Systems diagnosis in chronic disease: prediction and evaluation
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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. 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. 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 ω3/ω6), 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 $\text{iPF}_{2\alpha}$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 diabetes. 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 findings 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.

References

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
Gl-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
List of Abbreviations

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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
Gl-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, lysoosphatidic acid
LPC, lysoosphatidylcholine
LPE, lysoosphatidylethanolamine
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