722

3-Aminoisobutyric acid	-0.052	0.872	-0.132	0.095	-0.308	0.187
Serine	0.084	0.768	-0.120	0.152	-0.265	0.250
Cystathionine	-0.178	0.221	-0.095	0.355	-0.233	0.065
Pipecolic acid	0.315	0.070	-0.183	0.126	-0.009	0.963
Aspartic acid	0.180	0.562	0.173	0.585	0.109	0.577
s-Methylcysteine	0.005	0.988	0.066	0.528	0.243	0.327
Homocysteine	-0.063	0.839	0.145	0.072	0.340	0.498
O-Phosphoethanolamine	-0.127	0.493	0.052	0.588	0.097	0.430
Glutamine	0.109	0.751	-0.049	0.500	0.228	0.226
Glutamic acid	-0.248	0.413	0.014	0.861	-0.304	0.111
Methionine	0.048	0.844	-0.052	0.413	-0.061	0.735
Methionine sulfoxide	0.651	0.055	0.040	0.595	0.283	0.204
Phenylalanine	0.242	0.298	0.052	0.831	-0.012	0.951
l-Methylhistidine	0.528	0.113	0.101	0.123	0.284	0.127
Citrulline	-0.309	0.566	0.285	0.568	-0.392	0.326
Tyrosine	-0.091	0.763	-0.014	0.852	0.157	0.398

The log-transformed and autoscaled metabolites were put in a linear GEE on DAS28, ESR and CRP separately, together with possible influencing clinical parameters (age, gender, menopausal status, BMI, smoking status, alcohol consumption, concomitant csDMARDs (methotrexate, hydroxychloroquine, leflunomide, glucocorticoids) and non-DMARDs (statins, antihypertensive drugs, bisphosphonates and NSAIDs). Shown are the regression coefficients and p-values per metabolite, only of those metabolites that associated to either DAS28, ESR or CRP.

Figures

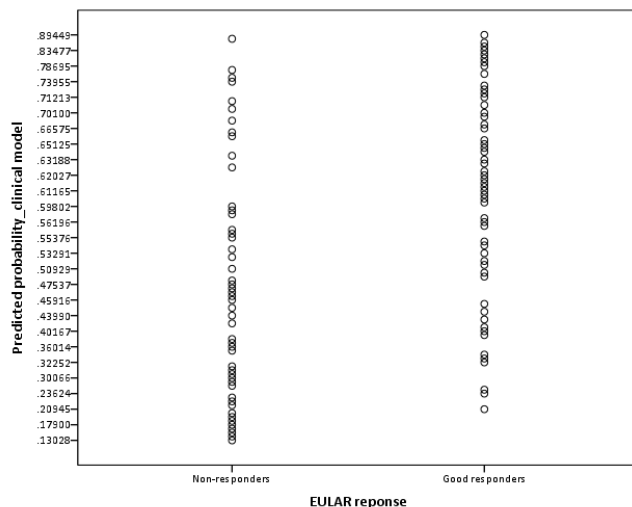


Figure S1. Scatter plot of the clinical model between good responders and non-responders to TNFi therapy. Patients were grouped according to their observed responses on the x-axis; whereas the y-axis represents the predicted probability calculated by the clinical model. The pseudo R-square, as a measure for the explained variance in the observed response by the model was 0.147 (Cox & Snell).

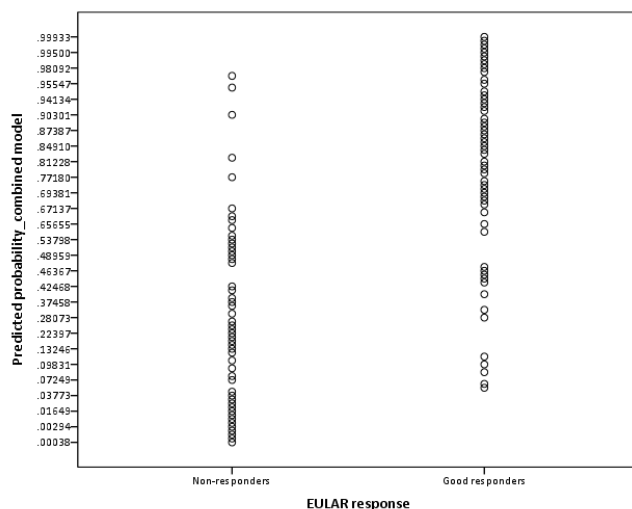


Figure S2. Scatter plot of the combined model between good responders and non-responders to TNFi therapy. Patients were grouped according to their observed responses on the x-axis; whereas the y-axis represents the predicted probability calculated by the combined model. The pseudo R-square, as a measure for the explained variance in the observed response by the model was 0.433 (COX & Snell)

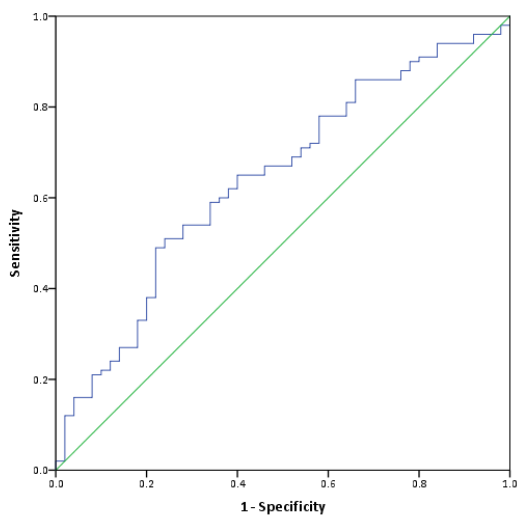


Figure S3. ROC curve for the clinical model containing non-, moderate- and good responders to TNFi therapy. The AUC-ROC was 0.641 (95% CI: 0.548-0.734).

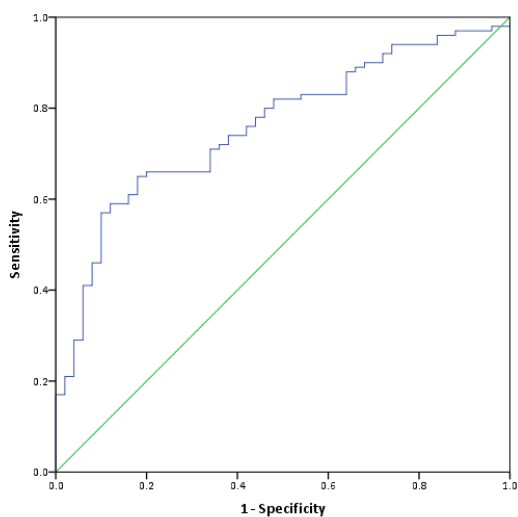


Figure S4. ROC curve for the combined model containing non-, moderate- and good responders to TNFi therapy. The AUC-ROC was 0.760 (95% CI: 0.682-0.837).

Chapter 3

3

Metabolomics profiling of the free and total oxidised lipids in urine by LC-MS/MS: application in patients with rheumatoid arthritis

Based on

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Metabolomics profiling of the free and total oxidised lipids in urine by LC-MS/MS: application in patients with rheumatoid arthritis

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Abstract

Oxidised lipids, covering enzymatic and auto-oxidation synthesised mediators, are important signalling metabolites in inflammation while also providing a readout for oxidative stress, both of which are prominent physiological processes in a plethora of diseases. Excretion of these metabolites via urine are enhanced through the phase-II conjugation with glucuronic acid, resulting in increased hydrophilicity of these lipid mediators. Here we developed a bovine liver- β -glucuronidase hydrolysing sample preparation method, using liquid chromatography coupled to tandem mass spectrometry to analyse the total urinary oxidised lipid profile including the prostaglandins, isoprostanes, dihydroxy-fatty acids, hydroxy-fatty acids, and the nitro-fatty acids. Our method detected more than 70 oxidised lipids biosynthesised from two non-enzymatic and three enzymatic pathways in urine samples. The total oxidised lipids profiling method was developed and validated for human urine, and was demonstrated for urine samples from patients with rheumatoid arthritis. Pro-inflammatory mediators $\text{PGF}_{2\alpha}$, $\text{PGF}_{3\alpha}$, and oxidative stress markers $\text{iPF}_{2\alpha}\text{IV}$, 11-HETE and 14-HDoHE were positively associated with improvement of disease activity score. Furthermore, the anti-inflammatory nitro-fatty acids were negatively associated with baseline disease activity. In conclusion, the developed methodology expands the current metabolic profiling of oxidised lipids in urine, and its application will enhance our understanding of the role these bioactive metabolites play in health and disease.

Introduction

Oxidised lipids are important signalling mediators in health and disease, capable of providing quantitative readouts relating to inflammatory and oxidative stress status. The *de novo* synthesis of oxidised lipids can be broadly divided into enzymatic and auto-oxidation pathways. The auto-oxidation pathway of oxidised lipids is interlinked with reactive oxygen species (ROS) or reactive nitrogen species (RNS), leading to the peroxidation of fatty acids in membrane bound phospholipids and producing the isoprostanes (IsoPs) ^{1,2} or nitro-fatty acids (NO₂-FAs) ³. The lipid peroxidation readout from IsoPs are considered the golden standard for measuring oxidative stress in biological systems ^{4,5}. Interestingly, NO₂-FAs potentiate diverse anti-inflammatory signalling actions regarded as beneficial within health and disease ³. These peroxidised lipids impair membrane and organelle integrity and are subsequently excreted from the cell into systemic circulation via cellular repair mechanisms ¹.

The enzymatic routes include: i). Cyclooxygenase-I/II/III (COX-I/II/III), synthesising the prostaglandins (PGs), ii). 5/12/15- Lipoyxygenase (5/12/15-LOX), synthesising leukotrienes, lipoxins and hydroxyl-fatty acids and lastly iii). Cytochrome P450 (CYP450) responsible for the synthesis of epoxy-fatty acids and dihydroxy-fatty acids ⁶. These enzymatically oxidised lipids are synthesised locally from essential free fatty acids and act as signalling mediators in immune modulation and inflammatory responses ⁶⁻⁹. Due to the potent biological signalling activity of enzymatically oxidised lipids, the active mediators are short-lived in systemic circulation where they are actively metabolised prior to excretion ¹⁰. Thus taking serum and/or plasma as a representative snapshot of the systemic circulation might not be the most suitable approach to study the oxidised lipid profile. Urine, on the other hand, is a non-invasive bio-fluid, which contains the collected excreted downstream metabolic products. These downstream metabolites provide an enriched systemic readout and are indicative of the presence of the active upstream mediators.

Urine does present some sample specific complications for analysis, such as rather large variations in metabolite concentration, limited solubilities of apolar metabolites and conjugation of some metabolites. These complexities are even more prominent during the analyses of lipid-like metabolites in urine. Due to the partial hydrophobic nature, oxidised lipids are often conjugated to increase their hydrophilicity, mainly by phase-II metabolism located in the liver ¹¹. Phase II metabolism comprises different enzymatic conjugation reactions, with oxidised lipids most commonly conjugated with glucuronic acid (GlcA) via UDP-glucuronosyltransferases ¹²⁻¹⁸. Effectively the oxidised lipids can be excreted in

different forms via urine: the unconjugated (free) and the conjugated species^{15,17}. Actually, the GlcA-conjugated oxidised lipids represent a more hydrophilic form of the metabolites.

Robust metabolic profiling of urinary oxidised lipids has been reported using either gas- or liquid chromatography coupled to mass spectrometry^{4,19–27}. However, these methods either detected metabolites in their free form (neglecting the conjugated forms) or focused on a subset of IsoPs and/or PGs. These methodologies lack the broad scope of compounds necessary for a more thorough understanding of disease pathology. Two recent methods reporting on the total (free + conjugated) IsoPs and PGs levels showed increasing urinary metabolite concentrations ranging from 36% to 100%^{15,28}, indicating the significant increases when measuring the total concentration, but these methods excluded the LOX and CYP450 oxidised lipid metabolites. Therefore, it is necessary to develop a method able to measure the total urinary oxidised lipids covering the three enzymatic synthesis routes as well as the auto-oxidation metabolites, broadening its biological range.

In the present study we developed and validated robust methods for measuring both the free and total levels of oxidised lipids in human urine samples, covering the PGs, IsoPs, hydroxyl-fatty acids, epoxy fatty acids, leukotrienes, lipoxins and NO₂-FAs. To measure the total oxidised urinary profile, we investigated the suitability of three different β -glucuronidase enzymes derived from *Helix pomatia*, *Escherichia coli*, and bovine liver. We evaluated these by determining the increase in free metabolites, metabolite stability and enzyme blank effect. Bovine liver derived β -glucuronidase was chosen as the preferred hydrolysing agent and was used in the total oxidised lipid method and validated concurrently with the free oxidised lipid method. Furthermore, we evaluated the benefit of total level oxidised lipids analyses in urine of rheumatoid arthritis patients. The methodology we established covers a broad scope of oxidised lipid, which enables further investigation of the function and mechanism of these lipids in both health and disease.

Materials and Methods

Chemicals and reagents

Ultra-performance liquid chromatography (UPLC)-grade acetonitrile, isopropanol, methanol, ethyl acetate, and purified water were purchased from Biosolve B.V. (Valkenswaard, the Netherlands). Acetic acid, ammonium hydroxide, ammonium acetate, and 2-propanol were acquired from Sigma-Aldrich (Zwijndrecht, the Netherlands). Sodium dihydrogen phosphate dihydrate, sodium hydrogen phosphate and sodium acetate were obtained from Merck (Darmstadt, Germany).

β-glucuronidase enzymes

β-glucuronidase (GUS) from (1) *Helix pomatia* (*H. pomatia*) type H-2 (Aqueous solution, ≥ 85000 units/mL), (2) *Escherichia coli* (*E. coli*) IX-A (Lyophilised power, 1000000-5000000 units/g), and (3) Bovine liver B-1 (Solid, ≥1000000 units/g), together with the exogenous substrate of GUS 4-methylumbelliferyl β-D-glucopyranoside (MUG) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Standards and internal standard solutions

Standards and deuterated standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA), Bio-mol (Plymouth Meeting, PA, USA), or Larodan (Malmö, Sweden). Standard and deuterated standard solutions were prepared in methanol containing butylated hydroxytoluene (BHT) (0.2 mg BHT/EDTA), stored at -80°C. Supplementary Table S1 lists an overview of the deuterated internal standards (ISTDs) used in this study.

Urine Sample collection

Collection of control urine for method development

Morning urine was obtained from 10 volunteers (5 males and 5 females) age 27 to 32. The urine samples were pooled, mixed and 400 µL were aliquoted into 2 mL Eppendorf tubes and immediately stored at -80°C prior to extraction.

Rheumatoid arthritis Patients

Oxidised lipid profiling was performed on urine samples derived from an observational study–BiOCURA²⁹. In BiOCURA, rheumatoid arthritis (RA) patients eligible for biological disease-modifying anti-rheumatic drugs (bDMARDs) were recruited, and urine samples were collected at random times as baseline samples before initiating bDMARD therapy. Clinical parameters and demographic data were also collected at the start of the study as baseline information, including disease activity measured in 28 joints (DAS28), C-reactive protein (CRP), age, sex, BMI, smoking status, alcohol consumption and concomitant DMARDs. The study was approved by the ethics committee of the University Medical Center Utrecht and the institutional review boards of the participating centres. Human material and human data were handled in accordance with the Declaration of Helsinki and written informed consent was obtained from each patient.

Our analysis was restricted to the BiOCURA patients with baseline (before bDMARDs treatment) disease activity score > 2.6 and good or no drug response after 3-month with

Etanercept (ETN) or Adalimumab (ADA) based on the EULAR response criteria. EULAR good response is defined as an improvement in DAS28 > 1.2 and a present DAS28 ≤ 3.2 , whereas a EULAR non-response is assigned to patients with an improvement of 0.6-1.2 with present DAS28 > 5.1 or patients with an improvement ≤ 0.6 . In the end, 40 subjects (20 good responders, 20 non-responders) with ETN and 40 subjects (20 good responders, 20 non-responders) with ADA were included in the present study. ETN and ADA are TNF- α inhibitors, which is the most widely used category of bDMARDs.

Methodology development for optimising enzymatic hydrolysis

Hydrolysis conditions were optimised for each of the three GUSs following the approach in Supplementary Fig. S1. Parameters included enzyme concentration (1000U, 1500U, 2000U per sample), incubation temperature (37°C, 55°C) and time (2h, 6h, 12h, 24h). The obtained experimental results for the three enzymes were used to determine the optimal hydrolysing conditions.

Enzymatic hydrolysing conditions

Literature was used to guide the selection of the optimal hydrolysing conditions for the three selected candidate enzymes with regards to the used buffer composition and pH^{18,30,31}. For both, *H. pomatia* and bovine liver GUS, a 200 mM acetate buffer (pH 4.5) was used for hydrolysis, and for *E. coli* GUS a hydrolysis buffer of 75 mM phosphate buffer (pH 6.8) was used. As a sensitive GUS substrate, MUG was added into each urine sample as a positive control for monitoring GUS activity.

Enzymatic hydrolysis procedure for GlcA-conjugated oxidised lipids

Ice thawed urine samples (400 μ L each) were immediately treated with 10 μ L antioxidants (0.2 mg BHT/EDTA) and spiked with 20 μ L internal standards (ISTDs) and 5 μ L MUG solution. Next, 200 μ L of the specific enzyme solution in its appropriate buffer was added to the sample, and the mixture was vortexed and incubated. After hydrolysis samples were put on ice prior to oxidised lipid extraction.

Total and free urinary oxidised lipid extraction

For both, total and free oxidised lipids, the extraction was performed by the same ethyl acetate liquid-liquid extraction procedure. For the analyses of the free oxidised lipids, 400 μ L urine were spiked with 10 μ L antioxidants and 20 μ L ISTDs, similar to the description of enzymatic hydrolysed (total) samples in ‘Enzymatic hydrolysis procedure for GlcA-conjugated oxidised lipids’. Subsequently, 200 μ L citric acid/phosphate buffer (pH 3) was

added to the total and free urine samples, and oxidised lipids were extracted by adding 1 mL ethyl acetate followed by shaking for 1 min. Samples were centrifuged at 13000 rpm for 10 min (4°C), after which 800 µL upper organic phase was transferred to a new Eppendorf tube. The LLE was repeated for a second time and the collected organic phase was evaporated to dryness in Labconco CentriVap concentrator (Kansas City, MO, USA). The residues were reconstituted with 30 µL solution of 70% methanol solution containing 100 nM 1-cyclohexyluriedo-3-dodecanoic acid (CUDA) as an external quality marker for the analysis. Afterwards, the extracts were centrifuged at 13000 rpm for 5 min (4°C), and transferred to LC autosampler vials.

Lipid chromatography-mass spectrometry analyses (LC-MS/MS)

The complete oxidised lipid target lists and corresponding ISTDs divided per pathway are shown in Supplementary Table S2-S5. The leukotrienes, hydroxyl-fatty acids, epoxy fatty acids and lipoxins were analysed by high performance liquid chromatography (Agilent 1260, San Jose, CA, USA) coupled to a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA), using an Ascentis® Express column (2.7 µm, 2.1 × 150 mm) as detailed in Strassburg *et al.*³².

For adequate PG and IsoP isomer resolution, together with sensitive detection of the NO₂-FAs, an optimised chromatographic method was developed in-house. UHPLC-MS/MS analysis was performed using the Shimadzu LCMS-8050 (Shimadzu, Japan) with a Kromasil EternityXT column (1.8 µm, 50 × 2.1 mm) maintained at 40 °C. The method used a three mobile phase setup with: A (H₂O with 5 mM ammonium acetate and 0.0625% ammonium hydroxide), B (methanol with 0.2% ammonium hydroxide) and C (isopropanol with 0.2% ammonium hydroxide), with a flow rate of 0.6 mL/min. The injection volume was 10 µL and all analytes eluted during a 10-minute ternary gradient with a starting percentage composition of 94.5:5:0.5 (A: B: C). A chromatographic gradient is provided in Supplementary Fig. S2.

The LCMS-8050 consisted of a triple quadrupole mass spectrometer with a heated electrospray ionisation (ESI) source. In negative ion mode, the source parameters were as follows: the heat block temperature was 400 °C, with the heating gas at 250 °C and a flow of 10 L/min. The nebulising and drying gas had a flow rate of 3 L/min and 10 L/min, respectively. The interface voltage was set at 4 kV with a temperature of 150 °C. The conversion dynode was set at 10 kV, and desolvation temperature was 250 °C. Analytes were detected in negative MRM mode.

Method validation

The targeted profiling of oxidised lipids has been previously validated for plasma samples and the performance characteristics linearity, intra- and inter-day precision and accuracy were reported ³². In the present study, we used the same chromatography parameters in term of the columns, mobile phase, gradient, etc. Therefore, the current validation was performed to determine recovery, matrix effect, and precision for the ISTDs for the reported extraction method.

Creatinine analysis

Urine samples of RA patients were collected at a random time of day, therefore the amount of liquids consumed influenced the concentration of the oxidised lipids in the samples. To eliminate this influence, levels of urinary creatinine were used to correct for dilution. Creatinine levels were determined based on a fast creatinine (urinary) assay kit (Item No. 500701, Cayman Chemical Company, Ann Arbor, MI, USA).

Data processing and statistical analyses

Peak determination and peak area integration were performed with Mass Hunter Quantitative Analysis (Agilent, Version B.04.00) and LabSolutions (Shimadzu, Version 5.65). The obtained peak areas of targets were first corrected by appropriate ISTD and creatinine concentrations (mg/dL), then normalised by log transformation. After data pre-processing, categorical principal component analysis (CATPCA, Supplementary methods) ³³ and multiple linear regressions (MLR) were applied to explore the relationships between oxidised lipids and clinical parameters. Cytoscape was used to visualise the associations ³⁴. All statistical analysis was performed using IBM SPSS Statistics 23.0 software (Chicago, IL, USA).

Results

Optimisation for the hydrolysis of IsoPs, PGs and NO₂-FAs

For determining the optimal enzymatic deconjugation procedure for urinary oxidised lipids, three GUSs derived from *H. pomatia*, *E. coli* and bovine liver were investigated. During the method development, critical parameters including enzyme concentrations, hydrolysis temperature and time were optimised. In order to simplify the method optimisation, we focused on the quantification of a pre-selected panel of metabolites to evaluate the method performance, which covers the most studied IsoPs, PGs and NO₂-FAs in human body fluids. The selected panel consisted of: F-series IsoPs (8-iso-PGF_{2α}, 8-iso-15(R)-PGF_{2α}, and 8-iso-13,14-dihydro-PGF_{2α}), F- and E-series PGs (PGF_{2α}, 13,14-dihydro-PGF_{2α}, PGE₁, and PGE₂)

and two NO₂-FAs mediators (NO₂-linoleic acid and NO₂-oleic acid) (see Supplementary Table S6).

For all three GUSs tested the optimal condition was 1000 U enzyme/400 µL urine incubated at 37 °C for 2 hours. No increase in metabolite levels were achieved through increasing the hydrolysis time beyond 2h, or from increasing the temperature (data not shown). Choosing the shortest possible hydrolysis time will also increase the throughput of the method. Fig. 1 presents the increase (or decrease) of the concentration (as reflected by the increase of the peak area) in the sample after hydrolysis compared to prior to the hydrolysis for a selected panel of compounds. Significant increases were observed for the F-series IsoPs—all three enzymes increased the metabolite levels by more than 50%. Some downstream metabolites, 8-iso-13,14-dihydro-PGF_{2α} and 13,14-dihydro-PGF_{2α}, were exclusively detected after GUS hydrolysis (Fig. 1B). No significant increase in the E-series PGs compared to the free levels were found after GUS hydrolysis. The NO₂-FAs mediators could not be analysed after hydrolysis due to their extreme temperature and enzymatic lability (see below for further discussion). No significant differences in the hydrolysis efficiency were found between the three GUSs for the pre-selected panel of compounds. Our final choice of the GUS for our protocol was finally made based on encountered blank effects of GUSs and metabolite stability in presence of the GUS (see below).

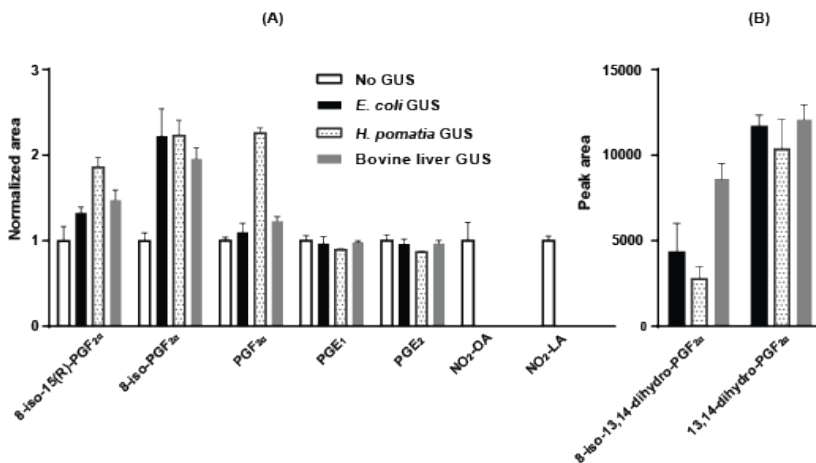


Figure 1. Changes in response of the selected panel of compounds in the urine samples after 2h enzymatic hydrolysis (at 37°C with *E. coli*, *H. pomatia*, or bovine liver GUS) compared to non-hydrolysed samples (no GUS). A – Y-axis represents the normalised peak area of a metabolite normalised to the mean area of the corresponding peak in non-hydrolysed urine. B – Y-axis represents the peak area without normalisation since 8-iso-13,14-dihydro-PGF_{2α} and 13,14-dihydro-PGF_{2α} were exclusively detected in GUS hydrolysed urine. Error bars indicate standard deviation.

The enzyme blank effect

We identified an important and so far unreported observation related to an oxidised lipid background present within the three GUSs, especially for *H. pomatia*. Evaluation of the enzyme blank samples, which consisted of water following the GUS hydrolyses sample workup, revealed the presence of an oxidised lipid background. In Fig. 2, we show the LC-MS/MS trace for PGF_{2α} and PGE₂ in the non-hydrolysed urine sample, GUS blank samples and procedure blank sample (water sample extracted by LLE, no enzyme added). Fig. 2A shows there is no signal for PGF_{2α} in the procedure blank sample while a high level of PGF_{2α} were found especially in the *H. pomatia* GUS blank sample. The levels of PGF_{2α} in *E. coli* and bovine liver GUS samples were lower compared to the high PGF_{2α} background present in *H. pomatia*. Similar observations are made for PGE₂ (Fig. 2B). For the selected panel of oxidised lipids, the enzyme blank effect is presented by the area ratio between the blank enzyme sample and the hydrolysed sample at 2h ($\text{Area}_{\text{in enzyme blank sample}} / \text{Area}_{\text{in 2h hydrolysed sample}}$). Inspection of the complete IsoP, PG and NO₂-FA target panel found that *H. pomatia* GUS contained the highest blank effect compared to *E. coli* and bovine liver (see Supplementary Table S7). Based on this observation *H. pomatia* was not considered as a suitable GUS candidate to measure the total urinary oxidised lipid profile.

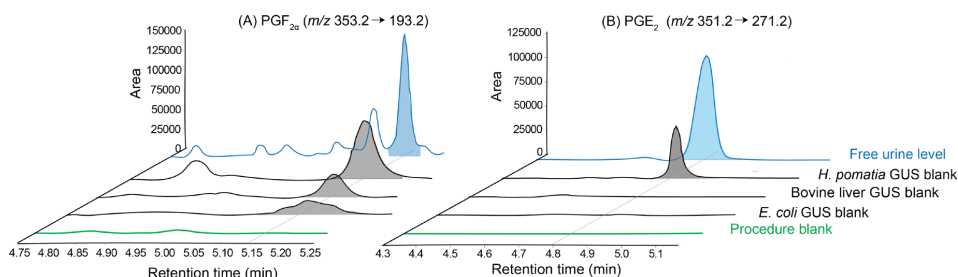


Figure 2. The enzymatic oxidised lipid background (enzyme blank effect). LC-MS/MS chromatograms representing the procedure blank (green), followed by the three enzyme blank samples (*E. coli*, bovine liver and *H. pomatia*), and the free urine levels (blue) are overlaid for (A) PGF_{2α} and (B) PGE₂, respectively. *H. pomatia* GUS shows a high oxidised lipid background. Samples were monitored for PGF_{2α} ($m/z\ 353.2 \rightarrow 193.2$) and PGE₂ ($m/z\ 351.2 \rightarrow 271.2$).

Internal standard stability

Beside the enzyme blank effect, the stability of the ISTDs during the 2h incubation at 37°C were investigated as representative for their respective endogenous metabolite classes. The percentage change for the ISTDs treated with the three different enzymes in 2h compared to 0h were determined and are shown in Fig. 3. ISTDs representing the F-series PGs and IsoPs were identified as stable at 37°C, over 2h with the addition of an enzyme included, showing

less than 10 % change in their levels. However, the E- and D-series PG ISTDs showed temperature sensitivity, especially PGD₂-d4. Reversely the A series PG ISTD PGA₂-d4 showed increasing concentrations, possibly due to PGD₂-d4 spontaneous dehydration forming PGA₂-d4. The 10-NO₂-Oleic acid d17 (NO₂-FAs ISTD) showed hypersensitivity to hydrolysis conditions (buffer and temperature), showing a 40% decrease in levels within 2h compared to non-hydrolysed sample. Furthermore, the addition of all three enzymes resulted in a more pronounced decrease (70% to 90%), explaining the above mentioned decrease of endogenous NO₂-FAs during enzymatic hydrolyses. Overall, the hydrolysis using GUS from *E. coli* showed the largest percentage change for the evaluated ISTDs, suggesting that the 75 mM phosphate buffer (pH 6.8) or *E. coli* GUS affect compound stability. Although, bovine liver GUS showed similar ISTD stability compared to *H. pomatia*, the latter's significant enzyme blank effect led to bovine liver being chosen as our preferred hydrolysing enzyme. Furthermore, bovine liver GUS also resulted in the inclusion of D- and A-series PGs in the target list. Therefore, we chose bovine liver derived GUS hydrolysing at 37°C for 2h as the optimal procedure for analysing the urinary oxidised lipid profile.

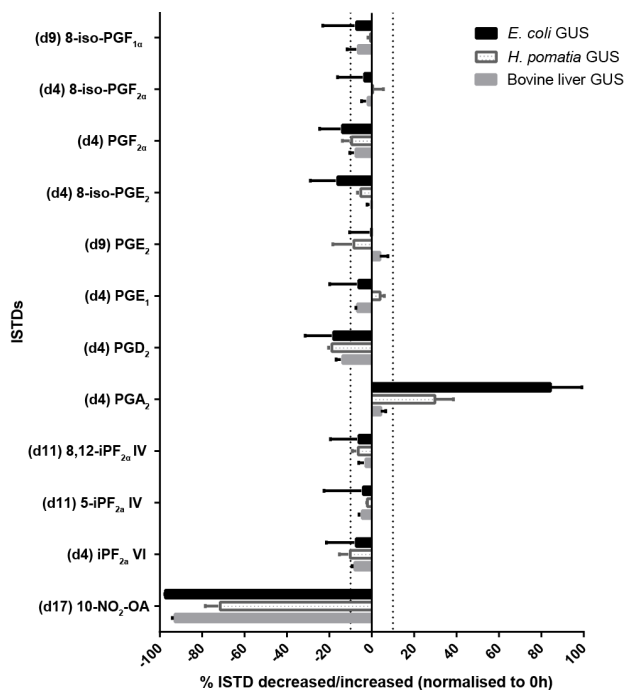


Figure 3. The stability of the IsoPs, PGs and NO₂-FAs ISTDs during the 2h hydrolyses. The percentage changes of ISTD levels (compare 2h to 0h) were investigated to evaluate the stability of each ISTD. Overall ISTDs with bovine liver GUS hydrolysis indicated the highest degree of stability. The vertical dotted lines indicate 10%

change. X-axis indicates percentage changes of ISTD areas between 2h and 0h, $(\text{Area}_{2\text{h-ISTD}}/\text{Area}_{0\text{h-ISTD}}) \times 100\%$. Error bars indicate standard deviation.

