A systems approach to sub-typing of rheumatoid arthritis

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PhD thesis with summary in Dutch

ISBN: 978-90-9027182-8

Printed by Wöhrmann Print Service, Zutphen, the Netherlands, 2012

Cover art was produced by Mike Thompson and Susana Camara for the AquaVita project, a joint project between the artists and the Netherlands Metabolomics Centre. A metabolomics analysis was carried out on 45 days worth of urine samples of both Susana and Mike. With this 'metabolic painting' the pattern of the body is imagined in time through strokes, similar to Chinese calligraphy.

A systems approach to sub-typing of Rheumatoid Arthritis

PROEFSCHRIFT

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P. F. van der Heijden

volgens besluit van het College voor Promoties

ter verdediging op 18 december 2012

klokke 12:30 uur

door

Hermann Adriaan van Wietmarschen

geboren te Oss in 1974

Promotiecommissie

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1. Introduction

Western medicine is based on a wide variety of disease management strategies that are employed with great success. For instance, the mortality of heart disease, stroke and cancer patients has dropped dramatically (Jemal 2009, Eheman 2012). Still, there is much room for improvement in various areas of medicine and care. Recently, within the American medical system medical interventions have become the leading cause of death with over 750.000 iatrogenic deaths each year (Null 2003). Alan Roses, past vice president of GlaxoSmithKline, reported in 2003 that 90% of the drugs work in only 30-50% of the patients. This means that large numbers of people receive medication that doesn't contribute to their health, leading to further hospitalizations due to adverse drug reactions and side effects.

Current Western drug development is based on the golden standard of randomized placebo controlled double-blind clinical trials. This type of study design is only suitable to discover average drug effects over large, homogenous groups of patients. However, when a drug enters the market it is largely unknown whether the drug is effective or toxic in the individual patient. On the other hand, drug candidates that might be very beneficial to a certain individual, will not reach the market when the average effect does not rise above a placebo effect in a large group of patients. In reality, drugs are developed for a statistically average patient which in reality doesn't exist, and not for the individual patient.

Personalized medicine

Personalized medicine has appeared on the horizon as a new drug development strategy, which aims to get the right drug, to the right patient, at the right time, in the right dosage, etc (van der Greef 2006) (Figure 1). The development of this type of medicine requires an entirely new way of conducting clinical trials and evaluating efficacy and effectiveness (Witt 2009). The effectiveness of drugs can be improved enormously when they are targeted to the right patients, and especially when they are developed for a specific sub-group of patients. Personalized medicine can only be developed in combination with proper diagnostic tools that are essential for matching individuals with a specific drug combination or treatment. This topic is extensively discussed in Chapter 2.

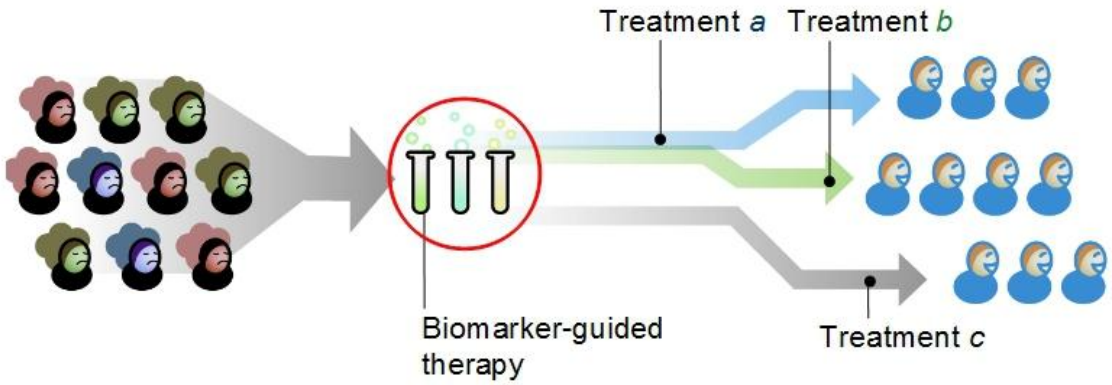

Figure 1. The concept of personalized medicine. Extensive diagnosis is the key for recognizing sub-groups of patients (differently colored unhappy characters on the left side of the figure). Medication is optimized for specific patient groups and eventually for the individual patient.

Better diagnostic tools can lead to better disease prevention strategies, and eventually to health promotion strategies. Health promotion is a largely unexplored area of science that for instance aims at improving the resilience of the system, and has the potential to offer enormous opportunities for improving quality of life and preventing diseases (Lindström 2005). Health promotion contrasts with current disease management strategies, which are mostly aimed at suppressing certain symptoms such as lowering cholesterol levels, reducing inflammation by suppressing the immune system, using insulin to control glucose levels, etc.

An intriguing challenge for scientists is how to measure health and health promotion. One step in this direction is the use of challenge tests such as the oral glucose tolerance test, exercise, or a high fat diet in healthy volunteers to measure the resilience of the system (Wopereis 2009). Not surprisingly, health is a very personal condition, each of us having to do with certain genetic predispositions, a constitution with certain strong and weak points, and more or less effective acquired behavioral patterns. Health can be promoted by certain life style changes that are generally good for everyone such as having a healthy diet and having a minimum of daily exercise. However, major improvements will be made possible by a personal targeted set of life style behaviors closely matched with the personal condition. Consequently, detailed diagnosis of the system is essential for the development of both personalized medicine as well as health promotion. Diagnosis is the topic of this thesis.

Systems thinking, systems biology & systems diagnosis

In the currently popular disease management mode of the health care system, people mostly wait to seek help until some serious symptoms are observed. For instance, when the pancreas function has already dropped dramatically, elevated blood glucose levels are detected, and diabetes type 2 can be diagnosed. This underlines that the body is quiet good at keeping processes within certain well-controlled boundaries without generating feelings of illness. This is called homeostasis. However, it might well be the case that other parts of the body are compensating for loss of function, while no changes in blood parameters are measured yet. In a disease management mode, it will be very hard to detect and prevent movements of the system towards unhealthy states.

It becomes increasingly clear that many chronic diseases are characterized by a combination of disturbances in various systems, such as the immune system, the autonomic nervous system, the gut microbiome and mental processes (Irwin 2011, Sterling 2011). For instance, deregulation of the cortisol circadian rhythm due to shift work or sleep disturbance has been found to increase cortisol levels in certain tissues, which can lead to immune system disturbances and diseases such as metabolic syndrome, cardiometabolic diseases and stroke (Cutolo 2012). In rheumatoid arthritis the role of gut microbiota in relation to joint problems 'the gut-joint axis' is becoming more and more important (Scher 2011). Studies such as these show that systems thinking, considering the interactions between various systems in the body, is essential for understanding complex diseases.

Systems thinking requires a shift in focus from objects to relationships. The quality of the organization of the system is more important than the quantity of the separate objects. At higher levels of the organization new properties emerge that cannot be observed at a lower level. For instance, cells working together in an organization called a liver show specific liver properties, which cannot be observed in the liver cells individually.

Current prevention strategies are generally based on treatment of populations at risk. For instance, the tendency is to prescribe statins for individuals with a 10 percent increased risk for cardiovascular diseases. This means that 90 percent of those individuals are receiving medication but will never develop the disease. However, these individuals might experience side effects. On top of that, statins work in only 25 percent of the patients and compliance is usually not very high, resulting in only one or two out of a hundred patients who would benefit from this prevention strategy. This leads to a medicalization of the society. Systems thinking is needed to design strategies for detecting early stages of disturbances of the body,

and finding the people who can really benefit from the drug. Changes in the relationships between processes are much more likely to be predictive of unhealthy conditions than single biomarker concentrations.

Systems biology is a biology-based interdisciplinary field that studies complex interactions in biological systems. Systems biology is based on the comprehensive measurement of processes for which a large variety of measurement techniques have been developed, ranging from genomics, proteomics and metabolomics to physiological measurements such as heart rate variability, blood pressure, ultra-weak photon emission and clinical chemistry parameters. Following the measurements, the data needs to be processed and analyzed, which requires specific statistical tools and approaches that deal with large numbers of features or variables compared to samples (Bijlsma 2006). Principal component analysis (PCA) is a key tool that is employed to search for the main sources of variation in datasets in which the variables are linearly related. Because most processes in nature are nonlinear, it is often more suitable to employ nonlinear principal component analysis techniques (Meulman 2003). Often unsupervised methods such as PCA are followed by supervised analysis methods. Classification and prediction of groups can be accomplished by applying for example partial least squares discriminant analysis (PLS-DA) (Barker 2003). Prediction based on large numbers of variables is susceptible to over-fitting (Westerhuis 2008), which requires extensive validation strategies such as double cross-validation and permutation testing (Hendriks 2007). The final step in the systems biology pipeline consists of the interpretation of the data analysis results. This non-trivial step usually involves extensive literature search, functional enrichment tools for -omics data and network analysis (Calvano 2005, Cline 2007, Bouwman 2012). The history, developments, and future perspectives in the field of metabolomics are discussed in Chapter 3.

The complex interactions in living systems, and between such systems and the environment are at the core of Chinese medicine. Chinese medicine evolved as a systems science with an emphasis on describing instead of explaining, which is the emphasis of the Western sciences. The following quotation from the Neijijng Suwen, the oldest classical Chinese medical text written around 3000 years BC, beautifully captures Chinese medical thinking:

'Huang Di stated, “People and nature are inseparable. In nature the cyclical movement of the heavenly bodies produces atmospheric influences that exert control over the rhythms of the seasons and is responsible for change to the myriad living and nonliving things. These cycles are repeated endlessly with patterns of predictability, and yet simultaneously with a tendency

towards chaos. It is this chaos in the macrocosm that upsets the balance of the delicate ecology within people that produces disease." (Ni 1995)

The Chinese descriptive approach is actually another systems biology approach in which patterns of complex organization are described and treated. For instance, in Chinese medicine a headache by itself is not treated, because it is a single symptom without context. Only when headache appears in a combination with other symptoms such as irritability, dry eyes, painful menstruation and an oppressed feeling in the chest, the pattern acquires a meaning and can be treated. More or less stable patterns of symptoms are recognized. In fact Chinese diagnosis can be termed a systems diagnosis approach. Treatment of these patterns or syndromes is aiming for a change or shift towards another more favorable or healthy state of the system. This concept of health closely relates to the attractor and bifurcation concepts from complexity theory (see introduction texts by Steven Strogatz (Strogatz 2001) and James Bassingthwaighte (Bassingthwaighte 1994)).

Further development of personalized and preventive medicine will require both Western and Chinese system thinking approaches. The key issue is how to communicate between the two sciences and how to translate and integrate the advantages of both sciences into one new future systems science. This is a major issue explored in this thesis. Two symbols of systems biology are shown in Figure 2 to illustrate this challenge. On the left side is a network model of the interactions between genes, proteins and metabolites measured in an atherosclerosis rat model using modern -omics techniques. On the right side a Chinese functional description of the body is presented in which the persons and the relationships between them represent organ functions and the relationships between the organs. Both sides of the picture show regulatory systems and the organization of regulation. The descriptive approach is therefore a nice addition to the more detailed explanatory approach.

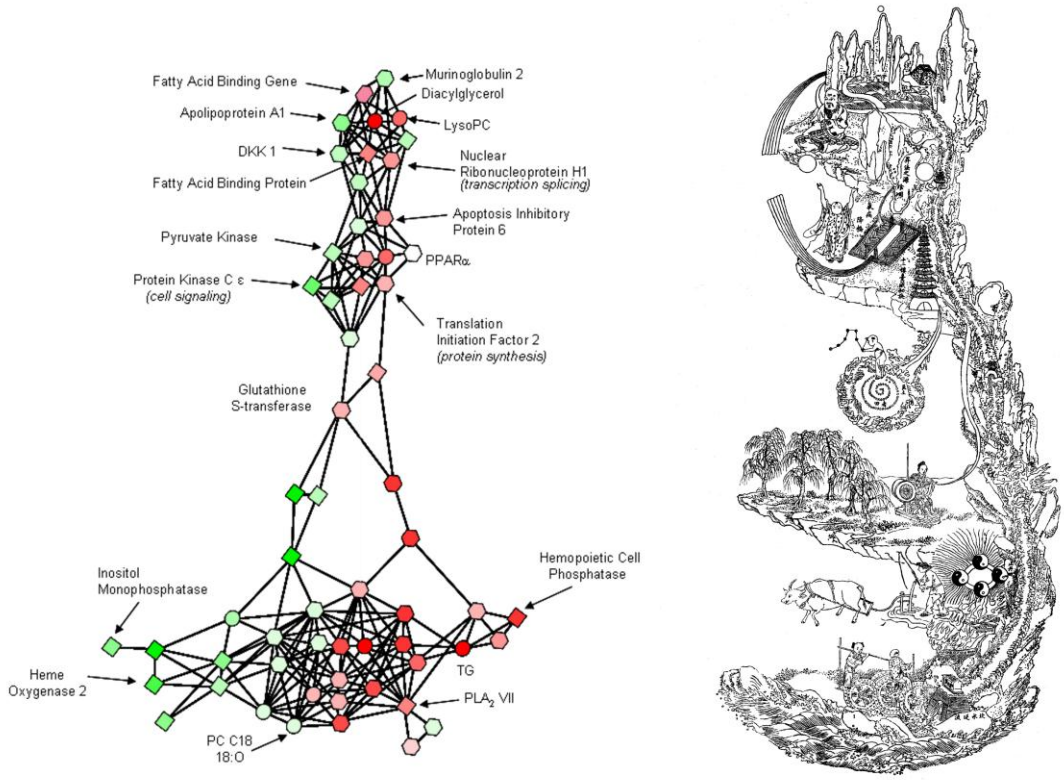

Figure 2. Illustration of systems thinking in Western science and the Chinese Taoist traditions. The left panel shows a network of correlations between metabolites, genes and proteins revealing the complex interactions related to the onset of atherosclerosis. The right panel shows the Neijing Tu, a chart of the inner landscape of the body from the White Clouds Taoist temple in Beijing. The Taoist school of Highest Clarity envisions the body as a complete world onto itself that is also a reflection of the world.

Sub-phenotyping approach for targeted care

In this thesis a step is taken in the direction of personalized medicine by integrating Chinese diagnosis with systems biology. The main focus of the research was to find relevant sub-types of rheumatoid arthritis (RA) patients. The patients in the studies were diagnosed according to the official American College of Rheumatology classification criteria for rheumatoid arthritis. On top of that a Chinese diagnosis was obtained for each patient and used for assigning patients to sub-groups. Systems biology, especially metabolomics and transcriptomics measurements were used to find biological differences between these sub-groups of patients.

The next step was to design new studies to test the clinical relevance of the possibly newly discovered sub-groups of RA patients.

Several sub-types of RA patients have been identified based on particular clinical and molecular features (van Baarsen 2010, van der Pouw Kraan 2007). Factors such as disease duration and age have been identified that predict response to treatment (Anderson 2000, Wolfe 1991). Although some molecular markers have been found to predict functional and structural outcomes, these markers rarely find their way into clinical practice. One reason is the difficulty to translate markers found in trial populations to routinely measurable and cost-effective predictors for individuals (Conaghan 2011). Another reason is that the discovery of novel relevant sub-types of RA patients is hampered by the unsupervised nature of the currently employed research strategies. Without prior knowledge it becomes very hard to sift through large amounts of gene expression, proteomics and metabolite information and come up with useful models and interpretations. This indicates that there is a need to develop new robust and reliable clinically applicable tools to identify sub-types of patients for rheumatoid arthritis.

Outline of the thesis

Chapter 2 gives an in-depth exploration of how personalized medicine can be envisioned and developed. Diagnosis is discussed as a key concept in this endeavor. A further integration of systems thinking into the life sciences and medical sciences is proposed aiming to further develop a systems science. Additionally, the value of Chinese systems thinking for personalized medicine is extensively discussed and methods for integration with Western science are described.

In Chapter 3 metabolomics as a key systems biology technology is reviewed in the light of 30 years of metabolomics at TNO. The chapter shows how metabolomics started out from a technology push that turned into a biology pull around the year 2000. A large variety of metabolomics applications that have been developed over 30 years illustrates this. The review is concluded with future perspectives for the metabolomics field and how the field can contribute to changing views on health care and healing.

Chapter 4 provides deeper insights in metabolic processes that are related to rheumatoid arthritis. This literature overview attempts to provide a comprehensive view of these processes that have been described over the last several decades. Subsequently functional

enrichment tools are used to integrate the information and find the dominant biological processes.

In Chapter 5 the first clinical study is described in which two sub-types of rheumatoid arthritis patients are found based on a Chinese diagnosis. The two sub-types of patients are characterized by gene expression profiles measured in CD4+ T-cells and by a GC-MS metabolomics analysis of plasma. This chapter provides the first evidence for biological differences between two sub-types of RA patients based on a combination of Western and Chinese diagnosis.

Chapter 6 describes an important step following the study presented in the previous chapter, namely the standardization of the Chinese diagnosis used to sub-type the patients. This step involves the development and application of a systems diagnosis questionnaire that allows the collection of extensive symptom patterns of arthritis patients. These symptom patterns are explored for 49 patients who filled in the questionnaire.

In Chapter 7 a more extended clinical study is described in which the same two sub-types of rheumatoid arthritis patients are explored. In this study the patients are classified by a Chinese medicine expert, but in addition the systems diagnosis questionnaire was completed by the patients as well. Extensive untargeted LC-MS metabolomics measurements were performed in addition to standard clinical chemistry measurements to characterize the sub-types of patients.

Finally, in Chapter 8 the contribution of this work towards personalized medicine is discussed. In addition ideas for future studies that could follow from this work are presented.

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2. Systems biology-based diagnostic principles as the pillars of the bridge between Chinese and Western medicine

Abstract

Innovative systems approaches to develop medicine and health care are emerging from the integration of Chinese and Western medicine strategies, philosophies and practices. The two medical systems are highly complementary as the reductionist aspects of Western medicine are favorable in acute disease situations and the holistic aspects of Chinese medicine offer more opportunities in chronic conditions and for prevention. In this article we argue that diagnosis plays a key role in building the bridge between Chinese and Western medicine. Recent advances in the study of health, healing, placebo effects and patient physician interactions will be discussed pointing out the development of a system-based diagnosis. Especially a systems biology based diagnosis can be used to capture phenotype information, leading towards a scientific basis for a more refined patient characterization, new diagnostic tools and personalized heath strategies. Sub-typing of rheumatoid arthritis patients based on Chinese diagnostic principles is discussed as an example. New insights from this process of integrating Western and Chinese medicine will pave the way for a patient-centred health care ecosystem.

Based on: van der Greef J, van Wietmarschen H, Schroën J, Wang M, Hankemeier T, Xu G (2010) Systems biology-based diagnostic principles as pillars of the bridge between Chinese and Western medicine. *Planta medica* 76(17): 2036-47.

Introduction

The health care system in Western societies and one of its pillars, modern medicine, are increasingly under debate. The increasing costs of health care are reaching the level where it will not be sustainable in the near future. Recent estimates for the US health care system show an increase from 17% of the gross domestic product (GDP) in 2009 to over 20% of the GDP in 2018, when the total cost will reach 4.35 trillion USD [1]. At the same time, hopes for developing medicines that are more effective are fading away, since the pharmaceutical industry produces fewer new chemical entities (NCE's) that reach the market every year.

In this era, crucial global issues have surfaced in various domains, including the financial, ecological, political, educational, religious/spiritual, and social realms. Mechanisms that previously functioned effectively must be replaced by new approaches. These challenges are not local, but global, and system thinking will be mandatory in the future. In such times of change, one must reflect and focus on the driving force of the system that needs to evolve. In health care, there must be a shift from an emphasis on economics, technological developments and political agendas back to a patient-centred health care environment.

In health care, the paradigm of system thinking has emerged as systems biology. It is limiting to develop interventions on how a single compound interacts with single target that is linked to a specific symptom. This "one-drug-fits-all" paradigm has shifted to an idea that patients require personalized medicines. Some successful examples have been used to treat cancer, including Herceptin® (trastuzumab), Gleevec® (imatinib mesylaat) and Iressa® (gefitinib). However, a thorough systems approach has not yet been applied to design new drugs. Moreover, the patient is not yet seen as a unique individual. Treatments focus on a disease or a disease phenotype rather than the person, even though effective treatments rely on a proper systems diagnosis.

In this paper, we describe how the global nature of challenges must be recognized to develop global solutions. These solutions must integrate diverse concepts and intuition to bring about a new understanding. Our discussion begins with a reflection on how Chinese and Western Medicine evolved over time. In the second step, we consider the diagnosis as the basis in each medical system, and then we consider new ways of merging knowledge from both systems. This information is used to provide a deeper insight into personalized health diagnostics, and we envision the development of a future system. This type of process requires multiple steps. As an example, we use rheumatoid arthritis (RA) to describe how to

diagnose and ultimately establish a personalized health care ecosystem.

Bridging Chinese and Western medicine: a philosophical and historical view

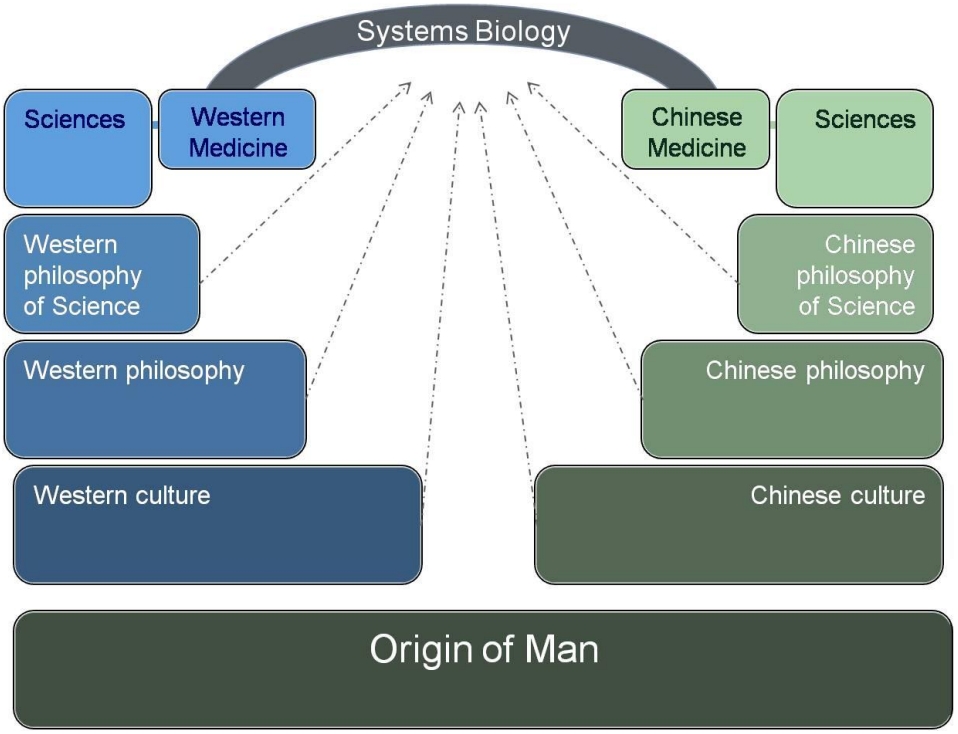

Figure 1. Systems biology as a bridge between Chinese and Western medicine. As migration moved populations apart in space and time, the Western and Chinese cultures evolve separately, giving rise to specific cultural philosophies. These philosophies gave rise to philosophies of science, which led to types of science. This evolution allowed different types of science to exist alongside one another (Figure 1) [2].

Medicine has been practiced since prehistoric times, and over time, a variety of medical traditions developed. In ancient Greece, biological theories were focused on solving one problem, that of the relationship between the "one" and the "many." This union between the many phenomena formed nature, which was rationalized in the theory of the four humours. In Hippocrates' and Galen's times, disease was still perceived as a natural or physiological process, and diagnoses were made by knowing this story of transformation [3]. Centuries later, Paracelsus developed a new concept of disease based on pathological, anatomical and metabolic changes, which resulted in a new diagnostic practice that was based on the classification of diseases [4]. Thus, the perspective of disease as a process and a relation

between the one and the many shifted towards viewing disease as a structure located in an individual.

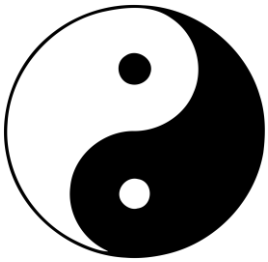

Figure 2. Tai Ji symbol

Chinese medicine developed in a Taoist, Buddhist and Confucian culture. Many of the key aspects of Chinese medicine are explored in one of the oldest classical Chinese texts, the Yi Jing, a book that is still considered essential when studying and practising Chinese medicine today [5]. The Yi Jing presents what we would today refer to as a systems view of life, health and society. This view is represented beautifully in the Tai Ji symbol (Figure 2). In the Tai Ji symbol, Yin (female, earth) and Yang (male, heaven) grasp each other but are also present within each other, as represented by the small dots. The Tai Ji symbol is the oldest known example of fractal thinking because the small dot of Yin within Yang is actually another Tai Ji symbol [6]. This relates to the view expressed in the Yi Jing that the world inside the body is a reflection of and thus connected with the world outside the body (Figure 3). In the classical text on Chinese medicine Neijing Suwen, Qi Bo answered the Yellow Emperor saying, 'The most important element in clinical diagnosis is to know the relationships between heaven, earth and humankind' [7]. Both the body and the world are complex, dynamic systems with fractal relationships to one another. In a healthy situation, Yin and Yang are in balance. This concept of balance, movement and connection was developed further in the Zang Fu (organ theory) and Five Phases theories.

Although the ancient Greek and Chinese ideas on health and medicine show many similarities, the variations in cultures and philosophies gave rise to the differences between the medical systems that we perceive today [2]. For instance, during the 17th century, Descartes proposed that there was a division between the mind and body, and this idea had an enormous impact on Western science and medicine. Western science evolved into an objective methodology where the scientist explained and analysed, but aimed not to be part of the studied object. Subjects related to matter became the province of science, while subjects

related to the mind and spirit entered the domain of religion [8]. Physicians were educated to treat patients as material systems, or physical machines, with medications and interventions for physical problems [9]. Illnesses and diseases were perceived as being located in the patient, rather than being embedded in networks, relationships, the environment and culture.

As a result, the modern Western scientific method was used to gather a tremendous amount of knowledge about, anatomy, physiology, cells, genes, proteins, metabolites , etc. Over the last decades, various thinkers, who were interested in how all of these aspects worked together, began combining theories from many disciplines into a systems view on life. One intriguing example is the Santiago theory of cognition in which the mind-body dualism is overcome by identifying cognition, the process of knowing, with the process of life and the body as the material structure to accommodate this process. Mental activity or the mind is an organizing function of living systems which is connected and interacting with the body, which plays a more structural role in living systems [10]. Additionally, the concept of health is shifting back to interpretations that are more comprehensive. For instance, the concept of salutogenesis defines health in terms of movement and connection [11].The capacity to move in a health-promoting direction is determined by comprehensibility, meaningfulness and manageability [12].

The systems approach to medicine that is now developing in the West has the potential to integrate with Chinese medicine [13]. In particular, the systems biology approach of patient profiling using modern genomics, proteomics and metabolomics technologies [14,15] is a perfect match for the systems diagnosis in Chinese medicine. Integration of these approaches may reveal different groupings or sub-phenotypes of patients, which require different treatments. Additionally, knowledge about the biological mechanisms behind the personalized herbal formulas used in Chinese medicine is expanding through modern herbal chemical profiling techniques [16,17].

