Systems diagnosis in chronic disease: prediction and evaluation
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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. In the past decades, the number of patients with chronic diseases and the cost of treatment increased rapidly. 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. In low- and middle-income countries, the accumulated cost for chronic diseases is estimated to be US$ 7 trillion in the period 2011-2025. 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. 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. 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. 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.

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. At present, the aetiology of RA is still unknown, while knowledge of the pathogenesis of RA is increasing. 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.

Rheumatoid arthritis related clinical measurements

A complex interaction of genetic and environmental factors is involved in RA. 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 1981 20. 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 mortality^{22}. 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 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 RA^{26}. However, the use of GCs is a double-edged sword—RA patients can benefit from the anti-inflammatory effects and slowing down of radiographic progression^{27} 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 RA^{30}. The mechanism behind these associations still needs to be elucidated further.

For patients not responding to the csDMARDs, bDMARDs are the second-line strategy^{25}. 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
month \(^3\). 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.

Table 1. Overview of biological agents used in the treatment of RA in Europe \(^3\).

<table>
<thead>
<tr>
<th>Tumour necrosis factor inhibitor</th>
<th>Full name</th>
<th>Structure</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infliximab (^a)</td>
<td>Chimeric human-murine monoclonal antibody</td>
<td>Block TNF(\alpha), prevent it from binding TNF(\alpha) receptor</td>
<td></td>
</tr>
<tr>
<td>Adalimumab (^b)</td>
<td>Recombinant human monoclonal antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golimumab (^b)</td>
<td>Human monoclonal antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Certolizumab pegol (^b)</td>
<td>Recombinant PEGylated humanized antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etanercept (^b)</td>
<td>Soluble TNF(\alpha) receptor-Fc of human IgG1 fusion protein</td>
<td>Block soluble and membrane-bound TNF(\alpha), lymphotoxin molecule</td>
<td></td>
</tr>
</tbody>
</table>

| 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 \(^a\) | Fe 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 costs \(^6\). In daily clinical practice, it would be ideal if a physician could tailor therapy based on the predicted therapeutic response by evaluating measurable 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 TNF\(\gamma\)-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. 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. Several studies have shown that positive RF and/or ACPA status were associated with positive therapeutic response. In addition, the endogenous TNF levels were found to be positively associated with response to infliximab and adalimumab. 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 (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. 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. 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. 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. From a systems perspective, the dynamic changes in time of biomarker patterns could contribute to the further understanding of the development of diseases. This approach may ultimately lead to the identification of new personalized markers that can predict treatment response for an individual.
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**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. High-throughput techniques, such as nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), have been widely applied in metabolomics studies. Especially MS-based platforms, which are capable of detecting picomolar concentrations, have been more extensively used for screening comprehensive metabolite profiles. Nowadays, MS has therefore become the most widely used technique and is applied in the characterization and diagnosis of various diseases.

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. Metabolites are regarded as downstream products of the genome, representing the interactions between gene-regulation and the environment of a dynamic living system. Metabolomic profiling could therefore provide a more direct readout of the physiological status of an individual when compared to genomics and proteomics analysis. 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. 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 compounds. 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 laboratories. Targeted metabolomics methods often produce quantitative results by including structural or isotope-labelled analogues as ISTDs as well as an established calibration model per metabolite. 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 model. 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 response. 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. 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. 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’, which is similar to the principles of systems biology. Diagnosis and treatment strategies are the two most important components of CM. According to CM-based diagnostic principles, symptoms and signs are recorded from patients by practitioners.
through in-depth observation, listening, questioning and pulse analyses. 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 medicine. Generally, a herbal formula contains several herbs, functioning through numerous active compounds targeting on multiple pathways. 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 effects. Chinese medicine is capable of phenotyping patients based on captured patterns and providing a very personalized treatment strategy. 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). 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 conditions. 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-syndromes, 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, etc. For each pattern of RA, there is a corresponding CM treatment strategy. 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. A large literature mining study suggests that Cold type of diseases are related to hormone disturbances whereas immune systems disturbances are Heat type related. 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
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. 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. 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). Principle components analysis is most widely used as a tool to analyse correlations between variables in complex numeric datasets, identifying potential phenotypes. 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. 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. 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). In addition, CATPCA with forced classification could also be used to identify relevant biomarkers for pre-defined groups of subjects, similar to PLSDA.

Multivariate logistic or linear regression models are frequently applied to predict outcomes in clinical trials. 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.
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 focuses on the relationships between variables. Additionally, sophisticated multivariate statistics provides more opportunities for identifying causal relationships\textsuperscript{89}. 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, oxylipin 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\textsuperscript{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.

References

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

Based on Junzeng Fu*, Bart V.J. Cuppen*, Herman A. van Wietmarschen, Amy C. Harms, Slavik Koval, Anne C.A. Marijnissen, Judith J.W. Peeters, Johannes W.J. Bijlsma, Janneke Tekstra, Jacob M. van Laar, Thomas Hankemeier, Floris P.J.G. Lafeber, and Jan van der Greef


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