Increasing the metabolite scope

Using the optimised bovine liver hydrolyses method, we investigated the potential to broaden the scope of the measurable oxidised urinary lipid profile. We targeted the metabolites from auto-oxidation, COX, LOX, and CYP450 pathways, and compared the amount of free oxidised lipids (from non-hydrolysed urine) to the total amount of oxidised lipids (from hydrolysed urine). There were 51 metabolites detected in both hydrolysed and non-hydrolysed samples, of which 23 metabolites were significantly increased by hydrolysis. More importantly, 27 additional oxidised lipids were detected exclusively in the total oxidised lipid analysis (Table 1). As shown in Table 1, we were able to increase the scope of the method by using an enzymatic hydrolysis approach to measure the total urinary oxidised lipid signature, providing a more complete picture for biological interpretation.

Table 1. Urinary oxidised lipids measured by bovine liver GUS hydrolysis and non-hydrolysis methods.

	RNS	ROS	COX	LOX	CYP450
Detected in both GUS hydrolysed and non-hydrolysed urine		11-HDoHE*	13,14-dihydro-15-keto-PGE ₂ *	12S-HEPE	12,13-DiHOME*
		14-HDoHE	15-keto-PGF _{1α}	20-carboxy-LTB ₄ [#]	12,13-EpOME*
		5- <i>i</i> PF _{2α} VI*	2,3-dinor-11b-PGF _{2α} *	5S,6R-LipoxinA ₄	14,15-DiHETrE*
		8,12- <i>i</i> PF _{2α} IV*	2,3-dinor-8-iso-PGF _{2α} *	11-HETE	8,9-DiHETrE*
		8-iso-15(R)-PGF _{2α} *	20-hydroxy-PGE ₂ [#]	11-trans-LTD ₄ [#]	9,10-DiHOME*
		8-iso-15-keto-PGF _{2α} *	Δ12-PGJ ₂ *	12-HETE	9,10-EpOME*
		8-iso-PGE ₁ [#]	Δ17, 6-ketoPGF _{1α} *	13-HODE*	
		8-iso-PGE ₂	PGA ₂	13-KODE	
		8-iso-PGF _{1α}	PGD ₁ [#]	15-HETE*	
		8-iso-PGF _{2α} *	PGD ₂ [#]	5-HETE	
			PGE ₁ [#]	9,10,13-TriHOME*	
			PGE ₂	9,12,13-TriHOME*	
			PGE ₃	9-HODE	
			PGF _{1α}	9-HOTrE	
			PGF _{2α} *	9-KODE	
			PGF _{3α}	LTD ₄ *	
			PGJ ₂ [#]		
			Tetranor-PGEM [#]		
Detected in GUS hydrolysed urine exclusively		10-HDoHE	13,14-dihydro-PGF _{2α}	15S-HETrE	20-HETE
		8-iso-13,14-dihydro-PGF _{2α}	13,14-dihydro-15-keto-PGD ₂	15-HpETE	12,13-DiHODE
		8-iso-15-keto-PGF _{2β}	13,14-dihydro-15-keto-PGF _{2α}	5S,15S-DiHETE	19,20-DiHDPA
		9-HETE	15-deoxy-Δ-12,14-PGD ₂	5S,6S-Lipoxin A ₄	11,12-DiHETrE
			15-keto-PGF _{2α}	5S-HEPE	11,12-EpETrE
			16-HDoHE	5S-HpETE	14,15-DiHETE
			bicyclo-PGE ₂	9-HEPE	17,18-DiHETE

	PGK ₂	5,6-DiHETrE
Detected in	NO ₂ -αLA	
non-	NO ₂ -LA	
hydrolysed	NO ₂ -OA	
urine		
exclusively		

*: significantly increase with hydrolysis

#: significantly decrease with hydrolysis

Method validation

Validation measurements were performed using pooled urine as a sample matrix. Recovery, matrix effect, and batch-to-batch precision were determined.

Recovery and ion suppression

The performances of the analytical methods (free and total oxidised lipid profiling) with respect to recovery (of the free forms) and ion suppression were validated for those metabolites which were available as deuterium labelled compounds, and which were usually used as ISTDs. For determining recoveries, samples were independently spiked before or spiked after the extraction procedure with ISTDs, and the area ratios between these samples ($\text{Area}_{\text{spike before}}/\text{Area}_{\text{spiked after}}$) were calculated as the recoveries. Fig. 4A demonstrates that recoveries are from 85% to 115% for most ISTDs, indicating the effectiveness of both procedures to extract the oxidised metabolome from urine samples. The non-hydrolysed samples (free levels) with a simpler sample handling procedure showed slightly higher recoveries compared to the hydrolysed procedure (total levels) except for the nitro fatty acid ISTD (10-NO₂-oleic acid-d17 as discussed in ‘Internal standard stability’).

To determine the ion suppression caused by the presence of compounds of the matrix, ISTDs areas in urine samples spiked after the total and free oxidised lipid extractions were compared to the ISTDs areas in injection solution. Ion suppression values shown in Fig. 4B, ranged between 0.5 and 1 for most of the metabolites; values below 1 indicate the presence of ion suppression caused by co-eluting compounds from the urine matrix that were not removed by the sample preparation and that affect the ionisation of those targeted metabolites³⁵. Three ISTDs with high ion suppression (lower than 0.5) were PGA₂-d4, 6-ketoPGF₁-d4 and TBX₂-d4. However, ion suppression effects are corrected through the deuterated ISTD normalisation ($\text{Area}_{\text{metabolite}}/\text{Area}_{\text{ISTD}}$) as both the metabolite and its corresponding ISTD experience similar ion suppression.

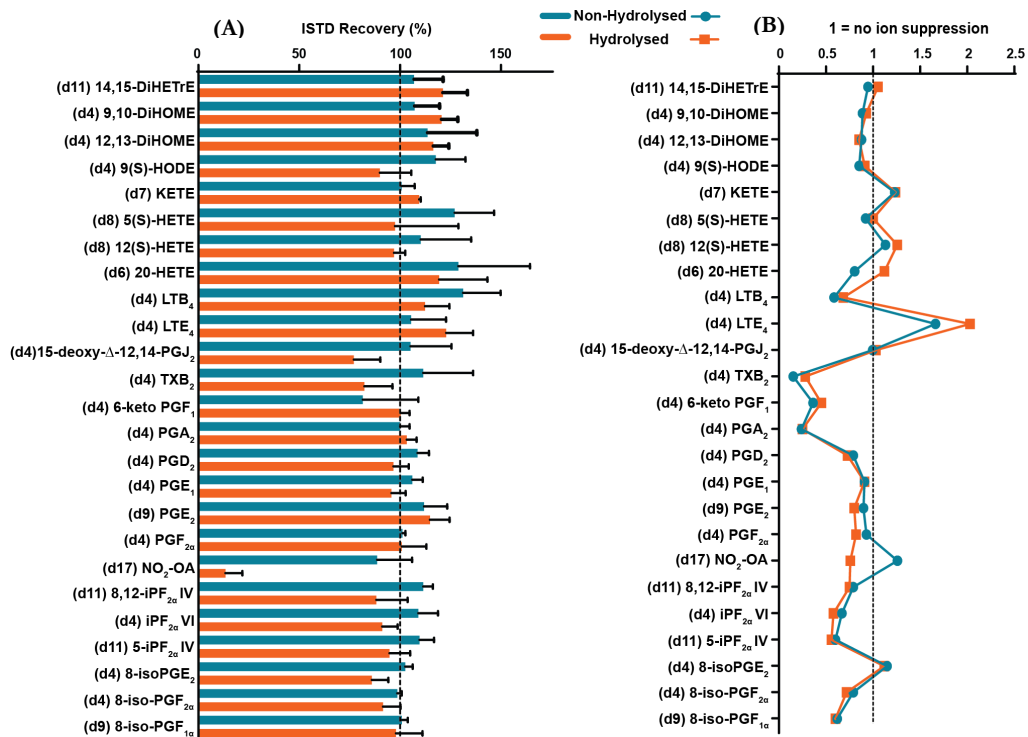


Figure 4. Performance characteristics of sample preparation. Deuterium labelled ISTDs were evaluated for (A) recovery and (B) ion suppression; values below 1 indicate presence of ion suppression.

Precision and batch-to-batch effect

To be able to reproducibly and accurately report the oxidised lipid metabolome from patient urine samples, it is important to estimate the analytical variation. Therefore, the intra-batch precision and inter-batch variability were assessed for all endogenous oxidised lipids. Precision is dependent on extraction reproducibility, injection variation and detector stability, while the inter-batch variability is influenced by the robustness of the chromatography and instrument stability across different measurement days ($n=3$).

In Supplementary Fig. S3A, the precision (intra-batch RSD) and in Supplementary Fig. S3B the batch-to-batch effect (inter-batch RSD) of endogenous metabolites obtained from hydrolysed/non-hydrolysed urine samples are shown. Investigating the precision for the non-hydrolysed procedure showed that 59 % of metabolites had the RSD < 15%, with a further 25% of metabolites having a RSD between 15% to 30%. The hydrolysed procedure showed improved precision with 66% of metabolites having the RSD < 15%, and 29% of metabolites having a RSD between 15% to 30%. The increased precision observed in the hydrolysed

procedure can be attributed to increased metabolites concentrations leading to more accurate detection, while the higher variation in the non-hydrolysed procedure was predominantly driven by low abundant compounds. Both procedures performed equally well in batch to batch effects, indicative of a stable and reproducible LC-MS analyses across three measurement days.

Application

Subjects

For the present study, baseline urine samples from RA patients who were treated with TNF- α inhibitors (ETN or ADA) were selected based on good response or non-response after three months of therapy. The baseline characteristics of 80 patients are shown in Table 2. Subjects had a mean age of 53.8 years, a median disease duration of 5 years, and had previously taken a mean of two concomitant disease-modifying anti-rheumatic drugs (co-DMARDs). Subjects were mainly female (58 females, 22 males). Good responders had higher baseline DAS28 and C-reactive protein (CRP) levels compared to the non-responders. There were no significant differences between the other parameters.

Table 2: Baseline characteristics of the study subjects (n=80), subdivided according to the therapeutic response at three months.

	All subject (n=80)	Non-response (n=40)	Good response (n=40)	<i>p</i> -value (good responders vs. non-responders)
Gender, female, n (%)	58 (72.5)	31 (77.5)	27 (67.5)	0.453
Age, mean (SD)	53.8 (11.0)	52.7 (11.3)	55.1 (10.8)	0.461
Disease duration, median (IQR)	5 (8.0)	5 (7.0)	6 (9.0)	1.000
Smoking currently, n (%)	23 (28.7)	12 (30.0)	11 (27.5)	0.805
Alcohol >7 units/week, n (%)	16 (20.3)	5 (12.5)	11 (27.5)	0.099
BMI, mean (SD)	26.9 (5.3)	27.1 (5.0)	26.7 (5.6)	0.874
RF, positive, n (%)	57 (71.3)	26 (65.0)	31 (77.5)	0.323
ACPA, positive, n (%)	57 (71.3)	26 (65.0)	31 (77.5)	0.323
Baseline DAS28, mean (SD)	4.5 (1.0)	4.2 (1.1)	4.8 (0.8)	0.006
CRP, median (IQR)	7 (10.0)	5 (7.8)	8 (11.0)	0.045

SD: standard deviation; IQR: interquartile range.

Detection of oxidised lipids in human urine

Applying total and free oxidised lipid profiling to randomly collected urine samples, we are able to detect 67 metabolites in hydrolysed urine samples and 44 in non-hydrolysed urine samples. There were 97% total and 95% free oxidised lipid metabolites measured with a RSD

< 30% (see Supplementary Table S8). Because the total oxidised lipid profiling provides a larger scope of metabolites, we focused on the total urinary oxidised lipid profiles (after hydrolysis), in addition to the free NO₂-FAs levels measured in the analyses without hydrolysis in the subsequent data analyses and interpretation.

Relationships between total oxidised lipids profiling and RA-associated parameters

To investigate the relationship between RA-associated parameters and total oxidised lipid levels, repeated assessments using multiple linear regression (MLR) models were performed on individual metabolite. Confounding factors (age, sex, BMI, smoking status, alcohol consumption, co-DMARDs) and RA-associated parameters (baseline DAS28, CRP and DAS28 improvement) were added into MLR as independent variables, and the oxidised lipid level was set as the dependent variable. RA-associated parameters which were correlated with oxidised lipid levels ($p < 0.10$) were selected (Table S9) and are visualised by Cytoscape in Fig. 5. CRP showed a positive association with 16-HDoHE ($p = 0.027$) and a negative association with PGD₃ ($p = 0.026$); DAS 28 showed a positive association with 14,15-DiHETE ($p = 0.036$) and 5S, 6R-LipoxinA₄ ($p = 0.005$); DAS28 improvement (DAS28_{month 0} – DAS28_{month 3}) showed significantly positive association with PGF_{3α} ($p = 0.017$), PGF_{2α} ($p = 0.018$), iPF_{2α} IV ($p = 0.021$), 11, 12-EpETrE ($p = 0.040$), 11-HETE ($p = 0.019$), 14,15-DiHETrE ($p = 0.017$) and 14-HDoHE ($p = 0.033$). Since NO₂-FAs were labile in the hydrolysing procedure, we included the free NO₂-αLA, NO₂-LA and NO₂-OA levels during the MLR. These three metabolites were all negatively associated with baseline DAS28 ($p < 0.05$).

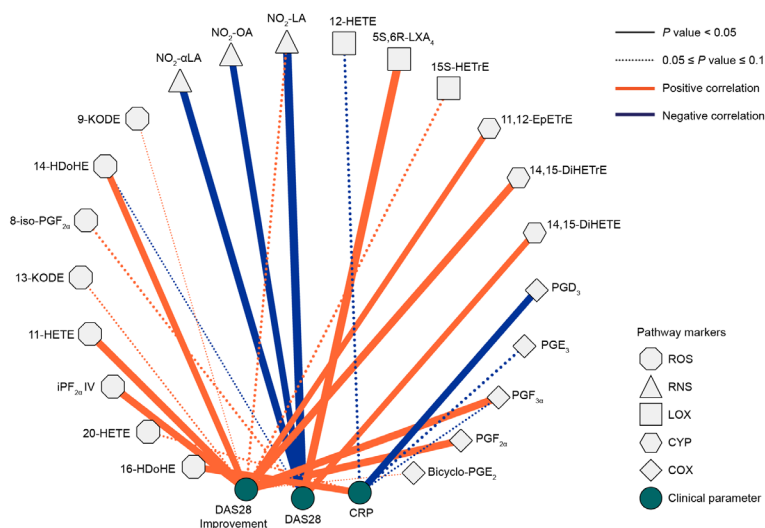


Figure 5. Associations between rheumatoid arthritis-associated clinical parameters and oxidised lipid levels with $p < 0.10$ based on multiple linear regression analyses.

Discussion

In the present study we optimised and validated a GUS based hydrolysis method to evaluate the oxidised urinary lipid profile. We demonstrated that the number of measured oxidised lipids were approximately two-fold increased after hydrolysing with bovine liver sourced GUS. Furthermore, we applied the method to urine samples from patients with RA and identified oxidised lipids associated with RA-associated parameters important in determining therapeutic response.

As a non-invasive biological matrix, urine is easily collected and can effectively be used as an oxidised lipids readout. However, urine does present some challenges including dilution effect (morning sample, 24h sample, random sample), glucuronidation of metabolites with low solubility, and a high salt concentration etc. Although some of these challenges can be addressed with proper experimental planning and standardised sample collection procedures, the choice between analysing free or total levels of oxidised lipids still needs to be addressed. Thus we developed a robust enzymatic method to hydrolyse the GlcA conjugated oxidised lipids, obtaining the total oxidised lipid profile for urine samples. During the method development, we found an enzyme blank effect in the three candidate enzymes (see Supplementary Table S7) which needs to be minimised in order to avoid adding artefacts which could interfere with the biological interpretation of the results.

Several different GUS are available commercially for research purposes, with each enzyme having its own specific characteristics properties relating to substrate affinities and efficiency. Our choice of candidate enzymes was guided by selecting those previously reported in literature^{18,30,31}. *H. pomatia* GUS was the most widely used enzyme but had a very prominent blank effect. The oxidised lipid background might be derived from inadequate purifying and cleaning procedures used during enzyme extraction and isolation. While this might not be true for all forms of GUS derived from *H. pomatia*, we decided against the use of *H. pomatia* as the background might influence our biological interpretation of the data.

Comparing the total and free oxidised lipid profiles revealed increased levels, especially in the F-series IsoPs, F-series PGs, hydroxy-fatty acids and dihydroxy-fatty acids, while having minimal effect on E-series PGs in the total oxidised lipid profile. The lack of effect on the E-series PGs could be explained in a study done by Little et al., where they investigated the different human recombinant UDP-glucuronosyltransferases and found that only one isoform UGT2B7 was capable of forming PGE₂ glucuronide¹⁷. UGT2B7 were exclusively found in

the colon, and faeces rather than urine might contain high levels of E-series glucuronide conjugates. The significant increase in LOX and CYP450 metabolites might be due to their increasing hydrophobic nature, with glucuronidation increasing their urinary excretion. Similar to our findings, Prakash *et al.*, reported significant increase in the level of 20-HETE, an CYP450 metabolite in urine treated with β -glucuronidases, and they concluded that CYP450 metabolites are predominantly excreted in conjugated form ¹³. Whereas the method reported by Newman *et al.*, needed 4 mL of urine to measure the free CYP450 oxidised lipid metabolites ²². Using our bovine liver hydrolysis method, we could evaluate the same CYP450 pathway metabolites by using 10 times less urine.

3 Optimising the extraction and analyses of the anti-inflammatory NO₂-FAs will aid us in studying the field of nitrosative stress. A disappointing finding was our inability to accurately measure the total NO₂-FAs levels, due to their labile nature. Nevertheless, we were able to effectively measure the free NO₂- α LA, NO₂-LA and NO₂-OA levels in urine. In work published by Salvatore *et al.*, they showed the presence of cysteine-NO₂-FAs conjugates in urine, and used a short chemical (HgCl₂) hydrolysis procedure at 37 °C to measure its total levels ³⁶. Thus, to accurately measure the total NO₂-FAs levels identification of the primary conjugated form needs to be done, followed with optimised chemical hydrolyses.

For the urine samples from RA patients, which were collected at a random time of day, we used the urinary creatinine level for correcting the oxidised lipids for sample dilution. Samples were retrospectively divided into good or no responders to therapy based on EULAR response criteria. However, the subjects could not be separated into non-/good response groups based on their baseline urinary oxidised lipids profile and clinical parameters using categorical principle components analysis (CATPCA, score plot is shown in Supplementary Fig. S4). After correcting for confounding factors (age, sex, BMI, smoking status, alcohol consumption, co-DMARDs), the urinary oxidised lipid readout did show associations between inflammation and oxidative stress, and clinical parameters (CRP, 3-month-DAS28 improvement, baseline DAS28). C-Reactive protein is an acute phase inflammatory protein, which showed a positive association with 16-HDoHE, a docosahexaenoic acid (DHA) lipid peroxidation metabolite. DHA showed efficacy as an anti-inflammatory treatment when used as a prophylactic treatment in a mouse RA model ³⁷, thus the increased peroxidation of DHA catalysed by ROS reduces the body's anti-inflammatory capacity. Oxidative stress has been identified as an aggravator of damage caused to bone in cartilage in RA ^{38,39}, so its association with CRP underscores this relationship.

DAS28 is the most widely used measurement for assessing the disease activity (swelling and tenderness in 28 joints, the ESR and VAS general health) in RA ⁴⁰. In the present study, good responders had higher baseline DAS28 compared to non-responders. Clinically it has been observed that a patient with severe RA has high DAS28 and is more likely to obtain therapeutic response ⁴¹. Veselinovic *et al.*, reported increased levels of RNS in patients with high disease activity in serum ³⁸, while we found in urine that the downstream anti-inflammatory NO₂-FAs correlated negatively with DAS28. This observation could be explained through the complex relationship between RNS species, the beneficial signalling abilities of RNS nitrated lipid metabolites (NO₂-FAs) and the body's anti-oxidant capacity. The positive association of DAS28 improvement with strong pro-inflammatory mediators PGF_{2α}, PGF_{3α}, and oxidative stress markers iPF_{2α}, 11-HETE and 14-HDoHE indicate the higher disease burden within these baseline patients. The association of DAS28 and its improvement with the anti-inflammatory CYP-450 dihydroxy-fatty acids metabolites and the LOX derived LXA₄ possibly reflects the intact innate anti-inflammatory pathways in these patients still trying to lessen the RA disease burden. The dihydroxy-fatty acids, 14,15-DiHETE, 11, 12-EpETrE, and 14, 15-DiHETrE are able to attenuate pro-inflammatory pathways through activating and signalling via the PPAR-gamma pathway ⁴². The urinary oxidised lipid profile of RA patients indicates the complex nature of the disease with oxidative stress and inflammation having an intimate relationship with clinically measured parameters.

Conclusions

In the present study, we thoroughly explored different deglucoronidation methods for urinary oxidised lipids and developed a bovine liver-GUS hydrolysing sample preparation method coupled with LC/MS to analyse the total urinary oxidised lipid profile. With bovine liver GUS hydrolysis, we are able to zoom into the urinary oxidised lipid metabolome, providing a readout for inflammation and oxidative stress. Our method detected more than 70 oxidised lipids in urine samples, biosynthesised from 2 non-enzymatic and 3 enzymatic pathways. The total oxidised lipid profiling method was developed and validated for human urine, and was demonstrated on patients with RA. The urinary oxidised lipid profile of RA patients indicates the complex nature of the disease with oxidative stress and inflammation having an intimate relationship with clinically measured parameters. In conclusion, the hydrolysed method developed here allows specific and sensitive quantitative assessment of more than 70 oxidised lipids, which expands the scope of compounds in urinary metabolic profiling, and may have wider applications in studies elucidating the role of these potent bioactive metabolites in human diseases.

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Supplementary information

Methods

Categorical principal components analysis

Categorical principal components analysis (CATPCA) is a nonlinear principal components analysis technique, which allows for different analysis levels (numeric, ordinal and nominal) for variables and is able to handle categorically and numerically measured variables. In our study, the variable “3-month response” (two categories: good responder, non-responder) was projected onto the model as a multiple nominal supplementary variable, which allows the observation of relationships between the therapeutic response and the other variables without interfering with the model.

Tables

Table S1. Overview of internal standards.

ISTD name	Precursor ion → Product ion (<i>m/z</i>)
(±) 12,13-DiHOME-d4	317.3 → 185.2
(±) 9,10-DiHOME-d4	317.3 → 203.2
10-Nitrooleate-d17	343.1 → 46.05
12(S)-HETE-d8	327.3 → 184.2
14,15-DiHETrE-d11	348.3 → 207.1
15-deoxy-D-13,14-PGJ2-d4	319.2 → 275.3
20-HETE-d6	325.3 → 279.2
5(S)-HETE-d8	327.3 → 116.1
5-iPF2a-VI-d11	364.2 → 115.05
6-keto PGF1a-d4	373.2 → 167.2
8,12-iso-iPF2a-d11	364.2 → 115.05
8-iso-PGE2-d4	355.3 → 275.25
8-iso-PGF1a-d9	364.2 → 320.25
8-iso-PGF2a-d4	357.3 → 197.15
9(S)-HODE-d4	299.2 → 172.1
iPF2a-VI-d4	357.2 → 114.9
KETE-d7	324.2 → 280.3
LTB4-d4	339.2 → 197.1
LTE4-d3	441.2 → 336.2
PGA2-d4	336.9 → 275.0
PGD2-d4	355.2 → 275.2
PGE1-d4	357.3 → 321.2
PGE2-d9	359.9 → 280.25
PGE2-d9	359.9 → 280.25
PGF2a-d4	357.3 → 197.2
TXB2-d4	373.2 → 173.1

Table S2. Complete COX pathway oxidised lipid target list

Metabolites	Precursor ion → Product ion (<i>m/z</i>)	ISTD	Formal name	Lipidmaps ID	InChIKey
11beta-13,14-dihydro-15-keto-PGF2a	353.2 → 113.2	(d4) PGF2a	9 η ,11 η -dihydroxy-15-oxo-prost-5 $\angle$ -en-1-oic acid	LMFA03010203	VKTI0NYPMSCHQI-KGLINJEGSA-N
11beta-PGE2	351.2 → 271.3	(d9) PGE2	9-oxo-11 η ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid	LMFA03010060	XEYBRNLFEZDVAW-YUOXZBOXSA-N
11beta-PGF2a	353.2 → 193.1	(d4) PGF2a	9a,11 β ,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid	LMFA03010036	PXGPLTODNUVGF-LZWAKLXPCSA-N
12S-HHTfE	279.2 → 179.2	(d8) 12(S)-HETE	12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid	LMFA03050002	KUKJHGXXXWHSBG-WBGSEGOAS-A-N
13,14-dihydro-15-keto-PGD1	353.2 → 209.1	(d4) PGD2	9a-hydroxy-11,15-dioxo-prost-1-oic acid	<i>na</i>	WTCAXDXNVHRHC-KURKYZTESA-N
13,14-dihydro-15-keto-PGD2	351.2 → 175.2	(d4) PGD2	9 η -hydroxy-11,15-dioxo-prost-5Z-en-1-oic acid	LMFA03010022	VSRXYLYXIXYEST-KZTWKYQFSA-N
13,14-dihydro-15-keto-PGE2	351.2 → 175.2	(d9) PGE2	9,15-dioxo-11 η -hydroxy-prost-5Z-en-1-oic acid	LMFA03010031	CUJMXIQZWPZMNQ-XYYGWQPLSA-N
13,14-dihydro-15-keto-PGF1a	355.2 → 193.2	(d4) PGF2a	9S,11R-dihydroxy-15-oxo-prostanoic acid	LMFA03010168	FVPKMMQYALWZHV-AKHDSKFAS-A-N
13,14-dihydro-15-keto-PGF2a	353.2 → 183.1	(d4) PGF2a	9 η ,11 η -dihydroxy-15-oxo-prost-5Z-en-1-oic acid	LMFA03010027	VKTI0NYPMSCHQI-XAGFEHLVSA-N
13,14-dihydro-PGF2a	355.2 → 275.3	(d4) PGF2a	9S,11R,15S-trihydroxy-5Z-prostenoic acid	LMFA03010079	LLQBSIQTCQVWTD-QPJXVBHSA-N
15-deoxy-delta-12,14-PGD2	333.2 → 271.2	(d4) PGD2	9 η -hydroxy-11-oxo-prosta-5Z,12E,14E-trien-1-oic acid	LMFA03010051	QUGBPWLPALUHDTH-PLGLXCLHSA-N
15-keto-PGF1a	353.2 → 221.1	(d4) PGF2a	9S,11R-dihydroxy-15-oxo-13E-prostanoic acid	LMFA03010150	QPXXPLNAYDQELM-QNXXGYPUA-N
15-keto-PGF2a	351.2 → 219.1	(d4) PGF2a	9S,11R-dihydroxy-15-oxo-5Z,13E-prostadienoic acid	LMFA03010026	LOLJEILMPWPILA-AMFHKTBMSA-N
1a,1b-dihomo-PGF2a	381.3 → 337.2	(d4) PGF2a	1a,1b-dihomo-9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	LMFA03010157	ZCTAOAWRUXSQQF-GWSKAPOCSA-N
2,3-dinor-11b-PGF2a	325.2 → 145.1	(d4) 8-iso-PGF2a	9a,11 β ,15S-trihydroxy-2,3-dinor-prosta-5Z,13E-dien-1-oic acid	LMFA03010011	IDKLJUJUUVJNR-KSYGFECSA-N
20-hydroxy-PGE2	367.2 → 287.2	(d9) PGE2	9-oxo-11 η ,15S,20-trihydroxy-prosta-5Z,13E-dien-1-oic acid	LMFA03010014	AZIGEYVZEVXWAD-NZGURKHLA-N
20-hydroxy-PGF2a	369.2 → 193.1	(d4) PGF2a	9S,11S,15S,20-tetrahydroxy-5Z,13E-prostadienoic acid	LMFA03010029	XQXUYZDBZCLAQO-UNKHNRNISA-N
6-keto PGE1	367.2 → 143.1	(d4) 6-keto PGF1a	6,9-dioxo-11R,15S-dihydroxy-13E-prostenoic acid	LMFA03010012	ROUDCKODIMKLNO-CTBSXBMHSA-N
6-keto-PGF1a	369.2 → 163.1	(d4) 6-keto PGF1a	6-oxo-9 η ,11 η ,15S-trihydroxy-prost-13E-en-1-oic acid	LMFA03010001	KFGOFTHODYBSGM-ZUNNUUQCSA-N
bicyclo-PGE2	333.2 → 113.2	(d9) PGE2	11-deoxy-13,14-dihydro-15-keto-11 η ,16 α ,xi-cycloprostaglandin E2	LMFA03010034	CGCZPJIMGKLV-TQ-PAJBVNRKSA-N
d12-PGJ2	333.2 → 233.1	(d4) 15-deoxy-D-13,14-PGJ2	11-oxo-15S-hydroxy-prosta-5Z,9,12E-trien-1-oic acid	LMFA03010020	TUXFWOHPFBNEJ-GJGEGAFSA-N
D17, 6-ketoPGF1a	367.2 → 163.1	(d4) 6-keto PGF1a	6-oxo-9S,11R,15S-trihydroxy-13E,17Z-prostadienoic acid	LMFA03010149	HFKNJQYMAQMXTX-CAPHXMBKSA-N
PGA1	335.1 → 273.15	(d4) PGA2	9-oxo-15S-hydroxy-10Z,13E-prostadienoic acid	LMFA03010005	BGKHCLZFGPIK-KU-LDDQNKHISA-N
PGA2	333.2 → 271.2	(d4) PGA2	9-oxo-15S-hydroxy-5Z,10Z,13E-prostatrienoic acid	LMFA03010035	MYHXHCUNDAAEOZ-FOSBLDSVSA-N