Figure 3 illustrates the Western systems biology view of the body and the Chinese view of the body as a landscape [15]. The correlation network in the left panel shows the relationships between metabolites, proteins and genes during an early stage of atherosclerosis. In the right panel, a drawing illustrates the relationships within the body. By zooming in elements can be found that have a relationship with Western perspectives. For example, the fire in the cauldron in the lower part in Figure 3 is called MingMen (the gate of life), which can be compared to the adrenal glands. MingMen catalyzes and supports processes in the body, such as temperature and metabolism, which is similar to adrenal hormone function.

Both Chinese and Western science describe life as a complex, dynamic, non-linear system. As Western scientists discovered the non-linear behavior of cytokine networks, Chinese practitioners have recognized non-linear patterns in how symptoms change in patients [18]. The fractal properties of the arteries, lungs and heart rate resemble the fractal thinking in Chinese medicine [19]. In both sciences, life is considered a self-organizing system that is far from equilibrium. Systems thinking can build the cultural, philosophical and scientific bridges that are necessary to share understanding between the two sciences. By studying tools and techniques developed in both the Chinese and Western medical systems, new insights will emerge that are necessary to heal the patient, his environment, and the world he lives in.

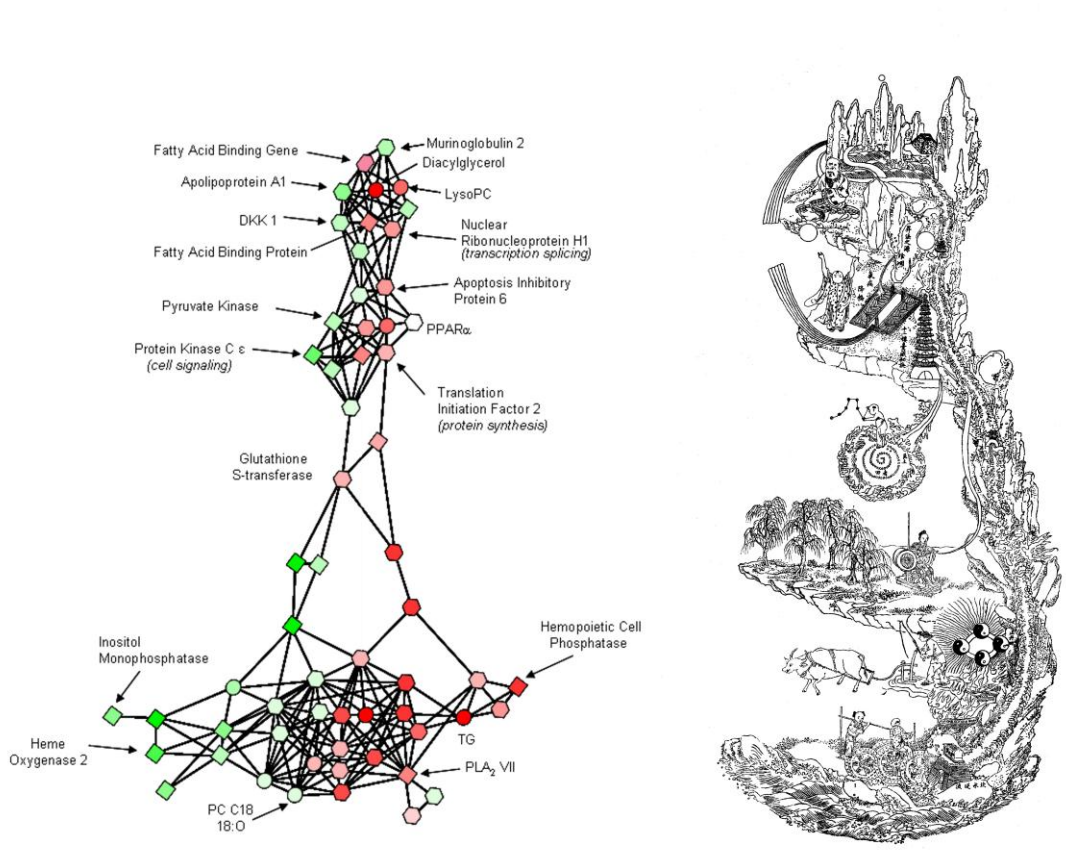

Figure 3. Illustration of systems thinking in Western science and in the Chinese Taoist traditions. The left panel shows a network of correlations between metabolites, genes and proteins revealing the complex interactions related to the onset of atherosclerosis. The right panel shows the Neijing Tu, a chart of the inner landscape of the body from the White Clouds Taoist temple in Beijing. The Taoist school of Highest Clarity envisions the body as a complete world onto itself that is also a reflection of the world.

Both Western systems biology and Chinese Taoist science consider the complete body and its relationships and connections to its environment. These similarities bring the two sciences closer together. Essentially, the world inside and outside the body are the same. Mapping relationship patterns has become an important strategy in modern systems biology research. For instance, advanced -omics technologies can be used to reveal diagnostic insights or indicate the impact of interventions [14].

Traditionally, the focus of the systems view in Chinese medicine has been directed towards health promotion. In recent Western terminology, this is known as strengthening the resilience of the homeostatic process, or salutogenesis. Typically, Western medicine has focused on disease management.

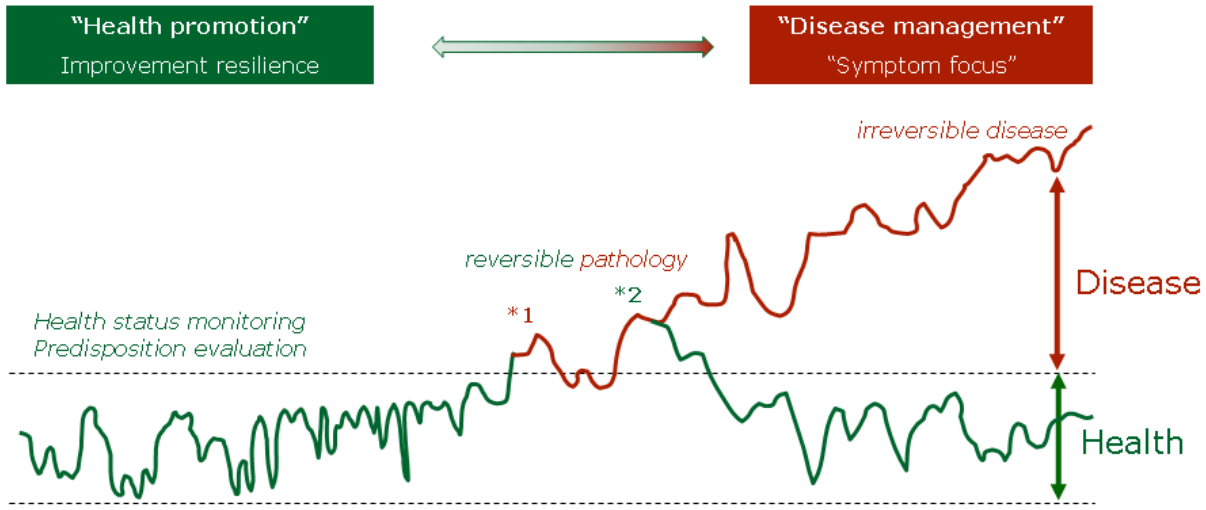

Figure 4. The schematic representation of a dynamic system losing its homeostasis and developing into a disease state. The effects of (*1) a reductionist intervention and (*2) a system intervention are illustrated.

Figure 4 illustrates a healthy dynamic situation that loses its control capability (homeostasis) and develops into a disease state [20]. The schematic is oversimplified, since the border between health and disease depends on the individual and may not always be clear. Moreover, the process is multidimensional, so the two-dimensional representation is limited for evaluation. The chart represents a typical readout if the diagnostic tool for monitoring health is based on a reductionist principle, such as blood pressure or glucose level measurements. When the system is incapable of maintaining health, a disease state occurs. In this scheme, a reductionist intervention (*1) can be applied to reduce the given symptom. While the reductionist intervention may appear promising, when the medication is stopped

the system cannot maintain control, and the disease state recurs [21]. In contrast, application of a system treatment (*2) allows the system to regain its resilience. Eventually, when the treatment is stopped, the system remains within the healthy range. Instead of monitoring a single variable, use of a system dynamics approach is more effective, which is noted especially in chronic conditions or during prevention strategies. In acute states, reductionist approaches can be very effective for releasing the immediate danger; however, strengthening the system provides a powerful additive value. For instance, during chemotherapy, Chinese medicine can protect or reduce potential side effects and strengthen immune function.

Therefore, Figure 4 illustrates the bridge between Chinese and Western medicine shown in Figure 1 from a different angle. In Figure 4, major emphasis from Western medicine is on disease management while the major emphasis on health promotion comes from Chinese medicine.

System-based diagnosis, building the bridge

Today, the term diagnosis has a wide variety of meanings depending on its context. The word originated from a combination of the Greek words “dia-,” meaning "apart” or “split" and “gnosi,” meaning "to learn” or “knowledge." Therefore, a diagnosis is related to a recognizable separation (or split) from the whole. In a medical context, a diagnosis is typically used to describe a certain condition or syndrome that is characterized by a combination of specific features that occur simultaneously and vary from “normal” functioning. A diagnosis can lead to the recognition of a specific disease or syndrome based on a group of symptoms or signs that characterize an “abnormal” condition. In Greek, the word “symptom” refers to a feeling of misfortune that has befallen a patient. The experience of confronting disease symptoms varies substantially in different cultures and in people with different worldviews, as discussed below. A differentiation can be made between symptoms that are described by the patient (subjective) and signs that are observed or measured by others (objective). For instance, a symptom could include pain sensations, and a sign could include glucose or cholesterol levels.

From a system view, there is an implicit danger while making a diagnosis because by characterizing a person using a selection of symptoms and signs and emphasizing the difference from what is believed to be normal or healthy, there is a loss of context information. As mentioned previously, this is one of the major differences between Chinese

and Western medicine. In Western medicine, the basis for developing therapeutic interventions often involves reducing a generic observation of a condition to the lowest number of possible combinations of symptoms and signs. For instance, type 2 diabetes is diagnosed by one sign, a person's glucose levels. As such, Western medicine has become successful in reducing symptomatic effects, which is relevant in acute or relatively threatening conditions. Corrective treatments focus on specific aspects rather than stimulating or guiding endogenous (homeostatic) regulatory processes, which is typical in systems-based approaches. The reductionist approach aims for simplified diagnosis-therapy protocols that have become the basis for quality management in health care.

This focus has advantages, but it also has clear limitations, which are evident in patients with chronic diseases or in situations requiring personalized medical approaches. Previously, these limitations were discussed from a systems perspective for drug discovery and drug development [22] and from a personalized medicine perspective [23]. At present, a clear diagnostic differentiation combined with a specific therapy has become a new driving force known as companion diagnostics or theranostics. This approach led to improvements in specific situations, such as cancer therapy, but it still follows a reductionist path as its basis. This trend will certainly continue in the coming years, and we will discuss this in detail below. However, to understand the limitations of this process and to reveal the unaddressed potential in these cases, we will first examine the diagnosis process in more detail.

A general schematic illustrating patient-provider interactions from a system's view can serve as the basis for understanding a number of important issues (Figure 5). The first step in the diagnosis process is recognition by a person that his/her ability to function in daily life is limited or challenged. These limitations may include a variety of physical and psychological features. In fact, a subjective shift in consciousness has already emerged that something is wrong, which is often followed by a feeling of fear that the condition will deteriorate in the future. Our thinking patterns often gravitate towards envisioning a future that is different from what we think is desired or essential (thinking based on pre-conceived ideas), and this is a first step towards becoming unhappy and stressed [24]. The next step is typically an appointment with a general practitioner. During the appointment, which is schematically depicted in Figure 5, many things happen simultaneously that have distinct influences on the diagnostic process.

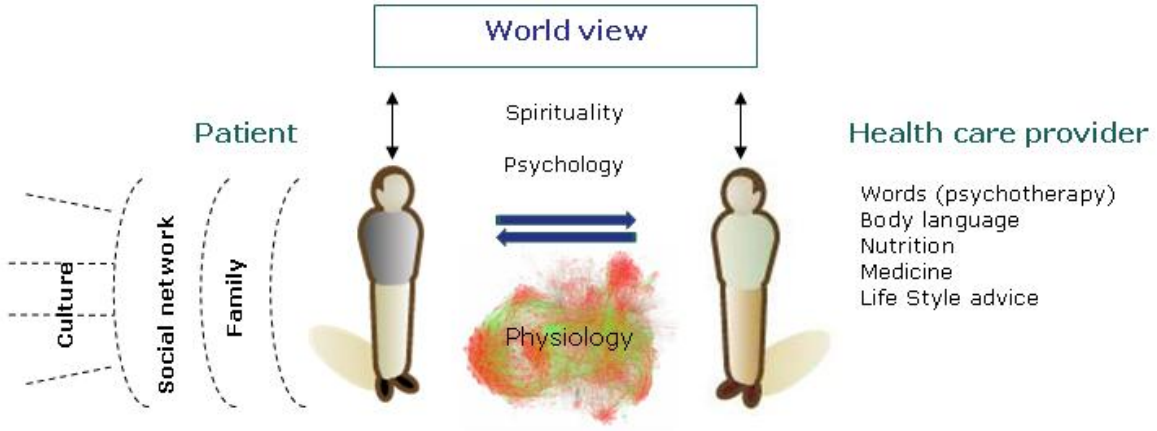

Figure 5. Schematic illustration of the interaction between a patient and a health care provider. The quality of the connection is of key importance for the diagnostic and therapeutic process.

Figure 5 reflects the essence of the meeting between the patient and physician and the importance of the connectivity for exchanging information. Typically, a physician will ask questions or invite the patient to explain his or her motivation for visiting. In the mean time, body language and intuitive processes are active. The process of anamnesis has begun, and the physician collects information that is sometimes combined with a physical examination, during which symptoms and signs are gathered. It is important to note that by asking questions, the consciousness and the awareness of the patient is already influenced and changed. Therefore, therapy and personal awareness has already begun.

The information collected relates not only to the patient's status at that point in time in isolation, but also to the person's social network, culture and history. These latter aspects are of key importance in the systemic view to fully understanding the patient. In fact, we should consider a biosphere that includes both the patient and the physician [25,26]. The physicians' biosphere must have the ability to communicate and the worldview (spirituality) of the patient and physician play a major role in this process. Although not the focus of this paper, we would like to emphasize here that the diagnostic process will follow different routes depending on different worldviews. For instance, when the belief is that a "misfortune" happened to the patient, this then leads to an intervention (physician-driven, corrective action). In contrast, if the situation is viewed as an "exploration" where decisions are made together about what the next step should be in the emerging future, then the patient accepts responsibility for his consciousness (reflective and process orientated) . Here the interaction is reflective and process-orientated, and the physician acts more like a coach.

In the anamnesis process in Western medicine, the physician provides direct advice to the patient or refers the patient to a medical specialist for a more detailed investigation. This process leads towards more reductionism in many cases. Additionally, the physician may prescribe or suggest an intervention with medicine. A clear shift has taken place in recent years, and medical advice has begun to comprise medicine along with suggestions for lifestyle changes, such as fitness, mental training and nutrition. This reflects a shift towards a more systemic approach, recalling the original Greek definition of the word "anamnesis," which means "remembering who you are" (in context of the whole).

Let us now examine how a broader diagnostic view can cause shifts in the development of modern medicine. Modern Western medicine is based on a reductionist view. It involves studying aspects or portions of physiology, while ignoring most of the influences of psychology or spirituality, with the exception of psychiatry.

In recent decades, this way of thinking has become more balanced. Much of this change in perception is due to placebo research. The placebo effect has been heavily debated for many years. The effect is typically considered as an annoyance in scientific medical research, since it hampers the ability to analyze the "pure" pharmacodynamic effects of a drug under development. The expression, "placebo effect," reflects the feeling that it is something unscientific and without value.

Even in surgical studies, placebo effects have been reported. Pain reduction experiments with "hidden treatments," demonstrated that the placebo effect is much smaller when patients were unaware that they are receiving medication. In depression research, placebo effects can account for at least 75% of the observed positive effects of medications [27,28]. According to Wayne Jonas (Placebo Symposium, Nov. 27-28 2009, Starnberg), placebo effects are influenced by: (1) pill colour, (2) number of pills or treatments, (3) compliance with treatment, (4) packet label, (5) form of treatment (pill, needle, heat, injection, laser), (6) location of the treatment (home, hospital or optimal healing environment (OHE)), (7) order or administration of treatments, (8) tone of treatment delivery and the authority of the physician, (9) information provided with delivery, (10) inherent social and cultural factors, (11) effectiveness of the drug, (12) effectiveness of other drugs with similar uses, and (13) combinations of the above factors.

In summary, placebo responses are observed frequently in clinical trials, and they vary considerably [29,30]. Therefore, it can be difficult to prove that the effectiveness of a drug is

superior to a placebo. When a cause or variable has such a strong effect on the clinical trials for developing medicines, the factor should be extensively studied. For the placebo effect, this is especially important, since the effect significantly affects both diagnostic and therapeutic medical practices. Additionally, a reverse placebo effect called the “nocebo effect” (Latin for "I will harm," and named by Walter Kennedy in 1961) has also been reported. The nocebo effect indicates a pessimistic view of a medicine under investigation.

In a recent placebo workshop (Starnberg 2009, Nov. 27-28 2009), Jonas argued that “for a better understanding we need to disentangle the “placebo effect” as the physiological, psychological and clinical effects of meaning and context (MAC) and learning” [31]. This is mandatory before taking the next step towards a systemic approach in health care.

To understand the placebo effect, scientists must separately study the effect of the placebo substance (ex. sugar) and the effect of administering the placebo (psychological responses induced by the health care environment). Often the natural cause of a disease is also taken into account. From a systemic perspective, this encompasses the movement of the whole, including psychological and spiritual factors. At this point, it is important to recall the split between body and mind in Western sciences, which was introduced by Descartes. This split led to the design of scientific experiments to reveal “pure” medication effects, which excluded the possible effect of the observer. This concept is now changing as the systemic view has emerged.

It is beyond the scope of this paper to go into further detail on placebo effects. We recommend that the interested reader consult the following authors as an entry to the placebo field: Fabrizio, Wallach, Kolls and Jonas. Additionally, we recommend exploring the references cited in the following papers: [32,33,34,35,36,37,29,38].

Following diagnosis, the relationship between the patient and the physician is crucial. The person’s worldview, including their family, social network, culture, and history, are necessary for context, as shown in Figure 5. Furthermore, the physiological network depicted in the figure is a reflection of the system response to all of these effects. In systems biology studies, this network is not always appreciated, and sources of unknown variation are not linked to other important effects. The abilities of a physician to connect to the world of the patient and use intuition and authority are extremely important. Despite the efficiency and cost-control pressures on medical health care systems today, excellent physicians are still able to use these skills. In modern medical education systems, relatively little, if any, attention is aimed at understanding the whole patient.

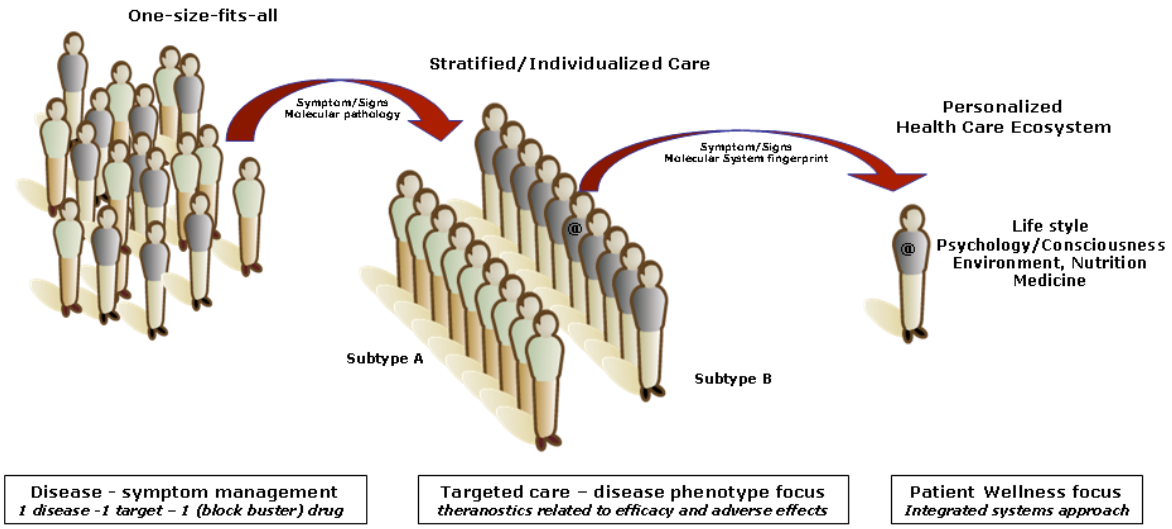

Figure 6. The steps from the current Western "one-drug-fits-all" concept to targeted care and eventually to a personalized health care system.

What does this mean for diagnostic strategies? From Figure 5, it is clear that from a systems view it is essential to consider the interactions of the person with his environment as a whole. The diagnosis is a description of a person at a given point in time, knowing the person's history and addressing his current condition, which is expressed as a disease. People with different cultural backgrounds will have different views, and consequently have different needs. The aim of the diagnosis is to find a way to support a person by relieving him from disabling factors or making him aware of necessary lifestyle changes, including changes in psychological thinking patterns.

In Chinese medicine, diagnosis is highly related to the above contextual description. A person is seen as unique, but also as part of a whole. Both the patient and the doctor grow during their interaction in their knowledge of life and its purpose. The modern technologies developed in Western medicine have become valuable for obtaining a deep insight into the physiological mechanisms of disease. Chinese medicine can be enriched enormously by adding this wealth of new diagnostic aspects. The systems biology technology has allowed for an additional bridge between the "seen and the unseen." In fact, the current situation is rich in opportunities. Descriptive diagnosis can be linked to a systems-based medical system. Additionally, technological capabilities have opened up new insights into biology. The next step in modern medicine will form from a combination of both strategies in a synergy of

global knowledge.

Taking this step requires that health care systems offer solutions that include patient participation. Whenever possible, the patient should remain responsible for his own health, and the health care process should follow a counselling trajectory. In a way, it can be compared to the modern view of how organizational changes can be guided through a deeper meaning (identity, soul or source of the organization) and not via a shortcut to a solution. The ultimate connection and quality of the relationship between those involved in the process is highly relevant, and a deeper understanding arises from reflection upon the whole. This approach, called "presencing," is expressed with other current management insights, such as Theory U [39]. These theories link to spiritual literature, such as the Chinese books of wisdom, the Tao Te Ching (The Book of the Way and its Virtue). Spiritual books are a source of inspiration, and the timelessness of the wisdom serves to help us discover ourselves and the purpose and meaning of our life. For instance, Lao Tzu (604 BC - 531 BC) said "If you do not change direction, you may end up where you are heading." This expression can be applied to health care as a reason to investigate current practice and envision the future. This will allow practical steps to be designed in a direction originating from current health care systems.

From a Western perspective, systems biology research can improve the "one-drug-fits-all" interventions based on reductionist diagnoses. In Figure 6, this is shown as the first step towards a more targeted approach in which subtype classes are recognized. Still the basis for this approach is disease-orientated; however, current medicines can be optimized for a better efficacy/safety ratio for a given subtype. The last and largest step towards personalized health care is diagnosis support to provide a patient-centred wellness approach. The health care ecosystem must fully utilize this potential for personalized medicine. To do so, a major driving force could be the combination therapy strategy outlined previously [40]. Chinese medicine is already based on a personalized approach, so improvements to this system must come from refining and expanding the current Western medicine options, focusing on quality control and providing scientific evidence to create a global acceptance of its practices outside Chinese culture.

In Western medicine, the step to improved subtyping (targeted care) can be strongly enhanced using knowledge from Chinese medicine. Currently, many studies are underway to detect subtypes by profiling cohorts with –omics technologies and systems biology platforms [14],

but these evaluations must be based on non-supervised procedures or drug response profiling for specific drugs. Gathering data from these methods is limited, since individual variation is high, as outlined above. Consequently, Chinese medicine is an attractive alternative to subtype cohorts diagnosed using Western diagnosis methods. For instance, rheumatoid arthritis (RA) or type 2 diabetes cohorts could be subtyped using pattern recognition techniques based on systems biology. This allows a subset of variables to be obtained and validated, which can serve as a new diagnostic principle when developing interventions. This strategy is the basis of the Sino-Dutch Centre for Preventive and Personalized Medicine. In the following example of subtyping in RA, we will demonstrate the strategy and describe the challenges in incorporating this method in practice.

Towards personalized medicine for rheumatoid arthritis

RA is the most common chronic, inflammatory joint disease, affecting approximately 0.5 -1% of the population worldwide [41,42]. The currently favoured treatment strategy focuses on reducing inflammation, and pain is generally believed to have a favourable effect on the disease course. However, assessments of disease activity and functional disability in patients do not always support this treatment strategy [43]. In addition, there is a high variability in treatment responses [44] For instance, nearly 30% of patients fail to respond positively to anti-TNF therapy, which is currently the most effective therapy for RA [45,46].

Predicting a patient's response to a treatment can help ensure that he receives the right treatment and minimizes the risk of side effects. Over the years, several clinical features and molecular markers have been identified that allow RA patients to be divided into subgroups that may respond differently to treatments [47,48].For example, van der Helm-van Mil and colleagues demonstrated that patients who were positive or negative for anti-citrullinated protein antibodies (ACPA) had distinctive RA risk profiles [49]. ACPA-positive RA patients had more inflamed joints and a higher level of joint destruction [50]. Van der Pouw Kraan and colleagues observed a large heterogeneity between the high and low INF-1 subtypes of RA patients in their gene expression profiles, but there was no clear difference between groups in terms of clinical features [51]. Unfortunately, the knowledge gained from these studies has not yet led to personalized health care strategies in clinical practice.

Since routine molecular markers and disease activity measurements have failed to predict treatment responses, patient phenotypes should be measured in more comprehensively.

Glocker and others advocate use of a systems biology approach to develop personalized medicine for RA [52,44]. This type of approach would simultaneously measure the interactions between genes, proteins, metabolites and symptoms [40,22,23]. The resulting patient profiles can then be used to diagnose and classify patients.

Interestingly, there are several health and medical traditions that use a systems approach, including Chinese medicine. A key aspect of Chinese medical diagnosis is that symptoms are collected from the patient by the practitioner through inquiry. The symptoms and signs deigned important for determining the correct treatment vary widely from the Western perspective. To diagnose RA, rheumatologists use seven criteria established by the American College of Rheumatology [53]. These criteria involve morning stiffness, arthritis of three or more joints, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, serum rheumatoid factor and radiographic changes of the joints. In contrast, the symptoms involved in the Chinese diagnosis include climate factors, emotional states, joint problems, types of pain, fever, swellings, digestive problems, and many others [54,55]. The Chinese diagnosis provides a more complete description of the entire body.

A second advantage in Chinese medicine is that its treatment strategies can be considered a systems biology approach. For thousands of years patients in China have been treated with personalized herbal formulas based on extensive diagnoses. Herbal formulas contain hundreds of compounds of which the function is often unknown. Interestingly, the combinations of compounds in herbal formulas work together to improve effectiveness and reduce side effects [13]. Additionally, an optimization strategy is often followed in which patients receive different or adapted herbal formulas after each consecutive consultation to improve the effectiveness or change the treatment target .