PGD1	353.2 > 273.2	(d4) PGD2	9 β ,15S-dihydroxy-11-oxo-prost-13E-en-1-oic acid	LMFA03010049	CIMMACURCPXICP-PNQRDRVSA-N
PGD2	351.2 > 271.2	(d4) PGD2	9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid	LMFA03010004	BHMBVRSPMRCCGG-OUTUXVNYSA-N
PGD3	349.2 > 269.2	(d4) PGD2	9S,15S-dihydroxy-11-oxo-5Z,13E,17Z-prostatrienoic acid	LMFA03010142	ANOICLBSJIMQTA-WXGBOIQSA-N
PGE1	353.2 > 273.2	(d4) PGE1	9-oxo-11R,15S-dihydroxy-13E-prostaenoic acid	LMFA03010134	GMVPRGQOIIMIDWKJAMRDSA-N
PGE2	351.2 > 271.2	(d9) PGE2	9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid, Prostin E ₂	LMFA03010003	XEYBRNLFEZDVAW-ARSRFYASSA-N
PGE3	349.2 > 269.2	(d9) PGE2	9-oxo-11R,15S-dihydroxy-5Z,13E,17Z-prostatrienoic acid	LMFA03010135	CBOMORHDZNRN-QLLOYDKTKSA-N
PGF1a	355.2 > 293.2	(d4) PGF2a	9S,11R,15S-trihydroxy-13E-prostaenoic acid	LMFA03010137	DZUXGQBLFALXCR-CDIPTNKSSA-N
PGF2a	353.2 > 193.1	(d4) PGF2a	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	LMFA03010002	PXGPLTODNULVGFL-YNNPMVKQSA-N
PGF3a	351.2 > 193.2	(d4) PGF2a	9 α ,11 α ,15S-trihydroxy-prosta-5Z,13E,17Z-trien-1-oic acid	LMFA03010138	SAKGBZWJALABSY-SAMSIYEGSA-N
PGJ2	333.2 > 233.1	(d4) PGD2	11-oxo-15S-hydroxy-prosta-5Z,9,13E-trien-1-oic acid	LMFA03010019	UQOQENZLBSFKO-POPZSFYSA-N
PGK2	349.2 > 205.1	(d9) PGE2	9,11-dioxo-15S-hydroxy-5Z,13E-prostadienoic acid	LMFA03010023	LGMXPVXISFPPTQ-DIUJBXLVSA-N
Tetranor-PGEM	327.1 > 309.2	(d9) PGE2	11R-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostan-1,20-dioic acid	LMFA03010032	ZIAZCYLYLVCSNH-JHJVBTASA-N
Tetranor-PGFM	329.2 > 311.2	(d4) PGF2a	9S,11R-dihydroxy-15-oxo-2,3,4,5-tetranor-prostan-1,20-dioic acid	LMFA03010139	IGRHJCFWWQYQE-SYQHCUMBSSA-N
TXB1	371.2 > 171.1	(d4) TXB2	9S,11,15S-trihydroxy-thrombox-13E-enoic acid	LMFA03030008	JSDWVNLJTCCSAV-RLBQWBROSA-N
TXB2	369.2 > 169.1	(d4) TXB2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid	LMFA03030002	XNRNNGPBEPNAR-IQBLCGNGSA-N
TXB3	367.2 > 169.1	(d4) TXB2	9S,11,15S-trihydroxy-thromboxa-5Z,13E,17Z-trien-1-oic acid	LMFA03030006	OYPPJMLKAYYWHH-NXJDUNG TSA-N

Table S3: Complete CYP pathway oxidised lipid target list

Metabolites	Product ion (m/z)	ISTD	Formal name	Lipidmaps ID	InChIKey
11,12-DHETE	337.2 → 167.2	(d11) 14,15-DHETE	(±)11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	LMFA03050008	LRPPQRCCHCFBPE-LZXKBWHHSA-N
11,12-EpETE	319.2 → 167.1	(d11) 14,15-DHETE	(±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid	LMFA03080014	DYOYQVHGIODSupplementary-LZXKBWHHSA-N
12,13-DHODE	311.2 → 293.0	(d4) 9(S)-HODE	(+/-)12,13-dihydroxy-9Z,15Z-octadecadienoic acid	LMFA02000046	RGRKFRAFZIQMS-OOHFSOINSA-N
12,13-DHOMe	313.2 → 183.2	(d4) 12,13-DHOMe	12,13-dihydroxy-9Z-octadecenoic acid	LMFA01050351	CQSLTKIXAJTQGA-GJGKEFFESA-N
12,13-EpOME	295.2 → 195.2	(d4) 12,13-DHOMe	(+/-)12(13)-epoxy-9Z-octadecenoic acid	LMFA02000038	CCPPLJZDQAOHD-FLIBITNWSA-N
14,15-DHETE	335.2 → 207.1	(d11) 14,15-DHETE	14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	LMFA03060077	BLWCDHEL VFRJY-4XQKDQKQSA-N
14,15-DHETE	337.2 → 207.2	(d11) 14,15-DHETE	(±)14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid	LMFA03050010	SYA WGTIVOGUZMM-KZTFMOQPSA-N
14,15-EpETE	317.2 → 207.1	(d11) 14,15-DHETE	(±)14(15)-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	LMFA03000003	RGZINZYRGZWDML-IXQDKQKQSA-N
14,15-EpETE	319.2 → 219.2	(d11) 14,15-DHETE	(±)14(15)-epoxy-5Z,8Z,11Z,17Z-eicosatrienoic acid	LMFA03080013	JBSCUHKPLGKXKH-KZTFMOQPSA-N
16,17-EpDPE	343.2 → 233.2	(d11) 14,15-DHETE	(±)16(17)-epoxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid	LMFA04000037	BCTXZWC PBLWCRV-QCAYAEICISA-N
17,18-DHETE	335.2 → 247.2	(d11) 14,15-DHETE	(+/-)17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03060078	XYDYGNAQQFWZEF-JPURVOHMSA-N
17,18-EpETE	317.2 → 259.2	(d11) 14,15-DHETE	(+/-)17(18)-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03000004	GPQVYVQEBXAKBJ-PURVOHMSA-N
19,20-DHIDPA	361.2 → 273.3	(d11) 14,15-DHETE	(±)19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	LMFA04000043	FFXKPSNQC PNORO-MBYQGORISA-N
20-HETE	319.2 → 289.2	(d6) 20-HETE	20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03060009	NNDIXBIHNL FUIP-DTLRTWKISA-N
5,6-DHETE	337.2 → 145.1	(d11) 14,15-DHETE	5S,6S-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid	LMFA03060018	UVZBUUTTYHTDRR-WAQVJNLQSA-N
5,6-EpETE	319.2 → 191.2	(d11) 14,15-DHETE	(±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid	LMFA03080017	VBQNSZQZAGRIX-GSKBNKFLISA-N
5S,6S-DHETE	335.2 → 115.1	(d4) LTB4	5S,6S-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid	LMFA03060018	UVZBUUTTYHTDRR-WAQVJNLQSA-N
8,9-DHETE	337.2 → 127.0	(d11) 14,15-DHETE	8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid	LMFA03050006	DCIBINATHQHPKO-TYAUIOURKSA-N
8,9-EpETE	319.2 → 155.1	(d11) 14,15-DHETE	(±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid	LMFA03080019	DBWQSCSXHFNTMO-ZZMPYBMWSA-N
9,10-DHOMe	313.2 → 201.1	(d4) 9,10-DHOMe	9,10-dihydroxy-12Z-octadecenoic acid	LMFA02000229	XEBKQSGNGRGDW-CIWPDPFNSA-N
9,10-EpOME	295.2 → 171.2	(d4) 9,10-DHOMe	(+/-)9(10)-epoxy-12Z-octadecenoic acid	LMFA02000037	FBUKMFXMZRG RB-KXIZPFPASA-N

Table S4: Complete LOX pathway oxidised lipid target list

Metabolites	Precursor ion → Product ion (<i>m/z</i>)	ISTD	Formal name	Lipidmaps ID	InChIKey
10S,17S-DiHDoHE	359.2 → 153.2	(d4) LTB4	10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid	LMFA0400047	CRDZYISQHCXHEG-XLBFCUQGS-A-N
11-trans-LTC4	624.3 → 272.1	(d4) LTE4	5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11E,14Z-eicosatetraenoic acid	LMFA03020020	GWNVDXQDILPJIG-GXMXCQGS-A-N
11-trans-LTD4	495.2 → 177.1	(d4) LTE4	5S-hydroxy-6R-(S-cysteinylglycyl)-7E,9E,11E,14Z-eicosatetraenoic acid	LMFA03020021	YEESKJGWJFYOOK-XILINCIBSA-N
11-trans-LTE4	438.2 → 333.2	(d4) LTE4	5S-hydroxy-6R-(S-cysteinyl)-7E,9E,11E,14Z-eicosatetraenoic acid	LMFA03020022	OTZRAYGBFWZKMW-VXBMJZGWSA-N
12-HETE	319.2 → 179.2	(d8) 12(S)-HETE	12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	LMFA03060088	ZNHVWPKMFKADKW-VXBMJZGWSA-N
12-KETE	317.2 → 273.3	(d7) KETE	12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid	LMFA03060019	GURBRQDZZKITB-VXBMIJZGYS-A-N
12S-HEPE	317.2 → 179.1	(d8) 12(S)-HETE	12S-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	LMFA03070008	MCRJLMXYVFDXLS-UOLHMMFTSA-N
12S-HpETE	317.2 → 273.3	(d8) 12(S)-HETE	12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	LMFA03060013	ZIOZYRSDNLNNI-VXBMJZGYS-A-N
13-HODE	295.2 → 195.2	(d4) 9(S)-HODE	13S-hydroxy-9Z,11E-octadecadienoic acid	LMFA02000154	HNICUWMFWBZBFP-BSZOFBHHSA-N
13-HpODE	311.2 → 113.2	(d4) 9(S)-HODE	(±)13-hydroperoxy-9Z,11E-octadecadienoic acid	LMFA02000034	JDSRHVWSAMTSSN-BSZOFBHHSA-N
13-KODE	293.2 → 113.1	(d4) 9(S)-HODE	13-keto-9Z,11E-octadecadienoic acid	LMFA02000016	JHXAZBBVQSRKJR-BSZOFBHHSA-N
14,15-LTC4	624.3 → 272.1	(d4) LTE4	15S-hydroxy-14R-(S-glutathionyl)-5Z,8Z,10E,12E-eicosatetraenoic acid	LMFA03020031	OBQVBASHEWLKCQ-JTLMKIFUSA-N
14,15-LTE4	438.2 → 333.2	(d4) LTE4	15S-hydroxy-14R-(S-cysteinyl)-5Z,8Z,10E,12E-eicosatetraenoic acid	LMFA03020033	ILJNENYVAVKECZ-HRXVJLLUSA-N
15-HETE	319.2 → 219.2	(d8) 5(S)-HETE	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	LMFA03060001	JSFATNQSRLKRBCL-VAEKSGLLSA-N
15-HpETE	335.2 → 113.1	(d8) 5(S)-HETE	15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	LMFA03060014	BFWYTORDSFIVKP-VAEKSGLLSA-N
15-KETE	317.2 → 113.2	(d7) KETE	15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid	LMFA03060051	YGTJUEISKATQSM-USWFWKISSA-N
15S-HEPE	317.2 → 219.2	(d8) 5(S)-HETE	15S-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid	LMFA03070009	WLKCSMCLEKGTIB-DBYSHIMFSA-N
15S-HETE	321.2 → 221.2	(d8) 5(S)-HETE	15S-hydroxy-8Z,11Z,13E-eicosatrienoic acid	LMFA03050007	IUKXMNDGTWNTPT-OAHXIXLCSA-N
20-carboxy-LTB4	365.2 → 347.2	(d4) LTB4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraene-1,20-dioic acid	LMFA03020016	SWXGWPVIGNOLNHT-VFLUTPKSA-N
20-hydroxy-LTB4	351.2 → 195.1	(d4) LTB4	5S,12R,20-trihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	LMFA03020018	PTJFJXLGRSTECQ-PSPARDEHSA-N
5-HETE	319.2 → 115.1	(d8) 5(S)-HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03060002	KGIHOYOSFUGPC-JGKLHWIESA-N
5-HETE	321.3 → 303.0	(d8) 5(S)-HETE	5S-hydroxy-6E,8Z,11Z-eicosatrienoic acid	LMFA03050005	LSADDRSUZRRBAN-FDSUASFTSA-N
5-KETE	317.2 → 203.2	(d7) KETE	5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03060011	MEASLHGILYBXFO-XTDASVJISA-N
5S,14R-Lipoxin B4	351.2 → 221.2	(d4) LTB4	5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid	LMFA03040002	UXVRTOKOJOMENI-WLPVFMORSA-N
5S,15S-DiHETE	335.2 → 115.1	(d4) LTB4	5S,15S-dihydroxy-6E,8Z,10Z,13E-eicosatetraenoic acid	LMFA03060010	UXGXCGPWGSUMINI-BVHTXILBSA-N
5S,6R-Lipoxin A4	351.2 → 115.1	(d4) LTB4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	LMFA03040001	IXAQOQZEQGMQIS-SSQFEBMSA-N
5S,6S-Lipoxin A4	351.2 → 115.1	(d4) LTB4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	LMFA03040001	IXAQOQZEQGMQIS-SSQFEBMSA-N
5S-HEPE	317.2 → 115.1	(d8) 5(S)-HETE	5S-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid	LMFA03070010	FTAGQROYQYQRHF-GHWNLOBHSA-N

5S-HpHETE	317.3 > 203.2	(d8) 5(S)-HETE	5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	LMFA03060012	JNUUNUQHXXOFDA-JGKLIHWIESA-N
6-trans-LTB4	335.2 > 195.2	(d4) LTB4	5S,12R-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid	LMFA03020013	VNYSSYRCGWBLHG-UKNWISKWSA-N
8-HETE	321.3 > 303.0	(d8) 12(S)-HETE	8S-hydroxy-9E,11Z,14Z-eicosatrienoic acid	LMFA03050011	SKIQVURLERJCK-RDCCVJQZSA-N
8S,15S-DiHETE	335.2 > 235.2	(d4) LTB4	8S,15S-dihydroxy-5Z,9E,11Z,13E-eicosatetraenoic acid	LMFA03060050	NNPWKSGGORTIM-HCCYKKOSA-N
9,10,13-TrHOME	329.2 > 171.1	(d4) 9(S)-HODE	E)9,10,13-Trihydroxy-11-octadecenoic acid	LMFA02000168	NTVQBHLSPGQ-BUHFOSPRSA-N
9,12,13-TrHOME	329.2 > 211.2	(d4) 9(S)-HODE	9S,12S,13S-trihydroxy-10E-octadecenoic acid	LMFA02000014	MDIUMSLCYIJBQC-MVFSOIOZSA-N
9-HODE	295.2 > 171.1	(d4) 9(S)-HODE	(±)-9-hydroxy-10E,12Z-octadecadienoic acid	LMFA02000151	NPDSHTNEKIQQU-ZJHFMPGASA-N
9-HOTE	293.2 > 171.1	(d4) 9(S)-HODE	9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid	LMFA02000024	RIGGEAZDTKMXSI-MEBVTUQTSA-N
9-HpODE	311.2 > 185.2	(d4) 9(S)-HODE	9S-hydroperoxy-10E,12Z-octadecadienoic acid	LMFA02000012	JGUNZIWGNMQSBM-IJUNYOVNOSA-N
9-KODE	293.2 > 185.2	(d4) 9(S)-HODE	9-oxo-10E,12Z-octadecadienoic acid	LMFA02000274	LUZSWWYKKLTDHU-ZJHFMPGASA-N
Hepoxilin A3	335.2 > 273.2	(d8) 12(S)-HETE	8-hydroxy-11S,12S-epoxy-5Z,14Z,9E-eicosatrienoic acid	LMFA03090005	SGTUOBURCVMACZ-SEVPPIGSA-N
LTB4	335.2 > 195.1	(d4) LTB4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	LMFA03020001	VNYSSYRCGWBLHG-AMOLWHMGSA-N
LTB5	333.2 > 195.1	(d4) LTB4	5S,12S-dihydroxy-6Z,8E,14Z,17Z-eicosapentanoic acid	LMFA03020010	BISQPGCQOHLHQK-HDNPQISLSA-N
LTC4	624.3 > 272.1	(d4) LTE4	5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11Z,14Z-eicosatetraenoic acid	LMFA03020003	GWNVDXQDILPJIG-SWQVEEFNSA-N
LTD4	495.2 > 177.1	(d4) LTE4	5S-hydroxy-6R-(S-cysteinylglycyl)-7E,9E,11Z,14Z-eicosatetraenoic acid	LMFA03020077	YEESKJGWJFYOOK-LDDGIIKSA-N
LTE4	438.2 > 333.2	(d4) LTE4	5S-hydroxy-6R-(S-cysteinyl)-7E,9E,11Z,14Z-eicosatetraenoic acid	LMFA03020002	OTZRAYGBFWZKMX-FRFVZSDQSA-N
Resolvin D1	375.2 > 141.0	(d4) LTB4	7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid	LMFA04000006	OIWTWACQMDFHUG-CCFUJAGSSA-N

Table S5: Complete ROS/RNS pathway oxidised lipid target list

Metabolites	Precursor ion Product ion (<i>m/z</i>)	ISTD	Formal name	Lipidmaps ID	InChIKey
NO ₂ -aLA (C18:3)	322.1 → 46.05	10-Nitrooleate-d17	nitro-9E, 12Z, 15Z-octadecadienoic acid	<i>na</i>	<i>na</i>
NO ₂ -LA (C18:2)	324.3 → 277.25	10-Nitrooleate-d17	nitro-9E, 12Z-octadecadienoic acid	LMFA01120001	LELVHAQTWXTCLY-XYWKCAQWSA-N
NO ₂ -OA (C18:1)	326.1 → 46.0	10-Nitrooleate-d17	nitro-9E-octadecenoic acid	LMFA01120003	WRADPCFZWXTOTI-BMRADRMISA-N
10-HDoHE	343.2 → 153.0	(d8) 12(S)-HETE	(+/-)-10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-docosahexaenoic acid	LMFA04000027	DDCYKEYDTGCKAS-SKSHMZPZSA-N
11-HDoHE	343.2 → 121.0	(d8) 12(S)-HETE	(+/-)-11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid	LMFA04000028	LTERDCBCHFKRI-BGKMTWLOSA-N
11-HETE	319.2 → 167.1	(d8) 12(S)-HETE	11R-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	LMFA03060028	GCZRCCHPLVMMJE-WXMXURGSA-N
13-HDoHE	343.2 → 281.0	(d8) 12(S)-HETE	(+/-)-13-hydroxy-4Z,7Z,10Z,14E,16Z,19Z-docosahexaenoic acid	LMFA04000029	SEVOKGDVLLJUMT-SKSHMZPZSA-N
14-HDoHE	343.2 → 205.0	(d8) 12(S)-HETE	(+/-)-14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid	LMFA04000030	ZNEBXONKCYFAF-BGKMTWLOSA-N
16-HDoHE	343.2 → 233.0	(d8) 12(S)-HETE	(±)16-hydroxy-4Z,7Z,10Z,13Z,17E,19Z-docosahexaenoic acid	LMFA04000031	CSXQXWHAGLFIH-VUARBIEWSA-N
17-HDoHE	343.2 → 281.3	(d8) 12(S)-HETE	(±)17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid	LMFA04000032	SWTVBBIUEPPYCX-VIIQGISXSA-N
18-HEPE	317.2 → 255.0	(d8) 12(S)-HETE	(±)18-hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid	LMFA03070033	LRWYBGFVSUIBWMO-UXNZXXPISA-N
2,3-dinor-8-iso-PGF2a	325.1 → 237.2	(d4) 8-iso-PGF2a	9a,11a,15S-trihydroxy-2,3-dinor-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110010	IDKLJIUUVJNR-JSEKUSAISA-N
20-HDoHE	343.2 → 299.0	(d8) 12(S)-HETE	(+/-)-20-hydroxy-4Z,7Z,10Z,13Z,16Z,18E-docosahexaenoic acid	LMFA04000033	YUZXOJOCNGKDNIL-FVREGEGBSA-N
4-HDoHE	343.2 → 281.0	(d8) 12(S)-HETE	(±)4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid	LMFA04000024	IFRKNPQVJFAQ-PQVBWYSWSA-N
(+/-) 5-IPF2a VI	353.3 → 115.05	(d11) 5-IPF2a IV	5,9S,11R-trihydroxy-6E,14Z-prostadienoic acid-cyclo[8S,12R]	LMFA03110011	RZCPXIZGLPAGEV-SUHLLOIRSA-N
7-HDoHE	343.2 → 281.0	(d8) 12(S)-HETE	(±)7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-docosahexaenoic acid	LMFA04000025	OZXAIGIRPOOTLI-XJAVJPOHSA-N
8,12-IPF2a VI	353.3 → 115.05	(d11) 8,12-IPF2a IV	(±)5,9e-trihydroxy-12a-prosta-6E,14Z-dien-1-oic acid	<i>na</i>	RZCPXIZGLPAGEV-DCOIXEBESA-N
8-HDoHE	343.2 → 189.0	(d8) 12(S)-HETE	(±)8-hydroxy-4Z,6E,10Z,13Z,16Z,19Z-docosahexaenoic acid	LMFA04000026	ZHBVYDMSPDDAKE-VITZNUJUSA-N
8-HETE	319.2 → 155.1	(d8) 5(S)-HETE	(±)8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	LMFA03060086	NLUNAYAEIUYXRB-HEJOTXCHSA-N
8-iso-13,14-dihydro-PGF2a	353.3 → 183.1	(d4) 8-iso-PGF2a	(±)8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	LMFA03110004	VKTIQNYPMSCHQI-JPRPWBOSBA-N
8-iso-15-keto-PGE2	349.1 → 287.2	(d4) 8-iso-PGE2	9S,11R-dihydroxy-15-oxo-5Z-prostaenoic acid-cyclo[8S,12R]	LMFA03110009	YRTJDWROBKPNV-RSNVZYGJSA-N
8-iso-15-keto-PGF2a	351.1 → 315.15	(d4) 8-iso-PGF2a	9,15-dioxo-11α-hydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110005	LOLJELMPWPILA-RLXHZABYSA-N
8-iso-15-keto-PGF2b	351.1 → 315.15	(d4) 8-iso-PGF2a	9a,11a-dihydroxy-15-oxo-(8β)-prosta-5Z,13E-dien-1-oic acid	<i>na</i>	<i>na</i>
8-iso-15(R)-PGF2a	353.3 → 193.2	(d4) 8-iso-PGF2a	9f,11a-dihydroxy-15-oxo-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110030	PXGPLTODNUVGFL-PGWUFSFSA-N
8-iso-PGA1	335.1 → 273.15	(d4) PGA2	9a,11a,15R-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110008	BGKHCLZFGPIKKU-DRSVPBQLSA-N
8-iso-PGA2	333.1 → 271.2	(d4) PGA2	9-oxo-15S-hydroxy-(8β)-prosta-5Z,10,13E-trien-1-oic acid	LMFA03110140	MYHXHCUNDAEIOZ-UKUWKSPLISA-N
8-iso-PGE1	353.3 → 317.2	(d4) PGE1	9-oxo-11a,15S-dihydroxy-(8β)-prost-13E-en-1-oic acid	LMFA03110002	GMVPRGQOIIIMT-JPCPGATGSA-N

8-iso-PGE2	351.1 → 271.15	(d4) 8-iso-PGE2	9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110003	XEYBRNLFZDVAW-CLQOMRTCSA-N
8-iso-PGF1a	353.3 → 311.1	(d9) 8-iso-PGF1a	9α,11α,15S-trihydroxy-(8β)-prost-13E-en-1-oic acid	<i>na</i>	DZUXGQBLFALXCR-PUCCXBQISA-N
8-iso-PGF2a (15-F2t-IsoP)	353.3 → 193.2	(d4) 8-iso-PGF2a	9α,11α,15S-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	LMFA03110001	PXGPLTODNUVGFL-NAPLMKITS-A-N
8-iso-PGF3a	351.1 → 307.15	(d4) 8-iso-PGF2a	9α,11α,15S-trihydroxy-(8β)-prosta-5Z,13E,17Z-dien-1-oic acid	<i>na</i>	<i>na</i>
9-HEPE	317.2 → 149.0	(d8) 12(S)-HETE	(±)-9-hydroxy-5Z,7E,11Z,14Z,17Z-icosapentaenoic acid	LMFA03070029	OXOPDAZWPWFJEW-IMCWFPBLSA-N
9-HETE	319.2 → 167.1	(d8) 12(S)-HETE	(±)-9-hydroxy-5Z,7E,11Z,14Z-icosatetraenoic acid	LMFA03060089	KATOYYZUTNAWSA-OIZRIKEUSA-N
iPF2a-IV	353.3 → 127.1	(d4) iPFa-VI	<i>na</i>	<i>na</i>	MZYVWZXTHYCVHQ-OXCZDIPSSA-N

Table S6 The selected panel of compounds

Pathway	Metabolites	<i>m/z</i>	Precursor ion → Product ion	ISTD
COX	13,14-dihydro-PGF2a	353.30 → 183.10	(d4) PGF2a	
	PGE1	353.30 → 317.20	(d4) PGE1	
	PGE2	351.10 → 271.15	(d9) PGE2	
	PGF2a	353.30 → 193.20	(d4) PGF2a	
ROS	8-iso-13,14-dihydro-PGF2a	353.30 → 183.10	(d4) 8-iso-PGF2a	
	8-iso-15(R)-PGF2a	353.30 → 193.20	(d4) 8-iso-PGF2a	
	8-iso-PGF2a	353.30 → 193.20	(d4) 8-iso-PGF2a	
RNS	NO2-LA (C18:2)	324.30 → 277.25	10-Nitrooleate-d17	
	NO2-OA (C18:1)	326.10 → 46.00	10-Nitrooleate-d17	

Table S7 The GUS blank effect compared to non-hydrolysed urine.

GUS	8-iso-15(R)-PGF _{2a}	8-iso-13,14-dihydro-PGF _{2a}	8-iso-PGF _{2a}	PGE ₂	PGE ₁	PGF _{2a}	13,14-dihydro-PGF _{2a}	NO ₂ -linoleic acid	NO ₂ -oleic acid
<i>H. pomatia</i>	42.15	0.00	41.76	2.64	0.06	128.39	0.00	0.00	0.00
Bovine liver	0.00	0.00	21.18	0.05	0.05	16.12	0.00	0.00	0.00
<i>E. coli</i>	0.00	0.00	0.00	0.06	0.00	16.80	0.00	0.00	0.00

The GUS blank effect is calculated by: Metabolite response_{Enzyme blank}/ Metabolite response_{Urine} ×100%

Table S8 RSDs of free and total oxidized lipids measured in RA patient urine

Compound name	RSD(%)	
	Total oxidized lipid	Free oxidized lipid
8,12-iPF2a IV	2.31	1.72
12,13-DiHOME	6.98	2.78
9,10-DiHOME	1.91	2.9
iPF2a Unknown	5.86	4.49
PGF2a	7.21	4.88
2_3-dinor-8-iso-PGF2a	12.36	5.49
5-iPF2a IV	2.8	5.71
9,10-EpOME	20.51	7.03
PGA2		7.31
9-HODE	5.04	7.37
9,12,13-TriHOME	9.95	7.71
D17, 6-ketoPGF1a	7.97	8.41
8,9-DiHETRe	4.43	8.64
13-KODE	9.12	9.12
12-HETE	9.35	9.4
13-HODE	7	9.96
8-iso-PGF1a	11.97	10.05
PGF1a	23.33	10.17
20-carboxy-LTB4	13.96	10.18
14,15-DiHETRe	4.24	11.04
8-iso-PGF2a	17.37	11.1
9-HOTRe	4.2	11.82
5-iPF2a-VI	11.46	11.85
PGE1		11.9
9,10,13-TriHOME	7.09	13.22
8-iso-15(R)-PGF2a	5.66	13.38
12,13-EpOME	32.38	13.8
2,3-dinor-11b-PGF2a	14.54	16.72
13,14-dihydro-PGF2a	13.49	18.19
bicyclo-PGE2	13.84	19.93

14-HDoHE	25.99	19.97
PGE2	11.63	20.86
11beta-13,14-dihydro-15-keto-PGF2a	16.83	20.97
11beta-PGF2a	21.92	23.49
NO2-LA		23.56
8-iso-PGF2a	23.8	23.85
5S,6R-LipoxinA4	14.72	25.29
9-KODE	25.86	25.6
5S-HEPE	6.66	26.29
NO2-aLA		26.33
12S-HEPE	19.08	26.57
PGF2a	9.49	26.83
NO2-OA		50.11
8-iso-PGE1		22.85
19,20-DiHDPa	5.1	
9-HEPE	6.96	
15S-HETrE	7.82	
11,12-DiHETrE	8.05	
PGK2	8.1	
5,6-DiHETrE	9.7	
17,18-DiHETE	10.24	
12,13-DiHODE	11.97	
PGF3a	12.63	
20-HETE	12.64	
PGE3	13.39	
20-HDoHE	14.14	
5-HETE	14.71	
2,3-dinor-11b-PGF2a	14.74	
9-HETE	14.86	
iPF2a	15.55	
8-HETE	16.58	
11,12-EpETrE	17	
10-HDoHE	17.14	
15-HETE	17.64	
13,14-dihydro-15-keto-PGE2	20.19	
11-HDoHE	20.44	
11-HETE	21.19	
PGD3	21.98	
14,15-DiHETE	22.37	
8_HDoHE	23.31	
5S,15S-DiHETE	23.9	
16-HDoHE	24.46	
13,14-dihydro-15-keto-PGF2a	41.36	

Table S9 Multiple linear regression models showing independent clinical parameters associated with oxidized lipid levels

Clinical parameter	Metabolite	Coefficient	p-value
CRP	PGF3a	-0.28	0.08
	12-HETE	-0.32	0.06
	16-HDoHE	0.26	0.03
	20-HETE	0.19	0.08
	PGD3	-0.63	0.03
	PGE3	-0.55	0.05
Baseline DAS28	8-iso-PGF2a	0.19	0.06
	14,15-DiHETE	0.26	0.04
	14-HDoHE	-0.39	0.09
	5S,6R-LipoxinA4	0.67	0.01
	NO2-aLA	-0.47	0.02
	NO2-OA	-0.26	0.03
	NO2-LA	-0.48	0.00
DAS28 improvement	PGF3a	0.26	0.02
	PGF2a	0.11	0.02
	iPF2a	0.12	0.02
	NO2-LA	0.18	0.06
	11,12-EpETrE	0.15	0.04
	11-HETE	0.15	0.02
	13-KODE	0.18	0.08
	14,15-DiHETrE	0.10	0.02
	14-HDoHE	0.31	0.03
	15S-HETrE	0.14	0.05
	9-KODE	0.14	0.10
	bicyclo-PGE2	0.14	0.09
	NO2-LA	0.22	0.03

Figures

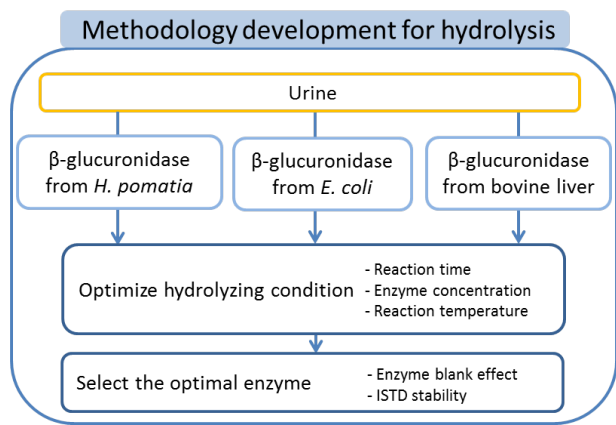


Figure S1. Method development overview

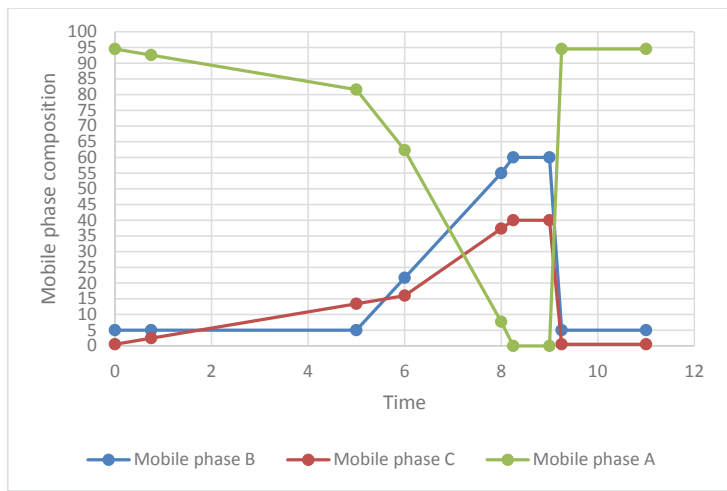


Figure S2. Ternary gradient of the used UHPLC-MS/MS method.

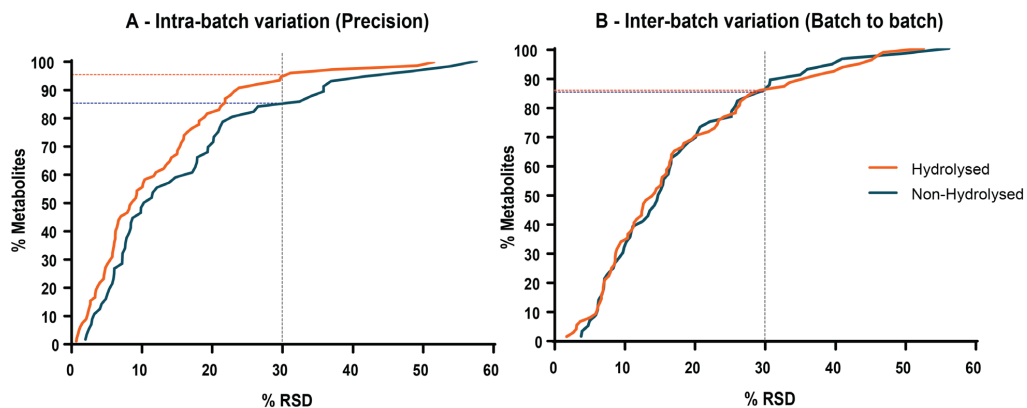


Figure S3. Detected endogenous oxidized lipids evaluated for A - precision and B - batch-to-batch effect. More than 90% of endogenous metabolites had an RSD below 30% for the hydrolysed procedure's precision, followed closely with 85% of metabolites in the non-hydrolysed procedure having a precision below 30%. Inter-batch variation indicated similar stable performance across three measurement days for both the hydrolyses and non-hydrolyses procedure.

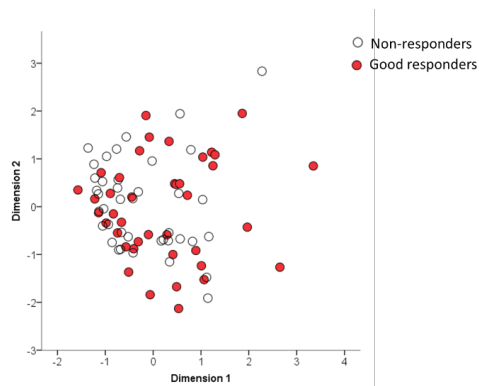


Figure S4. Score plot of the CATPCA model for good and non-responders to TNF- α inhibitor treatment of RA patients, depicting component score on Dimension 1 and 2.

Chapter 4

Systems approach for classifying the response to biological therapies in patients with rheumatoid arthritis in clinical practice

4

Based on

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Systems approach for classifying the response to biological therapies in patients with rheumatoid arthritis in clinical practice

Submitted for publication

Abstract

Background Biological therapies have greatly improved the treatment efficacy in rheumatoid arthritis (RA). However, in clinical practice a significant proportion of patients experience an inadequate response to treatment.

Objective To classify responders and non-responders to biological therapy in RA.

Methods Cold and Heat symptoms accessed by a Chinese medicine (CM) questionnaire and Western clinical data were collected as baseline data, before initiating biological therapy. Categorical principal components analysis with forced classification(CATPCA-FC) approach was applied to the baseline data set to classify responders and non-responders.

Results In this study, 61 RA patients were characterized using a CM questionnaire and clinical measurements. The combination of baseline symptoms ('preference for warm food', 'weak tendon severity') and clinical parameters (positive rheumatoid factor/anti-cyclic citrullinated peptide antibody, C-reactive protein, creatinine) were able to differentiate responders from non-responders to biological therapies with a positive predictive value of 82.35% and a misclassification rate of 24.59%. Adding CM symptom variables in addition to clinical data did not improve the classification of responders, but it did show 8.3% improvement in classifying non-responders.

Conclusions This study, for the first time includes CM symptoms into a classification analysis of RA patients' response to biological therapies. The adding of symptoms to the clinical parameters could improve the classification of non-responders. Although this improvement is not significant in the current study, we consider it worthwhile to investigate further the possible potential of symptom variables for reducing inefficient treatment.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that results in a systemic inflammation, affecting 0.5% to 1% population in Northern Europe and North America ¹. The pathogenesis of RA is not defined as yet, but there is no doubt that complex immune responses are highly related to inflammation and joint erosion ². In the late 1990s, tumor necrosis factor (TNF) inhibitors were introduced for the treatment of RA, followed by other kinds of biological agents including the anti-CD 20 agent, IL-6 inhibitor etc. By targeting cell-surface receptors or intracellular pathways, biological agents show powerful capabilities in the modulation of the immune response ³⁻⁵. Compared to the conventional RA medicines, biological agents can not only reduce disease activity but also decrease or prevent radiographic progression in RA ⁶.

Biological therapies can greatly improve the treatment outcome in RA, but there is a significant proportion of patients who have an inadequate response. Approximately 30% of the RA patients failed to respond after (at least) three-month biological treatment ⁷. For these non-responders this problem is compounded by high financial cost and significant side effects. Additionally in clinical practice for RA treatment, a biological agent is recommended only if the treatment outcome of non-biologic therapy is not reached with 6 months ⁸. Therefore, the opportunity to control the erosion of joints in early RA might be missed, further affecting the long-term outcomes of RA ⁹. Hence, it is necessary to better target this medication to patients who will benefit from the biological therapies ¹⁰, and to develop more personalized medication based a novel diagnostic principle.

As a practical medicine, Chinese medicine (CM) has a long history of development and optimization through observation in daily practice. Especially for complex diseases, CM could provide new possibilities for subtyping patients by pattern diagnosis ¹¹. In CM, RA is defined as a Bi-syndrome ¹². Bi-syndromes consist of multiple subtypes based on symptom patterns, including two basic patterns—‘Cold’ and ‘Heat’. For each pattern of RA, there is a different treatment strategy in CM ¹³. We previously reported differences in biological mechanisms between Cold and Heat RA patients, determined by metabolomics measurements of plasma and urine samples as well as gene expression analysis of CD-4 T-cells ^{14,15}. A large literature mining study suggests that Cold type of diseases is related to hormone disturbances whereas immune systems disturbances are Heat type related ¹⁶. Therefore, we hypothesize that the Cold or Heat patterns may be associated with the response to biological therapies of RA patients. According to CM, Cold or Heat diagnosis is based on integrating corresponding symptoms. However, in most cases patients diagnosed with Cold RA could also show Heat

RA related symptoms, besides dominant Cold symptoms; or the other way around with RA Heat patients.

Thus, it is difficult to find patients with exclusively Cold or Heat patterns in practice. Therefore, in the present study we focused on the individual Cold and Heat symptoms instead of patterns. Symptoms, which are important for the evaluation of patients' quality of life and disease burden, are more and more used as patient-reported outcome measures for the evaluation of treatment effects in clinical studies^{17,18}. Many factors from clinical and lab tests have been already reported as predictors/biomarkers of patients' response to biologic agents^{19–22}, but there is no study including baseline symptoms as potential markers.

In this study we used Eastern and Western diagnostic principles, including Cold and Heat symptoms as well as other baseline disease characteristics, to classify response to biological therapy in patients with RA. If we are able to better classify non-responders and responders at the start of biological therapy, patients can be treated with the best available drugs timely, avoiding side effects and unnecessary financial cost caused by trial-and-error practice.

Materials and Methods

Study design and participants

RA patients were selected from the observational BiOCURA, in which patients with RA starting a biological therapy were recruited. There was no particular intervention in this purely observational study. After three months of treatment with one of the following biological agents: Etanercept, Adalimumab, Golimumab, Certolizumab pegol, Rituximab, Abatacept or Tocilizumab, the outcome of the therapy was assessed according to EULAR response criteria. Re-inclusion after switching to a different biological agent was possible. The type of biological agent provided to each of the patients was decided by their own clinician as is done in routine clinical practice. The study was approved by the Medical Ethical Committee of UMC Utrecht and all subjects gave their written informed consent for participation in any procedure specifically for the study. Our study was restricted to the BiOCURA patients who completed a CM questionnaire at baseline.

Symptom questionnaire

This questionnaire was designed to measure symptoms based on a CM perspective of Cold/Heat patterns on arthritis, which was developed and tested recently¹⁴. The same questionnaire was used in the present study except translated into the Dutch language, which consists of 57 items separated into five categories (breathing, digestion, climate, quality of the

symptoms and pain). Most of the questions are on a Likert-scale, evaluating the severity and the frequency of symptoms from score 1 to 7. Score 1 was interpreted as never or not and score 7 was very severe or very often. The remaining questions were in yes/no format. The questionnaires were completed before initiating the biological therapy and used as baseline symptom data.

Demographics and clinical parameters

Before starting biological therapy, clinical parameters and demographic characteristic were obtained as baseline clinical data. The following demographics and clinical parameters of patients were collected at baseline: gender, age, body mass index (BMI), disease duration, smoking status, alcohol consumption, biological naivety, concomitant DMARDs, 28 tender joint count (TJC), 28 swollen joint count (SJC), 100 mm visual analogue score (VAS), disease activity score in 28 joints (DAS28), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), creatinine, hemoglobin, alanine aminotransferase (ALT), leukocyte count, rheumatoid factor (RF), platelet count, and anti-cyclic citrullinated peptide antibody (anti-CCP).

After three-month treatment, DAS28 was measured again as present DAS28. By combining the present DAS28 as well as the improvement of DAS28 after three-month treatment, a patient's response to a biological agent can be evaluated according to EULAR response criteria²³. A good response was defined as a present DAS28 ≤ 3.2 with a DAS28 reduction > 1.2 , whereas a reduction of DAS28 ≤ 0.6 or present DAS28 > 5.1 with a reduction ≤ 1.2 was defined as non-response. In between, a reduction > 1.2 with present DAS28 > 3.2 or a reduction between 0.6 and 1.2 with present DAS28 < 5.1 was specified as a moderate response. Since both good-and moderate responders achieved sufficient response, they were combined as responders in the following data analysis²⁴.

Data analysis

Firstly, univariate analyses including independent student's t-tests, chi-square tests, and Kruskal wallis H tests were applied to compare the differences of baseline clinical and demographic data between responders and non-responders. Subsequently, multivariate analysis was performed on combined data from questionnaires, clinical parameters, and demographic characteristics. Since the combined data sets contained variables with different measurement levels (nominal, ordinal, or numeric) that might be nonlinearly related to each other, categorical principal components analysis (CATPCA) was applied²⁵.

As a nonlinear principal component analysis technique, CATPCA allows different analysis levels (numeric, ordinal and nominal) for variables and is able to handle categorically and numerically measured variable²⁵⁻²⁷. In addition, variables can be given weights—the higher a variable weights, the more the solution will be influenced by the variable. When applying a large weight to only one variable, the solution will be dominated by that variable. This feature can be used to perform forced classification (FC)²⁸—by including a classification variable with a large weight in the analysis, the cases are forced into clusters according to the classification variable. Thus, variables with high component loadings indicate that they are highly related to the classification variable. In a previous study, CATPCA-FC has been used to identify the important symptoms related to Cold and Heat status of patients²⁹. Therefore, in the present study we use CATPCA-FC in an exploratory way to identify which baseline data related to treatment response, subsequently to classify responders and non-responders at baseline.

In the study, there were seven biological agents involved. Limited number of subjects for each treatment may lead to instability of CATPCA-FC model. Thus biological agents were grouped according to their mechanisms as TNF inhibitors (Etanercept, Adalimumab, Golimumab, Certolizumab pegol) and non-TNF inhibitors (Rituximab, Abatacept, Tocilizumab). RF and anti-CCP are clinically important parameters for the diagnosis and prognosis of RA patients³⁰. The combination of the two parameters may lead to a better classification of treatment outcome³¹. Therefore, RF and anti-CCP were combined in one variable—positive RF/anti-CCP (three categories: both positive, only one positive; both negative). EULAR response (two categories: responder, non-responder) was taken as the classification variable, which is derived from the patient's baseline DAS28 and current DAS28 scores. In addition, the DAS28 scores are calculated from the variables TJC, SJC, VAS and ESR³². Consequently, there is a strong correlation between DAS28, TJC, SJC, VAS and ESR and 3-month EULAR response. Therefore, these DAS28 related baseline clinical parameters were excluded from the CATPCA-FC analyses. The rest of baseline clinical parameters and demographic data (described in ‘Demographics and clinical parameters’) were taken as clinical data, which were merged with CM symptom to build a combined model. In the meantime, a symptom model and a clinical model were built separately.

After data screening and recoding, the processed data were further analyzed by CATPCA-FC. The number of components in CATPCA, which is required to restrict the component scores to groups, is set according to the classification variable. Since we have two classes (responders, non-responders) we used one component. The models were internally validated by a leave-one-out cross-validation (LOOCV) procedure²². During the LOOCV procedure, a predicted

response and a predicted score were computed for each subject. Details of statistics procedures are described in Supplementary Methods.

In order to determine the predictive ability of each model, the positive prediction value (PPV), negative prediction value (NPV), and misclassification rate (MR) were calculated based on the observed responses and the LOOCV predicted responses. In addition, the receiver operator characteristic (ROC) curve for each model was built with predicted scores as test variables and observed response as the state variable. The ROC curves were compared on the basis of the area under the receiver operator characteristic curve (AUROC)³³.

All procedures were applied similarly to the combined model, symptom model, and clinical model. Statistical calculations were performed with IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, N.Y., USA) and MedCalc for Windows, version 16.2.1 (MedCalc Software, Ostend, Belgium); the level of significance was set at 0.05.

Results

Subjects

A total of 65 RA patients, who completed 75 questionnaires, were enrolled in the study (8 patients were enrolled in the study twice, and one patient was enrolled three times). Each time before an RA patient restarted a new biological therapy, questionnaire data, demographic and clinical parameters were collected as baseline again. Consequently, those patients were regarded as new subjects in the following data analysis. Of those, 12 subjects were excluded due to lack of follow-up information in three months, and 2 subjects answering less than 50% questions in questionnaires were also removed. Finally, there were 61 valid subjects retained in the study: 37 responders and 24 non-responders.

Baseline characteristics and parameters of patients

As illustrated in Table 1, the majority of 61 subjects were female (77%), and all subjects had active rheumatoid arthritis (DAS28, 4.28 ± 1.32) before starting biological therapy. After 3-month of biological therapy, 37 subjects were EULAR responders, including 15 good responders and 22 moderate responders, whereas 24 subjects were non-responders. Baseline characteristics of non-responders versus responders differed in terms of DAS28 (mean = 3.64 ± 1.41 vs. 4.69 ± 1.10 , $p = 0.002$), SJC (median = 0.5 (0.0-1.0) vs. 2.0 (0.5-4.0), $p = 0.01$), ESR (median = 8.0 mm/hr (4.0-23.0) vs. 15.0 mm/hr (11.0-43.0), $p = 0.01$) and CRP (median = 3.0 mg/mL (2.0-8.0) vs 11.5 mg/mL (3.3-24.0), $p = 0.02$). Although responders had a higher proportion of positive anti-CCP and positive RF compared to non-responders, there were no

statistical differences ($p = 0.06$). There were also no significant differences in the rest clinical parameters, demographic data, and the proportion of subjects using DMARDs (Supplementary Table S1) between responders and non-responders.

Table 1. Baseline characteristics of responders and non-responders. P -values for comparisons among groups were calculated by independent t-tests ^a, Kruskal wallis H tests ^b and chi-squared tests ^c based on distribution of the clinical parameter. Bold values indicate significant p -values ($p < 0.05$).

	All (n=61)	Non-responders (n=24)	Responders (n=37)	p value
Age, yrs, mean $\pm$ SD ^a	56 $\pm$ 13	53 $\pm$ 12	57 $\pm$ 13	0.30
Disease duration, yrs, median (IQR) ^b	10.0 (5.0-18.0)	9.5 (3.5-17.5)	10.0 (5.0-19.5)	0.75
BMI, kg/m ² , mean $\pm$ SD ^a	27.0 $\pm$ 5.1	28.0 $\pm$ 5.1	27.7 $\pm$ 9.8	0.92
Female, n(%) ^c	47 (77)	17 (71)	30 (81)	0.37
Biologic-naïve, n (%) ^c	20 (33)	10 (43)	10 (26)	0.28
DAS28, mean $\pm$ SD ^a	4.28 $\pm$ 1.32	3.64 $\pm$ 1.41	4.69 $\pm$ 1.10	0.002
TJC, median (IQR) ^b	6.0 (2.5-11.0)	3.0 (0.0-10.3)	7.0 (4.0-11.0)	0.80
SJC, median (IQR) ^b	1.0 (0.0-3.5)	0.5 (0.0-1.0)	2.0 (0.5-4.0)	0.01
VAS, mean $\pm$ SD ^a	54 $\pm$ 24	50 $\pm$ 25	56 $\pm$ 23	0.41
ESR, mm/hr, median (IQR) ^b	13.0 (6.0-33.5)	8.0 (4.0-23.0)	15.0 (11.0-43.0)	0.02
CRP, mg/mL, median (IQR) ^b	6.0 (2.0-16.0)	3.0 (2.0-8.0)	11.5 (3.3-24.0)	0.01
Positive RF and anti-CCP, n (%) ^c	26 (42.6)	5 (21)	21 (57)	0.02
Positive anti-CCP, n (%) ^c	32 (52)	9 (37)	23 (68)	0.06
Positive RF, n (%) ^c	35 (57)	10 (43)	25 (67)	0.06
ALT, U/L, median (IQR) ^b	21.0 (17.0-31.0)	23.5 (19.0-30.8)	19.0 (17.0-35.0)	0.67
Platelets, 10 ⁹ /L, median (IQR) ^b	260 (206.5-342.5)	259.0 (208.8-320.8)	293.0 (204.5-352.5)	0.85
Leukocytes, 10 ⁹ /L, median (IQR) ^b	7.6 (6.15-9.3)	7.6 (4.9-9.4)	7.7 (6.3-9.3)	0.50
Creatinine, μ mol/L, mean $\pm$ SD ^a	74 $\pm$ 14	76 $\pm$ 12	73 $\pm$ 15	0.40
Hemoglobin, mmol/L, mean $\pm$ SD ^a	8.4 $\pm$ 0.8	8.6 $\pm$ 0.7	8.3 $\pm$ 0.8	0.12

$$\text{DAS28} = 0.56 \times \sqrt{\text{TJC}} + 0.28 \times \sqrt{\text{SJC}} + 0.70 \times \ln(\text{ESR}) + 0.014 \times \text{VAS}^{32}$$

ALT, alanine aminotransferase; Anti-CCP, anti-cyclic citrullinated peptide antibody; BMI, body mass index; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; IQR, interquartile range; VAS, 100 mm visual analogue scale; RF, rheumatoid factor; SD, standard deviation; SJC, 28 swollen joint count; TJC, 28 tender joint.

Systems approach for classifying the responders and non-responders to biological therapy by CATPCA

Data screening, recoding and discretization

After data screening, the inconsistent answers found in the questionnaires of 4 subjects were set to missing. In the recoding procedure, 13 variables related to 6 symptoms (shortness of

breath, dryness of the throat, tender abdomen, diarrhea, cold feeling, fever) were simplified and reorganized into 6 new variables. The variable ‘color of phlegm’ was with only one category, therefore, could not be used in the analyses. In total, there were less than 10% missing data identified in the symptom data set. Clinical variables with more than 15 continuous values were discretized by grouping the values into 15 categories. In total, 49 symptoms, 7 clinical parameters, and 12 demographics were taken as the baseline data for the further CATPCA. Details of included data are shown in Supplementary Table S2.

Multivariate analysis on baseline data—CATPCA-FC

In order to search for baseline data related to response, CATPCA-FC approach was employed. Baseline data of 61 subjects were included in the analyses with optimal analysis levels; EULAR response was set with large weight as the classification variable.

Variables with absolute component loadings > 0.30 were selected and included in the final models. For the final symptom model, two symptoms ‘weak tendon severity’ and ‘preference for warm food’ remained, and the final clinical model consisted of CRP, creatinine, and positive RF/anti-CCP. The final combined model comprised of the variables from both clinical and symptom model. The transformation plots and component loadings of variables in the combined model are shown in Table 2 and Figure 1, respectively. The transformation plot represents the transformed values of a variable based on its optimal analysis level, and the component loading indicates the (positive or negative) relationship between the transformed variable and the classification variable.

Table 2. Component loadings and analysis levels of selected variables in the final combined model

Variable name	Component loading	Analysis level
Creatinine	-0.349	Spline nominal
Preference for warm food	-0.312	Nominal
Positive RF/anti-CCP	0.355	Ordinal
Weak tendon severity	0.376	Nominal
CRP	0.406	Spline ordinal

CRP, C-reactive protein; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody

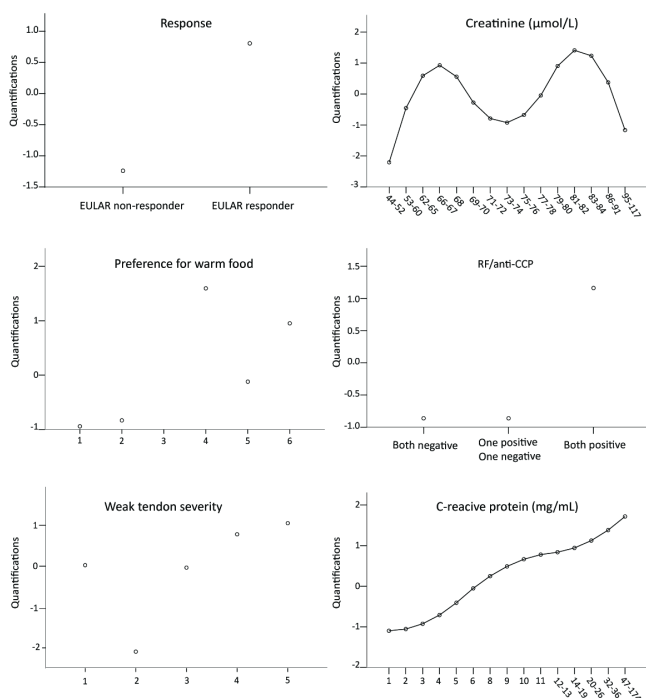


Figure 1. Transformation plot of the classification variable EULAR response and five classifier variables in the combined model. The categories of measured values are shown on the X-axis, and the quantification using optimal analysis is displayed on the Y-axis. The symptoms ‘weak tendon severity’ and ‘preference for warm food’ are with nominal analysis level; measured value 1 was interpreted as never or not and score 6 was very severe or very often. The clinical variables, RF/anti-CCP are with ordinal analysis level; C- reactive protein are with spline ordinal analysis level (degree 2, interior knots 2); creatinine is with spline nominal analysis level (degree 2, interior knots 2).

Classifiers ‘Positive RF/anti-CCP’, ‘weak tendon severity’ and CRP were found to have positive loading, indicating that the transformed values of these variables were higher in responders. Specifically, responders scored relatively high in the upper region of ‘weak tendon severity’ (4 through 5) and CRP ($> 8 \text{ mg/mL}$), while non-responders scored relatively low on these variables. For the positive RF/anti-CCP, the category of both positive (high score) was associated with responders while both negative or one negative (low score) were associated with non-responders. In contrast, of ‘preference for warm food’ with negative loading, responders scored in the low region (1 and 2), while non-responders had a high score in either 4 or 6. The transformation plot of creatinine (negative loading) shows a curve with a dip in the middle, to be interpreted as low, high, and intermediate values of creatinine being associated with responders, while non-responders tend to have moderate low and moderate

high values. None of the variables has a high loading in the model, suggesting that these variables by themselves could not give a clear-cut classification between response and non-response.

On the basis of the prediction results of the LOOCV procedure, prediction parameters PPV, NPC, MR and AUC were calculated for each model. As shown in Table 3, the combined model with both clinical and symptom classifiers had the best accuracy in differentiating responders from non-responders (MR = 24.59%) and predicting responders (PPV = 82.35%). Two observed non-responders, who were incorrectly classified as responders in the clinical model, were reclassified correctly as non-responders in the combined model, whereas no improvement in classifying responders were found. The reclassification of non-responders in the combined model was 8.3% $((18-16)/24 \times 100\%)$ better than the clinical model. The AUROC of the combined model is significantly larger than that of the symptom model ($p = 0.02$), whereas the differences of AUROCs between clinical and combined model were not significant (Supplementary Table S3).

Table 3. Prediction parameters of the combined model, symptom model, and clinical model

	Classified Observed			PPV ^a	NPV ^b	MR ^c	AUROC (95% CI)
		Non-responders	Responders				
Combined model	Non-responders	18	6	82.35	66.67	24.59	0.769
	Responders	9	28				(0.644 to 0.867)
Symptom model	Non-responders	12	12	68.42	52.17	37.70	0.574
	Responders	11	26				(0.441 to 0.700)
Clinical model	Non-responders	16	8	77.78	64.00	27.87	0.765
	Responders	9	28				(0.639 to 0.864)

PPV, positive predictive value; NPV, negative predictive value; MR, misclassification rate; AUROC, area under the receiver operator characteristic curve; CI, confidence interval.

^a Positive predictive values = true positive/(true positive + false positive) $\times 100\%$

^b Negative predictive values = true negative/(true negative + false negative) $\times 100\%$

^c Misclassification rate = (false positive+ false negative)/number of all subjects $\times 100\%$

Discussion

In this study, we described the application of CATPCA-FA on baseline data to classify response to biological therapies in patients with RA. The baseline data, which included symptoms from a Chinese diagnosis questionnaire, clinical parameters, and demographics data, were collected before the starting of the biological treatment. The combination of two

symptom and three clinical classifiers in CATPCA-FA model was able discriminate responders and non-responders with good accuracy (MR = 24.59%, AUROC = 0.769).

In order to compare the classification abilities of CM symptoms and clinical data, we developed and tested three CATPCA-FC models—one model using the symptoms only, one model using the clinical data only and one model using all the baseline data (the combined model). Although the combined model seems to perform slightly better than the clinical model in PPV, NPV and MR, no statistical differences were found in AUROC. However the AUROC of the combined model is significantly larger than that of the symptom model, suggesting that the combination of CM symptoms and clinical data has higher classification ability compared to the symptoms. Additionally, adding symptoms to the clinical data seemed to slightly improve the classification of non-responders. It is an interesting observation since this indicates that symptoms provide only limited extra information on classifying non-responders, besides the information captured with the clinical data. This might be explained by the complexity of this study cohort. Subjects were selected from a pragmatic-observational study, in other words our subjects were from a highly heterogeneous cohort reflecting real-life situations regarding RA. The subjects did not express clearly distinctive Cold or Heat symptom patterns, therefore the mixed Cold and Heat symptoms in a patient could have limited their classifying value. Besides, the CM symptom patterns reflecting the dynamic health of the entire system might not be suitable to classify the response to a Western symptom treatment such as blocking a single protein. A homogenous cohort of patients with more clear Cold and Heat symptom patterns is needed for the future research, which might provide a more clear view on the role of CM symptoms for classification.

It is the first time that a Cold/Heat symptom-based questionnaire was used to classify response to biological therapy in patients with RA. It is also the first time that CATPCA-FC is used as an exploratory classification method for discriminating the response to biological therapy in RA. The combined model consisted of two key symptom variables ('preference for warm food', 'weak tendon severity') related to EULAR response to biological therapy. Based on CM theory, 'Weak tendon severity' is a typical Heat pattern related symptom and 'preference for warm food' is a Cold pattern related symptom, and these two symptoms could be present in either Cold pattern or Heat pattern RA patients. According to the component loadings (Table 2), 'weak tendon severity' is positively related to EULAR response whereas 'preference for warm food' is negatively related to response. In other words, it seems that RA patients with evident Heat symptom and less evident cold symptom might respond better to biological therapy. However, a previous study reported more apoptosis of CD4⁺ T cell in RA Heat patients compared to RA Cold patients¹⁵. Another study showed the resistance of

synovial and inflammatory cells to apoptosis in RA patients, which is suggested to be the mechanism of RA ³⁴. Therefore, enhancing apoptosis might result in clinical improvement in RA. It is furthermore suggested that the use of anti-TNF- α antibody might induce apoptosis and clinical improvement ^{35,36}. Lu C et al. found that RA Cold patients responded better than RA Heat patients to combination therapy (using nonsteroidal anti-inflammatory drugs (NSAIDs) and DMARDs) ³⁷. Therefore, it is possible that patients with some obvious Heat symptoms may have more active apoptosis in the pathogenesis of RA, and might therefore respond better to biological therapy.

In the present study, we identified three clinical parameter (creatinine, positive RF/anti-CCP, CRP) related to treatment response. Several studies have used different strategies to identify clinical biomarkers that could classify/predict treatment outcome to biological therapies, in which positive RF/anti-CCP is consistently identified as predictive to response to biological therapy ³⁸, as well as a high level of CRP ^{39,40}. In addition, the clinical parameter creatinine is generally used as an indicator of renal function in drug studies ⁴¹. However the association between creatinine and treatment outcome of biological therapies are under-reported, hampering their biological interpretation. Up to now, none of the markers described above can be applied in clinical practice. This might be due to limitations in translating the results to the individual patient. Another reason might be that some (bio)markers are costly to measure and need equipment not readily available in each hospital. In our CM symptom questionnaire, the questions not only covered disease related aspects of rheumatoid arthritis, but also relevant symptoms about patients' reactions to the external environment, such as food and climate. Collecting this type of information in larger patient groups might increase our understanding of patients' responses to biological agents, and especially differences in response between patient subgroups. Furthermore, a CM symptom questionnaire is easy to implement in modern hospitals. This study suggests that a combination of Western medicine with Chinese medicine is a feasible road towards improving treatment strategies for rheumatoid arthritis and possibly other chronic diseases.

Conclusion

This study for the first time includes Chinese medicine symptoms into an analysis of RA patients' response to biological therapy. Adding symptom variables ('preference for warm food', 'weak tendon severity') in addition to clinical measures (creatinine, positive RF/anti-CCP, CRP) does not improve the classification of responders, but it does improve the classification of non-responders. Although this improvement is not significant, we consider that Chinese medicine symptoms are worthwhile to further explore for the differentiation of

non-responders and responders to biological therapies in the area of RA. Since clinical measurements and especially symptom measurements are cheap and easy to perform in clinical practice, these findings provide realistic avenues towards personalized medicine.

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Supplementary information

Tables

Table S1. Currently used treatments of all subjects and split for responders and non-responders.

Treatment	All (n=61)	Non-responders (n=24)	Responders (n=27)	p value
Initiated bDMARDs, n (%)				0.175
Adalimumab	12 (19.7)	6 (25.0)	6 (16.2)	
Etanercept	9 (14.8)	2 (8.3)	7 (18.9)	
Golimumab	9 (14.8)	2 (8.3)	7 (18.9)	
Certolizumab	9 (14.8)	6 (25.0)	3 (8.1)	
Abatacept	7 (11.5)	4 (16.7)	3 (8.1)	
Tocilizumab	8 (13.1)	1 (4.2)	7 (18.9)	
Rituximab	7 (11.5)	3 (12.5)	4 (10.8)	
Concomitant DMARDs, n (%)				
Methotrexate	39 (63.9)	14 (58.3)	25 (67.6)	0.418
Hydroxychloroquine	12 (19.7)	7 (29.2)	5 (13.5)	0.193
Leflunomide	17 (27.9)	10 (41.7)	7 (9.4)	0.082
Glucocorticoids	16 (26.2)	8 (33.3)	8 (21.6)	0.377

P values were obtained from Chi-square tests comparing the proportion of subjects using drugs between responders and non-responders.

bDMARDs: biological disease-modifying antirheumatic drugs.