The differences in the health, disease and treatment perspectives between Western doctors and Chinese experts makes it difficult to simply transfer herbal treatments to the Western medical practice. Several herbal treatments for RA have been studied with ambiguous results because important subtype information was ignored. For example Tripterygium wilfordii Hook. F. extracts are commonly and successfully used as a Chinese medicine intervention strategy for RA, and the extract has been studied extensively [56,57]. Preparations of Ganoderma lucidum (Leyss. Ex Fr.) Karst, an extract of multiple herbs called San-Miao-Wan [58] Celastrus aculeatus Merr. [59] and Forsythia suspense (Thunb.) Vahl [60] have been used as immune modulators in RA patients. In these studies, the benefits of using standard Chinese medicine preparations in RA patients were not always clear. We believe that it is

necessary to develop tools to determine which subgroups of patients will benefit from specific Chinese medicines before they can be effectively introduced in Western clinical practice. The key issue here is diagnosis.

In a study of 396 RA patients, a combination of Tripterygium wilfordii Hook. F. with a Yishen Juanbi tablet was effective in patients affected with joint pain and joint tenderness, who did not experience increased urination at night and joint stiffness [61]. Additionally a Western treatment consisting of diclofenac, methotrexate and sulfasalazine was found to be more effective in RA patients displaying joint tenderness and thirst, and less effective in RA patients with dizziness [61].These results indicate that a Chinese medical diagnosis can be used to identify RA subtypes. Specifically, Chinese medical diagnoses may identify RA patient subtypes that have different responses to Western treatments or require different Chinese herbal treatments.

The next section explores the Chinese medical diagnosis of RA patients in further detail. In Chinese medicine theory, RA is part of a group of syndromes, called the “Bi-syndromes”. The character 'Bi' means blockage or obstruction. In Chinese medicine, this term is used to denote 'obstruction of Qi and blood in the channels and collaterals' [54]. These obstructions mainly lead to pain, numbness and stiffness. The “Bi-syndromes” include other Western diseases, such as osteoarthritis, frozen shoulder, repetitive strain injuries (RSI), other rheumatic diseases, such as fibromyalgia and systemic lupus erythematosus, as well as conditions like bursitisand synovitis [54].

“Bi-syndromes” are caused by an attack of three out of four external pathogenic factors called “Heat”, “Cold”, “Dampness” and “Wind”. After its first superficial stage, the disease can develop into later stages that can be classified according to the affected “Fu organs” or the affected “Five Tissues” [7] A Chinese medicinal expert can determine the type of “Bi-syndrome” a patient is suffering from by employing diagnostic methods, such as inquiry, palpation, taking the pulse and looking at the tongue. Symptoms and signs related to the “Bi-syndromes” are collected, resulting in a pattern of relationships that can be interpreted and treated.

Figure 7. Network visualization of the "Bi-syndromes" based on Vangermeersch [54]. The Cytoscape network algorithm was used to calculate a network view where the length of the edges were minimized [62]. The red hexagonal nodes represent the "Bi-syndromes", and the yellow circles represent the symptoms and signs related to the "Bi-syndromes". The green lines (edges) represent the relationships between symptoms and syndromes, and thicker lines represent stronger relationships.

We can think about the patterns of symptoms and the relationships between symptoms and syndromes in terms of Western network theories and visualization methods. In Figure 7, the patterns of symptoms and syndromes that make up the "Bi-syndromes" are visualized using Cytoscape [63]. It is immediately clear that the "Bi-syndromes" (red hexagons) are intricately related and connected by certain specific symptoms (yellow circles). In fact, the syndromes act as nodes within the dynamic network of symptoms. While a single symptom, such as dry mouth, does not point toward a syndrome, a combination of symptoms, such as dry mouth, a warm feeling and an aversion to heat, suggest that the patient has a "Heat syndrome". Some symptoms are related to multiple "Bi-syndromes" and can indicate a change in the disease from one state to another. These symptoms can be referred to as bridge symptoms or

bifurcation points. For example, a patient with side pain, thirst, headache and cold feet over time could develop a new symptom like lower back pain. This indicates that the disease might suddenly shift from one stable state to another. In this case, the shift is from a liver disorder to a kidney problem, which reflects a more chronic stage of the disease.

Although the Western concept of RA is very different from the “Bi-syndromes”, there are some connections between the two disease classifications. We compared the symptoms and signs of RA described in the Merck manual with those related to the “Bi-syndromes” [64]. Only four symptoms were shared in both classifications: stiff joints, swollen joints, fever and pain with redness and swelling. Additionally, we compared the symptoms and signs of other rheumatic diseases with the “Bi-syndrome” symptoms. Again, there were few overlapping symptoms. Figure 8 shows the symptoms and signs that connect the “Bi-syndromes” (red hexagons) with RA and osteoarthritis (OA) (blue hexagons). Additionally, the symptoms connecting the various “Bi-syndromes” are drawn, and the symptoms connecting RA with OA are depicted. For simplicity, the symptoms unique to single diseases were left out of the network. In Chinese medical theory, all the symptoms that are related to the “Bi-syndromes” can occur in RA patients and in patients with other rheumatic diseases. However, it proves to be difficult to match the symptoms and signs used in Chinese and Western medicine. For instance, only a few of the symptoms and signs described in Chinese medicine as being related to arthritis were listed in the Merck manual [64]. The additional symptoms and signs recognized in Chinese medicine might point towards patient subtypes.

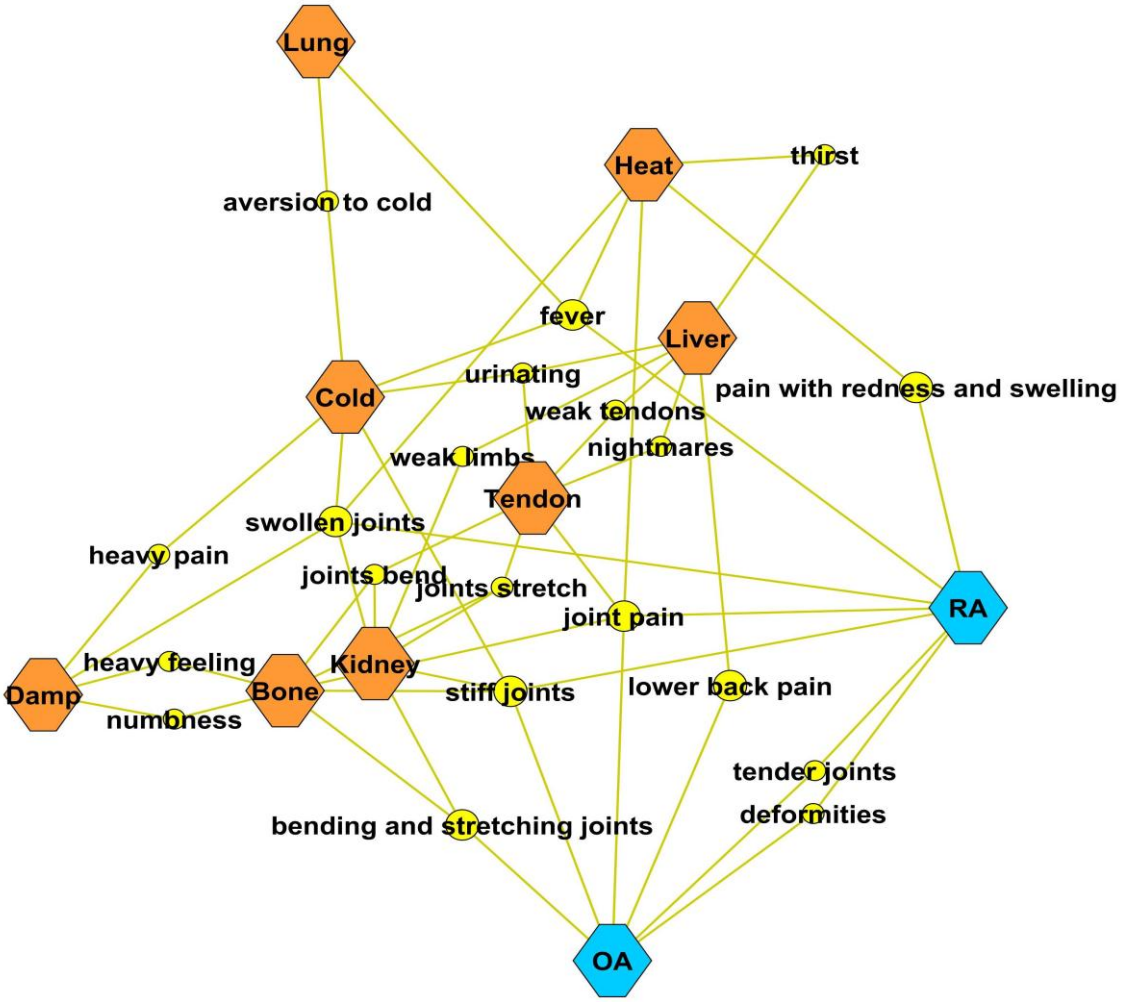

Figure 8. Network visualization of the connections between the "Bi-syndromes" (red hexagons) and RA and OA (blue hexagons). Only the symptoms (yellow nodes) that connect a "Bi-syndrome" with a Western rheumatic disease are labelled. For simplicity, the symptoms unique to single "Bi-syndromes" or diseases were left out of the network. Although in Chinese medical theory RA and OA are connected to all the symptoms of the "Bi-syndromes", only a few of these symptoms are recognized in Western medicine.

To test our hypothesis that a Chinese medicine diagnosis could point towards RA patient subtypes that can be interpreted with modern Western systems biology techniques, we designed a study that included 33 RA patients and 16 healthy volunteers. The patients were classified into "Cold" or "Heat" groups by a Chinese medicine expert, according to Chinese medical diagnosis. All patients had swollen and painful joints at the time of diagnosis and during collection of the blood samples. The blood samples were used to perform a gene expression analysis in CD4+ T-cells and for a broad-spectrum gas chromatography-mass

spectrometry (GC-MS) metabolomics analysis [65].

“Cold” and “Heat” were specifically chosen because these Chinese medical classifications are commonly used in Chinese medical schools. Therefore, there is little controversy over how to classify patients into the two categories although patients can express both “Cold” and “Heat” symptoms at the same time. According to Zhang and colleagues, approximately half of the RA patients in the general population fall into the “Cold” type while approximately 16% are classified as the “Heat” type [66]. We also selected these two groups because there are different Chinese herbal formulas for curing RA Cold and RA Heat patients. In this study, clearly defined “Cold” and “Heat” RA patients were asked to participate.

The “Cold” pattern can be described by severe pain in a joint or muscle that limits the range of comfortable movement with pain that does not move to other locations. The pain is relieved by applying warmth to the affected area, but increases with exposure to the cold. Loose stools are characteristic of this pattern, as well as clear profuse urine and an absence of thirst. A thin, white tongue coating is observable, combined with a wiry and tight pulse. In contrast, the “Heat” pattern is characterized by severe pain with hot, red, swollen and inflamed joints. Pain is generally relieved by applying cold to the joints. Other symptoms include fever, thirst, a flushed face, irritability, restlessness, constipation and deep-coloured urine. The tongue may be red with a yellow coating, and the pulse may be rapid [67].

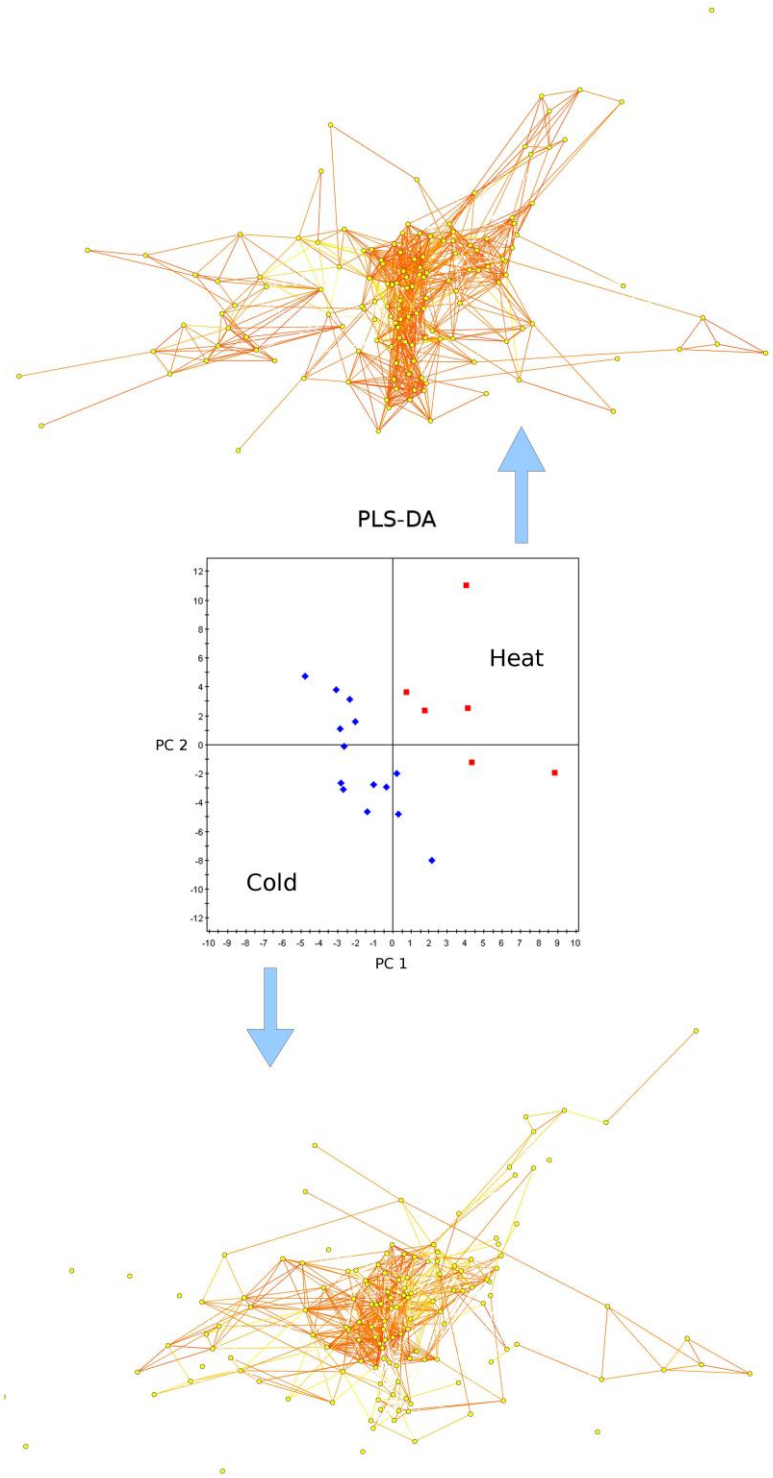

Figure 9. The centre panel shows a partial least square-discriminant analysis (PLS-DA) score plot in which all the RA patients are represented by a dot. The blue dots represent RA Cold patients, and the red dots represent RA Heat patients. The RA Cold patients and RA Heat patients are clearly separated. The PLS-DA model was validated using cross validation and permutation testing. Above and below the score plot are network views of correlations between blood plasma metabolites measured using GC-MS in the RA patients. The top network

shows correlations between metabolites >50% for RA Heat patients, and the bottom network shows correlations between metabolites >50% in the RA Cold patients. The nodes in both networks are the same and are composed of > 80% correlations in the RA Heat patients and > 80% correlations in the RA Cold patients. The topology of the network was generated using the Kamada algorithm, which was unweighed. The position of the nodes is equal in both networks, which means that the same metabolites (nodes) are represented in both networks, only the strength of the correlations are different.

This study revealed differences in gene expression profiles and metabolomics profiles between RA Cold and Heat patients (Figure 9 shows the metabolomics results). These differences involved apoptosis genes, and the RA Heat group showed more activity in apoptosis-stimulating genes than the RA Cold group. In contrast, several genes related to apoptosis resistance were more active in the RA Cold group than in the RA Heat group [65]. Thus, besides finding molecular differences between the two subgroups, there is also a biological explanation using Western chemical concepts that can explain the difference in subtypes that were distinguished through Chinese medical approaches.

Apparently, the Chinese concepts of “Heat” and “Cold” are relevant in understanding RA. A more general relationship between “Cold” and “Heat” and Western medicine was well described by Li and colleagues [68]. Using a database built from PubMed articles related to keywords Heat and Cold, the researchers calculated the co-citations of these keywords with hormones, cytokines and neurotransmitters. “Heat” symptoms were related more strongly with immune factors, while “Cold” symptoms were more strongly related to hormones. Neurotransmitters were equally related to “Cold” and “Heat” symptoms [68].

It is well known that patients with autoimmune diseases, including diabetes, RA and lupus, have autonomic nervous system dysfunctions [69].The autonomic nervous system – the sympathicus and parasympathicus – regulate basic physiologic processes, such as heart rate, blood pressure, respiratory rate, gastrointestinal motility and body temperature. Czura hypothesized that dysfunction of the cholinergic anti-inflammatory pathway may predispose some individuals to excessive inflammatory responses [70]. The cholinergic anti-inflammatory pathway normally provides a brake on the immune system that restrains cytokine production. If the brake becomes insufficient, due either to insensitivity to acetylcholine released by the pathway or to diminished signals in the pathway, cytokine responses can become excessive [70] Therefore, in RA, it is possible that there is both a disregulation of the autonomic nervous system and a disbalance in the immune system. Additionally, the extent to which each system is out of balance has relevance, which may be

understood by studying Chinese medicine diagnosis.

Li's study suggests that RA Cold patients suffer more from a hormone disbalance, and RA Heat patients suffer more from an immune system disbalance, although the two systems are closely related. Moreover, this suggests that RA Cold patients may respond better to a hormone treatment, such as corticosteroids, while RA Heat patients may benefit more from an immune related treatment, such as one of the new biologicals. Although this hypothesis has not yet been tested, this could be an excellent example of subtyping RA patients using Chinese medical diagnosis to personalize and improve the Western treatments for RA patients.

Conclusion and perspectives

During the last 10 years, systems thinking has been emerging in pharmaceutical and biomedical sciences. Consequently, a natural bridge can now be formed between holistic- and reductionist-focused philosophies. There are vast opportunities available through merging the Chinese and Western medical perspectives, since both are complementary to one another and each has its own merits. For example, reductionist strategies are favorable in acute (disease management) situations, while holistic approaches offer more opportunities for preventive (health promotion) and chronic conditions.

Figure 10. A composite photo made by merging two photographs [22] taken sequentially with different focal settings. The composite photo illustrates the wish to see both the whole and the details sharply in one glance

(holistic-systemic) in modern medicine.

In Figure 10, an analogy of the desired situation of combining holistic and reductionist perspectives is shown as a composite photo that displays a sharp view on the whole (moon) and the details (migrating cormorants). This view reflects the desire to merge the holistic and reductionist perspectives in health care. However, to create this image, two pictures were taken in sequence, each under different focal conditions, and then the two photos were merged. Today, such an image is impossible to obtain in a single shot even with advanced cameras, but the future may reveal new options in due time.

Diagnosis will play a major role in future developments, since it will serve as bridge between the different medical perspectives, and interventions can only be designed and exchanged based on a common diagnosis. The future points to an advanced personalized health care system in which personalized medicine is one of the key pillars. In Western medicine, the driving force is to improve the quality of life for patients as 90% of drugs work for only a limited number of patients. For instance, oncology medications have only a 35% response rate and the side effects are significant [71]. The economic resistance in leaving the blockbuster strategy has decreased, since the "one-drug-fits-all concept" is no longer a viable option. Combining subtyping and personalized medicine could bring more drugs into orphan drug status, which could be advantageous for drug development. When the efficacy/safety ratio improves, a higher return could be obtained for newly developed drugs within a smaller market. It is clear that combination therapy, the basic strategy in Chinese medicines [13] will create an important opportunity to generate the diversity needed for more personalized approaches.

Future trends in research and development will be more patient-centered, and the patient-physician relationship will be recognized more and more as a key element for providing the health care in the future. Health promotion and preventive approaches must be revised in Western medicine, since the development process is not geared towards health challenges where strengthening homeostasis is the key approach and subtle subtyping is the basis. Chinese medicine is likely to provide a variety of options based on the available diagnostic opportunities. In line with systems theory, the scale and complexity of a solution should match that of the problem. This theory indicates that combination therapy is an option, and diagnostic principles should utilize a personalized approach. Additionally, Chinese medicine can be used to strengthen the biological system, maintain a better basic health and reduce side

effects. These approaches can be developed further when Chinese-based diagnostic principles are scientifically proven and translated into biochemical fingerprints.

Systems biology-based diagnostics and particularly metabolomics will be essential in this merging process, since it captures relevant phenotype information. Studying the dynamics of systems might become mandatory to provide early, more preventive diagnoses. Health care is expected to change fundamentally in the next 25 years based on these new insights and the driving force from both the quality of life and health care cost perspectives. The Sino-Dutch Centre for Personalized and Preventive Medicine is contributing to these developments by stimulating education and through scientific research that applies metabolomics-based systems biology that is guided by the principles of Chinese medicine-based diagnostics. Disease subtyping is believed to improve the understanding of responder and non-responder challenges in Western medicine, to optimize cohort selection in clinical trials, and to underpin the current understanding of the combined use of Chinese and Western medicine in China. Most importantly, subtyping will provide a scientific basis to understand and globally integrate the complementary Chinese and Western medical systems.

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3. Looking back into the future: 30 years of metabolomics at TNO

Abstract

Metabolites have played an essential role in our understanding of life, health, and disease for thousands of years. This domain became much more important after the concept of metabolism was discovered. In the 1950s, mass spectrometry was coupled to chromatography and made the technique more application-oriented and allowed the development of new profiling technologies. Since 1980, TNO has performed system-based metabolic profiling of body fluids, and combined with pattern recognition has led to many discoveries and contributed to the field known as metabolomics and systems biology. This review describes the development of related concepts and applications at TNO in the biomedical, pharmaceutical, nutritional, and microbiological fields, and provides an outlook for the future.

Based on: van der Greef J, van Wietmarschen HA, van Ommen B, Verheij E (2012) Looking back into the future: 30 years of metabolomics at TNO. *Mass Spectrometry reviews*, submitted

Introduction to Metabolomics

Metabolites in ancient history

The word *metabolism* originates from the Greek “μεταβολισμός”, which means “change”. The concept of metabolism was mentioned by Ibn al-Nafis (1213-1288), who stated that “the body and its parts are in a continuous state of dissolution and nourishment, so they are inevitably undergoing permanent change.” Studies on individual changes during different daily activities were performed by Santorio in 1614 and were mentioned in his book *Ars de Statica Medecina* (Bing, 1971).

The measurement of single metabolites as a source of information related to health and disease has a long history that precedes the introduction of metabolomics and acronyms such as metabolomics, body fluid profiling, metanomics, metabonomics, metabolic profiling, etc. (Oliver et al., 1998; Lindon, Holmes, & Nicholson, 2003; Raamsdonk et al., 2001; Ramsden, 2009). The terminology is not relevant for the content. As stated by Juliet in Shakespeare’s Romeo and Juliet, *"What's in a name? That which we call a rose by any other name would smell as sweet"* (Shakespeare, 1594). From another perspective, metabolites have a long association with sweet flavors that extends to ancient times and predate the development of metabolic nomenclature. Ancient Chinese cultures (1500–2000 BC) recognized urine as an important source of health-related information and sweet-tasting urine as indicative of a disease (now known as diabetes). At that time, “clinical testing” involved actual urine tasting. Advanced “biosensor” ants could also be used to test differences between sample and reference urines. The association between sweet urine and disease was contemporaneously made in India by Ayurveda Hindus.

Diabetes as a disease state was described on 3rd Dynasty Egyptian papyri by the physician Hesy-Ra, who mentioned an additional symptom, frequent urination or polyuria. Approximately 1000 years ago, the Arabian physician Avicenna observed that an individual's urine changes during illness. In modern times, changes in the smell or color of urine are known to be related to changes in the concentration of chemical components and dysregulation of biochemical pathways that indicate certain metabolic diseases. For instance, blue urine can indicate Blue Diaper syndrome, caused by a defect in tryptophan absorption. Urine with a musty/mousey odor is suggestive of classical phenylketonuria (PKU), an autosomal recessive metabolic genetic disorder.

Metabolites in modern history (1900–present)

The discovery of enzymes by German chemist Eduard Buchner (1860–1917) at the beginning of the 20th century led to a focus on intracellular chemical reactions and inspired development of the field of biochemistry. The biochemical understanding of metabolism developed rapidly due to new insights into enzymatic reactions and intracellular biochemical pathways.

New technological developments in the early 20th century in the domain of mass spectrometry (MS) enabled researchers to measure the molecules involved in biochemical pathways, and to investigate their roles in disease states. As early as 1948, Williams and his associates identified important concepts of normality based on MS profiling of body fluids, and examined individual differences and pathologies in patients with alcoholism and schizophrenia. The correlation of emotional stress and physical exertions with urinary metabolite profiles were already emphasized by Ludwig et al. (Ludwig 1977). Gates and Sweeley performed a review of these early thoughts and experimental approaches (Gates & Sweeley, 1978). In their review, they mentioned some important hallmarks related to quantitative metabolic profiling. In particular, they referenced the works of Horning & Horning, who first introduced the concept of metabolic profiling by MS (Horning & Horning, 1971), and Pauling et al., who reported urine vapor and breath analyses with gas-liquid partition chromatography related to the effects of defined diet (Pauling et al., 1971).

To enable the routine measurement of molecules in biological matrices, researchers in the 1950s coupled gas chromatography (GC) with MS. Early commercial GC/MS instruments enabled practical clinical investigations and the measurement of profiles of urine and blood samples. Such profiles were typically limited to a specific class of compounds, such as organic acids, because the available technology required volatile components or compounds made volatile by chemical derivatization.

Opportunities for the direct application of GC/MS techniques came in the early 20th century. The British physician Sir Archibald Garrod (1857–1936) proposed the detection of changes in metabolic pathways caused by a single inherited gene defect. This concept became the basis for the field of inborn errors of metabolism. In clinical chemistry, body fluid profiles led to the discovery of numerous diseases related to single-gene defects (for more information, see reviews by Politzer, Dowty & Laseter, 1976, and Jellum, 1977). However, these types of

profiles were very limited from a systems perspective. Concentration changes in such genetic diseases are typically very large and more easily detected than changes in chronic diseases, in which time-dependent changes of regulatory processes must be observed. Consequently, there was a need for a broader characterization of systems and for sophisticated pattern-recognition tools to evaluate complex patterns.

In the 1970s, several developments occurred in The Netherlands that influenced research at TNO. Meuzelaar and Kistemaker developed an impressive pyrolysis-MS (PyMS) characterization methodology that allowed the pyrolyzation of bacteria and many other nonvolatile biomaterials (Meuzelaar & Kistemaker, 1973) . The resulting constituents were recorded as fingerprints and analyzed with multivariate statistical methods, such as nonlinear mapping and factor analysis (Windig, Kistemaker & Haverkamp, 1980). New multivariate tools became available in the field of GC profiling (Blomquist et al., 1979; McConnell et al., 1979; Rhodes et al., 1981). Although PyMS was a novel systems profiling method, it had some limitations: typically, only nonvolatile materials could be investigated, and thermal degradation products were not easily related to their precursor macromolecules. Despite these challenges, many successful investigations were achieved with PyMS in subsequent decades.

In the 1970s, the MS field expanded with the development of soft-ionization methods, such as field desorption and laser desorption (Schulten & Beckey, 1975) that enabled the analysis of low-volatile components. As part of his PhD project, under the guidance of Nico Nibbering at the University of Amsterdam, new electronics were developed to enable controlled desorption and reproducible biofluid profiling through field ionization kinetics/ field desorption (van der Greef, 1980). At the same time, new multivariate statistical tools became available in commercial packages such as Arthur (CPC, Seattle, WA, USA). However, interpretation of outputs from these tools was not straightforward; this time-period marked the beginning of the analysis of datasets with many more variables than objects. To illustrate this perspective, after a night of intense calculations, the Arthur program was known to return the following famous output: *"In controversial matters, my perception is rather fine. I always see both points of view: the one that's wrong and mine".*

Metabolomics at TNO

The body fluid profiling and pattern recognition project (early 1980s)

The developments of field desorption (FD) and the so-called emission-control device (van der Greef, 1980) overcame a major bottleneck in reproducible profiling of biofluid samples under controlled desorption conditions and presented a unique opportunity for researchers. In August 1980, the "body fluid profiling and pattern recognition" project was initiated at TNO, led by Jan van der Greef in the Instrumental Analysis group. Headed by Michael ten Noever de Brauw, these facilities held some of the most advanced MS capabilities worldwide at that time. The initial phase of the project involved construction of an ion source and an electronic control unit, equipped with photographic and electric detection, for the Varian 731 MS instrument. The design of the control unit was based on the original electronic design of Jim Dawson (University of Amsterdam).

The initial phase involved implementation of earlier designs, as well as production and successful operation of an automatic field-desorption emitter-production unit. The most complicated step was to interface the MS system with the PDP-8 computer because the high-voltage field-desorption ion source generated frequent sparking events. Software was developed to acquire, normalize, and import data to the Arthur software package for multivariate analysis. The multidisciplinary core team for this project included Jan van der Greef, Leo Bergman (electronics), Jaap Bouwman (computer hardware and software), Albert Tas (pattern recognition), and Michael ten Noever de Brauw (MS). An MS machine equipped with a field desorption source and emission-control electronics (van der Greef & Nibbering, 1977) coupled to computer data acquisition (Varian 620i, SS100 datasystem) with the Arthur package became operational in mid-1981 (Figure 1). This setup would be the basis for further technological developments at TNO in the years to come.

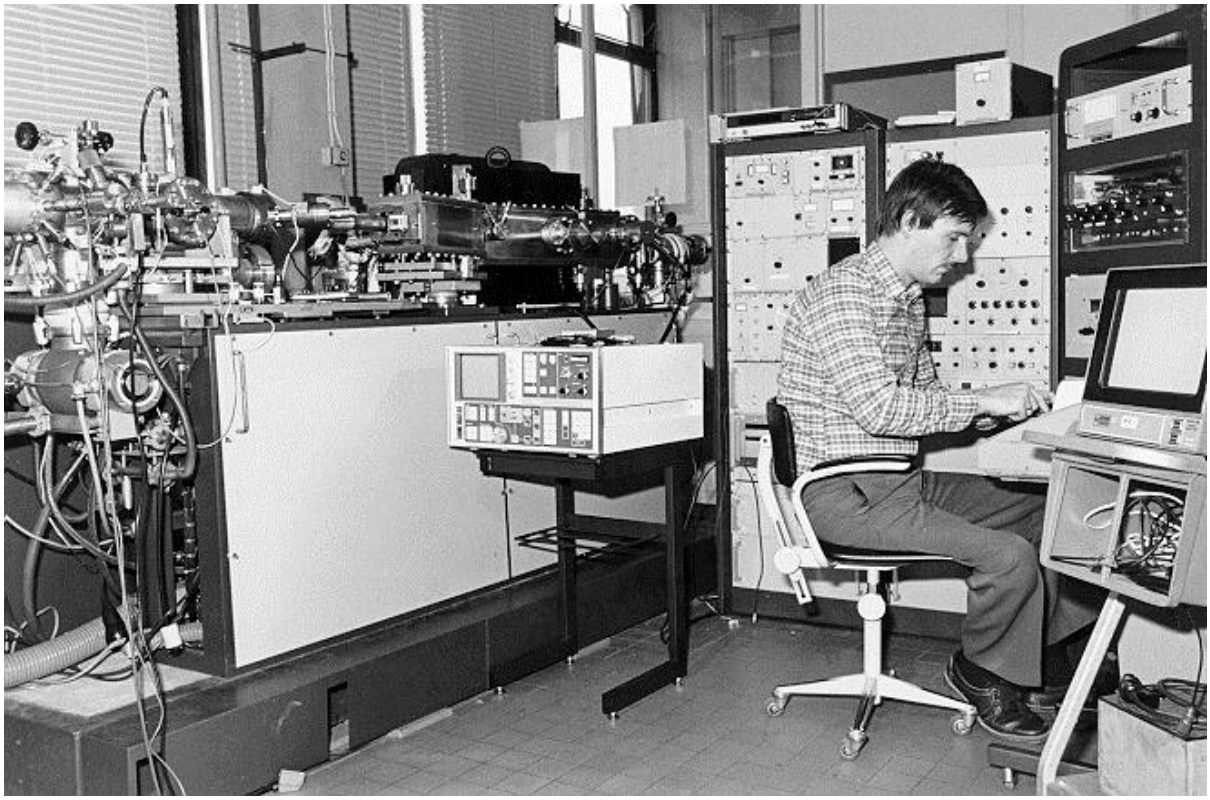

Figure 1. The first "body fluid & profiling" (metabolomics) instrument constructed at TNO in 1981. The instrument consisted of a Varian MAT 731 mass spectrometer equipped with a field desorption source and coupled to a 12-bit PDP-8 DEC computer, operated by Jan van der Greef (photo taken by M.C. ten Noever de Brauw).

The first important experiment performed with this system was measurement of human gender differences (van der Greef et al., 1983) with the unsupervised multivariate analysis of urine profiles in late 1981 (Figure 2). Clear differences between urine samples from different genders were observed. These differences were related, in part, to steroid conjugates. This result was later confirmed with high-resolution measurements with multichannel-analyzer methodology.

A major challenge presented by this system was desalting of body fluids and removal of abundant components (urea, etc.) while capturing as many components as possible. After investigating various options, it was decided that it was not feasible to use a single method to profile small molecules in body fluids. Thus, a multiplatform approach was developed. An additional challenge in analysis was normalization, and an inclusion/ exclusion approach (Fischer weighting) for MS peaks was chosen. Researchers recognized the complexity of datasets with many more variables (300-500) than objects (12-40), and the danger of using Fisher weighting (i.e., class information as the preselection filter). Validation procedures also

needed to be implemented. A practical solution to validate findings of this, and later, discriminant approaches, such as principal components discriminant analysis (PC-DA), was to classify unknowns or to use cross-validation to obtain a step towards validation. Soft independent modelling of class analogy (SIMCA) was evaluated for building models (Droge et al., 1987); however, at that time, the program was limited to the developmental stage.

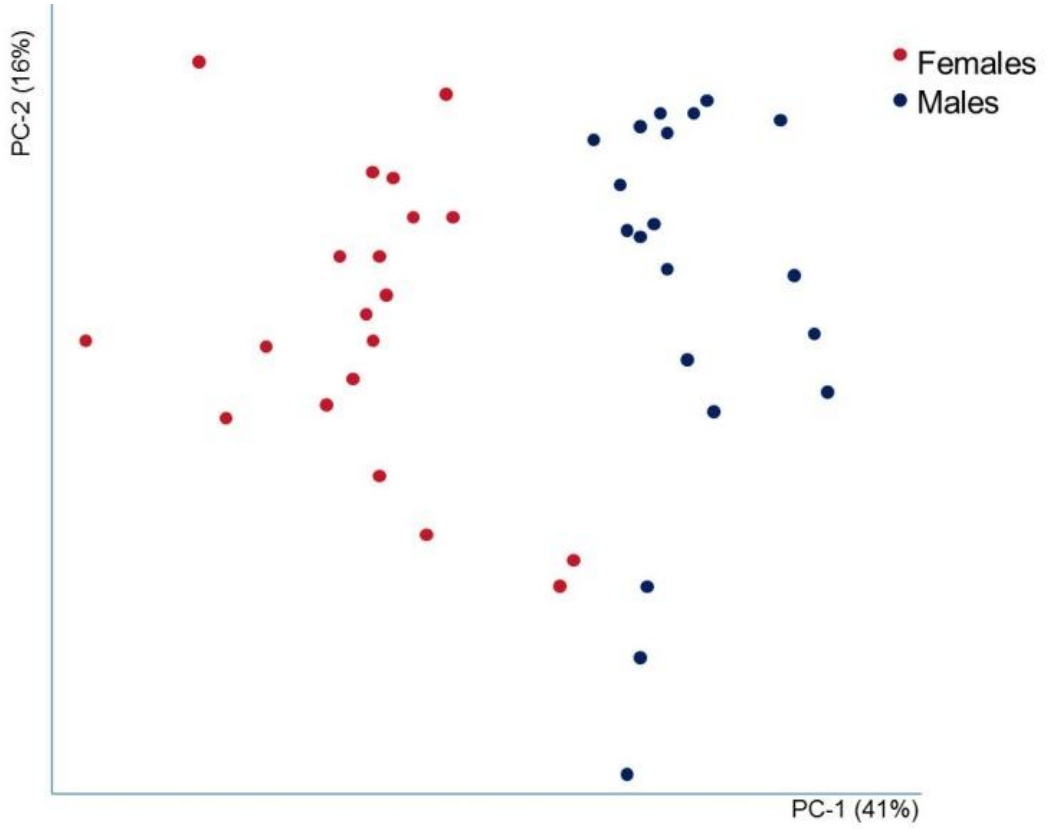

Figure 2. The first "metabolomics" outcome of an experiment performed in 1981. This experiment detected gender differences due, in part, to steroid conjugate profiles in human urine as analyzed with field desorption mass spectrometry combined with principal component analysis.

The fast atom bombardment (FAB) soft-ionization technique (Morris 1981) became available in the early 1980s. Although faster and easier than other methods, the routine application of FAB was limited because the robustness of FAB profiles was low due to background desorption matrices. FD and FAB were compared for metabolomics analysis with pattern recognition. The results were presented during the first International Chemometrics meeting in 1982 in Petten, The Netherlands, and published in *Analytica Acta* in 1983 (van der Greef et al., 1983).

Interestingly, the 1982 Chemometrics meeting focused on discussion of significant within-group variations, so-called interindividual differences. The discussion highlighted one of the strongest aspects of metabolomics as a phenotyping methodology, the potential for personalized medicine/ health approaches.

In 1991, Albert Tas developed direct chemical ionization (DCI)/MS as part of his PhD research (Tas, 1991). This robust method combined the soft ionization of volatile and low-volatility/ nonvolatile components (metabolomics part) with pyrolysis of biopolymer structures. This method became a routine methodology used in parallel with field desorption MS, and in the late 1980s it became the first method of choice until GC, GC/MS, and liquid chromatography (LC)/MS technologies were developed.

Technology-driven metabolomics research at TNO (1980–2000)

Figure 3 describes major metabolomics developments at TNO in the period 1980–2000. Research in this time frame was driven by technological development, combined with exploration of potential applications for these technologies.

Despite the experimental challenges of metabolomics profiling with field desorption MS, some intriguing concepts were developed in the early 1980s. Fast drug-metabolite detection was developed with the quotient spectra to correct for individual differences in backgrounds (van der Greef et al., 1984). The ratio of the metabolomic profiles before and after drug administration to rats was calculated, and the quotient spectra were statistically evaluated to improve drug-metabolite detection.

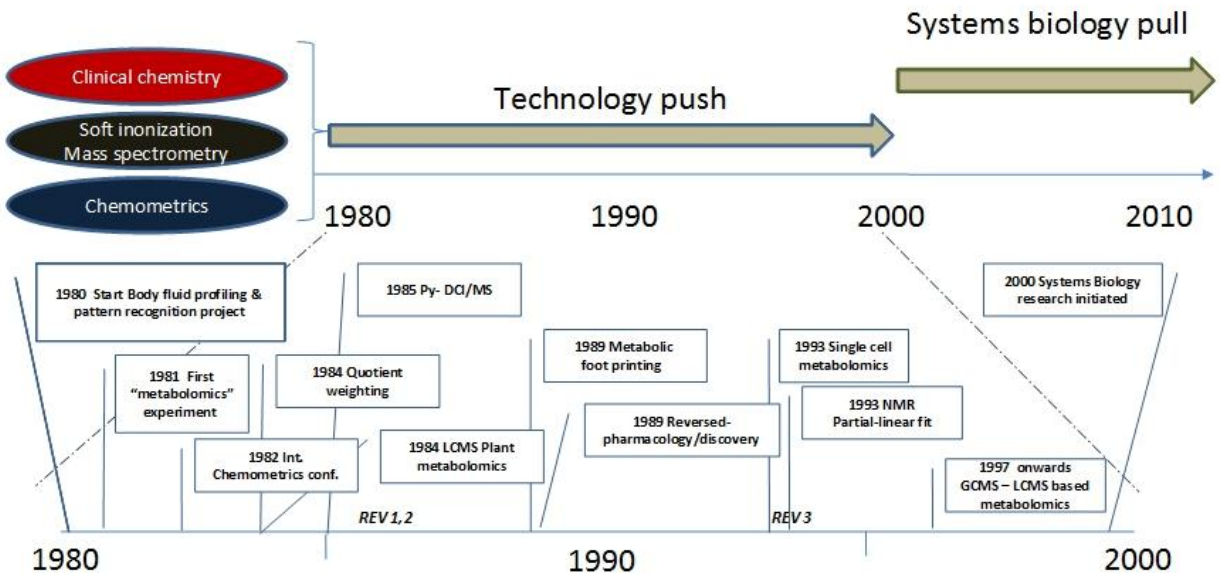

Figure 3. Metabolomics events with an impact on technology and applications from 1980 to 2000.

Interestingly, the approach revealed that levels of non drug-related compounds changed significantly. This concept underlies the contemporary use of biomarkers to assess system response. The system-response approach corrects for individual differences in homeostasis and focuses on changes, for instance in challenge tests. However, at that time of its development, this approach was not of major interest to the pharmaceutical industry because it revealed nontargeted mechanisms that were considered irrelevant. Investigations into new phenomena were believed to slow down the drug-development process. In hindsight, this approach provides a very relevant source of important information, not only for the discovery of drug mechanisms and off-target effects, but especially for recently very important systems toxicology effects. COMET I and II were important recent metabolomics projects in the area of drug safety as well as the US Critical Path Initiative and MetaMap Tox project run by BASF.

Interesting applications of TNO projects from 1980 to 2000 covered a wide range of topics, including microbiology related to food safety (bacteria, fungi, and algae), food production (barley, juice adulteration), feed, fermentation, doping, forensic toxicology, chemical industry, and pharmaceutical/ biomedical research. Microbiology was the first important field

that used Py-DCI/MS, and achievements of this research are summarized in a review (Tas, 1995). Other relevant reviews are indicated in Figure 3.

The concept of reversed pharmacology/ discovery was first described in 1989, based on a study that revealed the working mechanism of Lombazole inhibition of the yeast-hyphal conversion of the dimorphic fungus Candida albicans (Tas et al., 1989a). Profiling combined with pathway analysis allowed the targeted identification of the drug, as described in detail in a later review (van der Greef et al., 2003). This is a remarkable approach that, in the last 10 years, has formed the strategy of metabolomics-driven system research to identify multitarget pharmacology of complex herbal medicines. Metabolic foot printing, identification of biomarkers in supernatants of virus-infected cells, was another noteworthy tool developed at TNO (Tas et al., 1989b; Kell et al., 2005).

The use of nuclear magnetic resonance (NMR) technology for metabolomics became an option after an internal reorganization at TNO in 1990, when the NMR group was moved into the MS department. The first NMR-profiling results combined with pattern recognition were published in 1989 by Jeremy Nicholson's group (Bell, Brown & Sadler, 1989; Nicholson & Wilson, 1989). Since then, Nicholson's work has inspired the field of NMR-based metabolic profiling combined with pattern recognition globally in many application domains. At the turn of the century, Jack Vogels developed a new partial-linear fit algorithm for NMR data preprocessing to avoid unnecessary resolution loss due to the binning process. The NMR applications described by Vogels included characterization of wine, coffee, illegal use of growth promoters, biomarkers in multiple sclerosis, and hop suspension culture results, among others (Vogels, 2002). The NMR field at TNO remained important until about the year 2000, due to the high reproducibility and quantitative aspects of NMR. However, interest in this method dropped with the development of systems biology and the limited dynamic range of detected compounds with NMR. Although the concentration sensitivity limits of NMR result in a limited number of metabolites that can be monitored, the high concentration metabolites, including house keeping related molecules, may give an important view on high level system organization and might be very relevant especially when applied to prevention or early onset processes (Nicholson 2008). The focus in these areas is more on the balance between various regulatory processes than on detailed underlying metabolic processes.

After the appointment of Jan van der Greef as a professor at Leiden University in 1986, several new collaborations were initiated between TNO and Leiden University, specifically in the field of separation sciences and the domain of novel analytical MS techniques. A

landmark was development of single-cell metabolomics, which used peptide profiling by the matrix-assisted laser desorption/ionization (MALDI)-MS of single neurons in Lymnaea stagnalis (Jiménez 1994). This approach was used to identify proopiomelanocortin-processing products in melanotrope cells of the pituitary intermediate lobe of Xenopus laevis (Vázquez-Martínez 1999, Jespersen 1999, van Strien 1996).

An important project in the development of metabolomics field was the PhD project of Elwin Verheij (Verheij, 1993), the results of which formed a sound basis for unique LC/MS expertise. The combined use of LC/MS with field desorption was explored in the plant metabolomics profiling of the purgent principles of capsicum (van der Greef et al., 1985). Because TNO functioned as a testing location for new MS instruments produced by Varian MAT, many new technologies passed through the TNO MS group. In particular, field desorption MS was combined with LC/MS as early as 1984 with the moving-belt interface technology (van der Greef et al., 1984). The moving-belt interface LC/MS required substantial improvement for bioanalytical applications, and it was too limited for routine analysis and metabolomics-like applications.

The LC/MS technique became more applicable to metabolomics after introduction of thermospray (Heeremans, 1990), continuous-flow FAB LC/MS (Kokkonen, 1991), and electrospray combined with LC or capillary electrophoresis (Mazereeuw, 2000). Electromigration techniques such as micellar and chiral capillary electrophoresis and isotachophoresis were explored as separation and sample (stacking) pretreatment technologies (Storms et al., 2004). However, in spite of the attractiveness of electromigration methodologies, few metabolomics applications resulted from them so far, because of their limited sensitivity and lack of robustness in bioanalysis. A recent exception is Human Metabolome Technologies Japan which started to offer a CE-MS metabolomics service.

From 1980 onwards, many technological variations of GC/MS became available at TNO. The main limitation to the use of GC/MS and GCxGC/MS was (and continues to be) data handling. The reader is referred to a 2005 review that discusses the challenges involved in the symbiosis of mass metabolomics and chemometrics (van der Greef & Smilde, 2005). Of note, the step from direct profiling with field desorption and DCI/MS to separation techniques coupled with mass spectrometry (LC/MS and GC/MS) introduces the need for automatic corrections for peak shifts in the chromatography, baseline drifts, etc. The real application of chromatography-MS in metabolomics only became possible after software was developed that allowed automatic corrections for the above-mentioned issues. Solutions for these issues

were first developed in the late 1990s (Gaspari et al., 2001).

Biology-driven metabolomics research at TNO (2000–present)

Figure 4 describes major events in metabolomics development at TNO in the period 2000–present. From about 2000 forward, the major driver of metabolomics research at TNO has been biology, although technological developments have remained of major importance.

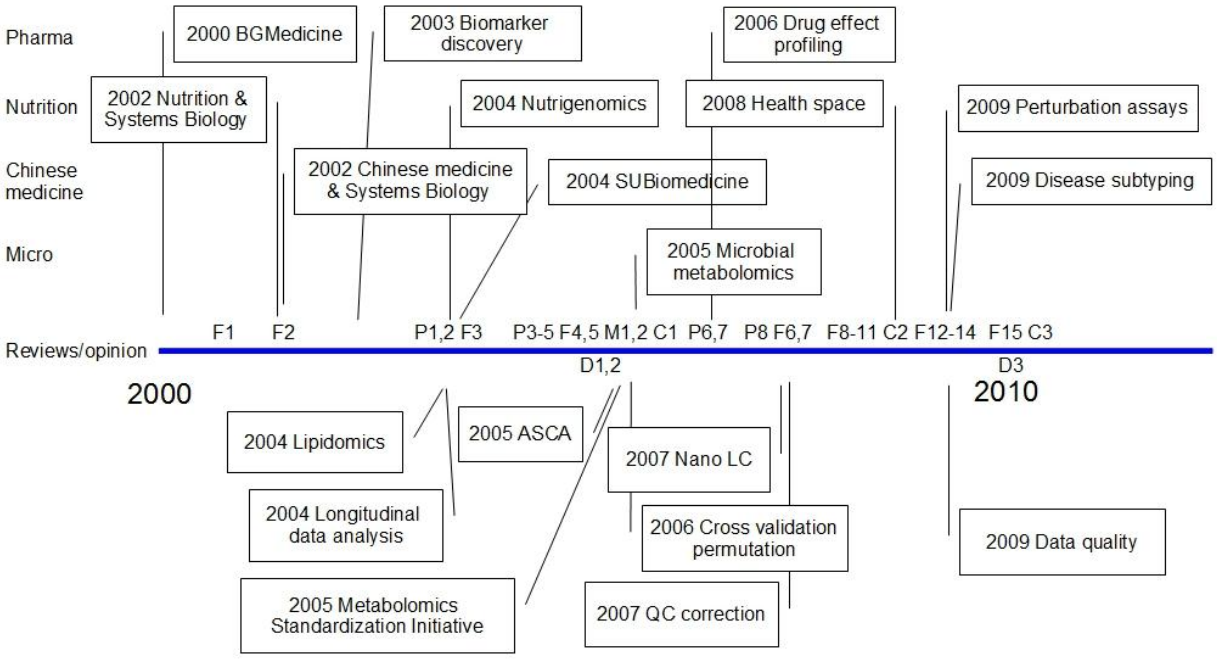

Figure 4. Metabolomics events from 2000 onwards. Reviews are divided into food (F), pharma (P), microbiology (M), Chinese medicine (C), and data analysis (D).

Systems biology at TNO

A working definition of systems biology in the year 2000 was "the study of biology as an integrated system of genetic, protein, metabolite, cellular, and pathway events that are in flux and interdependent" (Davidov et al., 2003). The concepts of systems diagnosis and systems intervention had been present at TNO from its inception.

The major drive for improvement of metabolomics research at TNO found its basis in a November 1999 discussion between Fred Regnier and Jan van der Greef on the future of biological and pharmaceutical research. Inspired by the development of first-generation proteomics tools at Purdue University, Regnier and van der Greef envisioned the integration of data at the transcript, protein, and metabolite levels. They shared this idea with Noubar

Afeyan of Flagship Ventures, and a business plan for integrative omics research was developed . In 2000, the first systems biology company in the world, Beyond Genomics [known later as BG Medicine (BGMD)], was born. In the year 2000, the famous Systems Biology Institute was founded by Leroy Hood. Research at this institute focused on systems modeling, but modeling at the metabolite level was not included in its analytical capabilities.

TNO served as the metabolomics research and development component of BGMD. Through this company, TNO collaborated in systems-biology projects with the pharmaceutical industry. In particular, the apolipoprotein E 3 (APOE3) Leiden transgenic mouse model was developed for studying atherosclerosis and as an initial proof-of-principle experimental model for systems biology. The results of the combined transcriptomics, proteomics, and metabolomics analyses of this transgenic model, which were based on and integrated with correlation networks, became available as early as 2001. This project was the first integrated in vivo systems biology experiment in the world of its kind.

To date, few published studies are available that measure all three biological levels in a single model with integration by a de novo correlation network. Most systems-biology studies are based on measurement of one or (at most) two levels (i.e., gene, transcript, or metabolite level), and literature data that concerns pathways are added to these findings. The results of the APOE3 Leiden transgenic mouse model study required substantial effort to publish because reviewers were very reluctant to accept this new approach. However, two related papers were finally published in 2004 (Clish et al., 2004; Oresic et al., 2004). In the meantime, various projects with the pharmaceutical industry delivered unique data, although most of these results had to remain confidential.

A 2005 review in Nature Reviews Drug Discovery (van der Greef & McBurney, 2005) explained the concept and thinking behind systems pathology and pharmacology. Several other reviews and opinion papers described the general concept of systems thinking (Morel et al., 2004; van der Greef, Stroobant & van der Heijden, 2004), (reversed)-translational research (van der Greef, 2005; van der Greef et al., 2006), systems toxicology (Heijne et al., 2005b), and connecting multicompartment omics (van der Greef, Hankemeier & McBurney, 2006; van der Greef et al., 2007).

The potential for systems biology in nutrition was recognized very early, as shown by the extensive literature describing this view from 2001 onwards (van der Werf et al., 2001; van Ommen & Stierum, 2002; van Ommen, 2004; Gibney et al., 2005; Corthésy-Theulaz et al., 2005; Joost et al., 2007; van Ommen, 2007; van Ommen et al., 2009). Ben van Ommen has

been the nutritional systems biology leader at TNO since 2001. In 2004, TNO coestablished and coordinated the European Nutrigenomics organization (NuGO), which has been instrumental to develop nutritional metabolomics technology (Kaput et al., 2005, 2006; Scalbert et al., 2009), concepts (Daniel et al., 2008; van Ommen et al., 2008a, 2008c, 2010a) and proof-of-principle studies (Cavalieri et al., 2009). This work became the basis of the nutrigenomics-based NuGO consortium (Baccini et al., 2008; Harttig et al., 2009). A key feature of nutritional metabolomics is the concept of the "biomarker of health," which is based on quantification of the stress-response curve after perturbation of homeostasis (van Ommen et al., 2009; Wopereis et al., 2009).

Metabolomics applications within Chinese medicine

Because systems biology encompasses a holistic perspective, the idea to use traditional Chinese medicine (TCM) as an entry to systems knowledge was born in 2002. With his background in natural product bioactive component detection in the spin-off company Screentec (now Kiadis) from his Leiden University group, Jan van der Greef arranged to meet with Mei Wang, a molecular biologist with knowledge of TCM and a strong network in China. In 2003, a lecture tour was organized to explain the new systems biology tools at TNO within the context of TCM. This collaboration created a strong link with the Chinese Academy of Sciences. Research was initiated at TNO to identify and elucidate the actions of synergetic active compounds (multidimensional or multitarget pharmacologic agents) within TCM (Wang et al., 2005). Through this research, many new insights were gained that complemented the Western disease-management approach with health-promoting knowledge. These discoveries led to the formation of the company SU ("Bridging the Seen and Unseen") Biomedicine in 2004.

A new research program was started between China and The Netherlands, known as the Sino-Dutch Center for Personalized and Preventive Medicine (SDPPM). The video message at the official opening of the SDPPM was delivered by the Chinese Minister of Health, Prof. Chen Zhu (see www.sinodutchcentre.nl). Zhu originally trained as a barefoot doctor, is a renowned scientist in the leukemia field. He understood the potential of systems biology, published with Lee Hood (Auffray, Chen & Hood, 2009), and his speeches always focus on the enormous complementarity of the two perspectives (systems biology and TCM).

The background ideas that unify systems biology and TCM areoutlined in a diagnosis-focused paper (van der Greef et al., 2010). This complementarity also holds promise for the

concept of personalized health/ medicine (van der Greef, 2011).