Table S2. Optimal analysis level of baseline data in the multivariate analyses

Type	Name	Analysis level
Symptoms	Dry mouth-Severity, Dry mouth-Frequency, Cough with phlegm, Amount of phlegm Sticky phlegm, Swollen or distended abdomen, Fullness of abdomen, Loose stools Thirst-Severity, Thirst-Frequency, Chills-Severity, Chills-Frequency, Warm feeling, Aversion to heat, Aversion to cold, Sweating at light exertion-Severity, Sweating at light exertion-Frequency, Preference for cold drinks, Preference for warm drinks, Preference for cold food, Preference for warm food, Heavy feeling of affected parts Numb and cold skin, Stiff joints, Swollen joints, Red joints, Warm joints, Swellings, Weak limbs, Weak tendons, Pain, Pain aggravates with fog, Pain aggravates at night Pain aggravates by cold, Pain with redness and swelling, Pain improves by warmth and movement, Soreness, Stabbing pain, Sharp pain, Deep pain, Heavy pain, Dull pain, Continuous light pain, Shortness of breath, Dry throat, Tender lower abdomen, Diarrhea, Cold feeling, Fever with chills	Nominal
Clinical parameters	C-reactive protein Hemoglobin Creatinine Alanine aminotransferase Plate count Leukocyte count	Spline ordinal
	Positive rheumatoid factor/anti-cyclic citrullinated peptide antibody	Ordinal
Demographics	TNF-alpha inhibitor, Leflunomide, Methotrexate, Hydroxychloroquine, Glucocorticoids, Biological naivety, Gender, Smoke status, Alcohol consumption	Nominal
	Body mass index, Age, Disease duration	Spline ordinal

Table S3. Pairwise comparison of ROC curves

	Combined model ~ Clinical model	Combined model ~ Symptom model	Clinical model ~ Symptom model
Difference between areas	0.0045	0.195	0.19
Standard Error	0.0495	0.0852	0.11
95% Confidence Interval	-0.0926 to 0.102	0.0278 to 0.362	-0.0255 to 0.406
z statistic	0.0909	2.286	1.728
Significance level	0.9276	0.0223	0.0839

Methods

Data pre-processing

Before the analysis, the questionnaire data were screened and recoded. Firstly the consistency of answers to the same symptom were checked, such as the estimation of severity and frequency of the symptom. The inconsistent answers were set to missing. Secondly, to avoid unstable results, the frequencies of the categories of the questionnaire variables were checked. The categories with a frequency less than 8 (the square root of the number of subjects) were merged with an adjacent category²⁵.

In the clinical dataset, the baseline DAS28 scores are calculated from the variables ‘TJC’, ‘SJC’, ‘VAS’ and ‘ESR’. The classification variable ‘Response’ is derived from the patient’s baseline DAS28 and current DAS28 scores, thereby there is a strong correlation among the baseline variables ‘DAS28’, ‘TJC’, ‘SJC’, ‘VAS’, ‘ESR’ and the classification variable ‘Response’. Therefore, these clinical variables were not included in the subsequent analysis

Categorical principle components analysis (CATPCA) procedures

CATPCA can handle both numeric and categorical variables, as well as non-linear relationships among them. All missing values in the dataset were handled by the option passive, which means all available data are used in the analysis without imputation and deleting data (i.e. without deletion of an entire case when missing on one or more variables).

Variable discretization

The clinical and demographic variables with numeric measurement levels were discretized by grouping, which uniformly recoded variable values into 15 categories.

Specifying optimal analysis options

Specifying an optimal analysis level for each variable is one of the most important features of CATPCA. The optimal scaling level, which is also called the optimal analysis level, can be chosen independently of the measurement level of a variable.

We do not have a priori idea of which level is optimal for each variable, so analyses with different levels were performed to determine which one resulted in the best balance between variance account for (VAF) and degrees of freedom (DF). First of all, a numeric scaling level was used for all variables, which is the most restrictive level (i.e., the least number of DF), and boils down to standard (linear) PCA. Then we compared the VAF per variable and the overall VAF of the linear analysis with results of analyses with more freedom in the

transformations: ordinal analysis levels and nominal (i.e., the most number of DF); for variables with many categories (e.g. BMI, age), ordinal or nominal spline levels were used. The level that resulted in relatively high total VAF and relatively low DF was selected as the optimal one for each variable.

Variable selection and model building

The respective contribution of CM symptoms, demographic and clinical variables to the follow-up response was studied by three separate models: a combined model, a symptom model, and a clinical model.

Firstly, for the combined model all variables were analyzed with optimal analysis levels in CATPCA with FC. The variables with loading > 0.30 were selected for the final model. Then, with the same procedure, models including only symptom variables or only clinical data were built separately.

Leave-one-out cross validation

In LOOCV, one subject is left out of the model (by specifying the test case as a supplementary case) as a test case. After running the model on the remaining subjects as the training set, the model is applied to the test case. For this test case, a predicted value was computed from the variables' transformed quantifications and component loadings. In the training model, observed scores for training cases were computed identically. This procedure is repeated N times, each time leaving out another subject and applying the training model to that case. In the meantime, in the training model a mean value (mean 1) of all responders' observed scores and a mean value (mean 2) of all non-responders' observed scores was calculated, and we chose the average of mean 1 and mean 2 as the cutoff value. Depending on this cutoff value and the predicted score, a test case will be predicted as a responder or a non-responder.

Chapter 5

Differences between serum lipid profiles of male and female rheumatoid arthritis patients in response to glucocorticoid treatment

5

Based on

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Differences between serum lipid profiles of male and female rheumatoid arthritis patients in response to glucocorticoid treatment

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Abstract

Objective As the pharmacological differences between males and females, and glucocorticoid (GC) treatment is associated with increased cardiovascular mortality rate in rheumatoid arthritis (RA) patients, it is important to study serum polar lipid profiles of male and female patients in response to GC therapy. Gender differences may require an adjustment to the treatment strategy for a selection of patients.

Methods Serum samples from 281 RA patients were analysed using a targeted lipidomics platform. The differences in GC use and gender on polar lipid profiles were cross-sectionally examined by multiple linear regression, while correcting for confounding factors.

Results Differences in polar lipids between GC-users and non-GC-users in females and males were merely restricted to lysophospholipids (lysophosphatidylcholines and lysophosphatidylethanolamines). Lysophospholipids in female patients treated with GCs were significantly higher than female patients not treated with GCs ($p = 6.0 \times 10^{-6}$), whereas no significant difference was observed in male GC users *versus* non-users ($p = 0.397$).

Conclusion The lysophospholipid profiles in response to GCs were significantly different between male and female RA patients, which may have implications for the cardiovascular risk of GC treatment.

Introduction

Rheumatoid arthritis (RA) is an auto-immune disease with unresolved aetiology which predominantly occurs in females ¹. Glucocorticoids (GCs) have been prescribed for the treatment of RA for decades, and are considered to be effective drugs in reducing inflammation and preventing joint destruction ². The role of gender as a crucial factor in drug studies is becoming increasingly appreciated ³. Several studies have investigated the effects of gender on clinical pharmacology for GCs on healthy volunteers, and showed gender specific differences in GC pharmacokinetics and pharmacodynamics ^{4,5}. However, these differences are balanced by complementary GC clearance and GC sensitivity. Therefore, these gender differences do not necessitate GC dose adjustments in clinical practice.

Glucocorticoids are known to undesirably affect triacylglyceride and fatty acid metabolism ⁶. It is thus conceivable that GC-induced changes of lipid profiles in RA patients also show gender dependence, apart from the gender differences in lipid metabolism seen in the general population ⁷. Because GC treatment is associated with increased cardiovascular mortality rate in RA patients ^{8,9}, it is important to study these gender-based lipid differences as they may require an adjustment in treatment strategy for a selection of the patients.

In the present study, we measured circulating polar lipids, such as lysophospholipids and free fatty acids, in the serum of RA patients using a targeted lipidomics platform, because polar lipids are crucial intermediates in lipid metabolism. Lipid profiles between GC users and non-GC users were examined and analysed for gender differences. Our results suggested that the lipid profile is more affected by GC treatment in female RA patients. In particular the levels of lysophospholipids were more elevated in female users compared to non-users. In males the differences of lysophospholipid levels in GC users were not significant compared to non-users.

Material and methods

Subjects in this study were participants of an observational study BiOCURA, in which RA patients eligible for biological therapy were recruited ¹⁰. Blood samples were collected before initiating the biological therapy and immediately processed into serum. Serum samples were stored at -80°C until used for lipidomic analyses. At the time of sampling, 42.35 % of patients were receiving GCs, including prednisolone and prednisone, at varying dosages (all medications are listed in Supplementary Table S1). The study was approved by the medical ethics committee of the University Medical Center Utrecht and the institutional review boards of the participating centres (see Acknowledgements). Written informed consent was obtained from each patient.

The operating procedures of the targeted lipidomics platform are optimised from the previously published method ¹¹. Polar lipids are extracted by methanol from serum samples, and analysed by liquid chromatography-mass spectrometry, covering the low abundance lipid species, including free fatty acids and lysophospholipids—lysophosphatidylcholines (LPCs) and lysophosphatidyl-ethanolamines (LPEs). Details of the procedures are described in the Supplementary Method.

A schematic overview of the statistical analyses is provided in Fig. 1. Initially, the obtained lipid data set was log transformed and standardised into Z-scores to produce normalised and auto-scaled data (mean = 0, SD = 1). Then, the differences in lipids between GC users and GC non-users were calculated and tested for significance by independent t-tests for male and female subjects separately. In parallel, principal component analysis (PCA) was performed on all detected lipids to elucidate the correlation structure of the metabolites. By combining the results of the t-tests, PCA, and prior biological knowledge, a decision was made on which lipids can be clustered into a new lipid score in order to have one overall outcome for subsequent analyses. For each patient, the score was computed by summing the standardized values of lipids and dividing this by the number of included lipids ($\sum_i^N \text{Standardised lipid } i / N$, with N = number of lipids clustered). This value represents both the average of lipids, as well as the patients' relative deviation from the mean lipid score in standard deviations (SDs). Multiple linear regression analysis was then conducted to study the effect of GC use between males and females on the lipid score. We entered the following subgroups in the model—female GC user (n = 77), female GC non-user (n = 136), male GC user (n = 42), and male GC non-user (n = 26) together with the clinical parameters (listed in Supplementary Table S1) as a full model, while setting the subgroup “Female GC non-user” as the reference group (i.e. the intercept of the regression model). To arrive at a final model, backward elimination was applied on the full model by excluding clinical parameters one by one on p-values (starting from highest to lowest p-value). Parameters were excluded only when the change in the regression coefficients after exclusion was < 10% for all four subgroups; otherwise the clinical parameter was kept in the model as a confounder. In order to explore the difference in lipid score between GC users and non-users in males, the reference group in the final model was switched to “male GC non-users”.

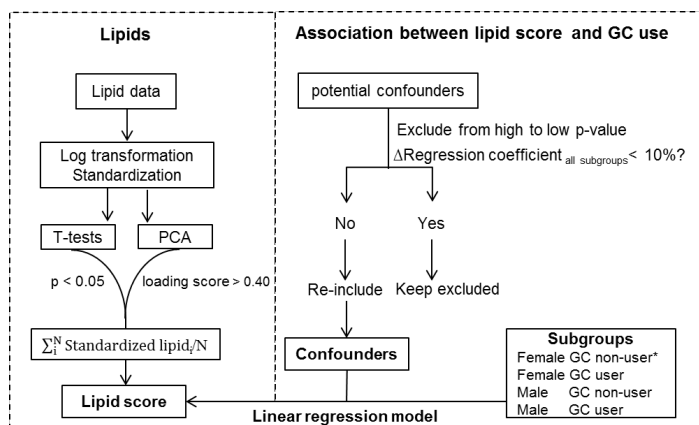


Figure 1. A schematic overview of the statistical analyses.

* the subgroup “Female-glucocorticoid non-user” is set as the reference group in the linear regression analysis.

Results and discussion

In the patient cohort (n = 281), there were more males taking GCs than females (61.8% vs. 35.8%, $p < 0.01$). There were no significant differences in disease activity among the relevant groups (Supplementary Table S1). However, the number of smokers was especially high in males ($p < 0.001$), positive rheumatoid factor was high amongst male GC users ($p = 0.004$), GC users used less non-steroidal anti-inflammatory drugs (NSAIDs) ($p = 0.010$) and more bisphosphonates (to reduce the risk of GC-induced osteoporosis) ($p < 0.001$)

By applying an established lipidomics platform, serum lipid profiles of the 281 RA patients were analysed, covering 44 lysophospholipids and 24 free fatty acids (Supplementary Table S2). T-tests between GC users and non-users were performed (Supplementary Table S3; a graphical representation is shown in Fig. 2). In females, we identified 10 LPEs and 22 LPCs which were significantly higher in GC users than in GC non-users ($p < 0.05$), whereas no differences were found in fatty acids. However, in male subjects only one LPE (LPE (20:5), $p = 0.029$) and LPC (*sn1*-LPC (18:2), $p = 0.027$), which were significant in females, were found significantly higher in GC users whereas one fatty acid (FA (20:3- ω 9), $p = 0.021$) was significant lower in male GC users among all measured FAs. These results suggest that the GCs have a more pronounced impact on female lysophospholipid profile compared to males.

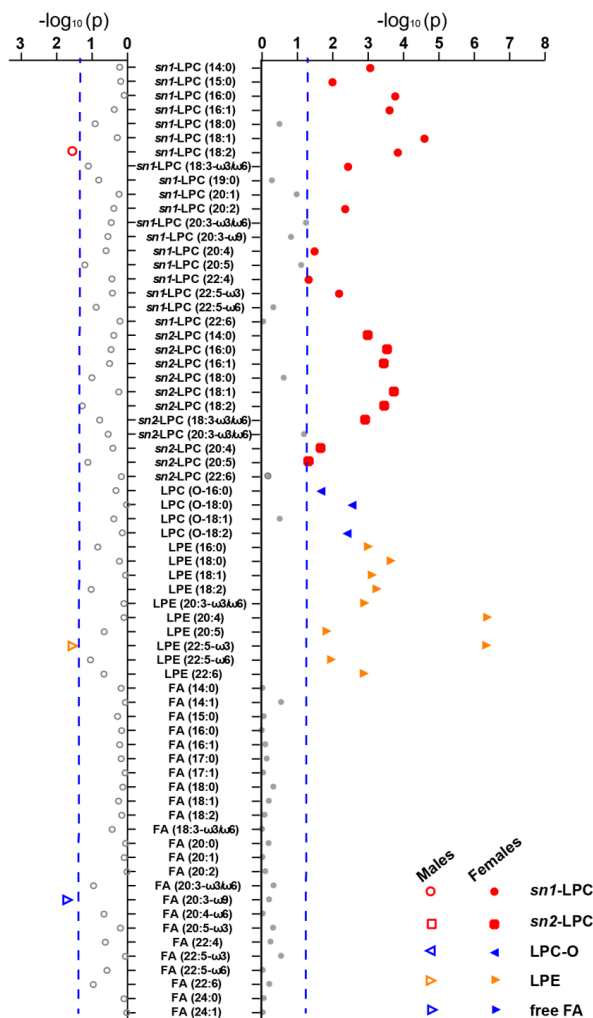


Figure 2 Graphical representation of t-tests results in lipid clustering. Independent sample t-tests were performed on all 68 metabolites on glucocorticoid (GC) users versus GC non-users, for both genders separately. The x-axis shows the logarithm of p-values per metabolite for males and females, the blue dash line represents p-value of 0.05.

The parallel PCA analysis on 68 metabolites showed that the loading scores of all 32 significant lysophospholipids were larger than 0.4 in the first component, thus highly correlated with each other (Supplementary Fig. S1, Table S4). A new score representing the lysophospholipid levels could therefore be computed by calculation of the mean of all significant lysophospholipids. As shown in Fig. 3, the absolute lysophospholipid scores were significantly different between female GC users and non-users (<0.001), whereas no

difference was seen in males ($p=0.450$). In addition, female GC users showed significantly higher values compared to male GC users ($p=0.041$), whereas no difference was seen between female and male non-users ($p=0.548$).

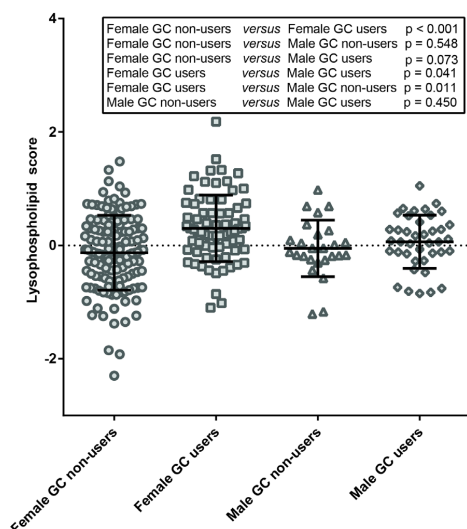


Figure 3. Lysophospholipid score in male patients without ($n = 26$) or with glucocorticoid (GC) treatment ($n = 42$), and in female patients without ($n = 136$) or with GC treatment ($n = 77$). Horizontal bars indicate mean values and standard deviation. One-way ANOVA with Fisher's LSD was used to compare the means of score among subgroups.

After backward elimination for potential confounders, a final model for the lysophospholipid score was established (Table 1). Subgroups of males and female GC users all had significant positive coefficients, which suggest that the lysophospholipid score of these subgroups was significantly higher compared to females not using GCs. In particular, females using GCs had a significant increase in mean lysophospholipid score of 0.398 ($p = 6E-6$), compared to females not using GCs. Interestingly, the difference between male and female non-users was not significant before correcting for confounders ($p = 0.548$, Fig. 3), while it was significant in the final model (coefficient = 0.310, $p = 0.041$). The difference between GC users and non-users in males was non-significant (0.122, $p = 0.397$; model with "male GC non-users" as a reference shown in Supplementary Table S5). Therefore, after correcting for confounders, the lysophospholipids score of female RA patients taking GCs was still significantly higher than the female patients not taking GCs, whereas in male RA patients no difference in lysophospholipid score was seen. This was consistent with the results of the t-tests on individual lipids and the uncorrected difference in lysophospholipid score.

Table 1. Final regression model investigating the association between gender and glucocorticoid (GC) use on the lysophospholipid score corrected for confounders. Shown is the difference in mean lysophospholipid score for subgroups compared to females not using GCs (as reference group).

Variables	Coefficients ^a (95%-CI)	p value
Female GC non-user (reference group)	-0.170 (-0.690 to 0.349)	0.519
Female GC user	0.398 (0.229 to 0.567)	6.0 E-6
Male GC non-user	0.304 (0.039 to 0.570)	0.025
Male GC user	0.427 (0.192 to 0.662)	4.1 E-4

^a Coefficients indicate the changes in mean lysophospholipid score, adjusting for: BMI, menopausal status, RF positive, log-transformed CRP, log transformed ESR, DAS28, and concomitant drugs (methotrexate, hydroxychloroquine, anti-diabetic drug and NSAIDs).

Patients with RA already have a higher cardiovascular disease risk and this elevated risk is only partly explained by the increased prevalence of traditional cardiovascular risk factors such as age, gender, dyslipidaemia, hypertension, smoking, obesity and diabetes mellitus ¹². In addition, systemic inflammation and genetic factors also play a role ¹². More recently, GC use has been directly related to an (dose dependent) increase in cardiovascular death in RA ⁹. However in this study, no effect on lipid profiles by different dosages was seen, as the factor low (< 7.5mg) *versus* moderate to high (>7.5mg) dosage was excluded during confounder selection. A possible protective effect can be expected from concomitant use of hydroxychloroquine, which significantly lowered lysophospholipid scores in our study (decrease in mean lysophospholipid score = 0.180, 95% CI (-0.347 to -0.013), p = 0.035; Table S5). It has also been reported to improve cholesterol levels, notably in those treated with GCs ¹³.

Lysophospholipids, including lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), are an abundant lipid species, mainly functioning as transporters for free fatty acids. The difference between LPC and LPE is based only on the functional head group—respectively choline or ethanolamine. The functions of LPEs are underreported, hampering their biological interpretation. Studies show that LPCs have properties resembling extracellular growth factors and signalling molecules ¹⁴. *In vivo*, LPCs are generated from phospholipase A1/A2 catalysed hydrolysis of phosphatidylcholines, the basic component of membranes ¹⁵. In addition, LPCs are released from phosphatidylcholines by the action of lecithin cholesterol acyltransferase in plasma ¹⁶. Most of the circulating LPC is bound to albumin, but they are also a major component of lipoprotein particles, where they are a known constituent of oxidised low-density-lipoproteins (LDL) ^{17,18}, a well-known risk factor for cardiovascular diseases ¹⁹. The lysophospholipid related gender differences are therefore

potentially relevant with respect to the risk of cardiovascular events in RA patients, which could eventually guide the adjustment of treatment strategies for either males or females.

As patients were included in BiOCURA based on the necessity of biological treatment and not GC treatment, it was only possible to use samples of users and non-users, but not before and after GC initiation. Future studies are needed to validate our results, preferably before and after initiation of GC treatment. Additionally the role of lipid profiles (including triglycerides, diglycerides, and sphingomyelins) in the association between GC use, gender and cardiovascular death, should be clarified in order to fully understand and (specifically) prevent unwanted clinical (side-)effects.

Conclusion

After correcting for confounding factors, lysophosphatidylcholines and lysophosphatidylethanolamines in female RA patients with GC treatment were significantly higher than in female patients not taking GCs, whereas in male RA patients these lysophospholipids levels were similar between GC users and non-users. These results could contribute to a better understanding and estimation of safety of GC drugs for male and female RA patients separately, particularly in relation to cardiovascular events.

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Supplementary information

Method—lipidomics profiling

For the detection of polar lipids, each 20 μL serum was spiked with internal standard (ISTD) mix and lipids were extracted by 440 μL methanol. After centrifugation, the supernatant was transferred to an Eppendorf tube and the solvent was evaporated using a Speedvac. The dried lipids were reconstituted in 145 μL isopropanol, 0.1% formic acid and transferred to autosampler vials. In total 8 μL was injected into ultra-performance liquid chromatography coupled to electrospray ionization-quadrupole time-of-flight (Agilent 6530 San Jose, A, USA) with an ACQUITY UPLCTM HSS T3 column (1.8 μm , 2.1 $\times$ 100mm). Lipids were detected in full scan in the negative mode.

Figure

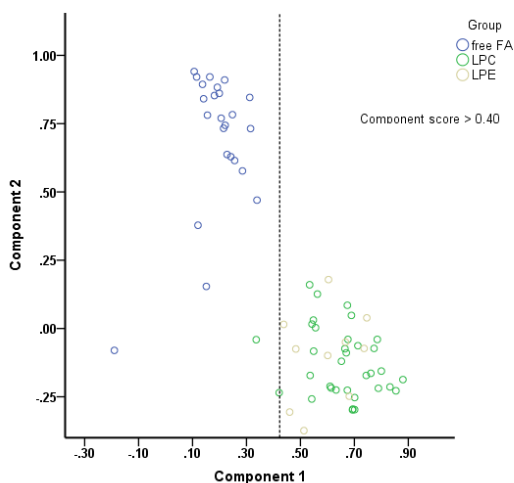


Figure S1. Graphical representation of loading scores of 68 lipids in component 1 and 2 by PCA analysis. All the lysophospholipids (green and yellow circles) are with loading scores > 0.40 in component 1 (on the right side of the dash line). FA: fatty acid; LPC: lysophosphatidylcholines; LPE: lysophosphatidylethanolamines

Tables

Table S1. Baseline characteristics of patients in the cohort (n = 281), split for glucocorticoid non-users and users, females and males.

Item	Females		Males		p-value
	glucocorticoid non-users (n = 136)	glucocorticoid users (n = 77)	glucocorticoid non-users (n = 26)	glucocorticoid users (n = 42)	
Age, years, mean $\pm$ SD ^a	53.2 $\pm$ 12.8	53.8 $\pm$ 14.1	54.2 $\pm$ 12.2	57.0 $\pm$ 11.7	0.428
Body mass index, kg/m ² , mean $\pm$ SD ^a	27.2 $\pm$ 5.8	27.0 $\pm$ 5.2	26.7 $\pm$ 3.4	25.0 $\pm$ 3.2	0.115
Current smoker, n (%) ^b	28 (20.6)	11 (14.3)	12 (46.2)	19 (45.2)	<0.001
Positive RF, n (%) ^b	91 (66.9)	38 (49.4)	15 (57.7)	34 (81.0)	0.004
Positive ACPA, n (%) ^b	90 (66.2)	49 (63.6)	17 (65.4)	31 (73.8)	0.726
CRP, mg/dL, median (IQR) ^c	6.0 (2.7-15.0)	5.0 (3.0-16.5)	5.5 (2.3-12.0)	10 (2.3-35.3)	0.487
Use of other DMARDs, n (%) ^b					
Methotrexate, n (%) ^b	99 (72.8)	48 (62.3)	22 (84.6)	32 (76.2)	0.115
Leflunomide, n (%) ^b	17 (12.5)	9 (11.7)	2 (7.7)	7 (16.7)	0.737
Hydroxychloroquine, n (%) ^b	21 (15.4)	22 (28.6)	9 (34.6)	10 (23.8)	0.050
Use of non-DMARDs					
NSAIDs, n (%) ^b	94 (69.1)	39 (50.6)	20 (76.9)	22 (52.4)	0.010
Anticoncepcion, n (%) ^b	10 (7.4)	5 (6.5)	0 (0.0)	0 (0.0)	0.162
Antidiabetics, n (%) ^b	5 (3.7)	4 (5.2)	1 (3.8)	1 (2.4)	0.892
Antihyperlipedemia, n (%) ^b	18 (13.2)	13 (16.9)	6 (23.1)	1 (2.4)	0.065
Bisphosphonates, n (%) ^b	34 (25.0)	58 (75.3)	3 (11.5)	30 (71.4)	<0.001
DAS28, mean $\pm$ SD ^a	4.6 $\pm$ 1.2	4.6 $\pm$ 1.2	3.9 $\pm$ 1.1	4.4 $\pm$ 1.4	0.071
TJC, median (IQR) ^c	8.0 (2.3-12.0)	7.0 (2.0-15.0)	4.0 (1.0-6.3)	6.0 (0.0-14.0)	0.069
SJC, median (IQR) ^c	2.0 (0.0-4.0)	2.0 (0.0-4.0)	1.0 (0.0-3.0)	1.0 (0.0-4.3)	0.885
VAS-GH, mean $\pm$ SD ^a	58.6 $\pm$ 24.2	56.4 $\pm$ 22.7	49.2 $\pm$ 25.1	57.1 $\pm$ 23.3	0.324
ESR, mm/h, median (IQR) ^c	19.0 (11.0-37.0)	22.0 (6.5-39.0)	15.5 (6.0-34.3)	19.0 (9.5-47.5)	0.786

P-values for comparisons among four subgroups were calculated by one-way ANOVA ^a, Chi-square tests ^b, and Kruskal wallis H tests ^c) based on distribution of the clinical parameter. Bold values indicate significant p-values (p < 0.05).

ACPA: anti citrullinated protein antibody, RP: c-reactive protein, DAS28: disease activity score based on a 28-joint count, DMARDs: disease-modifying antirheumatic drugs, ESR: erythrocyte sedimentation rate, IQR: interquartile range, NASID: non-steroid anti-inflammatory drug, RF: rheumatoid factor, SD: standard deviation, SJC: swollen joint count, TJC: tender joint count, VAS-GH: visual analogue scale-general health.

Table S2. List of detected metabolites in lipidomics profiling

Lipid Class	Lipid Maps	Metabolite species	Amount (n)
Free fatty acids (FA)	FA01	C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3- ω 3/ ω 6, C20:0, C20:1, C20:2, C20:3- ω 3/ ω 6, C20:3- ω 9, C20:4- ω 6, C20:5- ω 3, C22:4, C22:5- ω 3, C22:5- ω 6, C22:6, C24:0, C24:1	24
Lysophosphatidylcholine (LPC)	GP0105	<i>sn1</i> : C14:0, C15:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3- ω 3/ ω 6, C19:0, C20:1, C20:2, C20:3- ω 3/ ω 6, C20:3- ω 9, C20:4, C20:5, C22:4, C22:5- ω 3, C22:5- ω 6, C22:6 <i>sn2</i> : C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3- ω 3/ ω 6, C20:3- ω 3/ ω 6, C20:4, C20:5, C22:6	30
Lysophosphatidylethanolamine (LPE)	GP0205	C16:0, C18:0, C18:1, C18:2, C20:3- ω 3/ ω 6, C20:4, C20:5, C22:5- ω 3, C22:5- ω 6, C22:6	10
Plasmalogen (LPC-O) ^a	Lysophosphatidylcholine GP0106	C16:0; C18:0; C18:1; C18:2	4

The "*sn1*-" or "*sn2*-" prefix is used to indicate the position of the fatty acid chain esterified to the glycerol backbone of a lysophosphatidylcholine.

^a The 'LPC-O' is used to identify a plasmalogen lysophosphatidylcholine, where the fatty acid chain is attached via a vinyl ether linkage to the glycerol backbone. The ' ω 3', ' ω 6', ' ω 9' are used to indicate double bond (C = C) at the third, sixth or ninth carbon atom from the end of the fatty acid chain.

Table S3. Differences in 68 serum lipid levels between glucocorticoid (GC) users and non-users in males and females, respectively. The mean differences between GC non-users and users are shown. *P*-values were calculated by means of an independent sample t-test.

Metabolite name	Males (GC user vs. GC non-user)		Females (GC user vs. GC non-user)	
	Mean _{non-user} -Mean _{user}	p value	Mean _{non-user} -Mean _{user}	p value
<i>sn1</i> -LPC (14:0)	-0.106	0.602	-0.444	0.001
<i>sn1</i> -LPC (15:0)	-0.116	0.633	-0.346	0.010
<i>sn1</i> -LPC (16:0)	0.027	0.796	-0.301	< 0.001
<i>sn1</i> -LPC (16:1)	-0.181	0.422	-0.507	< 0.001
<i>sn1</i> -LPC (18:0)	0.289	0.122	-0.148	0.304
<i>sn1</i> -LPC (18:1)	-0.091	0.512	-0.368	< 0.001
<i>sn1</i> -LPC (18:2)	-0.283	0.027	-0.332	< 0.001
<i>sn1</i> -LPC (18:3- ω 3/ ω 6)	-0.343	0.077	-0.393	0.004
<i>sn1</i> -LPC (19:0)	0.252	0.153	0.091	0.494
<i>sn1</i> -LPC (20:1)	0.120	0.568	-0.207	0.099
<i>sn1</i> -LPC (20:2)	-0.153	0.408	-0.395	0.004
<i>sn1</i> -LPC (20:3- ω 3/ ω 6)	0.152	0.342	-0.226	0.054
<i>sn1</i> -LPC (20:3- ω 9)	0.284	0.276	-0.198	0.144
<i>sn1</i> -LPC (20:4)	0.183	0.244	-0.2	0.032
<i>sn1</i> -LPC (20:5)	-0.417	0.062	-0.237	0.074
<i>sn1</i> -LPC (22:4)	0.228	0.359	-0.283	0.047
<i>sn1</i> -LPC (22:5- ω 3)	-0.161	0.369	-0.373	0.007
<i>sn1</i> -LPC (22:5- ω 6)	0.357	0.128	-0.099	0.455
<i>sn1</i> -LPC (22:6)	0.107	0.610	-0.021	0.882
<i>sn2</i> -LPC (14:0)	-0.157	0.405	-0.427	0.001
<i>sn2</i> -LPC (16:0)	0.178	0.341	-0.501	< 0.001
<i>sn2</i> -LPC (16:1)	-0.237	0.308	-0.487	< 0.001
<i>sn2</i> -LPC (18:0)	0.211	0.098	-0.115	0.231
<i>sn2</i> -LPC (18:1)	-0.114	0.559	-0.443	< 0.001
<i>sn2</i> -LPC (18:2)	-0.395	0.052	-0.533	< 0.001
<i>sn2</i> -LPC (18:3- ω 3/ ω 6)	-0.362	0.161	-0.411	0.001
<i>sn2</i> -LPC (20:3- ω 3/ ω 6)	0.226	0.281	-0.263	0.062
<i>sn2</i> -LPC (20:4)	0.215	0.384	-0.331	0.021
<i>sn2</i> -LPC (20:5)	-0.421	0.075	-0.278	0.046
<i>sn2</i> -LPC (22:6)	0.092	0.666	-0.07	0.616
LPC (O-16:0)	0.157	0.466	-0.343	0.020
LPC (O-18:0)	-0.019	0.928	-0.419	0.003
LPC (O-18:1)	0.190	0.410	-0.152	0.299
LPC (O-18:2)	0.080	0.707	-0.398	0.004
LPE (16:0)	-0.279	0.143	-0.505	0.001
LPE (18:0)	-0.102	0.588	-0.587	< 0.001

LPE (18:1)	0.031	0.883	-0.449	0.001
LPE (18:2)	-0.349	0.093	-0.497	< 0.001
LPE (20:3-ω3/ω6)	-0.051	0.789	-0.469	0.001
LPE (20:4)	-0.062	0.785	-0.685	< 0.001
LPE (20:5)	-0.463	0.029	-0.352	0.012
LPE (22:5-ω3)	-0.328	0.089	-0.694	< 0.001
LPE (22:5-ω6)	0.294	0.215	-0.356	0.009
LPE (22:6)	-0.242	0.218	-0.512	0.001
FA (14:0)	0.089	0.654	-0.014	0.924
FA (14:1)	0.038	0.865	0.159	0.275
FA (15:0)	0.129	0.517	-0.027	0.855
FA (16:0)	-0.083	0.680	-0.006	0.966
FA (16:1)	0.113	0.600	0.042	0.770
FA (17:0)	-0.097	0.652	-0.057	0.692
FA (17:1)	0.041	0.852	0.023	0.872
FA (18:0)	-0.072	0.725	0.109	0.456
FA (18:1)	0.134	0.551	0.075	0.609
FA (18:2)	0.089	0.689	0.035	0.805
FA (18:3-ω3/ω6)	0.190	0.364	0.009	0.950
FA (20:0)	0.039	0.878	-0.071	0.620
FA (20:1)	0.054	0.803	-0.013	0.925
FA (20:2)	0.011	0.969	-0.042	0.761
FA (20:3-ω3/ω6)	0.377	0.109	-0.107	0.445
FA (20:3-ω9)	0.500	0.021	-0.074	0.595
FA (20:4-ω6)	0.250	0.212	-0.013	0.905
FA (20:5-ω3)	-0.105	0.628	-0.098	0.463
FA (22:4)	0.237	0.234	-0.077	0.543
FA (22:5-ω3)	-0.032	0.866	-0.143	0.271
FA (22:5-ω6)	0.247	0.261	0.017	0.901
FA (22:6)	0.371	0.107	0.072	0.585
FA (24:0)	0.071	0.783	0.028	0.838
FA (24:1)	-0.013	0.951	0.016	0.901

Table S4. Loading scores of 68 lipids in principle components analysis. Component loading scores of all lipids were presented for the first five components (with eigenvalue >1). Since the LPCs and LPEs showed high component loadings in one component (the first) and only with positive values, they all highly correlate in the same direction. Considering all LPCs and LPEs significantly different between glucocorticoid users and non-users, all loading scores were ≥ 0.438 .

Metabolite name	Component				
	1	2	3	4	5
<i>sn1</i> -LPC (14:0)*	0.701	-0.253	0.057	0.147	-0.357
<i>sn1</i> -LPC (15:0)*	0.610	-0.212	-0.159	0.053	-0.066
<i>sn1</i> -LPC (16:0)*	0.880	-0.187	-0.077	-0.080	-0.009
<i>sn1</i> -LPC (16:1)*	0.773	-0.073	0.152	0.044	-0.319
<i>sn1</i> -LPC (18:0)*	0.744	-0.172	-0.249	-0.082	0.295
<i>sn1</i> -LPC (18:1)*	0.854	-0.228	0.023	0.131	0.135
<i>sn1</i> -LPC (18:2)*	0.701	-0.297	-0.036	0.181	0.429
<i>sn1</i> -LPC (18:3- ω 3/ ω 6)*	0.694	-0.295	0.047	0.344	-0.100
<i>sn1</i> -LPC (19:0)	0.421	-0.235	-0.315	-0.029	0.291
<i>sn1</i> -LPC (20:1)	0.336	-0.041	-0.252	-0.132	0.203
<i>sn1</i> -LPC (20:2)*	0.800	-0.156	0.126	-0.108	0.108
<i>sn1</i> -LPC (20:3- ω 3/ ω 6)	0.665	-0.074	0.404	-0.312	-0.133
<i>sn1</i> -LPC (20:3- ω 9)	0.652	-0.120	0.345	-0.165	-0.213
<i>sn1</i> -LPC (20:4)*	0.674	0.085	0.154	-0.492	0.032
<i>sn1</i> -LPC (20:5)	0.543	0.016	-0.668	0.069	-0.296
<i>sn1</i> -LPC (22:4)*	0.676	-0.040	0.539	-0.326	0.026
<i>sn1</i> -LPC (22:5- ω 3)*	0.785	-0.040	-0.219	-0.022	0.014
<i>sn1</i> -LPC (22:5- ω 6)	0.556	0.003	0.569	-0.405	-0.068
<i>sn1</i> -LPC (22:6)	0.534	0.160	-0.574	-0.254	-0.084
<i>sn2</i> -LPC (14:0)*	0.674	-0.226	0.057	0.173	-0.343
<i>sn2</i> -LPC (16:0)*	0.832	-0.214	-0.050	-0.057	-0.051
<i>sn2</i> -LPC (16:1)*	0.713	-0.063	0.183	0.026	-0.314
<i>sn2</i> -LPC (18:0)	0.761	-0.164	-0.235	-0.079	0.283
<i>sn2</i> -LPC (18:1)*	0.789	-0.219	0.037	0.112	0.121
<i>sn2</i> -LPC (18:2)*	0.693	-0.298	-0.018	0.160	0.439
<i>sn2</i> -LPC (18:3- ω 3/ ω 6) *	0.614	-0.218	0.049	0.322	-0.104
<i>sn2</i> -LPC (20:3- ω 3/ ω 6)	0.670	-0.089	0.385	-0.302	-0.079
<i>sn2</i> -LPC (20:4)*	0.689	0.048	0.175	-0.463	0.024
<i>sn2</i> -LPC (20:5)*	0.548	0.031	-0.643	0.089	-0.315
<i>sn2</i> -LPC (22:6)	0.563	0.126	-0.551	-0.251	-0.070
LPC (O-16:0)*	0.632	-0.225	-0.219	-0.301	0.408
LPC (O-18:0)*	0.542	-0.258	-0.252	-0.242	0.411
LPC (O-18:1)*	0.536	-0.172	-0.241	-0.290	0.544
LPC (O-18:2)*	0.549	-0.083	-0.283	-0.209	0.406
LPE (16:0)*	0.681	-0.248	0.066	0.176	-0.278

LPE (18:0)*	0.746	0.039	0.082	0.184	-0.072
LPE (18:1)*	0.460	-0.306	0.210	0.558	0.173
LPE (18:2)*	0.513	-0.374	0.219	0.588	0.217
LPE (20:3- ω 3/ ω 6)*	0.601	-0.099	0.512	0.237	-0.167
LPE (20:4)*	0.667	-0.050	0.375	0.126	0.044
LPE (20:5)*	0.483	-0.075	-0.496	0.458	-0.328
LPE (22:5- ω 3)*	0.736	-0.073	0.161	0.340	-0.156
LPE (22:5- ω 6)*	0.438	0.015	0.672	-0.080	-0.177
LPE (22:6)*	0.604	0.179	-0.323	0.128	-0.235
FA (14:0)	0.192	0.883	0.066	0.221	-0.006
FA (14:1)	0.141	0.841	0.023	0.089	-0.061
FA (15:0)	0.215	0.733	0.102	0.210	0.065
FA (16:0)	0.206	0.770	0.190	0.294	0.183
FA (16:1)	0.164	0.921	0.067	0.020	0.000
FA (17:0)	0.221	0.744	0.069	0.239	0.151
FA (17:1)	0.219	0.910	0.031	0.068	0.043
FA (18:0)	0.120	0.378	0.218	0.392	0.259
FA (18:1)	0.106	0.941	0.057	0.073	0.130
FA (18:2)	0.137	0.894	0.012	0.087	0.206
FA (18:3- ω 3/ ω 6)	0.181	0.853	-0.044	0.183	0.119
FA (20:0)	0.151	0.154	0.105	0.336	0.209
FA (20:1)	0.115	0.921	-0.029	0.071	0.160
FA (20:2)	0.155	0.781	0.008	-0.129	0.112
FA (20:3- ω 3/ ω 6)	0.315	0.732	0.216	-0.184	-0.111
FA (20:3- ω 9)*	0.285	0.577	0.152	-0.208	-0.124
FA (20:4- ω 6)	0.256	0.615	-0.019	-0.349	0.074
FA (20:5- ω 3)	0.339	0.470	-0.686	0.085	-0.244
FA (22:4)	0.199	0.861	0.266	-0.164	-0.002
FA (22:5- ω 3)	0.312	0.846	-0.275	0.076	-0.065
FA (22:5- ω 6)	0.248	0.783	0.271	-0.237	-0.142
FA (22:6)	0.242	0.629	-0.494	-0.076	-0.163
FA (24:0)	-0.190	-0.080	0.319	0.392	0.553
FA (24:1)	0.228	0.637	-0.163	0.125	0.118

* Metabolites were also significantly different in independent t-tests between glucocorticoid users and non-users, in either females or males (Figure 2, Table S3).

Table S5. The final regression model investigating the association between gender and glucocorticoid (GC) use on the lysophospholipid score.

Variables	Coefficients ^a (95%-confidence interval)	p value
Male GC non-user (reference group)	-0.134 (-0.395 to 0.663)	0.618
Male GC user	0.122 (-0.161 to 0.406)	0.396
Female GC non-user	-0.304 (-0.570 to -0.039)	0.025
Female GC user	0.093 (-0.185 to 0.372)	0.510
Menopausal status	0.280 (0.106 to 0.453)	0.002
BMI (kg/m ²)	0.006 (-0.009 to 0.020)	0.448
RF positivity	-0.248 (-0.433 to -0.063)	0.009
ACPA positivity	0.182 (-0.008 to 0.372)	0.061
CRP (log transformed, mg/dL)	-0.122 (-0.192 to -0.051)	0.001
ESR (log transformed, mm/h)	0.058 (-0.031 to 0.147)	0.202
DAS28	-0.015 (-0.078 to 0.049)	0.654
Concomitant methotrexate	-0.023 (-0.180 to 0.135)	0.776
Concomitant hydroxychloroquin	-0.180 (-0.347 to -0.013)	0.035
Antidiabetics	-0.133 (-0.494 to 0.229)	0.470
NSAIDs	-0.068 (-0.215 to 0.079)	0.360

^a Coefficients indicate the changes in mean lysophospholipid score after correcting for confounders—BMI, menopausal status, RF positive, log-transformed CRP, log transformed ESR, DAS28, and concomitant drugs (methotrexate, hydroxychloroquine, anti-diabetic drug and NSAIDs).

BMI: body mass index; CRP: C-reactive protein; GC: glucocorticoid; RF: rheumatoid factor; ACPA: anti citrullinated protein antibody; ESR: erythrocyte sedimentation rate; DAS28: disease activity score based on a 28-joint count; NSAIDs: non-steroid anti-inflammatory drugs

Chapter 6

Systems response profiles to two *Rehmanniae Radix* formulae in metabolic syndrome patients

Based on

Junzeng Fu, Herman A. van Wietmarschen, Jan van der Greef, Yan Schroën, and Mei Wang
*Systems response profiles to two *Rehmanniae Radix* formulae in metabolic syndrome patients*
World Journal of Traditional Chinese Medicine, in press

Abstract

Objective To explore the effects of two *Rehmanniae Radix* formulae in patients with metabolic syndrome (MetS), a randomized controlled study was conducted.

Methods MetS patients were randomly assigned to receive either a classic *Rehmanniae Six Formula* (R6, or ‘Liu Wei Di Huang Wan’) or a novel multi-herbal *Rehmanniae Radix* containing formula SUB889 for 8 weeks. Western medicine related clinical parameters, Chinese medicine defined symptoms and syndromes as well as metabolomic profiles were evaluated at different time points.

Results R6 (n = 20) and SUB889 (n = 20) showed similar effects on MetS regarding the improvement of clinical parameters (waist circumference, body mass index, LDL-cholesterol, systolic blood pressure) and Qi/Yin deficiency ($p < 0.05$). Decreased levels of cholesteryl esters, phosphatidylcholines, triglycerides and sphingomyelins were found in the R6 group, while SUB889 formula resulted in increased levels of tricarboxylic acid cycle and glucose metabolism intermediates (malate, fumarate and pyruvate).

Conclusions R6 and SUB889 have similar effects on the treatment of MetS by improving Chinese medicine and Western medicine defined clinical outcomes. R6 is more effective in improving lipid profiles compared to SUB889. The exact mechanisms of the two formulae on MetS remain to be elucidated.

Introduction

Metabolic syndrome (MetS) is defined as a combination of central obesity, hyperglycemia, dyslipidemia and hypertension ¹. It is widely accepted that MetS is associated with an increased risk of cardiovascular disease and diabetes ². MetS, as defined by Western diagnostics, has no corresponding syndrome described in Chinese medicine ^{3,4}.

Chinese medicine (CM) is a descriptive medicine developed by the accumulation of empirical experience over thousands of years. In patients suffering from one disease defined by Western medicine (WM), a variety of CM syndromes may be observed ^{5,6}. Yin deficiency and Qi deficiency are two of the most generic CM syndromes. Syndrome differentiation is an essential diagnostic part of CM: a patient is diagnosed by observation, listening, questioning and pulse feeling. Diagnosis is followed by providing a CM therapy that corresponds to the syndrome, effecting multiple targets in the human body ⁷.

In contrast, WM is designed to work in a ‘one disease-one target’ mode. Often single biomarkers are used for diagnosis and monitoring disease progression (for example: fasting glucose level, blood pressure, cholesterol level) ⁸. Techniques such as metabolomics are developed for biomarker discovery in complex diseases which are not properly characterized by single biomarkers. Metabolomics is the systematic analysis of metabolites in body fluids, providing a comprehensive view of metabolic changes. Those patterns of change are potential biomarkers/biosignatures which can be used to optimize disease diagnosis and treatment ⁹. By unifying CM, WM and metabolomics technologies, different levels of organization within the body (metabolites, pathways, overarching processes, organ function, and even mental and emotional functioning) can be measured and integrated as individual system response profiles. Based on the response profiles, a better understanding of how patients respond to treatments can be obtained, and guidance for personalized treatment can be provided.

Herbal medicine is one of the most commonly administered CM therapies due to its ease of use, low costs and good compliance. An herbal formula consisting of multiple herbal constituents can be considered as a holistic and synergetic medicine. Rehmannia Six Formula (R6 or ‘Liu Wei Di Huang Wan’) has been used in clinical practice for thousands of years in China and is now widely used as a combination treatment for metabolic disorder related diseases including diabetes, hypertension, and hyperlipidemia ^{10–12}. R6 is made from a concentrated water extract of six herbs: Rehmanniae Radix Praeparata, Dioscoreae Rhizoma, Corni, Poria, Alismatis Rhizoma and Moutan Cortex in a proportion of 8:4:4:3:3:3 ¹³. According to Chinese medicine principles, R6 is known to promote the health of patients with Yin deficiency by nourishing ‘Kidney-liver Yin-deficiency’. Based on our previous study, R6

can improve the lipid profiles of MetS patients. Furthermore, the CM syndrome Yin and Qi deficiency were found to be improved by using the herbal formula over 8 weeks, and a specific sequence of events in the timing of those effects was discovered—Yin deficiency was found to be improved first after which Qi deficiency was improved¹³. There are no other studies reporting the use of R6 as a treatment for MetS, but there is a randomized controlled trial which reported using R6 to treat chronic kidney disease induced by MetS¹⁴. R6 is also reported as effective when used in combination with Western drugs to treat type 2 diabetes or hypertension^{10,11}. In addition, Huang et al. reported reduction of kidney failure risk among type 2 diabetic patients by taking R6¹⁵.

Based on CM guidelines and traditional practices, a novel formula SUB889 was designed for MetS subjects with Qi and Yin deficiency, but more focused on motivating ‘Yang’ without damaging ‘Yin’, an effect that is lacking in R6. SUB889 is a multi-component formula containing nine herbs: *Rehmanniae Radix*, *Polygoni Multiflori Radix*, *Cistanches Herba*, *Lycii Fructus*, *Polygonati Rhizoma*, *Ganoderma*, *Acanthopanax Senticosi Radix*, *Atractylodis Rhizoma*, *Ephedrae Herba* in a proportion of 12:6:6:5:5:5:5:3:1.

Even though the two *Rehmanniae Radix* formulae, R6 and SUB889, have different herbal compositions, both formulae contain *Rehmanniae Radix* as the ‘emperor herb’. In CM theory an emperor herb is the principle herb in a formula, which plays a major role in the treatment effect. However, in the preparation of R6, *Rehmanniae Radix* is processed by repeated steaming and drying, which is named *Rehmanniae Radix Preparata* (Shu Di Huang); in the preparation of SUB889 dried rehmannia root is used without any processing (Sheng Di Huang). The processing procedure changes the properties of *Rehmanniae Radix* from ‘releasing the Heat’ to ‘tonifying Yin’¹⁶. Besides that, the chemical composition is also changed by the processing procedure. Irodoids and oligosaccharides were reported to decrease while monosaccharides increase after processing^{17,18}. Dried *Rehmanniae Radix* was chosen instead of *Rehmanniae Radix Preparata* in SUB889 to focus on the blood-cooling and nourishing Yin, activating the pulse and eliminating stasis, to replenish the Kidney in a reasonable way. Except for *Rehmanniae Radix*, there is no overlap of herbs between R6 and SUB889. The ingenuity in SUB889 is that the combination of all herbs can nourish Yin and motivate Yang at the same time. The roles of other herbs of SUB889 were explained in the Supplementary Method ‘Roles of herbs in SUB889 except for *Rehmanniae Radix*’.

In order to compare the effects of the R6 and SUB889 formulae, MetS patients were randomized into two groups and subjected to an 8-week treatment with either R6 or SUB889. During this period, metabolite profiles, clinical parameters, CM syndromes and related

symptoms were measured at several time points up to 8 weeks after treatment. The measurements were analysed to evaluate treatment effects at different levels of organization within human body. The aim of the study is to combine all levels of data to explore the similarities and differences between MetS patients' response profiles to the two Rehmanniae Radix formulae. Furthermore, the response profiles will not only provide a better understanding of the treatment mechanisms involved using CM multi-herbal formulae (e.g. R6 and SUB889), but also improve our understanding of the biology of MetS.

Patients and Methods

The study design and subjects have been described by Wietmarschen et al. for MetS patients with R6 treatment ¹³, but also described here since it is essential for understanding the analysis presented in this paper.

Subjects

Human subjects were recruited from the Chengdu No. 1 People's Hospital in China. Subjects with MetS (waist circumference for men ≥ 90 cm, for women ≥ 80 cm; systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg) and diagnosed as Qi and Yin deficiency were recruited. Subjects with a diagnosis of another disease or using medication were excluded. This study followed guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the Ethics Committee of Chengdu No.1 People's Hospital. A doctor explained the details of the study to the subjects and ensured written informed consent of each subject.

Study design

The study was designed as a randomized controlled study in which two groups received an herbal formula for treating MetS, either R6 or SUB889 for 8 weeks. Qi /Yin deficiency and their related symptoms were recorded by one out of four CM doctors at the start of the study (T0), after 2 (T2), 4 (T4), 6 (T6) and 8 (T8) weeks of treatment. Fasting blood samples were collected and used to perform routine clinical chemistry (T0, T4, T8) and metabolic profiling (T0, T8).

Treatment

The R6 used in this study was an industrial and standardized product (batch number 4071161), manufactured by Beijing Tong Ren Tang Group Co., Ltd (Beijing, China). Daily intake of R6 was equivalent to active ingredients extracted from 9 g herbal mixture. The SUB889 was produced and tested for this study by Chengdu Jinhanfang Science and

Technology Service Co., Ltd (Chengdu, China). Daily intake of SUB889 was equivalent to active ingredients extracted from 12g herbal mixture (within the safety range of CM practice). In total 3 batches of SUB889 were produced for quality control, and one batch was used for the clinical study to minimize variation in the medication (batch number 050103). The procedure for producing SUB889 is described in the Supplementary Method section. Quality is controlled according to the Pharmacopeia of P. C. China 2010. No other Chinese herbal medication or Western medicine was allowed during this period.

Western medicine diagnosis: clinical parameters

Fasting blood samples were obtained for the measurement of glucose, insulin, triglycerides (TGs), total cholesterol (T-C), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glycated hemoglobin (HbA1c) and creatinine at T0, T4 and T8. Age and gender was documented. Weight, waist circumference, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured at T0, T2, T4, T6 and T8. All clinical measurements were conducted by the Chengdu No.1 People's hospital.

Chinese medicine diagnosis: syndrome differentiation

The Qi/Yin deficiency syndromes were diagnosed and corresponding symptoms were recorded by trained CM doctors. The typical symptoms of Yin deficiency are 'heat sensation in the chest, palms and soles', 'hectic fever and night sweat', 'head giddiness', 'tinnitus', 'insomnia and dreamful', 'dry mouth and pharynx', 'constipation', 'thin tongue shape', 'red tongue substance' (i.e. the color of tongue body), 'yellow tongue coating' and 'weak pulse'; Qi deficiency is characterized by the symptoms 'fatigue', 'spontaneous sweating', 'taciturn', 'weak joints and waist', 'head giddiness', 'tongue shape normal or enlarged', 'pale-red tongue substance' and 'thready and weak pulse' (i.e. soft, feels like a sewing thread, weak, without strength but not scattered by pressure) ^{19,20}. Symptoms were measured as ordinal or nominal variables, and Qi- and Yin-deficiency scores were calculated by summing the related symptom scores. The calculation rules are described in Supplementary Table S1.

Metabolic profiling

Plasma samples collected at T0 and T8 were measured by the Netherlands Organization for Applied Scientific Research (TNO) Zeist in the Netherlands. Extra plasma of the subjects was pooled and used to create quality control (QC) samples.

The gas chromatography/mass spectrometry (GC/MS) platform measures a large variety of metabolite classes important for the biological functioning of humans, such as amino acids, fatty acids and sugars. Briefly, 100 μ L of plasma was spiked with an internal standard (ISTD) mixture and extracted by methanol, followed by centrifugation and transferring the supernatant. After evaporating the supernatant to dryness, a two-step derivatization procedure was applied to the samples. The derivatized extracts were analyzed with an Agilent 6890N gas chromatograph equipped with an Agilent 5973 mass selective detector. Chromatographic separations were performed on a DB5-MS capillary column (30 m $\times$ 250 μ m i.d., 0.25- μ m film thickness; J&W Scientific, Folsom, USA), using helium as the carrier gas at a flow rate of 1.7 mL/min. The raw data were pre-processed using Chemstation G1701CA (Agilent, version D.01.02). The method used is described in detail by Koek et al ²¹.

The liquid chromatography/mass spectrometry (LC/MS) platform targets fatty acids, phosphatidylcholines, triglycerides and other lipid classes. In brief, 10 μ L of plasma was deproteinized by adding isopropanol containing an ISTD mix. This lipid profiling was conducted using TSQ Quantum Discovery Triple Quad mass spectrometer equipped with a Surveyor MS HPLC system. Chromatographic separations were performed with Alltech Prosphere C4 300Å column (150 $\times$ 3.2mm i.d., 5 μ m; Alltech, Lexington, USA) in combination with a Symmetry 300 C4 guard column (10 $\times$ 2.1mm i.d., 3.5 μ m; Waters, Milford, USA). The raw data were pre-processed using LCQuan (Thermo Fisher Scientific, version 2). The method used is described in detail by van Wietmarschen et al ¹³ and Draisma et al ²².

For each metabolite, the peak area was corrected by the appropriate internal standard (ISTD), and response ratios ($\text{Area}_{\text{analyte}}/\text{Area}_{\text{ISTD}}$) were then used in the subsequent analysis. An in-house developed algorithm was applied to compensate and correct for instrumental drift during the measurements. For each identified metabolite the standard deviation of its peak areas of the individual QC samples was computed relative to the average peak area over all QC samples (relative standard deviation, RSD) ²². The RSDs of the metabolites in the QC samples to assess the quality of the measurements of the targeted metabolites in each analytical platform. Subject samples were measured in duplicate and the average value was calculated for each metabolite. Log transformation (generalized logarithm transformation) and auto scaling (mean-centered and divided by the standard deviation of each variable) were performed to normalize the metabolomics data before statistical analysis.

Statistical analysis

The aim of the data analysis was to discover similarities and differences of subjects' response profiles between R6 and SUB889 therapy. Those similarities and differences are related to the various types of data—clinical parameters, CM syndromes and related symptoms and metabolite profiles. Statistical calculations were performed with MetaboAnalyst 3.0 (available at <http://www.metaboanalyst.ca/>)²³ and IBM SPSS Statistics for Windows, Version 23. The level of statistical significance was set at $p < 0.05$.

Within group, paired t-tests were used to identify which metabolites/clinical parameters were significantly changed after 8-week treatment. Change ratios of metabolites and clinical parameters (level of metabolite or clinical parameter_{T8} / level of metabolite or clinical parameter_{T0}) were calculated. Between groups, the independent t-test was used to identify which ratios were significantly different. To further determine the intergroup differences in the metabolite profiles, a hierarchical cluster analysis was performed and a heatmap was constructed by MetaboAnalyst to visualize changes of metabolite ratios.

To compare symptom changes after treatment, Marginal Homogeneity tests were applied to tongue and pulse related symptoms and Wilcoxon signed ranks tests were applied to other symptoms; to compare the symptom differences between groups, a Mann-Whitney U-test and a Chi-square test was performed to ordinal or nominal variables separately^{24,25}. For each subject, Qi-/Yin-deficiency scores were calculated by summing related symptom scores every two weeks. These summed scores are considered as Qi-/Yin-deficiency states.

Results and discussion

Subjects

In total 67 subjects were recruited in the study and randomly assigned to receive R6 or SUB889 treatment. Seven included subjects dropped out at various time points of the trial. In total 60 subjects completed the study of which 29 were in the R6 group, and the other 31 subjects in the SUB889 group. Metabolomics analyses were performed on 20 samples from each group due to budget constraints, therefore all of the following analyses were performed on these 40 subjects. Independent student t-tests and Chi-square tests were applied to test for significant differences between two treatment group, as illustrated in Table 1 (R6 vs. SUB889, T0), there were no significant differences in clinical characteristics between the two groups at baseline (0 week).

Changes of clinical parameters

The results of the comparisons between the R6 and SUB889 groups as well as within each of the treatment groups are shown in Table 1. SBP and Central obesity related parameters—waist circumference and BMI decreased significantly after 8-week treatment in both groups. The body weight declined (67.1 ± 12.4 kg vs. 65.0 ± 11.9 kg, $p = 0.001$) in the SUB889 group while in the R6 group the decrease was not significant (63.5 ± 5.7 kg vs. 62.7 ± 5.7 kg, $p = 0.055$). It seems that SUB889 has more effect on losing weight. In both groups, SBP and LDL-C decreased significantly. The decrease of T-C (5.4 ± 0.8 mmol/L vs. 4.6 ± 1.0 mmol/L, $p = 0.003$) and the increase of insulin level (9 ± 5 μ U/mL vs. 10 ± 5 μ U/mL, $p = 0.038$) were only found in R6 group. Therefore, R6 may improve the lipid profiles through affecting the insulin level in the human body. The change ratios of clinical parameters between the groups were similar (Table 1, R6 vs. SUB889). All the clinical parameter changes over time are shown as bar plots in Supplementary Fig S1.

Table 1. T-tests of clinical parameters within and between R6 (n=20) and SUB889 (n=20) group

	R6 (n=20)			SUB889 (n=20)			P value ^b
	T0	T8	P value ^a	T0	T8	P value ^a	
Age (years)	49 $\pm$ 10		-	50 $\pm$ 12		-	-
Gender (male/female)	5/15		-	8/12		-	-
Body weight (kg)	63.5 $\pm$ 5.7	62.7 $\pm$ 5.7	0.055	67.1 $\pm$ 12.4	65.0 $\pm$ 11.9	0.001	0.066
Waist circumference (cm)	88.9 $\pm$ 6.2	87.6 $\pm$ 5.7	0.001	91.0 $\pm$ 7.3	88.6 $\pm$ 6.6	0.001	0.168
Body mass index (kg/m ²)	24.9 $\pm$ 2.3	24.5 $\pm$ 2.2	0.005	25.7 $\pm$ 4.3	25.0 $\pm$ 4.1	<0.001	0.120
Plasma glucose (mmol/L)	5.4 $\pm$ 0.7	5.5 $\pm$ 0.8	0.93	5.6 $\pm$ 0.8	5.5 $\pm$ 0.7	0.803	0.815
Plasma insulin (μ U/mL)	9 $\pm$ 5	10 $\pm$ 5	0.038	9 $\pm$ 7	8 $\pm$ 6	0.435	0.071
Total cholesterol (mmol/L)	5.4 $\pm$ 0.8	4.6 $\pm$ 1.0	0.003	4.9 $\pm$ 0.8	4.7 $\pm$ 1.0	0.203	0.126
Triglyceride (mmol/L)	3.1 $\pm$ 2.7	2.4 $\pm$ 1.7	0.053	2.5 $\pm$ 1.4	2.2 $\pm$ 1.3	0.218	0.585
LDL-cholesterol (mmol/L)	3.4 $\pm$ 0.8	2.9 $\pm$ 0.8	0.024	3.2 $\pm$ 0.8	2.9 $\pm$ 0.9	0.009	0.452
HDL-cholesterol (mmol/L)	1.4 $\pm$ 0.3	1.3 $\pm$ 0.3	0.654	1.3 $\pm$ 0.3	1.3 $\pm$ 0.3	0.488	0.389
HbA1c (%)	5.7 $\pm$ 0.7	5.7 $\pm$ 0.4	0.216	5.4 $\pm$ 1.3	5.3 $\pm$ 1.3	0.824	0.637
Systolic blood pressure (mmHg)	135 $\pm$ 4	126 $\pm$ 7	<0.001	138 $\pm$ 8	127 $\pm$ 11	0.001	0.593
Diastolic blood pressure (mmHg)	75 $\pm$ 6	74 $\pm$ 6	0.56	76 $\pm$ 8	74 $\pm$ 7	0.417	0.782
Creatinine (μ mol/L)	64 $\pm$ 19	63 $\pm$ 18	0.877	70 $\pm$ 15	69 $\pm$ 13	0.785	0.919

^a Paired t-tests were applied to compare the clinical parameters between 0 week and 8 weeks within group;

^b Independent t-tests were applied to compare the change ratios of clinical parameters between R6 and SUB889.