The first activities in the field of TCM at TNO were related to the systems biology of herbal medicine (Wang et al., 2005). With metabolite profile measurements, Boelsma et al. showed that the skin blood flow-regulating properties of Ginkgo biloba depended on the baseline skin blood flow (Boelsma et al., 2004). Chang et al. used GC-MS to study the effects of different growth conditions of Rehmannia glutinosa on its constituents (Chang et al., 2006, 2011). Wang et al. developed LC-MS and MALDI-MS methods to study systems toxiclogy (Wang et al., 2009a) and alkaloid concentrations in Aconitum carmichaeli, which are important to control toxicity in this plant (Wang et al., 2009b). Bioconversion of ginsenosides was studied in an in vitro gastrointestinal tract model (Kong et al., 2009). Effects of various ages of Ginseng root extracts were compared in a diabetic rat model (Hu et al., 2011b). The APOE3 Leiden mouse model was used to test effects of Rimonabant and a Chinese herbal formula on lipid profiles (Hu et al., 2011c; Wei et al., 2012a). The Chinese formula was shown to reduce plasma cholesterol, cholesteryl ester transfer protein (CETP) levels, and CETP activity, and to increase high-density lipoprotein (HDL) cholesterol. Such studies are crucial for the quality control of herbal medicine - an important prerequisite for the introduction of Chinese herbal products to the European market and to modernize TCM.

An important recent activity of the SDPPM is the subtyping of patient groups based on TCM diagnosis. Focusing on rheumatoid arthritis (RA) and diabetes type 2, the aim is to discover subgroups of patients for whom treatment can be optimized. One step in the direction of personalized medicine was taken in a study that showed distinct gene expression and metabolomics profiles between Cold and Hot types of RA patients (van Wietmarschen et al., 2009). Different urine metabolite profiles were measured in two subgroups of prediabetes patients based on TCM diagnosis (Wei et al., 2012b). Further focus on standardization of TCM diagnosis revealed symptom clusters related to Cold and Heat in arthritis patients (van Wietmarschen et al., 2011). The principles of TCM will continue to contribute strongly to developments in systems thinking, health promotion, prevention, subtyping of disease, multifactorial therapeutics, and integrated medicine.

Metabolomics platforms at TNO and applications to improve human health

In the 21st century, metabolomics technology at TNO has increasingly been used. Figure 5 presents an overview of the metabolomics platforms developed over time and currently in use

(as of 2012) at TNO. A distinction is made among four types of platforms, which are used in combination and depend on the application type. Global platforms consist of unbiased methods especially useful for broad profiling and to discover new compounds and regulatory motifs. Metabolite class profiling methods typically use Fourier transform (FT) or orbitrap to measure classes of compounds, with the option to detect novel compounds in those classes. Targeted methods are specifically designed to measure a selection of known compounds of interest. Biomarker assays include accepted and validated assays that generally measure a single component per method.

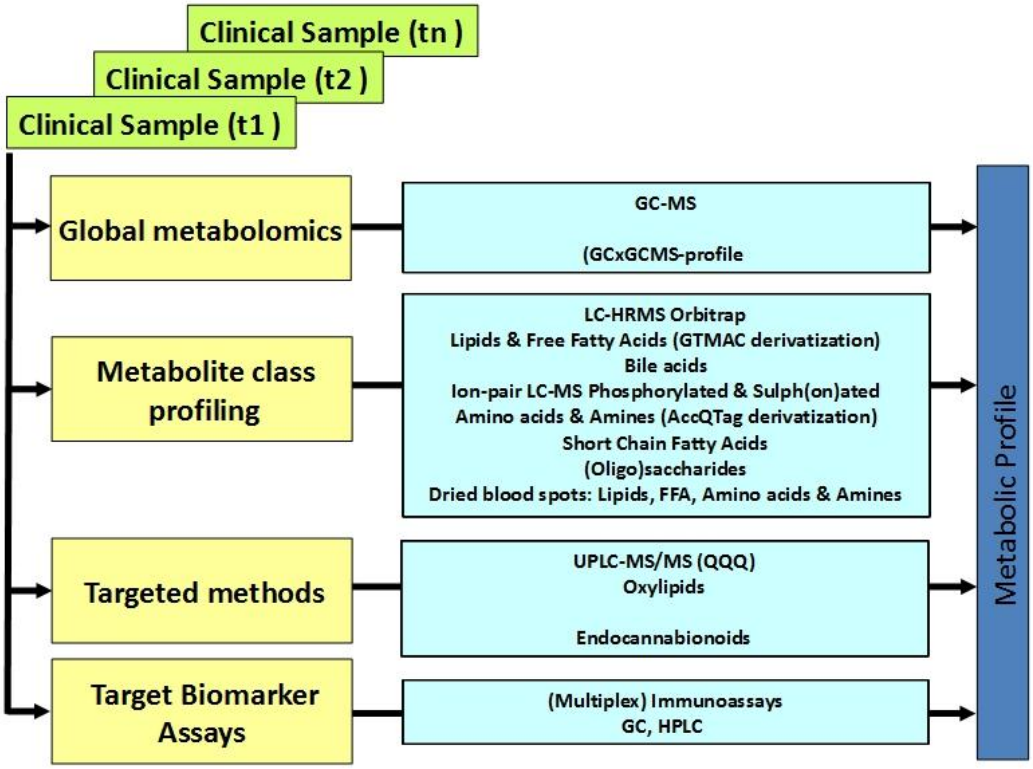

Figure 5. Overview of the currently used TNO metabolomics platforms.

The desire to measure complex changes in system organization required development of robust and sensitive measurement technologies, and extension of the types of body fluids and classes of metabolites that could be measured. Over the years a wide variety of instruments have been used. In the early days of LC-MS metabolomics quadrupole instruments were used, but quadrupole technology suffers from poor sensitivity in full scan data acquisition mode (duty cycle) and because of unit mass resolution (selectivity). The introduction of ion-trap systems improved the performance of many methods dramatically, resulting in improved

coverage of the metabolome despite the fact that selectivity is still poor on these insturments. A major step forward was obtained by applying high resolution mass spectrometers, e.g. ToF, FT-MS and Orbitrap MS as with these instruments metabolites at low concentration are typically resolved from intense background signals in LC-MS. A 2004 study found LC/MS lipidomics analysis to be the fastest method to categorize anti-inflammatory compounds (Verhoeckx et al., 2004b). In collaboration with Thomas Hankemeier at Leiden University, Hu et al. developed an extensive RPLC-ion trap-FTMS method for lipid profiling, which they used to measure over 160 lipids of eight different classes (Hu et al., 2008). In 2006, an effort was made to use capillary electrophoresis for urine sample profiling (Benavente et al., 2006). At the same time, a novel ion-pair LC-electrospray ionization-MS method was developed to measure several key classes of polar metabolites, such as nucleotides, coenzyme A esters, sugar nucleotides, and sugar biphosphates in microbes (Coulier et al., 2006).

Differences in bile-acid levels were detected in serum and liver samples of APOE3 mice on a high versus low cholesterol diet with a novel linear ion trap-Fourier transform-MS method (Bobeldijk et al., 2008). A 1H NMR method was developed to improve sensitivity in toxicology studies (Schoonen et al., 2007a, 2007b). An advanced GC × GC method was developed to improve the number of compounds that could be measured in a single analysis (Koek et al., 2011). This development was headed by Thomas Hankemeier in close collaboration with Mariet van der Werf, project leader for metabolomics-microbiology research at TNO. A comprehensive GC/MS method, which allowed detection of nearly 400 compounds, was developed to study microbial metabolomes (Koek et al., 2006; van der Werf et al., 2007). Host selection and target identification became an important analytical method (van der Werf, 2005; van der Werf, Jellema & Hankemeier, 2005). Other GC-MS and GC x GC-MS methods for metabolomics analysis have been reported (eg. Fiehn 2000, Jonsson 2004, O'Hagan 2007, Pierce 2006).

To measure synovial and cerebrospinal fluid (CSF), methods that could handle very small sample volumes needed to be developed. A nano-LC method was developed and applied to measure peptides in synovial fluid of osteoarthritis patients (Kamphorst et al., 2007). The first GC/MS method to profile mouse CSF was developed at TNO (Koek et al., 2010), which was followed by measurements of human CSF (Stoop et al., 2010). A set of stability studies showed changes in CSF compounds, such as transthyretin, after freeze/thaw cycles and in prostaglandin D synthase-derived peptides and certain amino acids after longer sample storage (Rosenling et al., 2009, 2011).

Disease biomarker discovery has become a hot topic globally in the last decade (Kussmann 2006, Schlotterbeck 2006). Research at TNO has led to identification of biomarkers in guinea pig urine (Lamers et al., 2003), patients and primates with multiple sclerosis ('t Hart et al., 2003, 2004), and patients with osteoarthritis (Lamers et al., 2005).

Metabolite profiles have been used to evaluate pharmacological effects of drugs. Early-responding biomarkers were found for the effects of thiazolidinediones in urine and blood samples of patients with type 2 diabetes (van Doorn et al., 2006). Early biomarkers for diabetic kidney disease were discovered (van der Kloet et al., 2011). When patients in two groups were treated with different statins, changes in the lipid profiles between groups were detected with metabolomics technology that could not be detected by classical clinical measurements (Bergheanu et al., 2008). Another study examined changes in the low-grade inflammatory status of obese subjects, with extensive metabolomics, proteomics, and genomics profiling to reveal a network of markers (van Erk et al., 2010). Metabolite profiles associated with drug-induced hepatotoxicity in rats were discovered with a novel NMR urine analysis approach (Heijne et al., 2003, 2004, 2005a, 2005b, 2005c, 2005d; Schoonen et al., 2007a, 2007b). The combination of metabolomics, proteomics, and genomics also revealed anti-inflammatory markers in macrophage cells stimulated with LPS and treated with beta-agonists (Verhoeckx et al., 2004a). Metabolomics has become a standard tool in research with mouse models in the area of metabolic and inflammatory disorders (Kleemann et al., 2007, 2010; Wopereis et al., 2012).

Nutrition is a major research topic at TNO. Of particular interest is the relationship between nutrition and metabolic syndrome, characterized by low-grade inflammation, excessive body weight, reduced insulin sensitivity, and other markers. Metabolomics integrated with other -omics technologies is a promising approach to study such early and subtle changes in the body (de Graaf et al., 2009). Altered lipid metabolism and markers of inflammation and tissue development were found in the APOE3 Leiden transgenic mouse model of atherosclerosis (Oresic et al. 2004). A high-cholesterol diet in these mice switched the liver from a resilient to a mainly inflammatory state (Kleemann et al., 2007; Radonjic et al., 2009a, 2009b). Another mouse model was used to study effects of starvation on lipid profiles (van Ginneken et al., 2007). Data from these and other studies led to the development a metabolite profile-based “health space” that spanned the dimensions of oxidative stress, inflammatory stress, metabolic disorders, and cell-cycle disorders (van Ommen et al., 2008b). This model

was used to visualize effects of an anti-inflammatory diet intervention in overweight subjects (Bouwman et al., 2012).

With the shift towards detection of early deviations from health, it is necessary to challenge the system and measure its resilience. Subtle metabolic changes in overweight subjects induced by a mild anti-inflammatory drug could be detected after administration of an oral glucose tolerance test (Wopereis et al., 2009) or a prostprandial challenge test that consisted of a high lipid, carbohydrate, and protein shake (Pellis et al., 2011). Baseline effects of the drug were also examined (Bakker et al., 2010). Dynamic-system measurement proved to be another approach to detect more subtle system changes (Kleemann et al., 2010).

Although TNO introduced metabolomics to improve food quality control in the 1980s, a more advanced multiplatform strategy was recently developed to measure sensory properties of tomatoes (Thissen et al., 2011). Metabolomics was used to show the enhanced immune reactivity of chickens fed with organic feed as compared to those fed normal feed (Huber et al., 2010). Metabolomics is a key technology for advanced microbial host selection. A substrate-oriented host-selection approach was shown to be superior to a product-oriented approach (van der Werf et al., 2008, Rumbold et al., 2009). Metabolomics has also been used to improve characterization of strain phenotypes, crucial in microbial production (Braaksma et al., 2009, Braaksma et al., 2011).

Plant metabolomics has played a minor role at TNO; therefore, this topic is hardly mentioned in this review. We refer the interested reader to a recent book (Hardy & Hall, 2012) that covers majors aspects of the application of metabolomics technology to plant biology.

Challenges of metabolomics data analysis

Datasets in the metabolomics field are characterized by a large number of variables compared to a relatively small numbers of objects. This feature greatly enhances the chance of overfitting the data, especially when supervised methods, such as discriminant analysis (DA), are used (Westerhuis et al., 2008). Several authors (Bijlsma et al., 2006; Hendriks et al., 2007) proposed the use of double cross-validation with permutation testing as a standard technique to validate supervised multivariate data analysis. This procedure was extensively tested for megavariate datasets, in which the number of variables was more than 10 times the number of objects (Rubingh et al., 2006).

Before data analysis can actually start, the quality of the data must be assured. Data quality can be compromised by various sources of analytical error, such as instrumental drift, ion

suppression, and metabolite concentration. A workflow was proposed that included the regular injection of pooled quality control samples and the use of multiple internal standards. Statistical techniques were developed to correct data with calibration samples and standards (van der Kloet et al., 2009). Extensive research has been done at TNO to develop comprehensive figures of merit that can give a reliable idea of the performance of a metabolomics platform (Smilde et al., 2009; Case et al., 2011). The next important step in the process of turning data into knowledge is the data scaling or transforming. Data transformation is used to reduce effects of noise, and can be employed in various ways, depending on the biological question (van den Berg et al., 2006). A discussion of the implications and applications of multivariate data analysis techniques on various types of GC × GC experiments was published (van Mispelaar et al., 2005).

Over time, metabolomics experiments have become increasingly complex. Particular experimental designs (Thissen et al., 2009), such as those that include different dosage groups or various time points per subject, require data analysis strategies that incorporate the study-design information. For example, batches of samples from a single study measured at different time-points cannot readily be compared; that fact has led to the development of data-fusion techniques (Draisma et al., 2008). In 2005, the first papers on analysis of variance (ANOVA)-simultaneous component analysis (ASCA) were published. This method combined ANOVA with principal component analysis (PCA) (Jansen et al., 2005b; Smilde et al., 2005a; Vis et al., 2007). Consensus PCA and canonical correlation analysis were developed to analyze relationships between a specific set of variables and the remainder of the data (van den Berg et al., 2009). Other methods that consider prior knowledge or experimental design are multiway partial least squares discriminant analysis (PLS-DA) (Rubingh et al., 2011), simplivariate models (Saccenti et al., 2011), two-mode clustering (Hageman et al., 2008), and subspace clustering (Damian et al., 2007). Development of analytical comparison methods, such as simultaneous component analysis (SCA) methods and quantile equating, allowed a comparison of different types of data or samples measured on different systems (van Deun et al., 2009; Draisma et al., 2010; Smilde et al., 2005b). Network theory was used to integrate data at the level of correlations, to allow visualization of changes in relationships between conditions or groups of subjects (Clish et al., 2004; Davidov et al., 2004; Adourian et al., 2008).

The introduction of longitudinal metabolomics data required yet another line of data analysis (van der Greef, 2005). The behavior of complex systems cannot be properly ascertained from

measurements at a single time point (Glass et al., 1988). Rhythms in the intensities of metabolites, proteins, and gene transcripts have been extensively measured, as have health problems associated with disturbances in these rhythms (Jonsson et al., 2006; Vis et al., 2010; Moser et al., 2006). A weighted PCA method was developed to tackle longitudinal data (Jansen et al., 2004), and multiway PLS was used to analyze longitudinal microbial metabolomics data (Rubingh et al., 2009). A multilevel component analysis strategy was applied to 29 time points of monkey urine data (Jansen et al., 2005a). A recent review discusses the longitudinal metabolomics data analysis strategies (Smilde et al., 2010).

Finally, as the metabolomics field has matured, there has been an increased need for the standardization of study designs and their reporting. Such standardization is essential when large multicenter studies are performed. The Human Metabolomie Database was founded to concentrate and curate metabolite information and make this information freely accessable (Wishart 2007). In 2003, the Standard Metabolomics Reporting Structures was founded, followed up by the Metabolomics Standards Initiative in 2005 (Lindon et al., 2005; MSI Board Members et al., 2007; Fiehn et al., 2007). Standardization was also followed through in the area of environmental metabolomics (Morrison 2007). Researchers at TNO specifically addressed the issue of standardization in the area of metabolomics (Fiehn et al., 2006; van der Werf et al., 2007).

Metabolomics in the Future

The changing landscape of health care

The definition of "health" was recently readdressed and emphasizes a person's ability to adapt and cope in the face of social, physical, and emotional challenges (Huber et al., 2011). The utopian definition of health provided by the World Health Organization (Callahan, 1973) as complete physical, mental, and social well-being is no longer useful in the face of a health care system that moves towards personalized medicine and health promotion. Health is no longer only a goal, but it is also a means to attain a good life as a well-functioning being in harmony with one's surroundings (Mordacci & Sobel, 1998). This shift away from health as a state and towards health as a process has important implications for research into wellness and health promotion.

The development of personalized prevention strategies based on health promotion requires

methods to measure health. Developing these methods is an enormous challenge because most biological knowledge is obtained from disease models and patient populations. Looking at healthy people raises questions about the variability of biological processes within the individual over time, between healthy individuals, and the subjectivity of experiencing health. Currently, few (if any) studies have assessed the variability of metabolomics profiles between and within healthy individuals.

In the 19th century, French physiologist Claud Bernard (1813–1878) proposed homeostasis as the regulatory model of the body. Homeostasis is characterized by the body's maintenance of stable conditions with feedback mechanisms. However, the body is a nonlinear dynamic system that must continuously adapt to new situations. Allostasis is the regulatory model in which the brain integrates prior knowledge with sensory data to optimize the resources distribution (Sterling, 2011). Figure 6 illustrates the dynamic processes of regulation. As long as the system is able to respond effectively to a challenge (i.e., the stress level goes up), the system can be called "healthy". However, when the system is no longer able to respond sufficiently to stress, a disease state occurs.

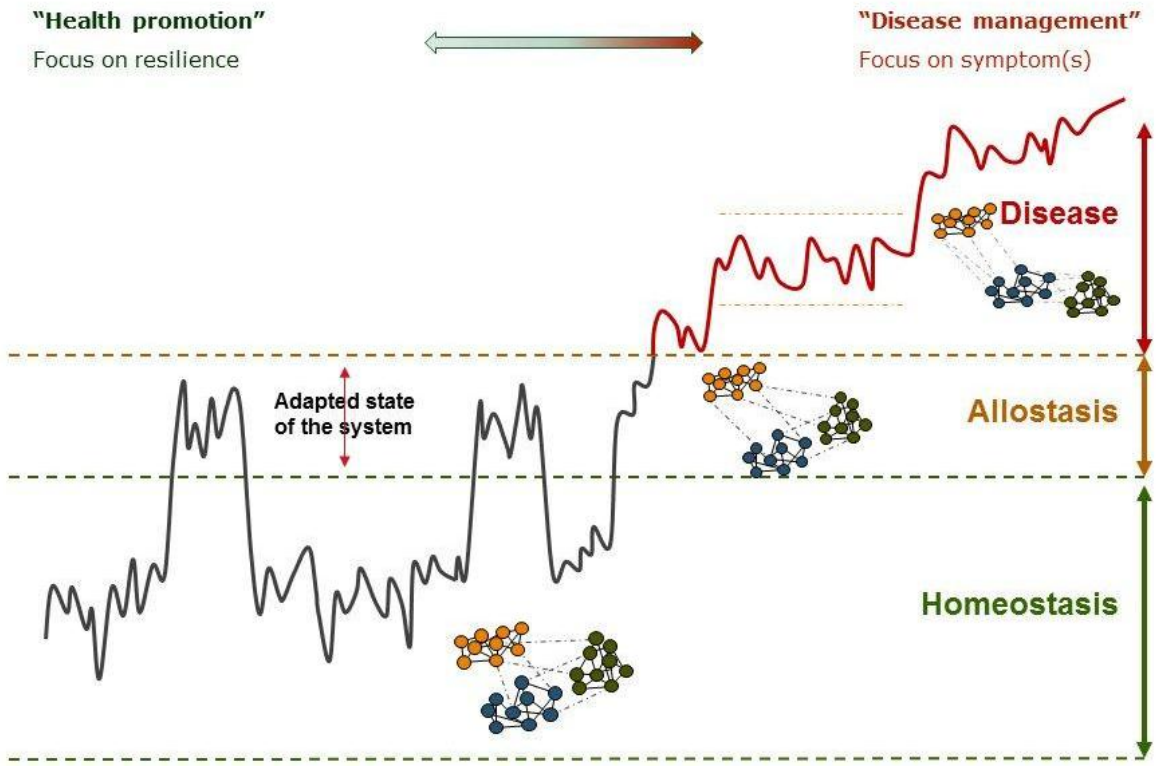

Figure 6. Illustration of dynamic system regulation. A system that is able to respond effectively to a challenge ("allostasis" area in the figure) can be called "healthy". When the system is no longer able to respond sufficiently, a disease state develops.

Systems science, complexity theories, and nonlinear dynamic system theories are specially designed to account for system properties, such as connectivity, emergence, self-organization, stability, and flexibility (Prigogine, 1980; Capra, 1997). Systems science will allow a proper investigation of many healing arts that are based on holistic concepts of health, such as mind-body interventions, herbal medicine, etc. Processes of healing can be elucidated and the right treatment options can be found for the right situations.

For additional insight, we can look to other health traditions, such as Asian medicine, which are founded on systems thinking. These traditions incorporate, for example, organization of symptoms into clusters, sudden changes of symptom patterns, irregular healing processes, and multitarget herbal medicine, among others (Wang et al., 2005; van der Greef et al., 2010).

The integration of these medicine approaches will complement the Western model of reductionist science and disease management strategies. A recent study in the Annals of Internal Medicine found that RA patients treated with standard therapy complemented with Trypterigium wilfordii Hook F had a significantly higher response rate than the group complemented with sulfasalazine (Goldbach-Mansky et al., 2009). As a simpler example, a patient's good physical condition before cardiac surgery greatly improves how well the person recovers from surgery (Jack, West & Grocott, 2011).

A movement towards health promotion invites the question of who is responsible for which part of health care. A shift towards a more equal patient–practitioner relationship, which is already occurring, will place the doctor in the relative role of health coach. Health promotion strategies will be much more integrated in daily life by, for example, health "apps" on mobile phones, courses in stress-reduction techniques, personalized nutrition advice, etc.

As health care becomes more patient-centered, integrated, preventive, and personalized, the role of scientists must change. Science conducted in multidisciplinary teams, including patients, consumers, and health care organizations, will allow development of health-care products that are actually desired by patients and consumers. The scientist will act as a knowledge organizer and integrator. Discoveries will more easily lead to strategies that can enter clinical practice and reach the consumer. The future health of the society and its members will emerge from the many relationships among the various actors and the inspiration evoked by these relationships.

Role of metabolomics in preventative health care strategies

Metabolomics will be a major research area to develop dynamic system-monitoring tools that lead to diagnosis. Static, single time-point biomarkers will be replaced by dynamic biomarkers that can identify whether the system is moving towards or away from health. There is ample literature that relates disease to disturbances of the daily, seasonal, yearly, or other rhythms in the human body. For example, inflammation and depression seem to be related to sleep disturbances and, especially, to perturbations of the body's activity/ rest cycles (Cutolo, 2012). This knowledge has resulted in glucocorticoid chronotherapy with optimal medication at 3 am. Shift work has been shown to increase one's risk to develop breast cancer (Moser et al., 2006). Several studies in the area of chronobiology have revealed metabolic dynamics and their relationships with disease (Eckel-Mahan et al., 2012; Bass & Takahashi, 2010; Froy, 2010). Promotion of "healthy" rhythms and measurement of these rhythms in the body will be crucial in the future.

Figure 7. Contemporary nature photography that emphasizes dynamics and emotion with time-lapse integration. This image, obtained with a shutter speed of 1/8 s, is of a herring gull taking off from a Norwegian fjord (photograph by Jan van der Greef). The same principle of visualization of dynamic living systems is the key challenge for the next generation of metabolomics methodologies.

The focus on dynamics, movement, and emotion, with less emphasis on structure and form, in systems-based metabolomics has its parallel with developments in art history, when figurative painting developed into expressionism and impressionism. Figure 7 shows an example of this concept in contemporary nature photography. The use of slow shutter speeds allows time integration and provides insights into the dynamics and movement of (in this case) a herring gull taking off in a Norwegian fjord. The ripples on the water and the shimmering stream of water from its legs, illustrating the transition from floating on the water to the freedom of movement in the air, creates an ethereal image in which dynamics, emotion, rhythm, and direction of movement are captured.

Another approach to obtain a more dynamic picture of health is to measure the reaction of a system to physical, mental, spiritual, and other challenges (van der Greef et al., 2007). A classic example is the oral glucose tolerance test, used to detect early changes in the regulation of blood glucose levels and other metabolic processes (Shaham et al., 2008). Other examples are the high fat-load challenge (Pellis et al., 2011), exercise challenge (Lehmann et al., 2010), and stress tests. New challenge tests in the areas of spiritual, mental, physical, environmental, and social domains of resilience would be valuable additions.

Almost all data-analysis techniques used to describe such biological processes utilize linear models and specific linear statistical tests. However, many of the nonlinear dynamic features of biological systems cannot be captured by such techniques. The future of systems science will see a rapid rise of nonlinear techniques, such as nonlinear PCA, network analysis, and fractal calculations (Meulman, 2003; Zhou, 2012; van Wijk et al., 2010). With such techniques, it will be possible to analyze changes in symptom clusters and to treat these clusters.

Health is related to myriad aspects (e.g., spiritual, psychological, and physical conditions) that are reflected over many levels of organization (e.g., molecular, cellular, organ, whole body, and societal levels). At each level, new properties emerge that cannot be detected at a lower level of organization. Metabolomics can measure several levels of organization, although its coverage must increase. Plasma metabolome measurements reflect the system's response to keep certain processes within specific boundaries. Numerous stress and immune system components can also be detected (Bouwman, 2012). Urine is informative about what is processed by the system, whereas single-cell metabolomics will eventually allow a more differentiated view of cellular processes (Svatos, 2011). The metabolomics analysis of breath is an interesting development because it is noninvasive (Haviland et al., 2011). Gut

microflora metabolomics adds a very important dimension to this field because the gut is one region where the inside and outside of the body connect (Nicholson et al., 2012; Wikoff et al., 2009).

It is clear that the human body is an open system which continuously needs input of food, oxygen and water. Without this input the system would certainly fall apart in a certain amount of time. Such a system is called an autopoetic system, an open system far from equilibrium (Maturana 1980). The composition and quality of nutrients that enter the body is therefore paramount for maintaining optimal health as well as for the capacity of the body to store and utilize fuel when it is needed. This dynamic relationship between the self and the environment in the area of nutrition is termed metabolic flexibility (Galgani 2008). Improving metabolic flexibility is a relatively new target for preventing life style related disorders such as metabolic syndrome and diabetes. For instance reducing insulin resistance will improve the response to an oral glucose tolerance test. Metabolic flexibility as a measure of health can therefore be used to test the effects of nutritional interventions (Suhre 2010). However, the large variations in metabolic make-up of people needs to be taken into account to optimize nutritional interventions for the individual (Gieger 2008, Illig 2010).

A challenge in the area of data analysis is the fusion of the data obtained from these measurements. A good quality of the quantitative data is essential for the optimal integration of information (MSI Board Members et al., 2007). A great step forward in this area is the recently developed standard reference plasma available from the National Institute of Standards and Technology.

Metabolomics must be integrated with physiological measurements, such as heart rate variability, ECG, EEG, and ultra-weak photon emission, which measure higher levels of organization (McCraty et al., 2009; van Wijk, van Wijk & Bosman, 2010). To obtain an integrated view of an individual within its environment, information about psychological processes and coping mechanisms are needed (Antonovsky, 1987). Psychobiology is a growing field of science that aims to integrate physical with psychological measurements (Rossi, 2002). The effects of the environment on the immune and central nervous system regulation are slowly becoming common knowledge (Irwin & Cole, 2011). The discovery of early immediate genes, which are activated within minutes of an environmental stimulus and have profound effects on various biological processes, have given new direction to the nature versus nurture debate (Pérez-Cadahía, Drobic & Davie, 2011). Medical-imaging techniques are currently being employed to study gene expression throughout the living body (Lok,

2001). Altogether, measurement techniques allow a more-comprehensive view of processes in the body and between the body and its environment.

A better understanding of biology from a systems perspective will be the driving force for the next generation of metabolomics technologies, with a focus on low-cost, high-throughput capabilities to enable longitudinal studies with improved coverage. Miniaturization and electromigration technologies might be instrumental in this development (Lindenburg, 2012). Advanced multivariate statistics and nonlinear dynamic modeling are vital to obtain new insights into living systems. Metabolomics will provide new opportunities to guide industrial, applied, and research activities in the life sciences and to build the future of health care.

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4. Metabolite space of rheumatoid arthritis

Abstract

Metabolites play numerous roles in the healthy and diseased body, ranging from regulating physiological processes to providing building blocks for the body. Therefore, understanding the role of metabolites is important in elucidating the etiology and pathology of diseases and finding targets for new treatment options. Rheumatoid arthritis is a complex chronic disease for which new disease management strategies are needed. The aim of this review is to bring together and integrate information about the various roles that metabolites have in rheumatoid arthritis.

An extensive PubMed search is conducted to collect the relevant manuscripts. The metabolites are discussed in relation to rheumatoid arthritis. Subsequently, the metabolites are organized according to levels of system organization. In the last section an integrated pathway analysis of the metabolites conducted with Ingenuity Pathway Analysis software is presented.

Literature search resulted in information about vitamins, eicosanoids, fatty acids, lipids, hormones and peptides. The metabolites could be related to metabolic processes, oxidative stress processes and inflammatory processes. Cell death, lipid metabolism and small molecule biochemistry were found by the pathway analysis to be the top functions, characterized by the metabolites arachidonic acid, ascorbic acid, beta-carotene, cholecalciferol, hydrocortisone, keratan sulfate, melatonin, palmitic acid and stearic acid. These nine metabolites are highly connected to a number of canonical pathways related to immune functions, the production of nitric oxygen and reactive oxygen species in macrophages and pathways involved in arthritis.

This review indicates groups of metabolites that could be interesting for metabolomics studies related to rheumatoid arthritis. Circadian rhythms of metabolite levels are found to be important for understanding and treating rheumatoid arthritis. In addition, some key processes and pathways are found by integrating the metabolite data. This might offer new ideas for studies into the mechanism of and possible treatment options for rheumatoid arthritis.

Based on: van Wietmarschen HA, van der Greef J (2012) Metabolite space of rheumatoid arthritis. *British Journal of Medicine and Medical Research* 2(3): 469-483.

Introduction

Metabolites, the intermediates and products of metabolism, play numerous roles in the healthy and diseased body, ranging from regulating physiological processes to providing building blocks for the body. Together with genomics, transcriptomics and proteomics, metabolomics provides the biochemical building blocks for understanding the body as a complex and dynamic system of interactions (Wishart et al. 2007). Metabolites occupy a special place in this systems biology approach in that they are usually close to the phenotype (vd Greef et al. 2007). Therefore understanding the role of metabolites is very important in elucidating the etiology and pathology of diseases and finding targets for new treatment options. Metabolic profiles of cells, tissues or body fluids can provide snap-shot information about the current condition of (part of) the system. However, the future will be more determined by dynamic metabolic profiling. Measuring changes in metabolism over time and in response to challenges to the system provides a more profound insight not only in the status of the living system but also in it's capacity to regulate and adapt.

Especially for chronic diseases such as Rheumatoid Arthritis (RA) of which the etiology is unclear but with both genetic and environmental factors playing a role, it is interesting to study the metabolome (Glocker et al. 2006). RA is a chronic systemic autoimmune disease characterized by inflammatory polyarthritis which affects approximately 0.5 -1% of the population worldwide (Hochberg et al. 2007). Although the currently favored treatment regime using disease-modifying anti-rheumatic drugs (DMARD's) in an early stage of RA is believed to have a favorable result on disease course by influencing inflammation, patients assessment of disease activity and functional disability do not always support this result (Welsing et al. 2005). Even 30% of the RA patients initiating the most effective treatment option available, anti-TNFα therapy, fail to respond (Smolen 2005). New disease management strategies are therefore needed.

In this review the focus is on metabolites involved in RA. Various hypotheses about the role of certain metabolites in the etiology and progression of RA are reviewed. Secondly, an attempt is made to elucidate relevant interactions between the metabolites identified in the RA literature making use of pathway analysis tools. Finally we show the relevance of metabolites in the RA field and provide ideas for new insights on tackling this disabling disease.

Metabolites and metabolic processes involved in RA

An extensive literature search of the PubMed database was performed using several key words related to metabolites in combination with the keyword 'rheumatoid arthritis'. The results were screened and categorized, leading to a number of topics with sufficient material to be discussed for this review. In this section research is discussed concerning vitamins, eicosanoids, fatty acids, lipids, hormones and cartilage breakdown products. Although trace elements are not considered metabolites, information about this particular group of micronutrients is included because of the importance for RA.

Vitamines

Vitamin D deficiency is related to several autoimmune diseases including type 1 diabetes, multiple sclerosis, SLE and rheumatoid arthritis (Arnson et al. 2007). Vitamin D (a secosteroid) is transformed in the liver and kidney into the hormone 1,25 dihydroxyvitamin D (1,25(OH)2D3), the active form that stimulates the intestinal absorption of calcium and phosphor. Vit D receptor is expressed on mononuclear cells, dendritic cells, activated T and B cells, antigen presenting cells. Dendritic cells produce vit D hormone which inhibits T-cell proliferation, maturation of dendritic cells, secretion of IL-2 and INF-y by CD4+ T-cells, secretion of cytokines by macrophages and B-cell antibody production (Arnson et al. 2007). Low blood levels of vit D hormone also result in increased blood levels of insulin-like growth factor (IGF-1), a potent stimulator of cell growth and inhibitor of apoptosis (Huynh et al. 1998, O'Connor et al. 2008). In short, Vit D hormone stimulates an anti-inflammatory environment and particularly affects Th1 driven autoimmunity (Cutolo et al. 2007).

There is some controversy concerning the association of vit D intake with RA risk. However, Cutolo et al. suggest that these differences in results arise from the various ways of measuring vit D intake. Vitamine D intake should be measured in serum and not by dietary questionnaires (Cutolo et al. 2011). When this is taken into account, low levels of vitamin D can be associated with an increased disease risk as well as increased disease activity (Merlino et al. 2004, Oelzner et al. 1998). Several factors can result in lower blood vit D hormone levels. Animal protein has been shown to block the kidney enzyme responsible for converting vit D to the active vit D hormone by promoting blood acidity (Breslau et al. 1988, Langman 1989). Secondly, high blood calcium levels decreases vit D hormone which regulates blood calcium levels (Chan et al. 1998).

Interestingly a circannual rhythm in Vit D levels was found which correlates inversely with DAS28 scores of RA patients, which could also provide difficulties in interpreting measurements (Cutolo et al. 2006). Taking this into account lower serum levels of vit D seem to be associated with higher disease activity. This suggests that vit D might play a role in the differences in RA prevalence between northern and southern countries (Cutolo et al. 2006).

Other vitamins with antioxidant activity such as vitamin C, vitamin E and β-carotene have also been implicated in RA. Pattison et al. found a threefold increased risk of developing inflammatory polyarthritis when vitamin C intake was very low (Pattison et al. 2005). Low serum levels of β-carotene have been detected in blood bank samples of people who later on developed RA (Comstock et al. 1997). Low levels of plasma pyridoxal-5-phosphate (PLP) the active form of vitamine B6 have been found in RA patients (Roubenoff et al. 1995). In general, a low antioxidant status increases the risk of developing RA (Heliovaara et al. 1994). No relationships have been found between the progression of RA and plasma vitamin D, vitamin C, vitamin E and β-carotene levels.

Eicosanoids and fatty acids

RA patients have been shown to improve on a diet low in arachidonic acid (AA) and supplemented with N-3 fatty acids (Adam et al. 2003). Arachidonic acid and other fatty acids are precursors for eicosanoids which are important mediators of inflammation. Interestingly N-6 and N-3 fatty acids are competitively metabolized into N-6 and N-3 eicosanoid families with different functions. An N-6 dominant diet is implicated in increased prevalence of thrombotic vascular events and inflammatory diseases. The modern diet contains over 50 times the required amount of N-6 fatty acids which markedly shifts the n-6/n-3 ratio (Cleland and James 1997). N-3 fatty acids are less pro-inflammatory and have been shown to reduce TNF-α and IL-1β levels in RA patients, by competitively inhibiting the production of leucotriene B4 (LTB4) from arachidonic acid (Kremer et al. 1990).

Arachidonic acid is metabolised in several classes of compounds denoted as eicosanoids including prostaglandins, thromboxanes, leukotrienes, hydroxy- and epoxy-fatty acids, lipoxins and isoprostanes (Funk 2001). Leukotriene B4 and 5-hydroxy-eicosatetraenoic acid (5-HETE) are two strong chemotactic eicosanoids found in synovial fluid of RA patients (Klickstein et al. 1980). Dietary AA is exclusively derived from animal based foods of which 80% is metabolized into eicosanoids. Endogenous AA synthesis from linoleic acid also

occurs in the body, which is inhibited by poly-unsaturated fatty acid (Adam et al. 2003). These prostaglandin synthesis pathways are the basis of NSAID's and are therefore essential in current RA management strategies (ACR 2002).

Lipids

An atherogenic blood lipid profile has been reported in persons who more than 10 years later developed RA, characterized by higher total cholesterol, triglyceride and apolipoprotein B levels and lower high-density lipoprotein (HDL) cholesterol levels (van Halm et al. 2007). Contradictory observations have been reported concerning changed lipid profiles in RA. Dursunogh reported higher levels of Lp(a) lipoprotein, triglyceride (TG), lower levels of HDL cholesterol and no changes in total cholesterol and low-density lipoprotein (LDL) cholesterol (Drusunoglu et al. 2005). Lazarevic also reported increased TG and decreased HDL-c levels, but found lower total cholesterol (TC) and LDL-c levels (Lazarevic et al. 1993). Lakatos in contrary reported lower TG and higher TC and LDL-c levels, but also reported lower HDL-c levels (Lakatos and Hárságyi 1988). Only higher lipoprotein Lp(a) and lower HDL-c levels are consistently reported.

Ethnic, racial and geographic differences are suggested as possible causes for these differences in lipid profiles. Both genetic and dietary factors may contribute to higher Lp(a) levels, especially increased fat and cholesterol consumption (Cesur et al. 2007). Blood cholesterol levels increase most effectively by consuming animal protein, and to a lesser extend by consuming saturated fat and cholesterol. In contrast, plant based foods contain no cholesterol and decrease blood cholesterol levels (Kritchevsky et al. 1982).

A high LDL/HDL ratio, which indicates a less favorable lipid profile, has been associated with the pro-inflammatory molecules CRP, IL-6 and TNF-α (Hulthe and Fagerberg 2002). In contrast HDL cholesterol associated with apolipoprotein A-1 has anti-inflammatory effects. Apo A-1 is reported to inhibit IL-1, TNF-α and interactions between T-cells and monocytes (Burger and Dayer 2002, Hyka et al. 2001). This suggests that a less favorable lipid profile stimulates a pro-inflammatory status which might be related to RA disease activity and RA susceptibility.

Hormones

Cortisol is the strongest endogenous anti-inflammatory substance in the human body. Inflammation activates the immune system and the hypothalamic-pituitary-adrenal (HPA) axis, leading to the secretion of cortisol. However, in RA patients cortisol levels are not sufficiently increasing in response to inflammation (Neeck et al. 1990). Even in the presence of high serum levels of circulating cytokines, plasma cortisol levels in RA patients are similar to that of control subjects. A result of the relatively low plasma cortisol levels and presence of vasodilatory cytokines such as TNF-α is an increase in sympathetic activity. This is needed to stabilize blood pressure, systemic circulation and glucose homeostasis. However, the higher sympathetic tonus does not lead to an increase of noradrenaline, which has an anti-inflammatory effect, and therefore doesn't compensate the relatively low cortisol levels (Capellino and Straub 2008).

Inflammatory cytokines stimulate the conversion of anti-inflammatory adrenal androgens into estrogens. Elevated estrogen and estrone levels in the synovium of RA patients sustains a pro-inflammatory state. This is thought to be related to the increased prevalence of RA in women (Capellino and Straub 2008).

It is well known that RA symptoms such as stiffness and pain can differ greatly during the day and even during the year (Labreque at al. 1995). Cutolo extensively studied metabolic processes underlying such circadian, circamensual and circannual rhythms in RA (Cutolo et al. 2005). Cortisol, melatonin and prolactin show a marked daily rhythm. Melatonin stimulates Th1 type cytokines while cortisol stimulates Th2 type cytokines with a diurnal rhythmicity (Cutolo 2008). In patients with a high disease activity cortisol is downregulated during the evening and night, leading to an increase of inflammation in the morning. Low cortisol levels reduces its IL-6 and TNF inhibitory effects and consequently drives an increase in TNF, IFN-γ, IL-2, IL-12 and IL-6 levels. In RA patients, a melatonin plateau of 2-3 hours is observed and prolactin levels are significantly higher during the night. As a result, a Th1 response is induced during the night (Cutolo and Straub 2008). Rhythms in concentrations of inflammatory metabolites have to be considered when time-points for measurements and treatment are being determined.

Cartilage breakdown products

RA is characterized by extensive degradation and synthesis of joint cartilage which consists for a large part of type II collagen fibers, other types of collagen and proteoglycans. Consequently, cartilage breakdown products of type II collagen have been extensively studied in the synovium, serum and urine of RA patients (Charni et al. 2005). An immunoassay is described that detects C-terminal crosslinking telopeptide of type II collagen (CTX-II) (Christgau et al. 2001). Elevated urine CTX-II levels have been associated with more rapid disease progression in OA and RA patients. Another urine marker for early RA and progression of RA is HELIX-II, a sequence arising from degradation of the helical region of type II collagen (Charni et al. 2005).

Another collagen breakdown product that has been used as a biomarker in synovial fluid and serum is cross linked carboxyterminal telopeptide of type I collagen (ICTP). Markers for collagen synthesis that have been used are the aminoterminal propeptides of type I procollagen (PINP) and of type III collagen (PIIINP) (Aman et al. 1999). One study reported an increase of serum PIIINP and ICTP levels in 50% of the RA patients. Additionally PINP and PIIINP were both increased in synovial fluid indicating collagen synthesis (Hakala et al. 1995). A disturbed balance of collagen breakdown and synthesis seems to be evident in inflamed joints.

Apart from various types of collagen, cartilage also contains large proteoglycans including the glycosaminoglycans keratan sulfate (KS) and chondroitin sulfate (CS). KS and CS containing fragments have been measured in synovial fluid as biomarkers of cartilage metabolism. In RA patients, the level of 846 fragment of CS is reported to be increased in serum and more so in synovial fluid, indicating aggrecan synthesis in the inflamed joint. Serum KS levels were reduced in RA (Poole 1994). It is important to note that metabolite concentrations and changes can differ markedly between synovium and serum, providing multiple sources of information concerning local and systemic aspects of the disease.

Trace elements

Zinc and copper are two trace elements of which plasma concentrations decrease during acute inflammation (Halsted and Smith 1970, Lewis 1984). Plasma zinc levels are found to be decreased in RA patients. Especially T-cell function is impaired by reduced zinc availability

leading to reduced T-cell numbers and the Th1/Th2 ratio shifting towards Th2 (Honscheid et al. 2009). Zinc and copper have antioxidant properties. They are constituents in cytoplasmic superoxide dismutase (SOD), a metalloenzyme with anti-inflammatory properties, and copper is also found in the serum antioxidant ceruloplasmin (Honkanen et al. 1991). Intracellular SOD activity has been shown to be decreased in RA patients (Rister et al. 1978) which corresponds with decreased plasma zinc levels. However, the ceruloplasmin levels in serum of RA patients is increased. An increase in zinc consumption is correlated with a decrease in copper absorption by the intestine (Honkanen et al. 1991). This decrease of copper absorption might be a reason for the contradictory results found in studies into the effects of dietary zinc supplementation in RA (Rasker and Kardaun 1982, Mattingly and Mowat 1982, Tudor et al. 2005).

Selenium (Se) is another trace element that plays a role in regulating ROS through incorporation into selenoproteins. Glutathion peroxidase for example catalyzes the degradation of peroxides, is involved in arachidonic acid metabolism and can affect leukotrine and prostaglandine synthesis (Kalpakcioglu and Senel 2008). Low levels of Se have been reported in plasma and cells of RA patients (Tarp et al. 1985). Selenoproteins can have various effects on the immune system such as stimulating a Th1-type response more than a Th2-type response. However Se supplements only seem to boost the immune system for people who already had low Se levels (Hoffmann and Berry 2008). Much still needs to be learned about the functions of the various selenoproteins.

Metabolites and levels of system organization

Van Ommen and others described a health space based on metabolomics phenotypes to organize metabolite data (van Ommen et al. 2008). The metabolites reviewed in the previous sections can be organized according to this approach in what might be called levels of system organization. In this review a distinction could be made between metabolites involved in metabolic processes, especially those located in the inflamed joints, oxidative stress processes and inflammatory processes (Table 1). As could be expected a number of compounds are upregulated or downregulated in RA patients that are involved in the inflammatory process. In addition, oxidative stress factors also seem to play an important role in RA. The metabolism aspects are mostly represented by the changes in the inflamed joints of RA patients.

Table 1. Organization level of metabolite changes in rheumatoid arthritis

	*Metabolite**	*Matrix***	*Effect*
Inflammation	Vitamin D ↓	p	IGF-1 ↑, Th-2 ↑
	AA ↑	p	Inflammation ↑
	LTB-4 ↑	s	Chemotaxis ↑
	5-HETE ↑	s	Chemotaxis ↑
	LDL ↑	p, s	CRP ↑, IL-6 ↑, TNF-α ↑
	HDL-c ↓	p, s	CRP ↑, IL-6 ↑, TNF-α ↑, CVD risk ↑
	Lp(a) ↑	p	CRP ↑, IL-6 ↑, TNF-α ↑
	Cortisol ↓	p	Symp ↑, TNF ↑, IFN-γ ↑, IL-2 ↑, IL-6 ↑, IL-12 ↑
	Estrogen ↑	p	Inflammation ↑
	Prolactin ↑	p	Th-1 ↑
	Melatonin	p	
	n-3 FA ↓	p	TNF-α ↑, IL-1β ↑
	n-6 FA ↑	p	TNF-α ↑, IL-1β ↑
	VLDL ↑	s	
Oxidative stress	Vitamin B6↓	p	RA risk ↑
	Vitamin C ↓	p	RA risk ↑
	Vitamin E ↓	p	RA risk ↑
	β-Carotene ↓	p	RA risk ↑
	Copper ↓	p	SOD ↓
	Zinc ↓	p	SOD ↓
	Selenium ↓	p	ROS ↑, Th-2 ↑
	Lactic acid ↑	p, s	p: oxidative damage ↑,s: anaerobic metabolism ↑
	Glucose ↓	s	Anaerobic metabolism ↑
Metabolism	CTX II ↑	u	RA progression ↑
	HELIX II ↑	u	RA progression ↑, collagen breakdown ↑
	ICTP ↑	p, s	Collagen breakdown ↑
	PIIINP ↑	p, s	Collagen synthesis ↑
	KS ↑	p	
	CS ↑	p, s	Cartilage synthesis ↑

* Changes in metabolite levels found in serum of RA patients are given with the effects due to these changed levels.
** Matrix in which the metabolite was measured: p=plasma, u=urine, s=synovium

RA and metabolomics

Metabolomics techniques are recently becoming popular for biomarker discovery, also in the field of rheumatoid arthritis. Seven studies were found in PubMed in which metabolomics platforms were used to find metabolite profiles that distinguish rheumatoid arthritis patients from healthy controls. Four of these papers are dealing with synovial fluid samples (Williamson et al. 1989, Naughton et al. 1993a, Naughton et al. 1993b, Meshitsuka et al. 1999), two papers with human serum samples (Lauridsen et al. 2010, Wietmarschen et al. 2009) and one paper with mouse serum samples (Weljie et al. 2007).

The synovial fluid of healthy people show elevated lactate levels, indicating a hypoxic status

in the joint. In RA patients this level of lactate is more elevated, glucose levels are diminished and ketone bodies are detectable indicating an even more hypoxic state and the utilization of fatty acids for energy. The inflamed joint of RA patients also contained lipoprotein associated fatty acids such as VLDL triacylglycerols, LDL, HDL, cholesterol and HDL phospholipids, which are not able to enter the joint space in healthy conditions (Naughton et al. 1993a).

In serum of K/BxN arthritis mice several metabolic changes have been found compared to healthy control mice using NMR spectroscopy. Some lipid, fatty acid and carbohydrate metabolism markers were found: Glycerol, Choline, 2-hydroxybutyrate, acetylcarnitine, TMAO. Energy metabolism markers 2-oxoglutarate, acetylcarnitine were detected. The markers xanthine, hypoxanthine, uridine, uracil, TMOA are related to nucleic acid metabolism. Glutamate, serine, phenylalanine, glycine, methionine, asparagine are related to amino acid metabolism. Methionine, glycine, taurine, xanthine, hypoxanthine, TMAO, acetylcarnitine are important for dealing with oxidative stress (Weljie et al. 2007).

Plasma from human RA patients has been shown to contain elevated lactate and cholesterol levels and decreased HDL levels. Elevated plasma lactate levels have been associated with increased oxidative damage and lowered synovial pH. Lower levels of HDL and consequently higher levels of cholesterol support the increased risk of cardiovascular diseases in RA patients (Lauridsen et al. 2010). The only study in which gas chromatography mass spectrometry (GC-MS) was used to detect metabolic differences between RA patients and healthy controls reported higher levels of heptanoic acid, l-Alanine, 2-oxy-butanoic acid, l-asparagine, palmitic acid and lower levels of 2-butenoic acid, undecanoic acid, d-glucuronic acid, ribitol, stearic acid in RA patients (van Wietmarschen et al. 2009).

Connecting the dots

In the following section we will discuss the literature findings concerning metabolites and RA in an attempt to uncover relationships between metabolites and biological mechanisms relevant for studying RA. The interpretation of metabolite information from various sources is challenging for several reasons. For instance, publications often describe the role of classes of metabolites instead of specifying individual metabolites with appropriate database identifiers. Pathway analysis tools are not able to handle groups or families of metabolites. A second challenge is that many measured metabolites are not yet present in the databases connected to pathways analysis software. Therefore the actual list of metabolites that can be

analyzed by pathway construction tools usually represents only part of what is actually known.

For 20 of the 25 metabolites related to human plasma changes in RA an identifier could be found in the Kyoto Encyclopedia of Genes and Genomes (KEGG). These metabolites were imported in the Ingenuity Pathway Analysis software and used for the subsequent analysis (Gehlenborg et al. 2010). Metabolites with higher levels in RA patients were given a fold change value of 2 while the metabolites with lower levels in RA were given a fold change value of minus 2 because the actual values could not be determined and compared between studies based on the literature.

Several of the compounds were found to be involved with the key molecular and cellular functions cellular development, cellular growth and proliferation, cell death, lipid metabolism and molecular transport. In addition, the set of compounds was found to be mostly related to metabolic disease, inflammatory response, cardiovascular disease, endocrine system disorders and gastrointestinal disease.

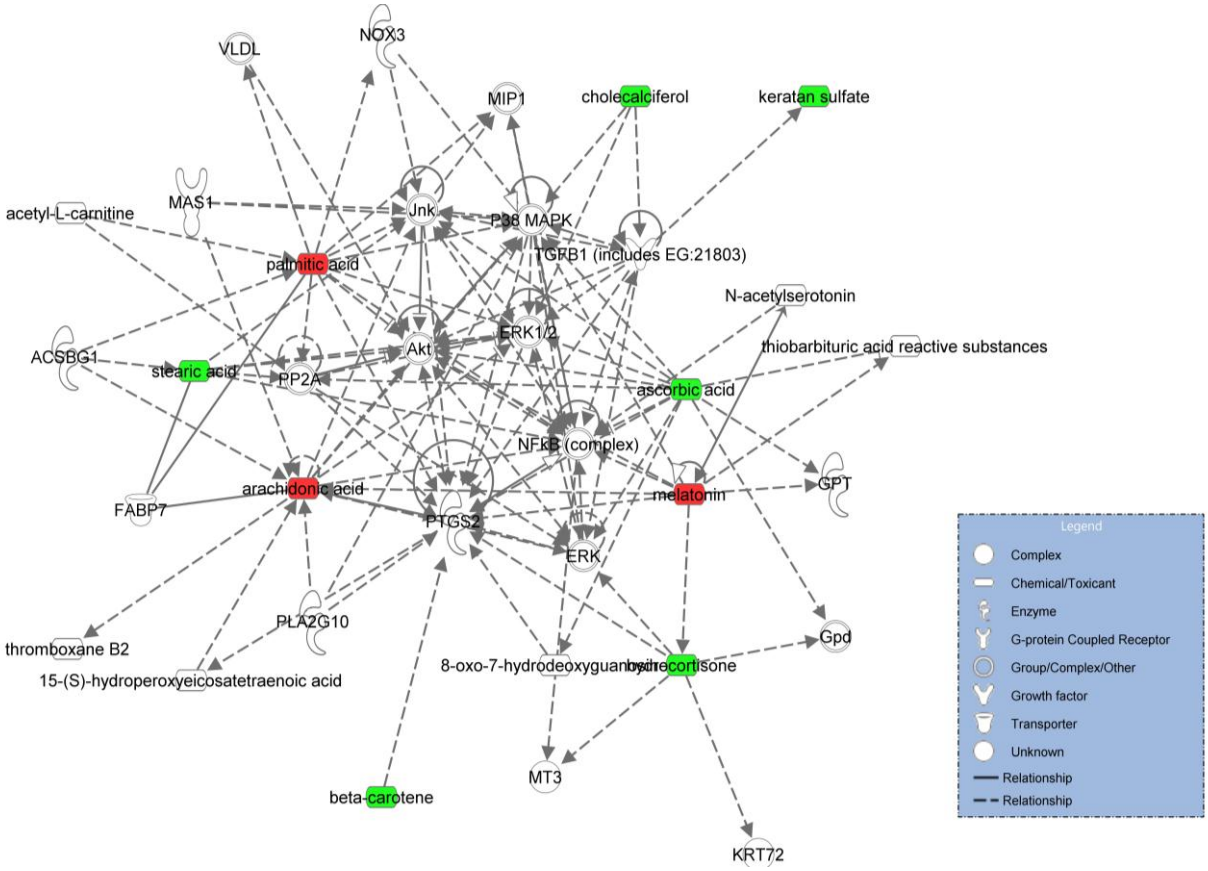

Figure 1. Ingenuity pathway analysis of the metabolites discussed in this review that are related to rheumatoid arthritis. Relationships are based on literature mining. Red colored nodes indicate up-regulated metabolites and green colored nodes indicate down-regulated metabolites.

The network (Figure 1) most closely related to the data set was characterized by the top functions cell death, lipid metabolism and small molecule biochemistry containing 9 of the reviewed metabolites: arachidonic acid, ascorbic acid, beta-carotene, cholecalciferol, hydrocortisone, keratan sulfate, melatonin, palmitic acid and stearic acid. These nine metabolites are highly connected to a number of canonical pathways related to immune functions, the production of nitric oxygen and reactive oxygen species in macrophages and pathways involved in arthritis. Specifically the role of macrophages, fibroblasts, endothelial cells, osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis are connected to this network as well as the role of IL-17 in arthritis and IL-12 signaling and production in macrophages.