Changes of symptoms and syndromes

According to CM, treatment effects can be observed as improvements of the corresponding CM syndrome. The operational definitions of the clinical diagnosis are not mutually exclusive, for example Qi and Yin deficiency contain overlapping symptoms (head giddiness, thready and weak pulse). Furthermore, a patient can show mixed syndromes, and syndromes can change during treatment. In this study, syndromes were represented by deficiency scores, and these scores were calculated by summing related symptom variables (Fig 1). The higher the deficiency score, the worse the patient is with regard to the corresponding syndrome. For each treatment time point the scores are plotted in Fig 2. It shows that both Rehmanniae Radix formulae reduced the deficiency scores after 8 weeks.

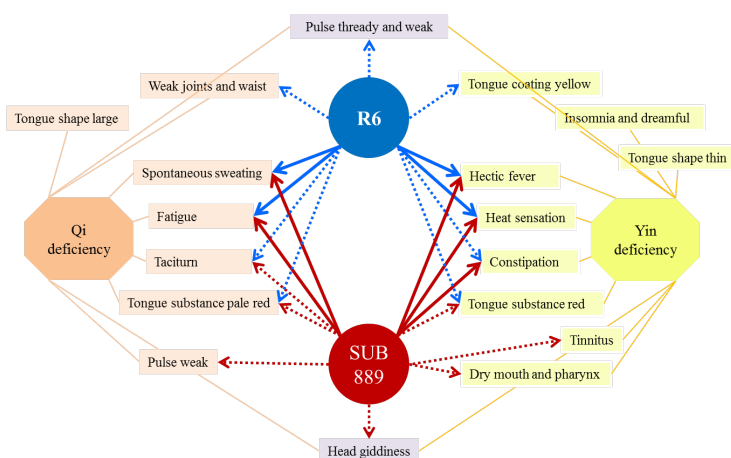


Figure 1. The treatment effects of R6 and SUB889 on CM symptoms related to Qi or Yin deficiency, comparing week 0 with week 8. Blue arrows indicate a symptom significantly improved with the R6 treatment ($\rightarrow$, $p < 0.01$; $\cdots\rightarrow$, $p < 0.05$); red arrows indicate a symptom significantly improved with SUB889 treatment ($\rightarrow$, $p < 0.01$; $\cdots\rightarrow$, $p < 0.05$). Symptoms with light orange, light yellow or grey colors are related to Qi deficiency, Yin deficiency and both deficiencies respectively.

R6 is traditionally used to treat Kidney- and Liver-Yin deficiency, leading to a decrease of Yin-deficiency. However, in our study Qi-deficiency scores also decreased. This phenomenon may be explained by the observation that Qi- and Yin deficiency was present in all patients in the study, and moreover there was a significant correlation between these two deficiency scores (Pearson correlation coefficient = 0.362, $p < 0.001$). Furthermore, according to CM theory, Deficiency is the results of an imbalance in the human body, which can be restored with the use of herbal formulae⁷. As both Qi and Yin are essential components to maintain this balance, the reduction of Yin deficiency may cause the improvement of Qi deficiency. This can explain that the improvement of Qi-deficiency scores together with Yin-deficiency

scores during the R6 treatment. However, the mechanism behind it still needs to be further studied. Interestingly two different formulae demonstrated similar effects on CM syndrome deficiency scores—although the sequential events on improvement of symptoms were not exactly the same.

Mann-Whitney U-tests were applied to compare the R6 and SUB889 treatment in the deficiency scores, no significant differences were found at all the time points. Therefore, R6 and SUB889 have a similar effect over 8 weeks when considering the syndrome level. However, the decrease of the Yin deficiency score between T2 and T4 (Yin deficiency score_{week 2} - Yin deficiency score_{week 4}) during SUB889 treatment was larger than that during R6 treatment ($p = 0.020$). Both Qi- and Yin-deficiency scores decreased after 8-week treatment ($p < 0.001$), which means R6 and SUB889 have significant effects in treating MetS based on CM diagnosis.

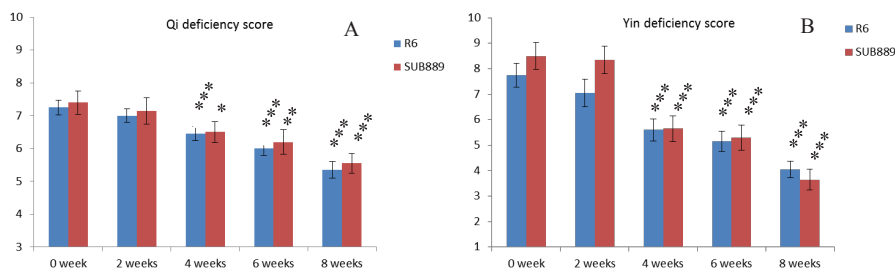


Figure 2. R6 and SUB889 decrease deficiency scores significantly. Effect of two formulae on Qi deficiency score (A) and Yin deficiency score (B) in MetS patients at time points of week 0, 2, 4, 6 and 8 (blue bar: the R6 group; red bar: the SUB889 group). Data represent the mean $\pm$ standard error of 20 patients and were analyzed by Wilcoxon signed rank test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 0 week). At each time point Qi or Yin deficiency scores were similar between R6 and SUB889 group

Associations between WM diagnosis and CM diagnosis

To investigate the associations between WM diagnostic clinical parameters and CM diagnostic scores for Qi/Yin deficiency, correlation analyses (Spearman's rho) were applied to the clinical parameters and deficiency scores containing all subjects with the R6 and SUB889 treatment ($n=40$) in week 0, 4 and 8. The results are shown in Supplementary Table S2. Qi- and Yin-deficiency scores were found to be highly correlated to systolic blood pressure (SBP) (Fig 3). Blood pressure is the diagnostic standard for hypertension according to WM, and the regulation of blood pressure involves a system intervention including the kidney, and the central or peripheral nervous systems in WM²⁶. There is no concept of hypertension in CM, but according to symptoms it could be divided into three major CM

syndromes, including deficiency syndrome²⁷. There are many studies pointing out the relationship between hypertension and Yin-/ Qi-deficiency. Although the mechanism behind this relationship is still unknown, deficiency in general may be a basic pathogenesis related to hypertension^{28–30}.

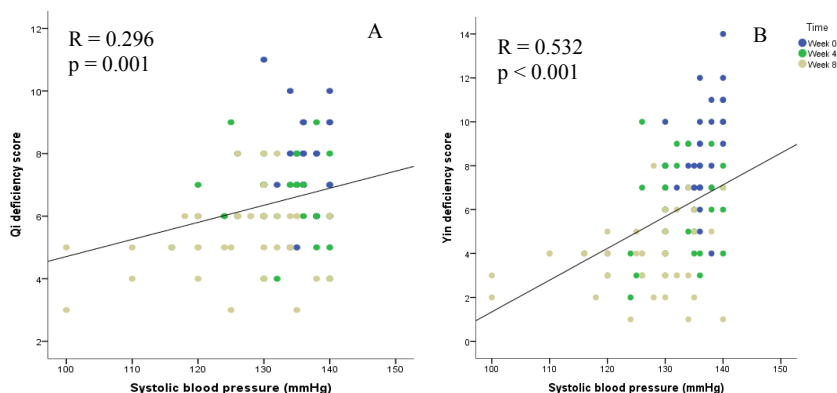


Figure 3. Relationship between systolic blood pressure and Qi deficiency score (A) and Yin deficiency score (B) during 8-week treatment of the two *Rehmanniae Radix* formulae. Graphs depict linear regression lines as well as Spearman correlation coefficients and their p values.

Changes of metabolites

Fasting blood samples collected from subjects treated with R6 (n=20) and SUB889 (n=20) at T0 and T8 were analyzed with a LC/MS method to obtain lipid profiles and with a GC/MS method to obtain global profiles. There were 95 and 127 metabolites measured with the LC/MS and GC/MS platforms respectively. After data pre-processing and quality checking, three statistical outliers were detected and therefore removed—one from the R6 group (patient ID: 37) and two from the SUB889 group (patient ID: 18, 40).

Paired t-tests and independent t-tests were applied to the normalized metabolomics data of the remaining 37 patients, and results are shown in Supplementary Table S3 (R6: week 8 vs. week 0; SUB889: week 8 vs. week 0). In the R6 group, 75 metabolites changed significantly after 8 weeks of treatment ($p < 0.05$), and most of the decreased metabolites were lipids, which consisted of six classes: cholesteryl esters (ChEs, n=8), fatty acids (FAs, n=7), phosphatidylcholines (PCs, n=12), triglycerides (TGs, n=16), diglycerides (DGs, n=2) and sphingomyelins (SPMs, n=18). The downregulated lipids measured by metabolomic profiling are consistent with the decrease of clinical parameter T-C and LDL-C during the R6 treatment. In the SUB889 group, 13 metabolites were found significantly upregulated, and

most of them were organic acids, including malic acid, fumaric acid, pyruvic acid and a few FAs, while 5 metabolites including glucose were found to be significantly downregulated.

A hierarchical cluster analysis was performed on the top 30 metabolite ratios of metabolites_{T8/T0} (Supplementary Table S3, Ratio) ranked by the independent t-tests, since these metabolites show the largest difference in response to R6 versus SUB889. Euclidean distance was used as the similarity measure and average linkage (clustering uses the centroids of the observations) was used as the clustering algorithm. The clustering results were visualized as a heatmap with the samples as the rows and metabolites as the columns, and each colored cell corresponding to a normalized metabolite ratio value (Fig 4). Most patients were found to be clustered correctly in their respective treatment group, and the misclassification rate was 16.22% (6 subjects misclassified). Comparing the two groups, metabolites in the R6 group, especially SPMs, PCs and FAs, were downregulated (color blue), while in the SUB889 treatment group the metabolites from these classes were relatively stable or slightly increased (color red).

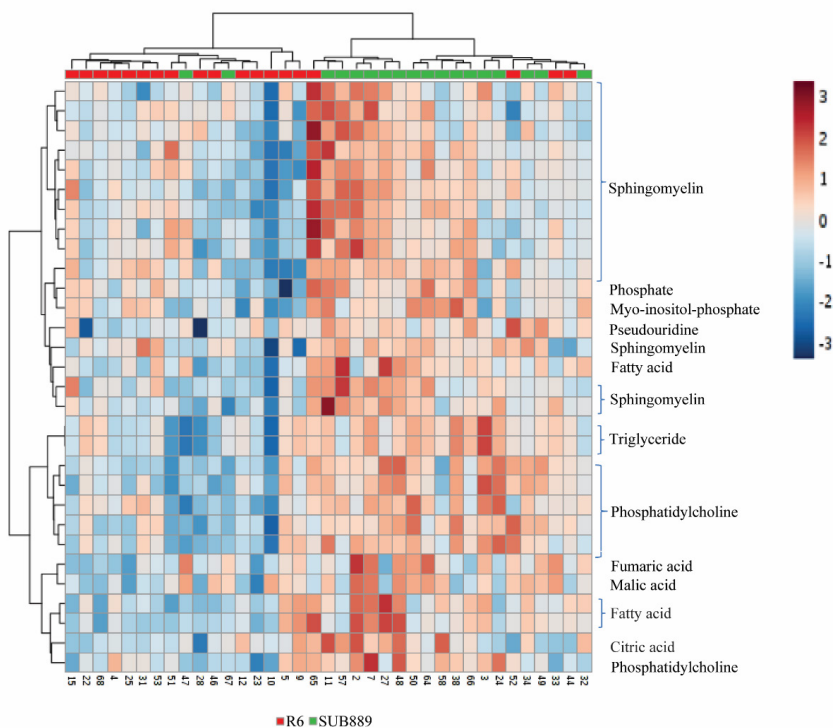


Figure 4. Heatmap of the R6 group and the SUB889 group based on the top 30 metabolite ratios (T8/T0) of the selected metabolites. The horizontal axis represents the patients: patient IDs were listed at the bottom and

grouped by treatment; the vertical axis represents 30 selected metabolic ratios, in which similar metabolites cluster in rows.

Hypothetical mechanisms of the two *Rehmanniae Radix* formulae

In the present study, responses of subjects to R6 or SUB889 were measured at multiple levels, and it is interesting to investigate the mechanisms of these two formulae by combining metabolomics data and clinical parameters. Metabolites which were changed after 8 weeks (Supplementary Table S3, T0 vs. T8) of treatment were selected. These selected metabolites and clinical lipid parameters (TG, HDL-C, LDL-C) were mapped on metabolite pathways taken from KEGG ³¹.

R6 treatment significantly decreased lipid related metabolites (FAs, SPMs, TGs, DGs, PCs and ChEs) and clinical parameters (T-C and LDL-C) (Fig 5A). The decrease of lipids may be explained by the increased insulin level. Insulin can activate the synthesis of lipids and inhibit their degradation ³². R6 treatment might stimulate insulin secretion, leading to reductions of the plasma lipids and LDL-C; downregulated FAs indicated that R6 may also promote the β -oxidation of FAs for energy generation. Since the lipid levels substantially declined during the R6 treatment, the BMI and waist circumference were reduced after 8 weeks. In addition, decreased SPMs could lead to changes in cell membrane composition and related signaling pathways. Many studies have already revealed the role of SPMs and related signaling pathways in obesity, insulin resistance, and other metabolic disorders ^{33–35}. It is reported that R6 may affect endocrine and excretory systems related pathways, such as peroxisome proliferator-activated receptor (PPAR) signaling pathway and aldosterone-regulated sodium reabsorption, which play important roles in lipid metabolism, glucose uptake and blood pressure regulation ^{36,37}.

However, lipid levels were not substantially changed during the SUB889 treatment, only a few lipid metabolites were slightly altered (Fig 5B). Malic acid/malate and fumaric acid/fumarate, which are important intermediates of the tricarboxylic acid (TCA) cycle, increased dramatically after 8 weeks, indicating that the TCA cycle may be stimulated with the SUB889 treatment. Besides, as an important intermediate of gluconeogenesis and glycolysis, pyruvic acid/pyruvate was also upregulated significantly, which may also induce the TCA cycle. Therefore, we hypothesize that SUB889 did not change the lipid homeostasis in plasma, but it might treat MetS by regulating glucose metabolism (gluconeogenesis, glycolysis) and the TCA cycle to achieve an improvement of clinical outcomes in MetS patients.

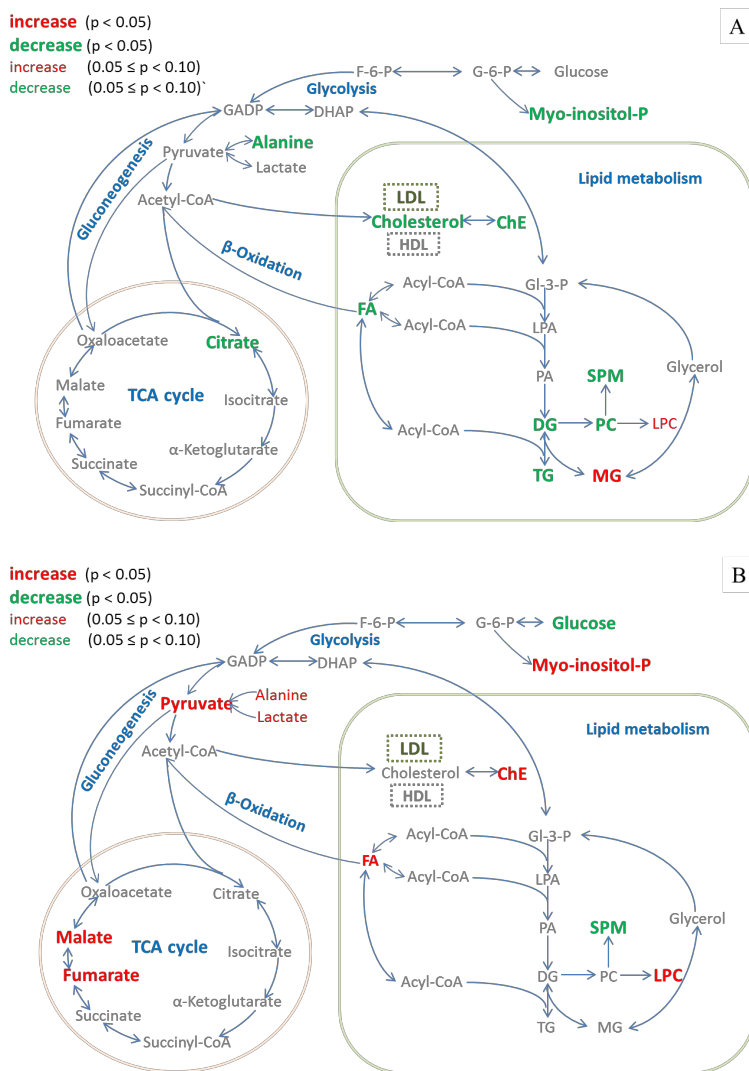


Figure 5. Pathway analysis of metabolites which were affected by 8-week treatment of R6 (A) or SUB889 (B).

It is likely that the formulae both lead to improvement of MetS even though the herbal components have effects on a variety of different metabolic targets. This indicates that these pathways are interconnected in the regulation of human physiological states. Multi-herbal formulae consisting of numerous active components inducing complex interactions within the human body, might therefore be especially suitable for treating complex diseases (e.g. MetS) and offer valuable alternatives to Western single target medicines. Since the R6 and SUB889 formulae have very similar effects with respect to CM, we assume that the regulation of lipid metabolism, glucose metabolism or the TCA cycle by different formulae may result in the

same therapeutic outcomes. From a Chinese medical point of view, one Western medical defined disease can be treated with different approaches, much according to the CM statement ‘Tong Bing Yi Zhi’ (same disease, different treatment). However, in this study there was no placebo group included. The similar effectiveness may be due to other factors (e.g. placebo effect) than the herbal formula in the treatment of MetS. Therefore, a placebo-controlled randomized study is needed to validate our findings in the future.

Conclusions

In conclusion, this study shows that R6 and SUB889 have similar effects on the treatment of MetS by improving CM deficiency syndromes and WM clinical outcomes (central obesity and systolic blood pressure). R6 is more effective in decreasing lipid profiles compared to SUB889, indicating that the two formulae target different metabolic pathways in the patients. Even though the two multi-herbal *Rehmanniae Radix* formulae induce different metabolic changes, the therapeutic effect can be similar according to both CM and WM diagnosis. In the future, SUB889 may be developed as a candidate treatment for MetS patients or the prevention of cardiovascular disease. Further studies are needed with larger cohorts as well as healthy control groups to elucidate the mechanism of actions for the two *Rehmanniae Radix* formulae in MetS.

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Supplementary information

Method

Procedures for producing SUB889

Rehmanniae Radix, Polygoni Multiflori Radix, Cistanches Herba, Acanthopanax Senticosi Radix, Atractylodis Rhizoma and Ephedrae Herba were refluxed with 80% ethanol for 2h, filtered through a gauze. The residue and three other herbs were extracted with boiling water twice, 2h each time. Then all filtrates were pooled and evaporated with rotary evaporation under vacuum at 70°C. In the end, soluble starch and dextrin (1:1) were added as the excipient into the herbal extract to produce the SUB889 pills.

Roles of herbs in SUB889 except for Rehmanniae Radix

In SUB889, Polygoni Multiflori Radix and Cistanches Herba were chosen as ‘minister herbs’; Lycii Fructus, Polygonati Rhizoma, Ganoderma and Acanthopanax Senticosi Radix were chosen as ‘assistant herbs’. The ‘emperor herb’, ‘minister herbs’ and ‘assistant herbs’ all work together to increase the nurturing. Polygoni Multiflori Radix replenishes the vital Essence and Blood, expel Wind and counteract toxicity, as well as moistens the intestine and stimulates purgation. Cistanches Herba is used to warm Kidney and help Yang, improve vitality and reinforce Essence, moisten the intestine and stimulate purgation, to enhance the vital Yang and vital Yin power of Kidney. Lycii Fructus, Polygonati Rhizoma, Ganoderma and Acanthopanax Senticosi Radix could reinforce Kidney, replenish vital energy and benefit Essence. Furthermore, the ingenuity in this formula is that Atractylodis Rhizoma and Ephedrae Herba were chosen as the ‘envoy herbs’ to improve the effectiveness of all the ingredients together and complement each other without side effects.

Tables

Table S1 Calculation rules of Qi and Yin deficiency scores

Symptom score criteria	Symptom name	Qi- or Yin- deficiency related
Main symptoms (0= mild or no symptom, 2= moderate symptom, 4= sever symptom)	Fatigue	Qi
	Heat sensation	Yin
	Hectic Fever	Yin
Secondary symptoms (0= mild or no symptom, 1= moderate symptom, 2=	Spontaneous sweating	Qi
	Taciturn	Qi

sever symptom)	Joints & Waist weakness	Qi
	Head Giddiness	Qi & Yin
	Tinnitus	Yin
	Insomnia & Dreamful	Yin
	Dry mouth & Pharynx	Yin
	Constipation	Yin
Tongue and pulse diagnostic symptoms (0= no symptom, 1= with symptom)	Tongue substance pale red	Qi
	Tongue substance red	Yin
	Tongue shape normal or enlarged	Qi
	Tongue shape thin	Yin
	Tongue coating yellow	Yin
	Pulse thready and weak	Qi
	Pulse weak	Yin

Symptoms were grouped as main, secondary and tongue and pulse diagnostic symptoms. All symptoms were scored by CM doctors according to the symptom score criteria. For example, each main symptom can be scored 0, 2 or 4, in which 0 indicates ‘mild or no symptom’, 2 indicates ‘moderate symptom’ and 4 indicates ‘sever symptom’. For each patient, Qi- and Yin-deficiency scores were calculated by summing the related symptom scores separately.

Table S2. Spearman’s correlation between Chinese medicine and Western medicine diagnosis

Clinical parameter	Qi deficiency score		Yin deficiency score	
	Correlation coefficient	p value	Correlation coefficient	p value
Body weight	0.07	0.35	0.14	0.05
Waist circumference	0.09	0.23	0.24	<0.001
BMI	0.08	0.25	0.06	0.41
Systolic blood pressure	0.23	<0.001	0.54	<0.001
Diastolic blood pressure	0.10	0.14	0.09	0.22
Plasma glucose	-0.04	0.66	0.09	0.35
Total cholesterol	0.02	0.85	0.02	0.80
Triglycerides	0.03	0.78	0.05	0.62
HDL-cholesterol	-0.06	0.49	-0.02	0.81
LDL-cholesterol	0.04	0.69	0.00	0.96
HbA1c	0.02	0.87	0.06	0.62
Plasma insulin	-0.08	0.41	0.04	0.68

p values calculated with paired t-tests (0 week v.s. 8 weeks)

Table S3. The differences of metabolites with and between R6 and SUB889

Class	R6: week8 vs. week0			SUB889: week 8 vs. week 0			Ratio: R6 vs. SUB889	
	Metabolite	p value ^c	Regulation	Metabolite	p value ^c	Regulation	Metabolite	p value ^d
FA ^a	C16:0	0.015	Down	C10:0	0.018	Up	C10:0	0.001
	C16:1	0.007	Down	C17:0	0.020	Up	C16:0	0.034
	C17:0	0.010	Down	C20:1	0.023	Down	C16:1	0.025
	C18:0	0.002	Down				C17:0	0.047
	C18:1	0.003	Down				C18:1	0.020
	C20:1	0.016	Down				C14:0	0.100
	C22:6	0.049	Down				C18:2	0.052
	C10:0	0.054	Down				C20:1	0.087
	C14:0	0.074	Down					
	Heptadecaspshing-4-ene/FAC24:0	0.009	Down	Sphingosine/FAC18:0	0.026	Down	Heptadecaspshing-4-ene/FAC24:0	0.019
SPM ^a	Hexadecaspshing-4-ene/FAC18:0	0.047	Down	Sphingosine/FAC17:0	0.069	Up	Hexadecaspshing-4-ene/FAC20:0	0.020
	Hexadecaspshing-4-ene/FAC20:0	0.020	Down				Hexadecaspshing-4-ene/FAC22:0	0.023
	Hexadecaspshing-4-ene/FAC24:1	0.003	Down				Hexadecaspshing-4-ene/FAC24:1	0.016
	Sphingadiene/FAC24:0andC24:1	0.015	Down				Sphingadiene/FAC24:0andC24:1	0.024
	Sphingosine/FAC24:0andC24:1	0.001	Down				Sphingosine/FAC17:0	0.031
	Sphingadiene/FAC20:0	0.016	Down				Sphingosine/FAC22:0	0.040
	Hexadecaspshing-4-ene/FAC16:0	0.067	Down				Sphingosine/FAC24:0andC24:1	0.026

Oth- ers ^a	HexadecaspHING-4-ene/FAC22:0	0.086	Down			Spingadiene/FAC20:0	0.008
	Sphingosine/FAC17:0	0.058	Down			HeptadecaspHING-4-ene/FAC16:0	0.073
	Sphingosine/FAC18:0	0.058	Down			HexadecaspHING-4-ene/FAC16:0	0.077
	Sphingosine/FAC18:0	0.074	Down			Sphingosine/FAC16:0	0.055
						Spingadiene/FAC16:0	0.062
	MG ^a 1-Monolinoleoylglycerol	0.041	Up				
	2-Hydroxybutanoic acid	0.008	Down		D-Glucose	Citric acid	0.001
	Alanine	0.012	Down		Fumaric acid	D-Ribulose-orD-Xylulose	0.036
	Cholesterol	0.030	Down		L(-)Malic acid	Fumaric acid	0.005
	Citric acid	0.002	Down		L-Cystine	Glycerol	0.032
Oth- ers ^a	D-Glutamic acid	0.044	Down		Myo-inositol	L(-)Malic acid	0.011
	HypoXanthine	0.046	Down		Myo-inositolphosphate	L-Threonine	0.042
	Myo-inositol-1,2-cyclicphosphate	0.048	Down		Pseudouridine	Monomethylphosphate	0.042
	Myo-inositolphosphate	0.026	Down		Pyruvic acid	Myo-inositolphosphate	0.027
	Phosphate	0.043	Down		1-Palmitoyl-L-alpha-lysophosphatidic acid	N-Acetylaminomalonic acid	0.041
	VitaminE	0.008	Down		3-Methyl-2-oxo-valeric acid	Phosphate	0.019
	3-Methyl-2-oxo-valeric acid	0.073	Down		1,5-Anhydro-D-Glucitol	Pseudouridine	0.020
	4-Deoxyglucose	0.072	Down		2-Amino-1-butyric acid	2-Hydroxybutanoic acid	0.047
	Creatinine	0.092	Down		Beta-Alanine	Aminomalonic acid	0.042
	D-Ribose	0.062	Down		D-Maltose	4-Methyl-2-oxovaleric acid	0.052
	Monomethylphosphate	0.058	Down		Lactic acid	Alanine	0.078
	Ureum	0.069	Down		L-Threonine	Cholesterol	0.084

C46:0	0.029	Down		C56:6	0.025
C46:1	0.049	Down		C50:3	0.076
C48:1	0.008	Down		C52:5	0.099
C48:2	0.013	Down		C56:5	0.068
C48:3	0.042	Down			
C50:1	0.013	Down			
C50:2	0.006	Down			
C50:3	0.007	Down			
C50:4	0.037	Down			
C52:2	0.007	Down			
C52:5	0.048	Down			
C54:2	0.030	Down			
C56:4	0.002	Down			
C56:5	0.028	Down			
C56:6	0.002	Down			
C44:0	0.093	Down			
C50:5	0.052	Down			
C52:3	0.061	Down			
C54:3	0.099	Down			
C14:0	0.006	Down	C15:0	C14:0	0.024
C14:1	0.006	Down	C24:0	C14:1	0.029
C15:0	0.030	Down		C15:0	0.049
C16:0	0.005	Down		C16:0	0.022
C16:1	0.014	Down		C16:1	0.032
C18:0	0.014	Down		C22:0	0.005
C18:1	0.030	Down		C23:1	0.026
C22:0	0.030	Down		C24:1	0.039

SPM^b

C23:1	0.032	Down		C24:2	0.025
C24:1	<0.0001	Down			
C24:2	0.005	Down			
C32:0	0.001	Down		C32:0	0.033
C32:1	0.001	Down		C32:1	0.006
C34:0	0.019	Down		C34:1	0.007
C34:1	0.001	Down		C36:3	0.014
C34:3	0.038	Down		C36:4	0.013
C36:1	0.009	Down		C36:5	0.012
C36:3	0.011	Down		C38:5	0.015
C36:4	<0.0001	Down		C34:3	0.073
C36:5	0.001	Down		C36:1	0.086
C38:4	0.001	Down		C38:4	0.067
C38:5	0.001	Down			
C38:6	0.002	Down			
LPC ^b					
C18:2	0.093	Up	C18:1	0.018	Up
C16:1					0.043

a: analyzed in GC/MS platform

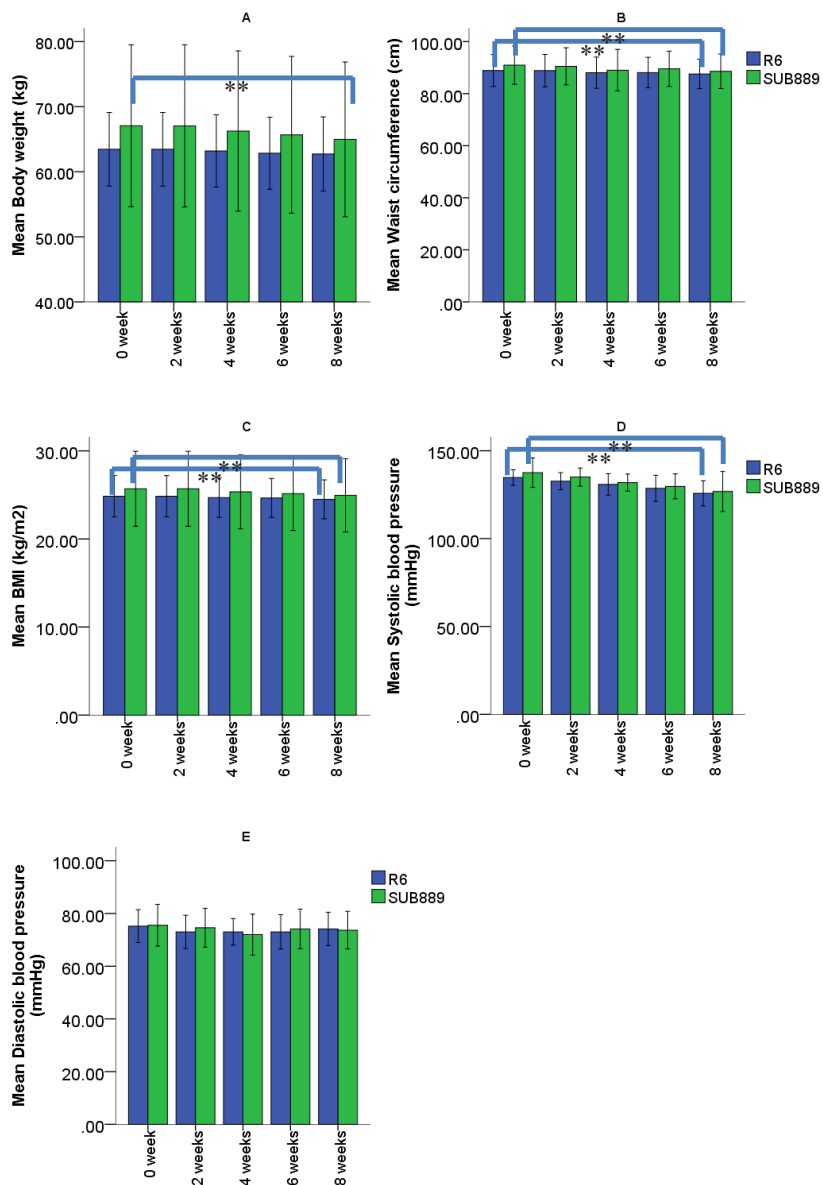
b: analyzed in LC/MS platform

c: p values calculated with paired t-tests

d: p values calculated with independent t-tests

ChE, cholesteryl ester; DG, diglyceride; FA, fatty acid; LPC, lysophosphatidylcholine; MG, monolinoleoglycerol; PC, phosphatidylcholine; SPM, sphingomyelin; TG, tri-glyceride.

Figures



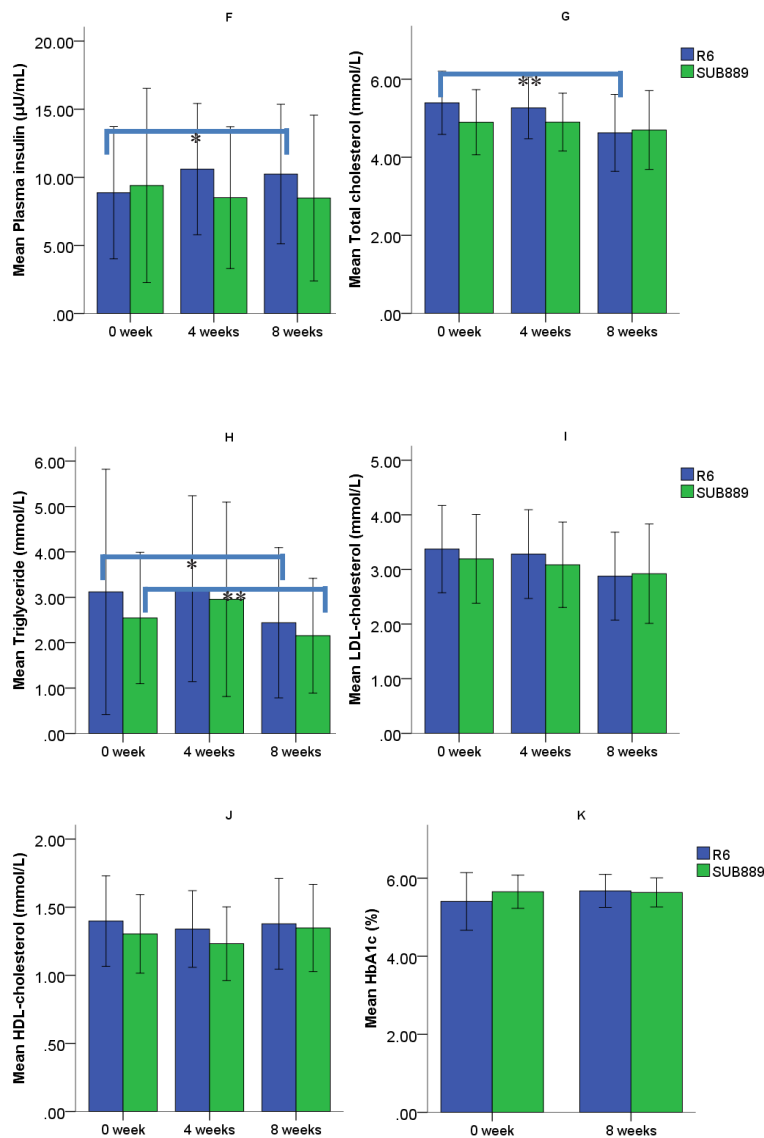


Figure S1. Bar plots of clinical parameters changing with treatment time in R6 and SUB889 group. A, body weight; B, waist circumference; C, BMI; D, systolic blood pressure; E, diastolic blood pressure; F, plasma insulin; G, total cholesterol; H, triglycerides; I, LDL-cholesterol; J, HDL-cholesterol; K, glycated hemoglobin

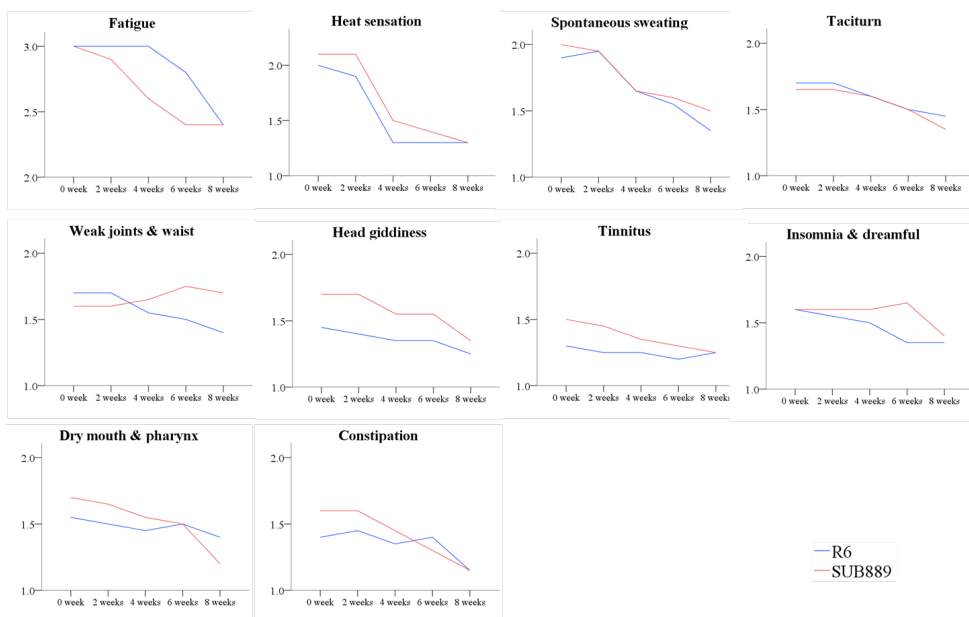


Figure S2. Symptom changes with treatment time. X-axis displays the treatment time, Y-axis display the symptoms scores.

Chapter 7

Conclusions and perspectives

Conclusions

Chronic diseases are the leading cause of mortality and loss of quality of life, resulting in a growing financial burden in health care around the world ¹. Due to the complex mechanisms related to disease initiation and development, the ‘one-size-fits-all’ approach of Western medicine (WM) has reached the limits of its success with managing chronic diseases, while systems approaches focusing on personalized medicine are more promising for the individual patient. The development of system approaches can provide new solutions for optimal treatment, leading to cost-effective therapies for patients in clinical practice. In light of this idea, this thesis focuses on applying systems diagnosis (e.g. metabolomics and Chinese medicine based diagnostic principles) to predict and evaluate the response to medication of patients with chronic diseases, and rheumatoid arthritis (RA) in particular.

A major part of the research presented in this thesis (Chapter 2 to 5) involves data obtained from the observational study BiOCURA (Biologicals and Outcome, Compared and predicted in Utrecht region, in Rheumatoid Arthritis), in which RA patients who started biological therapies were enrolled between 2009 and 2015 from eight hospitals in the Utrecht region in the Netherlands ². The goal of the study is to identify RA subpopulations with specific characteristics which benefit from biological therapies, to allow a more patient tailored treatment and balancing of efficacy, preventing unnecessary high costs and possible side effects for those who do not benefit from treatment. The recruited patients had been receiving concomitant drugs, such as conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and nonsteroidal anti-inflammatory drugs (NSAIDs). Clinical and demographic information, Chinese medicine (CM)-based questionnaires, blood as well as urine samples were collected before patients started the biological therapy (i.e. at baseline).

In Chapter 2 we investigated the possibilities of using baseline serum metabolomic profiles to predict the response to TNF- α inhibitors (TNFi). Serum samples were analysed on three targeted liquid chromatography-mass spectrometry (LC-MS) platforms, covering a large range of metabolites, i.e lipids, oxylipins and amines. Multivariate logistic regression models were built and validated to identify predictors from the metabolomic data for good-responders and non-responders in patients receiving TNFi (n=124). Four metabolites *sn1*-LPC(18:3- ω 3/ ω 6), *sn1*-LPC(15:0), ethanolamine, and lysine showed significantly added value over clinical parameters in predicting therapeutic responses, and the addition of metabolite characteristics improved the reclassification of non-responders by 30% (i.e. by adding metabolomic predictors to the model an additional 30% of ‘observed non-responders’ were correctly classified as ‘predicted non-responders’). This is the first time that serum

metabolomics profiles analysed by LC-MS have been demonstrated to predict therapeutic response to biological treatment in RA. In addition, we explored the associations between metabolomic profiles and the disease activity (as defined by relevant parameters) of RA patients starting either a TNFi or a non-TNFi treatment ($n = 231$). This analysis was done by generalized estimating equation (GEE) with a linear regression model, resulting in 88 metabolites (lysophospholipids, fatty acids, oxylipins, and amines) significantly associated with disease activity according to the EULAR criteria. Besides, in this chapter we reported that the metabolite profiles of patients could be influenced by certain concomitant drugs. As a follow up, the effects of one class of concomitant drugs, namely glucocorticoids (GCs), are reported in Chapter 5.

In addition to blood, urine is another commonly collected biological matrix in clinical trials and widely used for metabolomic profiling, as metabolic profiles of urine can be considered as the waste products of the human body metabolism. In Chapter 3 we developed a bovine liver- β -glucuronidase hydrolysing sample preparation method for a subsequent LC-MS analysis to profile the total urinary oxidised lipids, i.e. free and conjugated oxidized lipids, including the following classes of compounds: prostaglandins, isoprostanes, dihydroxy-fatty acids, hydroxy-fatty acids, and nitro-fatty acids. Oxidised lipids, which can be detected in both urine and blood, play crucial roles in prominent physiological processes in RA. Since oxidised lipids are short-lived in systemic circulation, the downstream metabolic products excreted in urine are more easily measured. The total oxidised lipids profiling method was developed and validated for human urine, and was then applied to urine samples from 80 RA patients starting with TNFi therapies. But no single urinary metabolite or metabolite profile was found to reliably predict treatment outcome, but pro-inflammatory mediators $\text{PGF}_{2\alpha}$, $\text{PGF}_{3\alpha}$, and oxidative stress markers $\text{iPF}_{2\alpha}\text{IV}$, 11-HETE and 14-HDoHE were positively associated with an improvement of DAS28. Furthermore, the anti-inflammatory nitro-fatty acids were negatively associated with baseline DAS28. The developed methodology extends the current metabolic profiling of oxidised lipids in urine, and its application will enhance our understanding of the role that these bioactive metabolites play in health and disease.

Except for metabolomics, we also explored the added value of CM based diagnostic principles for subgrouping responders and non-responders of RA patients to biological therapies. In Chapter 4, CM defined symptoms as well as WM characteristics were used to classify response to biological therapy in RA patients ($n = 61$). According to CM, Cold or Heat patterns are based on integrating corresponding symptoms. The symptoms were measured by questionnaires designed to capture these Cold and Heat related symptoms. Categorical principal components analysis (CATPCA) was applied to integrate data from CM

and WM diagnosis, including therapeutic response as the classification variable. The combination of baseline symptoms ('preference for warm food', 'weak tendon severity') and clinical parameters (positive rheumatoid factor/anti-cyclic citrullinated peptide antibody, CRP, creatinine) were able to differentiate responders from non-responders to biological therapies with a positive predictive value of 82.35% and a misclassification rate of 24.59%. Adding CM-defined symptom variables in addition to clinical data showed 8.3% improvement in classifying non-responders (i.e. by adding CM-defined symptoms, 8.3% more of the 'observed non-responders' were correctly classified as 'predicted non-responders'). Therefore, we consider it worthwhile to investigate further the possible potential of a description of symptom variables, or disease patterns for predicting treatment outcome. This study for the first time includes CM-defined symptoms into a classification analysis of RA patients' response to biological therapies.

In addition to predicting the therapeutic outcome and monitoring disease activity, metabolomics is a powerful tool for evaluating the response to on-going medical interventions, and can assist in understanding the mechanisms of action of a drug as well as potential side effects of the drug. We have reported that certain concomitant drugs could influence metabolomic profiles in Chapter 2. Therefore, in Chapter 5 we specifically investigated the effects of one class of concomitant drugs, glucocorticoids (GCs), on the serum lipidomic profiles of RA patients. Interestingly, after correcting for potential confounders, the lysophospholipid level was significantly higher in the GC users compared to non-users in females, whereas in males the difference between GC users and non-users in lysophospholipid level was similar. As a constituent of oxidized low-density lipoproteins, the lysophospholipid level could be related to cardiovascular risk. The lysophospholipid related gender differences are therefore potentially relevant with respect to the risk of cardiovascular events in RA patients, which could eventually guide the adjustment of treatment strategies for either males or females.

In the last chapter of the thesis we combined metabolomics, CM as well as WM diagnosis as systems diagnosis to monitor the characteristics of patients with metabolic syndrome (MetS) receiving herbal formulae treatments during 8 weeks. Metabolic syndrome is a chronic disease and highly associated with an increased risk of cardiovascular diseases and diabetes³. A randomized controlled study of forty MetS patients was designed to compare the systems effects of two *Rehmanniae Radix* formulae (R6 and SUB889). Serum metabolomics by targeted LC-MS and untargeted GC-MS platforms, CM diagnosis (Qi/Yin deficiency, evaluated by trained CM physicians) as well as WM diagnostic parameters were integrated to investigate the effects of the two treatments in patients with MetS. The analysis revealed that

R6 (n = 20) and SUB889 (n = 20) have similar effects on MetS regarding the improvement of WM routine clinical parameters (waist circumference, body mass index, LDL-cholesterol, systolic blood pressure) and WM syndrome diagnosis (Qi/Yin deficiency). This result corresponds to the CM saying: different treatment could work on the same disease to achieve the same therapeutic effect. Actually, different effects of the two formula were found at the metabolomic levels. Decreased levels of cholesteryl esters, phosphatidylcholines, triglycerides and sphingomyelins were found in the R6 group, while the SUB889 formula resulted in increased levels of tricarboxylic acid cycle metabolites and glucose metabolism intermediates (malate, fumarate, and pyruvate). Both formulae showed similar effects on the treatment of MetS as assessed by improving CM and WM defined clinical characteristics, while R6 is more effective in decreasing lipid profiles compared to SUB889. However, the exact mechanisms of the two formulae on MetS remain to be elucidated in future studies.

Future perspectives

In this thesis metabolomics approaches were applied in most of the chapters (Chapter 2, 3, 5, and 6). Metabolomics is still a developing field, and with high-throughput techniques emerging, metabolomics can systematically characterize patients by analysing a large range of small molecules in biological samples. Therefore it could offer new opportunities for personalized medicine in chronic diseases. In the BioCURA study targeted metabolomic platforms were applied. Serum (Chapter 2) and urinary metabolomic profiles (Chapter 3) were able to predict the response to biological therapies as well as to monitor disease activity of RA patients. Besides, polar lipid profiles showed gender-related differences in response to concomitant GC (Chapter 5). Moreover, in Chapter 6 targeted and untargeted profiling was combined to monitor metabolomic changes with treatments on MetS patients, and provide insight into the understanding of mechanisms of action for two *Rehmanniae Radix* formulae. In Summary, these findings suggest that metabolomics has great potential for predicting treatment outcome and monitoring disease activity as well as measuring the response of patients to medications.

Although metabolomic profiling is powerful in reflecting the reactions of an organism to interventions, the profiling can be directly or indirectly interfered with many factors. For example, metabolomic profiles can be changed by diet, age, gender, body mass index, disease durations, concomitant medications, etc. As an observational study, the inclusion criteria of BiOCURA were very broad, which led to a highly heterogeneous dataset. Although in regression analyses the interaction factors were marked and treated as potential confounders, the heterogeneity is still an issue probably jeopardizing the predicting abilities of metabolites.

Even though we could not correct for diet, we still showed that the predictive and evaluative ability of metabolomic profiles can be quite high in a cohort of RA patients resembling daily clinical practice. It would be worth studying how these metabolites could be used for predicting therapeutic response to TNFi therapies in other studies, thereby potentially optimizing the treatment strategy for patients with RA.

Recently, a multi-centre, randomised and double-blind study called U-ACT-EARLY has been carried out in the Netherlands focussing on patients with early RA (disease duration less than one year) ⁴. This study compared three treatment strategies for freshly diagnosed RA: i) biological-monotherapy with tocilizumab, an interleukin-6 receptor blocker; ii) tocilizumab with methotrexate, and iii) methotrexate monotherapy. Fasting blood samples were collected before the start of the very first treatment of the RA, and metabolomic profiling will be used to predict sustained drug free remission (i.e. a DAS28 of less than 2,6 and a swollen joint count of four or fewer joints of the 28 joints assessed) within two years. Because of the strict inclusion criteria in the design of U-ACT-EARLY, the interaction factors were minimalized as much as possible. The expectations are that metabolomics applied on this study will provide new evidence for choosing treatment strategies for early RA patients and possibly reveal useful biomarkers for predicting response to treatment, which could significantly improve the quality of life in the later stages of RA.

In this thesis, Chinese medicine (CM)-based study of symptom patterns was explored as another systems diagnosis approach for studying response to treatment. In Chapter 4 we explored the possibility of subtyping RA patients in responders and non-responders using a combination of CM-defined symptoms and Western-based typical clinical parameters. These symptoms were evaluated by questionnaires which were designed on the basis of diagnostic principles of CM. All the questions are standardized and cover crucial symptoms relevant to Cold/Heat patterns. There are a few challenges in this study. Firstly, for using questionnaires as CM diagnostic tool to subtype responders and non-responders in RA, patients could interpret each question differently based on their subjective understanding, and may lead to incomparable answers. Secondly, many patients have a mixture of patterns in practice. As a pattern is diagnosed by integrating related symptoms, in this study most RA patients who completed the questionnaire did not express clearly distinctive Cold or Heat patterns. Additionally, a CM expert evaluation of Cold and Heat patterns on the basis of all the questionnaires did not result in an improvement of the predictive power of the patterns. Therefore, instead of Cold and Heat patterns, we explored the predicting abilities of the individual symptoms, but still the contribution of adding symptoms was not significant. Furthermore, prior use of drugs (from Western Medicine) might significantly change the CM

symptom patterns, comparable to their changes of the metabolomic profiles making prediction of treatment outcome more difficult as discussed in Chapter 2. Therefore, the CM defined symptoms or patterns may reflect the dynamic health (resilience) of the entire system but might not be suitable to classify the response to a specific Western treatment such as blocking a single protein. Nevertheless, based on previous findings⁵ and the above mentioned observations we consider it worthwhile to investigate further the potential of symptom or pattern variables for reducing inefficient treatment. A homogenous cohort of patients with more clear Cold and Heat patterns is needed for basic future research, which might provide new possibilities in phenotyping patients for optimal therapy. In contrast, in the MetS study (Chapter 6) Qi/Yin deficiency was diagnosed by CM practitioners followed by Chinese herbal medicines treatment. The aim was not to predict response to treatment, but to monitor the effects of treatment. Therefore, the responses to the herbal medicines were objectively measurable as changes in CM-defined patterns. In this case, practitioners had good agreement on the diagnosis. However, in a large patient cohort we would rather use questionnaires instead of CM experts.. These studies showed that both CM questionnaires and practitioners could be applied in the CM pattern diagnosis for treatment response to patients with a WM defined disease.

In summary, each systems approach has its advantages and drawbacks, and combining the systems approaches with modern WM could probably offer a more detailed systems diagnosis in the study of complex chronic diseases. Systems diagnosis is powerful in predicting response to therapies and monitoring diseases activities. Additionally, it can be applied in studies into the effects of drugs or lifestyle (physical exercise or diet). The development of new metabolomic approaches used in combination with CM-based symptom pattern diagnosis of health and disease will most probably provide increasing opportunities for personalized medicine in healthcare.

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List of Abbreviations

ACPA/anti-CCP, anti-citrullinated protein antibody
ADA, adalimumab
ALT, alanine aminotransferase
aOR, adjusted odds ratio
AUC, area under the curve
AZA, azathioprine
bDMARDs, biological disease-modifying antirheumatic drugs
BMI, body mass index
CATPCA, categorical principal components analysis
ChE, cholesteryl ester
CI, confidence interval
CM, Chinese medicine
COX, cyclooxygenase
CRP, C-reactive protein
csDMARDs, conventional synthetic disease-modifying antirheumatic drugs
CYP450, cytochrome P450
CUDA, 1-cyclohexyluriedo-3-dodecanoic acid
DAS28, disease activity score based on a 28-joint count
DG, diglyceride
DHAP, dihydroxyacetone phosphate
DMARDs, disease-modifying antirheumatic drugs
ESR, erythrocyte sedimentation rate
ETN, Etanercept
EULAR, European League Against Rheumatism

F-6-P, fructose 6-phosphate
FA, fatty acid
FC, forced classification
G-6-P, glucose 6-phosphate
GADP, glyceraldehyde 3-phosphate
GC, glucocorticoid
GC/MS, chromatography coupled to mass spectrometry
GEE, generalized estimating equations
GI-3-P, glycerol 3-phosphate
GlcA, glucuronic acid
GUS, β -glucuronidase
HbA1c, glycated hemoglobin
HCQ, hydroxychloroquine
HDL-C, high-density lipoprotein cholesterol
ISTD, internal standard
IsoPs, isoprostanes
IQR, interquartile range
LC, liquid chromatography
LDL-C, low-density lipoprotein cholesterol
LEF, leflunomide
LLE, liquid-liquid extraction
LOD, limitation of detection
LOOCV, leave-one-out cross-validation
LOX, lipoxygenase
LPA, lysophosphatidic acid
LPC, lysophosphatidylcholine
LPE, lysophosphatidylethanolamine
MetS, metabolic syndrome
MG, monolinoleoglycerol
MS, mass spectrometry

MUG, 4-Methylumbelliferyl β -D-glucopyranoside
MTX, methotrexate
NO₂-FA, nitro- fatty acids
NSAID, Non-steroidal anti-inflammatory drug
NPV, negative predictive value
PA, phosphatidic acid
PC, phosphatidylcholine
PCA, Principal component analysis
PG, prostaglandin
PK/PD,
Pharmacokinetics/pharmacodynamics
PPV, positive predictive value
QC, quality control
R6, Rehmannia Six Formula
RA, rheumatoid arthritis
RF, rheumatoid factor
RNS, reactive nitrogen species
ROC, receiver operator characteristic
ROS, reactive oxygen species
SD, Standard deviation
SJC, 28 swollen joint count
SPM, sphingomyelin
T-C, total cholesterol
TCA, tricarboxylic acid
TG, triglyceride
TJC, 28 tender joint count
TNFi, tumor necrosis factor inhibitor
VAS-GH, visual analogue scale on general health

Samenvatting
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Samenvatting

Chronische aandoeningen zijn de belangrijkste oorzaak van sterfte en een verminderde kwaliteit van leven. Tegelijkertijd nemen de kosten van de zorg laatste decennia snel toe. Hierdoor is er een grote urgentie voor het vinden van nieuwe en betere strategieën voor het aanpakken van chronische aandoeningen. De complexiteit van de mechanismen rond het ontstaan en de progressie van chronische aandoeningen hebben de ontwikkeling van effectieve behandelwijzen geremd. Zo reageert slechts ongeveer een derde van de reumatoïde artritis (RA) patiënten positief op een behandeling met biologicals. Een meer persoonlijke benadering is daarom nodig voor mensen met chronische aandoeningen. In dit proefschrift wordt onderzoek gepresenteerd waarbij een systemische diagnose ingezet is voor het vinden van persoonlijke interventies. Metabolomics en diagnostische concepten uit de Chinese geneeskunde zijn gebruikt als een systeemdiagnose voor het evalueren en voorspellen van de respons op medicatie in patiënten met een chronische aandoening, met de nadruk op reumatoïde artritis.

In **Hoofdstuk 2** is de voorspellende waarde onderzocht van een baseline serum metabolomicsprofiel voor de respons op een behandeling met TNF- α inhibitors (TNFi). Een grote verscheidenheid aan metabolieten werd gemeten (lipiden, oxylipiden en amines), waarvan de vier metabolieten sn1-LPC(18:3- ω 3/ ω 6), sn1-LPC(15:0), ethanolamine, en lysine een significant toegevoegde voorspellende waarde lieten zien boven de klinische parameters op de therapeutische respons. De toevoeging van de metabolieten verbeterde het voorspellen van non-responders met 30% bij patiënten die TNFi kregen (n=124). Dit is de eerste studie waarin serum metabolomics profielen gemeten met LC-MS de respons op een behandeling met biologicals kon voorspellen in reumatoïde artritis. Daarnaast werden de relaties tussen de metabolomicsprofielen en de ziekteactiviteit onderzocht van RA patiënten die startten met een TNFi of een niet-TNFi behandeling (n=231). 88 Metabolieten (o.a. lysophospholipids, vetzuren, oxylipinen, en amines) bleken significant gerelateerd te zijn aan relevante parameters voor ziekteactiviteit. In dit hoofdstuk wordt ook de invloed van aanvullende medicijnen gebruik op de metabolieten profielen gerapporteerd. In **Hoofdstuk 5** wordt in nog meer detail gekeken naar het effect van glucocorticoïden (GC's).

Naast bloed is urine een biologische matrix die veel verzameld wordt in klinische studies, omdat urine metabolieten profielen inzicht geven in de afvalproducten van het lichaam. In **Hoofdstuk 3** wordt een lever- β -glucuronidase hydrolysering als monstervoorbewerking-smethode beschreven voor een LC-MS platform voor het meten van het totale geoxideerde lipidenprofiel in urine. Dit profiel bestaat uit vrije en geconjugeerde geoxideerde lipiden,

waaronder de klassen prostaglandinen, isoprostanen, dihydroxy-vetzuren, hydroxy-vetzuren en nitro-vetzuren. Geoxideerde lipiden die in zowel bloed als urine kunnen worden gedetecteerd, spelen een cruciale rol in belangrijke fysiologische processen in RA. Omdat geoxideerde lipiden slechts kort in de systemische circulatie zijn, is het gemakkelijker om de afbraak producten ervan in urine te meten. De geoxideerde lipiden methode is ontwikkeld en gevalideerd voor menselijke urine en werd vervolgens toegepast op 80 urinemonsters van RA patiënten die net met een TNFi behandeling waren begonnen. Helaas kon er geen metabolietenprofiel gevonden worden die een betrouwbare voorspelling kon doen over behandel-effect. Wel werd een positieve associatie gevonden tussen de pro-inflammatoire mediators PGF2 α en PGF3 α , de oxidatieve stress markers iPF2 α IV, 11-HETE en 14-HDoHE, en een verbetering op de DAS28. Bovendien werd een negatieve associatie gevonden tussen de anti-inflammatoire nitro-vetzuren en baseline DAS28. De nieuw ontwikkelde methodologie is een uitbreiding op de bestaande metabolomics-methoden voor geoxideerde lipiden in urine. De toepassingen ervan zullen het begrip van de rol van deze bioactieve verbindingen in gezondheid en ziekte vergroten.

De toegevoegde waarde van diagnostische principes gebaseerd op Chinese geneeskunde voor het subtyperen van RA patiënten in patiënten die wel of niet goed reageren op biologicals werd onderzocht. In **Hoofdstuk 4** werden zowel Westerse als in de Chinese diagnostiek bekende symptomen gebruikt voor het classificeren van RA patiënten (n=61). In de Chinese geneeskunde is de indeling van patiënten in Koude en Hitte typen essentieel. Deze typen worden vastgesteld door het uitvragen van de bijbehorende symptomen door middel van een vragenlijst. De combinatie van de baseline symptomen ('voorkeur voor warm eten', 'mate van zwakke pezen') en klinische parameters (reumafactor/ anti-cyclic citrullinated peptide antibody positief, C-reactive protein en creatinine) gaf de beste positief voorspellende waarde voor response (82.35%) met een misclassificatie percentage van 24.59%. Het toevoegen van Chinese symptomen aan de klinische data liet een verbetering zien in het classificeren van de responders van 8.30%.

Naast het voorspellen van het aanslaan van een therapie en het monitoren van ziekteactiviteit is metabolomics ook een krachtig instrument voor het monitoren van reacties op medicatie gedurende een interventie. Het kan bijdragen aan een beter begrip van werkingsmechanismen van medicijnen en van mogelijke bijwerkingen. In **Hoofdstuk 2** werd gerapporteerd dat bepaalde aanvullende medicijnen een invloed hebben op metabolietenprofielen. De effecten van glucocorticoiden op serum lipidenprofielen van RA patiënten werd onderzocht en wordt beschreven in **Hoofdstuk 5**. Na correctie voor potentiële confounders werd een significant hoger lysophospholipide niveau gevonden bij de vrouwelijke GC gebruikers vergeleken met

de vrouwelijke niet-GC gebruikers, terwijl bij de mannelijke GC en niet-GC gebruikers dit niveau gelijk was. Als één van de componenten van geoxideerd LDL zou lysophospholipide gerelateerd kunnen zijn aan cardiovasculair risico. De gevonden verschillen in lysophospholipide-niveaus tussen mannen en vrouwen zouden daarom relevant kunnen zijn voor cardiovasculaire risico's in RA patiënten en eventueel kunnen leiden tot aangepaste behandelstrategieën voor mannen en vrouwen.

In het **laatste hoofdstuk** van dit proefschrift werd metabolomics gecombineerd met Chinese en Westerse diagnostische kenmerken tot een systeemdiagnose, waarmee gedurende 8 weken de effecten van twee kruidenformules op kenmerken van metaboolsyndroom patiënten werden gevolgd. Metabool syndroom is een chronische ziekte, die sterk geassocieerd is met verhoogd risico op cardiovasculaire aandoeningen en diabetes. Een gerandomiseerde gecontroleerde studie met 40 metaboolsyndroom patiënten werd uitgevoerd om de effecten van twee *Rehmanniae Radix* formules (R6 en SUB889) te vergelijken. Serum metabolomics profielen werden gemeten met een gericht LC-MS platform en een globaal GC-MS platform, gecombineerd met Chinese diagnose (Qi/Yin deficiëntie vastgesteld door ervaren Chinese artsen) en Westerse diagnostische kenmerken. R6 (n=20) en SUB889 (n=20) bleken vergelijkbare effecten op metaboolsyndroom te hebben wat betreft verbeteringen op Westerse klinische parameters (buikomvang, BMI, LDL en systolische bloeddruk) en Chinese kenmerken (Qi/Yin deficiëntie). Echter op het niveau van metabolietenprofielen werden verschillende effecten gevonden tussen de twee formules. In de R6 groep gingen niveaus van cholesterylesters, fosfatidylcholinen, triglyceriden en sphingomyelinen naar beneden, terwijl in de SUB889 groep tricarboxylic zuurcyclus en glucose metabolisme-intermediären (malaat, fumarat en pyruvaat) verlaagd waren. Dus beide formules lieten vergelijkbare klinische effecten op metaboolsyndroom zien, waarbij R6 een positief effect had op het lipiden profiel vergeleken met SUB889.

Het combineren van systeembenaderingen met moderne Westerse technologieën kan bijdragen aan een meer gedetailleerde systeemdiagnose van complexe chronische aandoeningen. Systeemdiagnose is een krachtig instrument voor het voorspellen van respons op een therapie en het monitoren van ziekteactiviteit. Daarnaast kan systeemdiagnose worden ingezet voor het monitoren van effecten van medicijnen en leefstijlinterventies. De ontwikkeling van nieuwe metabolomics technieken gecombineerd met symptoompatronen uit de Chinese diagnostiek biedt nieuwe mogelijkheden voor de ontwikkeling van persoonlijke therapieën en zorg.

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Curriculum Vitae

Junzeng Fu was born on February 26th, 1987 in Zibo, P. R. China. After graduating from Zibo No.4 Middle school of Shandong Province in 2002, she studied Biotechnology in Shandong Normal University and got her bachelor degree in 2006. In the same year, she started her master study under the supervision of Prof. dr. Guoan Luo and Prof. dr. Gang Bai in Department of pharmacology, Nankai University, majoring in pharmacokinetics of Qishenyiqi dropping pills in rats. After finishing her master study, in November 2012 she started her Ph.D. in Analytical Biosciences, Leiden Academic Center for Drug Research, Leiden University. From 2012 to 2016, she studied personalized medicine for patients with chronic diseases, especially rheumatoid arthritis.

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