Final remarks

Many metabolites have been reported to play a role in rheumatoid arthritis. This review indicates groups of metabolites that could be interesting for metabolomics studies into the mechanism of and possible treatment options for rheumatoid arthritis. Eicosanoids, fatty acids, lipids, trace elements, vitamins and several hormones are interesting candidates for elucidating the mechanism of RA. Pathway analysis has provided an indication of biological processes related to the discussed collection of RA related metabolites. This review suggests that more clinical studies are needed to elucidate the effects of vitamin supplementation on RA activity and progression. In addition, circadian rhythms in hormone production and other metabolite levels are important to consider. For instance, the timing of glucocorticoid treatment is essential for obtaining an optimal effect (Cutolo 2008). This review also shows the complexity of the immune and hormonal processes that are changed in rheumatoid arthritis patients, suggesting that a move from single-target therapy towards multi-target therapy is needed. Finally, a more personalized medicine approach is needed to address the differences in the expression, severity, progression and metabolic changes of RA for each individual.

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5. Systems biology guided by Chinese Medicine reveals new markers for sub-typing rheumatoid arthritis patients

Abstract

Background. Complex chronic diseases such as rheumatoid arthritis have become a major challenge in medicine and for the pharmaceutical industry. New impulses for drug development are needed.

Objective. A systems biology approach is explored to find sub-types of rheumatoid arthritis patients enabling a development towards more personalized medicine.

Methods. Blood samples of 33 RA patients and 16 healthy volunteers were collected. The RA patients were diagnosed according to CM theory and divided into two groups, the RA Heat and RA Cold group. CD4+ T-cells were used for a total gene expression analysis. Metabolite profiles were measured in plasma using gas chromatography/mass spectrometry (GC/MS). Multivariate statistics was employed to find potential biomarkers for the RA Heat and RA Cold phenotype. A comprehensive biological interpretation of the results is discussed.

Results. The genomics and metabolomics analysis showed statistically relevant different gene expression and metabolite profiles between healthy controls and RA patients as well as between the RA Heat and RA Cold group. Differences were found in the regulation of apoptosis. In the RA Heat group caspase 8 activated apoptosis seems to be stimulated while in the RA Cold group apoptosis seems to be suppressed through the Nrf2 pathway.

Conclusions. Rheumatoid arthritis patients could be divided in two groups according to CM theory. Molecular differences between the RA Cold and RA Heat groups were found which suggest differences in apoptotic activity. Subgrouping of patients according to CM diagnosis has the potential to provide opportunities for better treatment outcomes by targeting Western or CM treatment to specific groups of patients.

Based on: van Wietmarschen H, Yuan K, Lu C, Gao P, Wang J, Xiao C, Yan X, et al. (2009) Systems biology guided by Chinese medicine reveals new markers for sub-typing rheumatoid arthritis patients. *Journal of clinical rheumatology* 15(7): 330-7.

Introduction

Complex chronic diseases such as rheumatoid arthritis have become a major challenge in medicine and for the pharmaceutical industry. New impulses for drug development are needed. Rheumatoid Arthritis (RA) is a chronic systemic autoimmune disease characterized by inflammatory polyarthritis of unknown etiology, affecting approximately 0.5 -1% of the population worldwide.[1]

Although the currently favored treatment regime is believed to have a favorable result on disease course by influencing inflammation, patients assessment of disease activity and functional disability do not always support this result.[2] The target-centric approach seems to have reached its limit and a more personalized, system-based strategy provides a potential for a paradigm shift in drug discovery and development.[3, 4] An important bottleneck in moving towards patient stratification is diagnosis.

Over the years several clinical features and molecular markers have been identified to sub-type RA patients.[5, 6] Anti-citrullinated protein antibodies (ACPA) positive or negative status is found to be related to distinctive RA risk profiles.[7] More inflamed joints and a higher level of joint destruction was reported in ACPA positive RA patients.[8] A large heterogeneity between the INF-1 high and low sub-typed have been found in gene expression profiles of RA patients, but this is not very clear in the clinical features.[9] All this knowledge has yet to lead to more personalized health strategies in clinical practice.

A systems biology approach has been advocated for managing RA.[10] Systems biology aims to increase our understanding of biological systems by looking at the many interactions between hundreds of genes, proteins and metabolites simultaneously.[3, 11, 12] Interestingly a systems approach already exists for several thousands of years in the practice of Chinese Medicine (CM), but the underlying theory is not yet understood from a biochemical basis. In CM theory diseases are called syndromes, which are based on patterns of various symptoms expressed by the entire body.[13] Treatment is usually personalized although more general strategies are also used. Systems biology could therefore be a bridge between CM and western medicine.[14][15]

However simply copying CM treatments to the western practice is not feasible. Extracts of Tripterygium wilfordii Hook. F. is one of the commonly and successfully used CM intervention strategies for RA that has been studied extensively.[16, 17, 18]. Other examples are the use of preparations of Ganoderma lucidum (Leyss. Ex Fr.) Karst, an extract of

multiple herbs named San-Miao-Wan[19], Celastrus aculeatus Merr[20] and Forsythia suspense (Thunb.) Vahl[21] as immune modulators in RA. In these studies the benefits of standard CM preparations given to RA patients in general are not always that clear.[19] We think that the key issue here is the different diagnostic practices between CM and western medicine.[22]

Several studies show that the diagnostic methods used in CM cannot be separated from the treatment strategies used.[23] A study with 396 RA patients shows that the treatment of RA patients with Tripterygium wilfordii Hook. F. is more effective when the patients were affected with joint pain and joint tenderness, but didn't have more urination at night and joint stiffness. Additionally a Western treatment consisting of diclofenac, methotrexate and sulfasalazine was found to be more effective in RA patients displaying joint tenderness and thirst, but less effective in RA patients with dizziness. These results also indicate that CM diagnosis can be used to discover sub-phenotypes of RA.[16]

According to CM theory RA patients would fall in the Bi Zheng (Bi-syndromes), a collection of different syndromes that is characterized by obstruction of Qi and Blood in the Channels and Collaterals. The Bi-syndromes encompass diseases with Western descriptions such as myalgias, osteoarthritis, RA, repetitive strain injuries and nerve pain. Bi syndromes are described in CM theory as the result of an attack by three out of the four external pathogenic factors: Wind, Cold, Damp and Heat.[24] Several syndromes or patterns are therefore used for patients in CM such as a Wind pattern or a Cold pattern. Using the Bi syndrome patterns RA patients can be differentiated into various groups, which are also treated very differently with Chinese herbal medicine. In this study the two very distinct CM patterns cold and heat were chosen to divide the RA patients into an RA Cold and RA Heat group. These two patterns were chosen because the symptoms are very different and the patients are treated very differently based on Chinese herbal medicine practice.

The Cold pattern can be described as severe pain in a joint or muscle that limits the range of comfortable movement which doesn't move to other locations. The pain is relieved by applying warmth to the affected area, but increases with exposure to cold. Loose stools are characteristic as well as an absence of thirst and clear profuse urine. A thin white tongue coating is seen, combined with a wiry and tight pulse. In contrast the Heat pattern is characterized by severe pain with hot, red, swollen and inflamed joints. Pain is generally relieved by applying cold to the joints. Other symptoms include fever, thirst, a flushed face, irritability, restlessness, constipation and deep-colored urine. The tongue may be red with a

yellow coating and the pulse may be rapid.[25]

In this exploratory study genomics and metabolomics tools were used to find biomarkers that could indicate new sub-phenotypes of RA, related to the differentiation of RA patients into Cold and Heat pattern according to CM diagnosis.

Patients and methods

33 Female RA patients visiting the Institute of Traditional Chinese Medicine in Beijing for the first time and 16 healthy female volunteers, all residents of Beijing, age 24 to 64 years old (μ=43.6) participated in the study. All participants gave consent and the study was approved by the ethics board of the Institute of Basic Research In Clinical Medicine, China Academy of Chinese Medical Sciences. RA patients were eligible to participate if they had met the American College of Rheumatology (ACR) criteria for rheumatoid arthritis for at least one year with functional Class at level I, II, or III.[26] All patients completed a 115 item questionnaire and a tongue and pulse diagnosis was taken by a CM practitioner. The questions were related to joint issues, pain, response to weather, and other symptoms such as fever and thirst. Using this information the CM practitioner then classified the RA patients as heat pattern and cold pattern as described above. These groups are very different in the symptoms they expressed, for example the RA Heat patients experience severe pain in hot weather or when heat is applied and RA Cold patients in cold conditions. The two groups, the RA Cold and RA Heat patients, did not differ in mean age and erythrocyte sedimentation rate (tested with Mann-Whitney U test). For the control group healthy women living in Beijing, coming to the hospital for a regular health exam, were included if they had no diagnosed diseases. After the study the RA patients received CM treatment based on the diagnosed heat or cold pattern and some of them also received Western treatment, as is the regular practice in the CM hospital in China.

Patients continuously receiving NSAID's, corticosteroids for over 6 months, or receiving the above mentioned medicines within one month were not included in the study. Also the patients were not on any CM medication yet. The patients with severe diseases of the cardiovascular system, lung, liver, kidney, mental and blood system, women who were pregnant, breast-feeding or planning pregnancy in the next 8 months, were excluded from the study.

Genomics

Analysis of blood samples

For the genomics analysis 8ml venous blood was collected in anticoagulation tubes before breakfast. CD4+T cells play a key role in inflammatory processes in RA.[5, 6] It is suggested that an altered CD4+T cell homeostasis in RA may contribute to the autoimmune response as well as to the immunodeficiency in RA patients.[27] Therefore CD4+T lymphocytes were extracted. The CD4+T cells were collected referring to StemSep® Rhesus CD4 T Cell Enrichment Cocktail Kit manual(StemCell Technologies, Inc. Canada).

Total RNA was extracted from the 33 RA samples and 12 control samples that were at least 95% purified, using the TRIzol kit (Life Technologies, Inc) in accordance with the manufacturer's procedure. Purity was checked by flow cytometry. Gene chips containing 23232 cDNAs were hybridized according to the Micromax ASAP RNA labeling kit procedure (PerkinElmer). Reverse transcription was done following the manufacturer's protocols of SuperScript ⅡFirst-strand Synthesis System (Invitrogen, Life Technologies). The Gene chips were scanned using Genepix4000B scanner. Picture information scanned was transformed into data using GenePix®Pro Micrroarray Image Analysis Software.[28, 29]

Data analysis

Data normalization to correct for technical variation among individual microarray hybridizations was conducted using a two-step procedure described in detail by Jarvis and colleagues.[30, 31, 32]

Gene expression profiles of controls were compared with RA patients and the profiles of RA Cold patients were compared with RA Heat patients. The differences in gene expression levels were considered significant when the signal was increased or decreased more than 1.4 compared to control ($p<0.05$ in Student's t-test). More than 1.4 increase in signal comparing controls with RA or RA Cold to RA Heat was recorded as up regulated, a decrease of more than 1.4 as down regulated. Also the gene expression fold change must be present in more than 50% of the patients. To determine differences in gene expression profiles between the Controls and RA patients as well as between the RA Cold and RA Heat patients hierarchical cluster analyses were performed using Cluster 3.0 and TreeView software (available at http://www-stat.stanford.edu/~tibs/SAM/faq.html).[33]

Metabolomics

Analysis of blood samples

For the metabolomics analysis blood samples of 21 RA patients and 16 healthy volunteers were used. The other 12 RA samples were used in another analysis. Blood plasma was treated as described elsewhere except this time a sample vs. acetonitrile ratio of 1:2 was taken.[34] 5 µL decanoic acid (internal standard) was added to each 200µL sample followed by the addition of 65 µL of DMF and 65 µL of MTBSTFA (derivatization agent).GC-MS analysis was performed according to Yuan et al.[35]

Data analysis

Metabolomics experiments usually result in a large number of measured metabolites, in this study 255 metabolites were measured. Specific multivariate statistical techniques are needed to analyze this kind of data. We first used Principal component analysis (PCA), a commonly used technique which is used to explore the data (SIMCA-P version11.0, Umetrics AB, Umea, Sweden). A PCA model attempts to project the maximum amount of variation in as few dimensions (principal components) as possible.[36, 37]

Before applying PCA analysis all variables in the data set were scaled. This is to prevent certain variables from dominating the resulting model. The means of the variables were set to zero and the values were also divided by the standard deviation. After this step partial least squares discriminant analysis (PLS-DA) was employed to find the variables that contribute most to the distinction between controls and RA patients [38]. PLS-DA was also used to find the most important variables that contribute to the distinction between the RA Cold and RA Heat group and then identified by mass spectrometry.

Cross-validation and permutation tests were used to validate the PLS-DA models. For the RA Cold versus RA Heat PLS-DA model a jackknifing procedure was followed to remove noisy variables.

Results

Genomics

Panel A in Figure 1 shows the 146 genes that were expressed significantly different between the RA patients and the control participants. The hierarchical cluster analysis results in a clustering of the control samples at the left and a clustering of RA patient samples at the right. In the first section of Table 1 the 10 most upregulated and downregulated genes are shown.

In Panel B of Figure 1 the significant differences in gene expression between the RA Cold and RA Heat group is shown. The cluster analysis of the 64 genes involved shows different gene expression profiles for the RA Cold (left), RA Heat patients (middle) and the control participants (right). In the second section of Table 2 the 10 most upregulated and downregulated genes are shown.

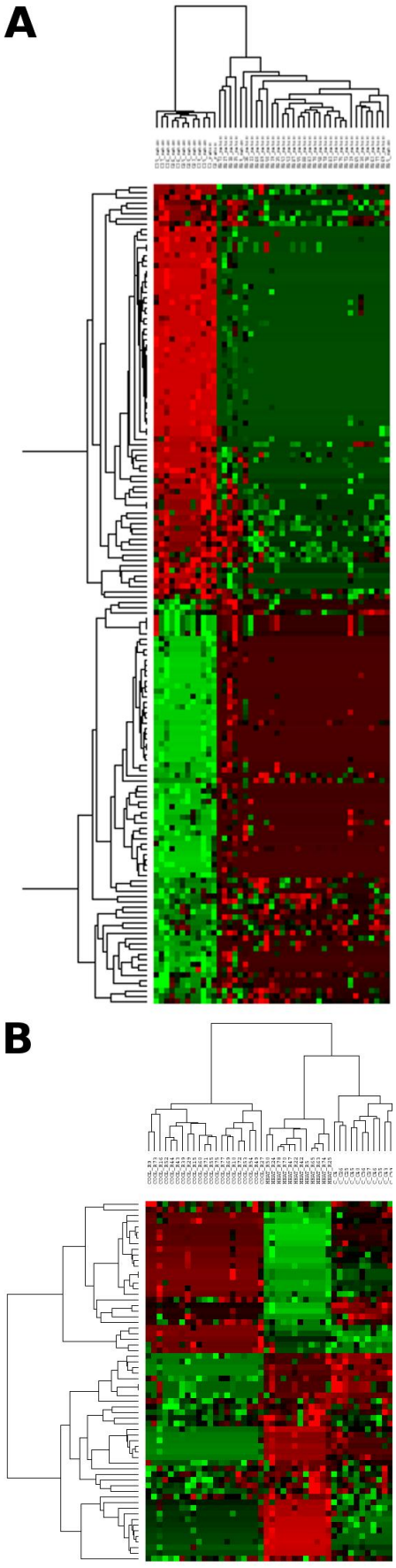

Figure 1. Panel A shows a hierarchical cluster analysis of the expression of all genes (146

genes) on the RA gene expression array. The tree structure reflects the similarity of the gene expression profiles. The horizontal axis represents the samples, and the vertical axis represents the gene expression. Red color in the figures means up-regulation and green color means down-regulation.12 control samples cluster together into a group on the left, while the remaining 33 RA samples at the right form a second group. Panel B shows a similar hierarchical cluster analysis of the expression of all genes (64 genes) on the cold-heat RA gene expression array. At the left side the RA Cold subjects are grouped, in the middle is the RA Heat group and at the right side are the control subjects.

Table 1. A selection of differentially expressed genes

RA versus control (↑ means more expressed in RA)	
DTW domain containing 1	↓
Leukocyte-associated Ig-like receptor 2	↓
Signal transducer and activator of transcription 5B	↓
Related RAS viral (r-ras) oncogene homolog	↓
Quiescin Q6	↓
Spastic paraplegia 20	↓
Chromosome 20 open reading frame 121	↓
Mitochondrial ribosomal protein L45	↓
Cell division cycle 25C	↓
Ubiquitin specific protease 20	↓
S100 calcium binding protein A8 (calgranulin A)	↑
Cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6	↑
KIAA0082 protein	↑
Myeloid cell nuclear differentiation antigen	↑
HSPC141 protein	↑
Transcobalamin I (vitamin B12 binding protein, R binder family)	↑
S100 calcium binding protein A9 (calgranulin B)	↑
Ribosomal protein L34 pseudogene 2	↑
Ficolin (collagen/fibrinogen domain containing) 1	↑
Interferon-induced protein with tetratricopeptide repeats 1	↑
RA Cold versus RA Heat (↑ means more expressed in RA Cold)	
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)	↓
H2A histone family, member X	↓
Basic leucine zipper transcription factor, ATF-like	↓
Zinc finger protein 22 (KOX 15)	↓
CHCHD8	↓
Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	↓
RGC32 protein	↓
rab11 family interacting protein 4 (class ii)	↓
Kelch-like 14	↓
Aspartyl-tRNA synthetase	↓
Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	↑
MSTP043 protein	↑
Leucine-rich repeat protein, neuronal 3	↑
G protein-coupled receptor 12	↑
ATPase, Ca++ transporting, plasma membrane 1	↑
Peptide transporter 3	↑
Heme oxygenase (decycling) 1	↑
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 20, 103kD	↑
Proline-serine-threonine phosphatase interacting protein 2	↑
Human clone 23695 mRNA sequence	↑

Metabolomics

Figure 2 panel A shows a PCA score plot containing all the participants of the study. Panel A indicates a grouping of control participants (more in the upper part) and RA patients (below the control participants).

The PLS-DA score plot in Figure 2. panel B shows that the RA patients and control participants could be divided into two groups based on their metabolite profiles. Cross-validation and 200 permutation tests show that the model, and thus the classification into a RA and Control group, is significant.

Figure 2 panel C shows a PLS-DA score plot in which RA Cold patients are separated from RA Heat patients. Cross-validation and 200 permutation tests show that the model, and thus the classification into a RA Heat and RA Cold group, is significant. Panel D of figure 2 shows the contribution of the metabolites to the RA Heat and RA Cold classification.

Identification of the metabolites that contribute most to the distinction between RA and control subjects resulted in 10 potential biomarkers. Additionally 7 potential biomarkers were identified related to the separation between the RA Cold and RA Heat groups (Table 2).

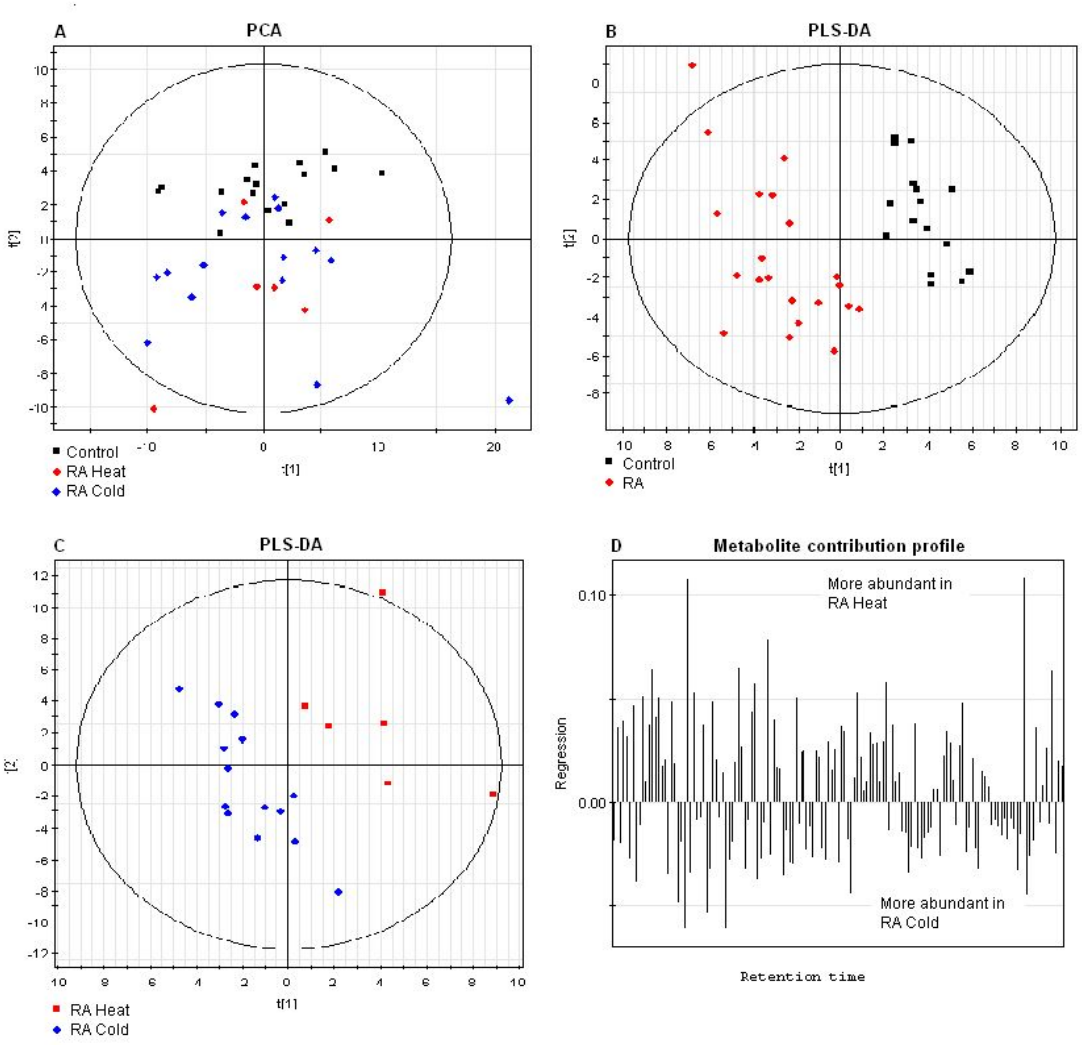

Figure 2. Panel A shows a PCA score plot in which all the RA patients and control participants are represented by a point. Different colors and symbols are used to visualize the control, RA Cold and RA Heat participants. Panel B shows a PLS-DA score plot for the first two principal components. The RA group and control group are clearly separated. In Panel C the RA cold and RA heat group are separated in a PLS-DA score plot. Panel D shows the contribution of the metabolites to the RA Heat and RA Cold classification.

Table 2. Potential biomarkers from metabolomics data

RA versus control	
Heptanoic acid	↑
2-Butenoic acid	↓
L-Alanine	↑
2-Oxy-butanoic acid	↑
Undecanoic acid	↓
L-Asparagine	↑
Palmitic acid	↑
D-Glucuronic acid	↓
Ribitol	↓
Stearic acid	↓
RA Cold versus control	
L-Leucine	↑
Inositol	↓
RA Heat versus control	
L-Proline	↑
5-Oxo-proline	↑
Urea	↑
RA Heat versus RA Cold	
3-Oxy propanoic acid	↑
L-Proline	↑
Urea	↑
L-Leucine	↓
5-Oxo-proline	↑
Ribitol	↑
Inositol	↑

Discussion

The comprehensive systems analysis consisting of genomics and metabolomics profiling described in this paper reveals a number of features that distinguish rheumatoid arthritis patients from healthy volunteers, and those that discriminate these RA patients into two subgroups called the RA Heat group and RA Cold group. Figures 1 and 2 suggest that a biological basis underlies the CM differentiation into these subgroups.

To understand more about the biology underlying RA and the sub-groups RA Heat and RA

Cold, we determined functionally related genes using DAVID's functional gene annotation tool (http://david.abcc.ncifcrf.gov/).[39] An interesting functional cluster of genes containing immune system processes was found, using the RA versus Control gene data. From the genes involved in this cluster a network was created using Cytoscape.[40] Additionally a cluster of genes involved in apoptosis was found using the RA Cold versus Heat gene data, and was added to the network and connected with dashed edges (Figure 3). The length of the edges connecting the nodes (genes) is determined by Kappa values, which expresses the strength of the relationship between the genes.

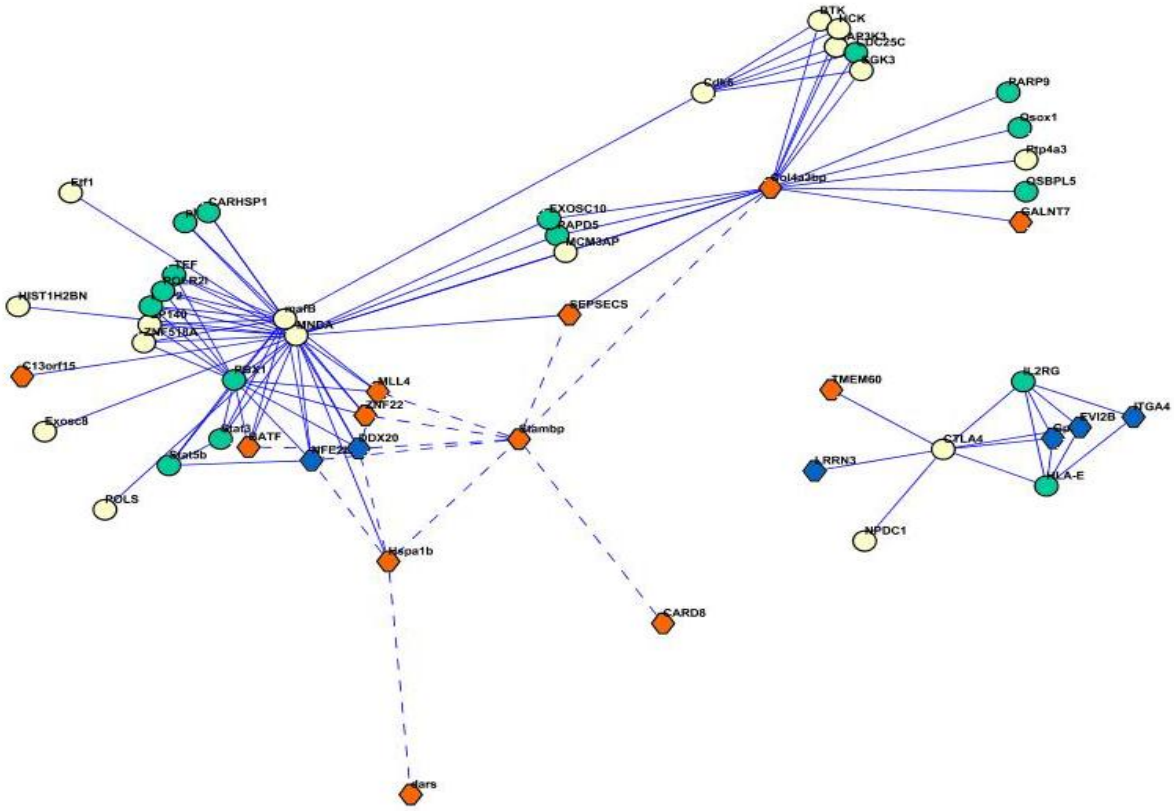

Figure 3. A network illustrating the functional relationships between genes involved in processes of the immune system (solid lines) and apoptosis (dashed lines). Genes colored dark green are upregulated in RA patients, light green colored genes are downregulated in RA patients. Octahedrons denote genes differently expressed in the RA Heat and RA Cold groups, blue means more expression in RA Cold and red means more expression in RA Heat. The weight of the edges is determined by the relatedness between the nodes. A clustering of genes active in RA Cold are clustering in the upper left network. Many genes upregulated in RA Heat are involved in apoptosis.

The great number of dark green nodes in the network shows that a lot of the genes involved

in immune processes are up-regulated in the RA patients in this study. Additionally the network shows that many of the genes up-regulated in RA Heat patients cluster together. Also 4 genes up-regulated in RA Cold patients group together in a small cluster separated from the main network. Interestingly 9 of the 11 up-regulated genes involved in apoptosis (dashed lines in Figure 3) are up-regulated in the RA Heat patients (red octahedrons), suggesting that activation of apoptosis plays a more significant role in the RA Heat subtype than in the RA Cold subtype.

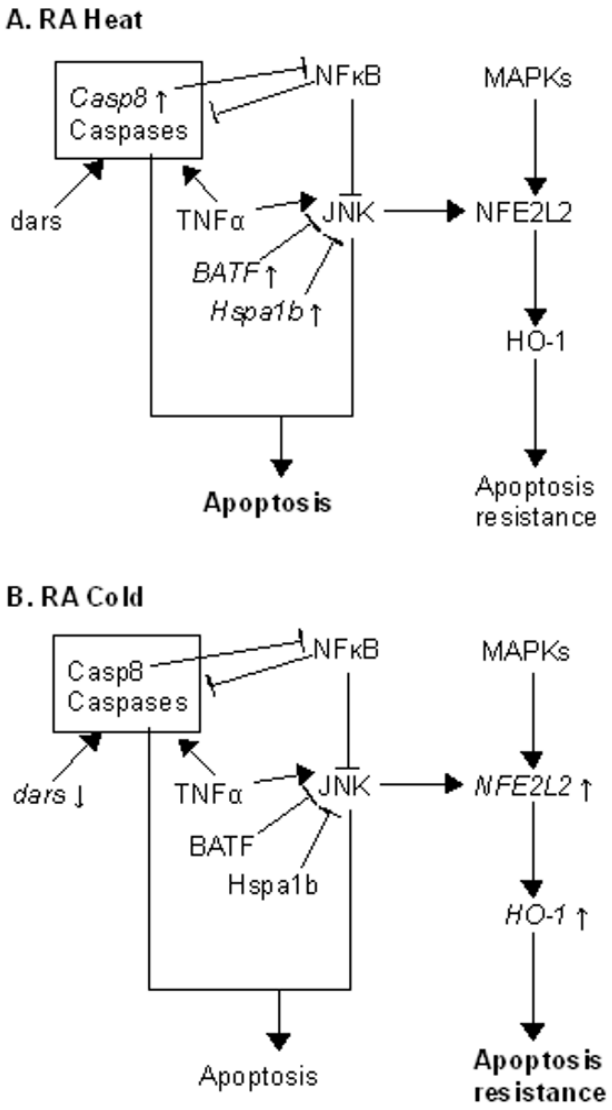

Figure 4. Apoptosis regulation in RA Heat and RA Cold patients. In RA Heat patients the expression of Caspase 8 was increased which resulted in a stimulation of apoptosic processes. In RA Cold the combination of no enhanced expression of Caspase 8 and the increased expression of NFE2L2 (Nrf2) and HO-1 activated apoptosis resistance events. Arrows denote activation, lines with flat ends denote inhibition. (The gene symbols used correspond with: dars: Aspartyl-tRNA synthethase, BATF: basic leucine zipper transcription factor, Hspa1b: Heat Shock 70kda protein, NFE2L2: Nuclear factor erythroid 2-related factor 2, HO-1: Heme oxygenase1.)

Figure 4 shows how apoptotic pathways are differently affected by the gene expression patterns in RA Heat and RA Cold patients. The discovery of the up-regulation of Caspase 8 recruitment domain in RA Heat patients points to the death receptor pathway. Apoptosis via this pathway occurs through ligation of death receptors such as TNF receptor and Fas [41]. Activation of the death receptors leads to the recruitment of Caspase 8 which can then activate Caspase 3 or Bid, both leading to downstream apoptotic events.

In contrast, two genes involved in apoptosis resistance, Heme oxygenase-1 and Nrf2 are up-regulated in RA Cold patients.[42] There is no up-regulation of Caspase 8, BATF and Hspa1b. NfκB and JNK are important regulators of apoptosis in which JNK can play both a stimulating and inhibiting role.[43, 44] In RA Heat patients Caspase 8 will inhibit NfκB[45], which will in turn reduce the inhibiting activity of NfκB on JNK. At the same time the up-regulated BATF and Hspa1b inhibit JNK activity.[46] The down-regulation of Aspartyl-tRNA synthetase in RA Cold patients, which is needed for the production of the aspartic acid rich caspases, is another finding that indicates reduced apoptosis activity. It could be that JNK is more active in RA Cold patients which could stimulate apoptosis resistance through the Nrf2 pathway.[47] RA Cold could be considered a more severe disease state as a lack of apoptosis in synovial fibroblasts, macrophages, fibroblasts, lymphocytes, neutrophils and osteoclasts has been proposed to contribute to the persistence of RA.[46]

Metabolomics shows an increased urea production in RA Heat patients, indicating more protein breakdown than in RA Cold patients. Also Proline and oxo-Proline are increased in RA Heat patients which is abundantly released during collagen breakdown. Additionally in RA Cold patients L-Leucine levels are raised. On one hand this can indicate protein synthesis, which is in agreement with normal urea levels found in RA Cold patients. On the other hand it can indicate a state of inflammation, which is the case in all RA patients but perhaps with varying metabolic effects.

The promising results of this study based on a limited number of patients were evaluated carefully. First a statistical evaluation was applied to show significance, but a major validation came from the coherent biological information found in this study. Further validation in future research will encompass also possible differences between patients from different cultural backgrounds.

In conclusion, rheumatoid arthritis patients could be divided into two groups according to CM theory. Molecular differences between the RA Cold and RA Heat groups were found

which validates the subtyping. Both the gene and metabolite profiles have elucidated relationships between several of the markers, revealed insight in the mechanism of RA and have provided the first stepping stones for a biological interpretation of the CM groups RA Cold and RA Heat. This biological interpretation is a second validation of the subtyping of RA patients, which could also lead to an increased understanding and improvement of CM intervention strategies specifically directed at alleviating Cold and Heat symptoms. Further studies are needed to gain knowledge about the biology behind other sub-groups of RA patients. The different subtypes of RA patients might be considered in studying Western treatment of RA to improve treatment response and provide opportunities for more personalized treatment.

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6. Sub-typing of rheumatic diseases based on a systems diagnosis questionnaire

Abstract

Background: The future of personalized medicine depends on advanced diagnostic tools to characterize responders and non-responders to treatment. Systems diagnosis is a new approach which aims to capture a large amount of symptom information from patients to characterize relevant sub-groups.

Methodology: 49 patients with a rheumatic disease were characterized using a systems diagnosis questionnaire containing 106 questions based on Chinese and Western medicine symptoms. Categorical principal component analysis (CATPCA) was used to discover differences in symptom patterns between the patients. Two Chinese medicine experts where subsequently asked to rank the Cold and Heat status of all the patients based on the questionnaires. These rankings were used to study the Cold and Heat symptoms used by these practitioners.

Findings: The CATPCA analysis results in three dimensions. The first dimension is a general factor (40.2% explained variance). In the second dimension (12.5% explained variance) 'anxious', 'worrying', 'uneasy feeling' and 'distressed' were interpreted as the Internal disease stage, and 'aggravate in wind', 'fear of wind' and 'aversion to cold' as the External disease stage. In the third dimension (10.4% explained variance) 'panting s', 'superficial breathing', 'shortness of breath s', 'shortness of breath f' and 'aversion to cold' were interpreted as Cold and 'restless', 'nervous', 'warm feeling', 'dry mouth s' and 'thirst' as Heat related. 'Aversion to cold', 'fear of wind' and 'pain aggravates with cold' are most related to the experts Cold rankings and 'aversion to heat', 'fullness of chest' and 'dry mouth' to the Heat rankings.

Conclusions: This study shows that the presented systems diagnosis questionnaire is able to identify groups of symptoms that are relevant for sub-typing patients with a rheumatic disease.

Based on: van Wietmarschen HA, Reijmers TH, van der Kooij AJ, Schroën J, Wei H, Hankemeier T, et al. (2011) Sub-typing of rheumatic diseases based on a systems diagnosis questionnaire. *PloS one* 6(9): e24846.

Introduction

Pharmacological disease management strategies for rheumatoid arthritis (RA) are for an important part based on trial and error. In general less than 53% of RA patients with a disease duration of less than one year show a positive ACR20 response to treatment [1]. This number goes down to 38% for patients with 5-10 years of RA. Even 30% of RA patients initiating the most effective and expensive treatment option available, anti-TNF therapy, fail to respond [2]. Non-responders are switched to other drugs until one is found that gives the desired effect [3]. A similar trial and error approach is often used for the treatment of osteoarthritis and fibromyalgia as well. The result is that a considerable number of patients experience no benefits from a treatment but just the side effects.

Rheumatoid arthritis patients as well as patients with other rheumatic diseases could benefit substantially from a shift towards a personalized medicine approach which aims to get the right treatment to the right patient, in the right dose, at the right time and via the right route [4,5]. In the traditional approach, patients are classified as according to criteria specified by the American College of Rheumatology (ACR). A single disease management strategy that is specifically developed for treating the particular type of rheumatic disease will then be applied. A more personalized approach will go beyond the ACR classification and will require much more information about the patient and his or her environment [6]. Specific individual patient situations require specific types of treatment, which can consist of specific drugs, life-style changes, psychological support and other interactions depending on the wish of the patient [7].

The challenge for personalized medicine is to characterize groups of patients and relate these groups to certain treatment options. Modern systems biology technologies such as genomics, proteomics and metabolomics [8-10] are currently able to generate an enormous amount of data, which can be seen as signs defined as manifestations that are measured. Several clinical features and molecular markers have been identified for example to sub-type RA patients [11,12]. Anti-citrullinated protein antibodies positive or negative status is found to be related to distinctive RA risk profiles [13]. More inflamed joints and a higher level of joint destruction was reported in Anti-citrullinated protein antibodies positive RA patients [14]. A large dissimilarity has been found in gene expression profiles of INF-1 high and low sub-types of RA patients, but this is not very clear in the clinical features of patients [15]. Unfortunately, this knowledge has not resulted in personalized health strategies in clinical

practice yet, which illustrates that searching for clinically relevant subtypes without clear indications of what to look for and based mostly on signs is difficult.

Symptoms, manifestations that are observed by the patient himself, are on the other hand a subjective type of information which is actually much closer to the phenotype of the patient than signs. Symptoms therefore provide an extra dimension of information. A wide variety of symptoms can be collected related to physical manifestations but also to the psychology, the family, the environment, and worldview of the patient which is well known to play a large role in arthritis [16-19].

Diagnosis is the key process in which symptoms and signs are used by a medical practitioner to distinguish the state of a person as different from the 'normal' situation. It is used to differentiate one disease from another and it is used to base treatment on. A move towards personalized medicine needs an optimization and refinement of this diagnostic process. One way is to expand it by including more symptoms than currently in use [20].

Chinese medicine diagnosis is a systems diagnosis approach that takes into account a broad spectrum of symptoms (as reported by the patient) as well as signs observed by the practitioner by listening to the body, feeling the body and observation of the patient [21]. Constitutional, behavioral and social aspects are also considered in the diagnosis and the choice of treatment. In Chinese medicine, rheumatoid arthritis as well as other rheumatic diseases fall into a group of diseases termed Bi-syndromes. A Bi-syndrome is characterized by the presence or absence of over 100 symptoms [22].

Symptoms are related to one or more symptom patterns which are called syndromes in Chinese medicine. These patterns or syndromes lead to treatment principles on which particular treatments are based. Recently two particular patterns of symptoms have been studied more closely [23]. These patterns are called Cold and Heat and are general patterns of symptoms much used in Chinese medicine [21,24]. The Cold pattern can be described as severe pain in a joint or muscle that limits the range of comfortable movement, the pain does not move to other locations. The pain is relieved by applying warmth to the affected area, but increases with exposure to cold. Loose stools are characteristic also, as well as an absence of thirst and clear profuse urine. A thin white tongue coating is seen, combined with a wiry and tight pulse. In contrast, the Heat pattern is characterized by severe pain with hot, red, swollen and inflamed joints. Pain is generally relieved by applying cold to the joints. Other symptoms include fever, thirst, a flushed face, irritability, restlessness, constipation and deep-colored urine. The tongue may be red with a yellow coating and the pulse may be rapid [25].

In a recent study [26] differences have been found between RA Cold and Heat patients when looking at symptoms, gene expression, and metabolomics profiles. Interestingly, the differences turned out to be related to apoptosis, an important biological process. The RA Heat group showed more activity of apoptosis related genes than the RA Cold group. Lu and others found that RA patients with the Cold pattern responded better to biomedical combination therapy (diclofenac, methotrexate, sulfasalazine) than did patients with RA with a Heat pattern, at 12 weeks and 24 weeks of treatment [27]. These findings show that sub-grouping of RA patients using knowledge from Chinese medicine diagnosis can lead to more personalized treatment in RA. Especially the Cold and Heat groups are promising for optimizing treatment.

The objective of the study is to analyze similarities and differences between patients with a rheumatic disease with respect to their symptoms. A questionnaire was therefore designed to establish a systems diagnosis of patients with a rheumatic disease based on a range of symptoms that are used in Chinese and Western medicine. A second objective is to analyze the Cold and Heat status of these patients based on the questionnaire results and an evaluation of the questionnaires by Chinese medicine experts.

Network analysis concepts were used to visualize the relationships between the symptoms and the corresponding syndromes according to Chinese medicine theory, as well as the relationships observed in patients. Categorical principal component analysis was used to find similarities and differences between the patients. The results were interpreted using theoretical and expert knowledge about symptoms and syndromes.

The following part of the analysis focused on the absence and presence of Cold and Heat related symptoms in the patients. Two Chinese medicine experts were asked to rank the Cold and Heat status of each patient on a seven point scale, based on the questionnaire results. These Cold and Heat rankings were introduced as an extra source of information in the categorical principal component analysis. The results were interpreted using Western and Chinese perspectives on arthritis-like diseases. Finally, several suggestions for creating diagnostic tools using the presented systems diagnosis approach will be discussed, which can lead to new opportunities to advance personalized medicine for rheumatic diseases.

Materials and Methods

Design of the questionnaire

The questionnaire was designed to establish a systems diagnosis of patients with rheumatic diseases. Symptoms described as related to Bi-syndromes [28] and reviewed by two Chinese medicine experts were used to create a list of 106 questions related to these symptoms divided into nine areas: location of the symptoms, breathing, climate, digestion, emotions & behaviour, quality of the symptoms, changes in the symptoms, pain, and urination. For most of these questions the 7-point Likert scale [29], was used to assess the frequency or the strength of a symptom. A score of 1 means never or not present, while a score of 7 would mean very frequent or very strongly present. Some of the questions were in binary, yes or no, format.

The questionnaire also represents a number of symptoms used in Western medicine to assess disease activity and the response to treatment, for example 'stiff joints', 'joint pain' and 'swollen joints' [30]. In addition to the questions related to symptoms, some general questions were included concerning disease history, medication, and arthritis related blood factors. The full questionnaire is added as supplementary information.

The questionnaire was designed to reflect Chinese thinking and diagnosis by extensive discussions with two Chinese medicine experts. Additionally, the questionnaire was reviewed by the scientific committee of the two Dutch professional organizations for Chinese medicine practitioners in the field of acupuncture (Nederlandse Vereniging voor Acupuntuur) and Nederlandse Artsen Acupunctuur Vereniging).

The medical ethical committee of the Leiden University Medical Center was notified of the study before the questionnaire was send out to patients and waived the need for further approval of the study by the medical ethical committee. All participants were informed about the study the data was going to be used for either by e-mail or verbal communication. All participants gave an informed consent in an e-mail or verbally to this use of the data.

Study sample

People with one or more rheumatic diseases were invited to participate in this study by completing a questionnaire. A small invitation text was published on the website of the

Osteo- and Rheumatoid Arthritis Foundation in Amsterdam, the Netherlands, http://www.reuma-stichting.nl. The invitation was also published in one of the newsletters of the foundation. Additionally, the questionnaire accompanied with an explanation of the purpose of the study was send to all members of the Netherlands Acupuncture Society.

A total of 91 people requested to participate in the study. A questionnaire was send of which 52 were returned. Three questionnaires could not be used for the analysis: one was missing a full page of answers, of another one only the first page was completed and a third questionnaire contained 11 incorrectly filled answers. Table 1 summarizes the types of rheumatic diseases most prevalent in the respondents and the most used medication.

Table 1 Characteristics of the respondents

Rheumatic disease*		Medication	
Osteoarthritis	24	Diclofenac	11
Rheumatoid arthritis	11	Ibuprofen	7
Fibromyalgia	5	Paracetamol	6
Systemic lupus erythematosus	2	Prednison	6
Missing entry	4	Methotrexate	5
Other rheumatic disease	10	Hydroxychloroquine	4
		Tramadol	4
		Etoricoxib	3
		Naproxen	2
		Celecoxib	2
		Other medication	15
		No medication	6
		No entry	5

* 10 respondents have a combination of two rheumatic diseases.

Data screening and recoding

Before data analysis, the consistency of the data was checked. Inconsistent answers to follow-up questions or to questions requiring a severity and frequency score for the same symptom were converted to missing. For example, if the answer to the question 'Do you feel cold?' is no, the answer to the follow-up question 'Is this cold located mostly in the feet or legs?' should only be no, which is not always the case. The question labeled 'sighing' was removed because multiple interpretations were possible and 'affected parts heavy' was completely covered by 'heavy feeling' and therefore also removed.

Most questions contain categories in which only a few patients had a score, which is unfavorable in CATPCA because it may lead to unstable results [31]. Therefore, we merged categories with a frequency less than 7 (the square root of the number of patients) with an adjacent category. This resulted in 10 variables with only a single category which were therefore removed: 'vomiting', 'nightmares', 'joints bend', 'joints stretch', 'tired with slight exertion', 'symptoms wander', 'upper part affected', 'symptoms appear suddenly', 'pain appears slowly', 'pain with redness and swelling'. The variable 'joint pain' was removed because one of the two categories had only four observations. The final dataset used for the CATPCA analysis contained 93 variables.

Network analysis

In Chinese medicine the focus is more on relationships between symptoms than on the symptoms themselves. The symptoms involved in the Bi-syndromes form a network of relationships. Network theory [32-34] therefore offers a perfect set of concepts to visualize and analyze the network properties of the Bi-syndromes. Cytoscape version 2.6.3 [35], an open source network visualization and annotation package, was used to create a network (graph) of the questionnaire. The position of the nodes was calculated using an algorithm [36] implemented in Cytoscape which minimizes the total length of the edges in the graph. An example is given of how the symptom scores of a patient can be mapped on the network. The questionnaire scores are represented in the graph as the weights of the relationships (edges) between symptoms and the corresponding syndromes. Such a symptom fingerprint can be used by a practitioner to get an overview of the symptom patterns for a particular patient.

Categorical principal component analysis

To explore the similarities and differences between the patients with respect to the set of 93 variables, Principal Component Analysis (PCA) would be the appropriate method. However, since our data are categorical, standard PCA is not suitable. Nonlinear Principal Component Analysis (NLPCA) is a method that can deal with categorical data and is therefore the method of choice. NLPCA finds the parameters of the PCA model in an iterative process in which "Optimal Scaling" is incorporated. Optimal Scaling is a technique that finds optimal quantifications for categorical variables. The quantifications are optimal in the sense that the percentage of variance of the *quantified* variables accounted for by the principal components is maximal. NLPCA is available in SAS [37] as PRINQUAL [38,39], and in SPSS [40] as CATPCA [41,42]. In this study we have used CATPCA in SPSS version 17.0.

The number of principal components was determined using parallel analysis [43,44] with permuted data [45]. We used 100 data sets with random permutation within all variables. Because CATPCA maximizes the eigenvalues of the first P components (with P the number of components specified by the user), solutions with different numbers of components are not nested. For instance, the two components in a two-component solution are not equal to the first two components of a three-component solution. Therefore, multiple parallel analyses might be required: If the parallel analysis for the one-component solution shows a significant eigenvalue (greater than the 95^{th} percentile of the random eigenvalues) for the second component, the parallel analysis is repeated for the two-component solution. Then the significance of the third eigenvalue is checked, etc., until the $(P+1)^{th}$ eigenvalue of a P-component solution is not significant, then P is the chosen number of components.

After determining the number of principal components, variables with a total variance accounted for (VAF) $\geq$ 50% and another set of variables with a total VAF $\geq$ 60% were selected for further analysis. With these two sets of variables the procedure of determining the number of components as described above was repeated (Figure 1).

The loadings resulting from the two CATPCA models were compared with the theoretical relationships between symptoms and syndromes. The two resulting models were interpreted by a Chinese medicine expert. The interpretability determined which of the two sets of variables were the most appropriate for further analysis.

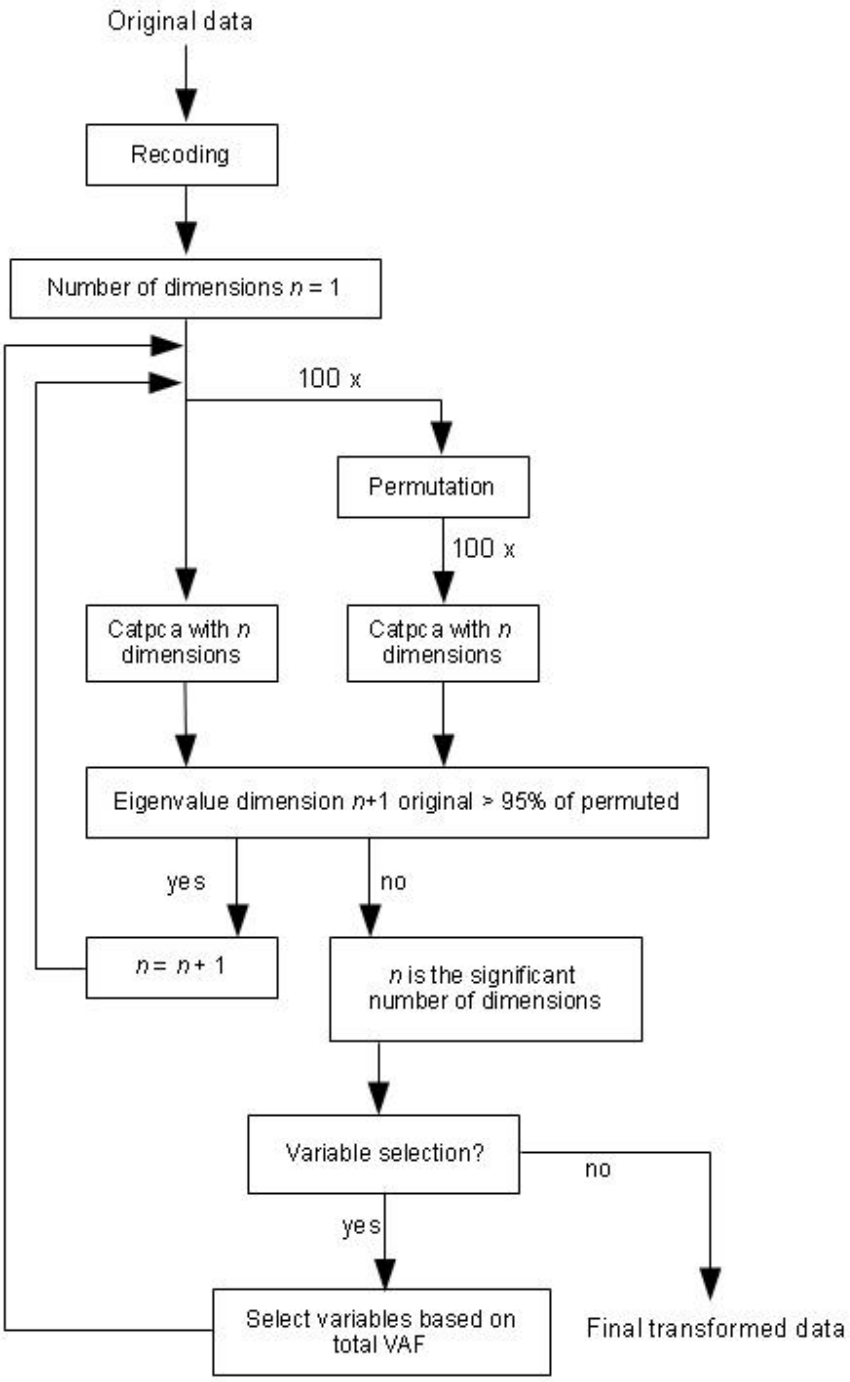

Figure 1. CATPCA data analysis strategy flowchart.

The next part of the analysis focused on the Cold and Heat status of patients and the ability of the questionnaire to provide information on this status. Two Chinese medicine experts (expert 1 and expert 2) were asked which symptoms they deemed most important for Cold and Heat. The two Chinese medicine experts were also asked to determine the Cold and Heat status of

each patient based on the questionnaire scores. In the subsequent analysis therefore there are four sources of information: 1) the patient scores on the questionnaire, 2) theoretical Cold and Heat related symptoms, 3) Cold and Heat related symptoms according to experts and 4) Cold and Heat ranking of each patient questionnaire by two experts.

The symptoms that the experts used to rank the Cold and Heat status of the patients were compared with the symptoms connected with Cold and Heat according to theory. Furthermore, to examine the relationship between the symptoms and the Cold and Heat ranking by the experts, these rankings were plotted as ordinally scaled supplementary variables in the CATPCA solution [46]. Supplementary treatment of variables implies that they are projected into the component space, but do not participate in defining the component space. The locations of the Cold and Heat rankings of both experts were then examined respective to each other and the other variables.

To find out which symptoms are most related to the experts Cold and Heat ranking, a semi-supervised analysis was performed. In this analysis the Cold and Heat rankings of the two experts did participate in the model building with a large weight (the ranking variables were included a large number of times in the model) [46]. For this analysis the number of principal components was also determined using the permutation testing approach described above. Due to the large weight of the four ranking variables, they almost completely determine the solution, causing the VAF of the other variables to decrease compared to the unsupervised solution. Questionnaire variables with a total VAF $\geq$ 25% in one of both dimensions were selected to build a final model.

Results and discussion

Similarities and differences between patients with a rheumatic disease

The theoretical relationships between symptoms used in the systems diagnosis questionnaire and syndromes were visualized as a graph (Figure 2) [20]. The resulting Bi-syndrome network is a bi-partite graph consisting of two types of nodes, the syndromes (red hexagons) and the symptoms (yellow circles). This graph visualizes the relationships between symptoms and syndromes according to Chinese medicine theory. Some symptoms are related to more than one syndrome which might in some instances be referred to as bridge symptoms or bifurcation points. The appearance of such a bridge symptom can indicate a strengthening of

the pattern itself, an upcoming change towards another pattern or a complication of the pattern. Many other symptoms are unique for particular syndromes. Certain related syndromes according Chinese medicine theory are positioned close together in the graph. For example prolonged Bone Bi develops into Kidney Bi, two syndromes which are closely related in theory and thus close together in the network. Heart Bi can develop after a long period of Vascular Bi. Intestine and Bladder Bi are two late stages of the disease related to the Hollow Organs [28].

To visually explore the symptom pattern of a patient, the scores on the questionnaire can be mapped on the network. An example of one patient is shown in Figure 2. Edges that are thicker and darker blue denote high scores while light and thin edges represent low scores.

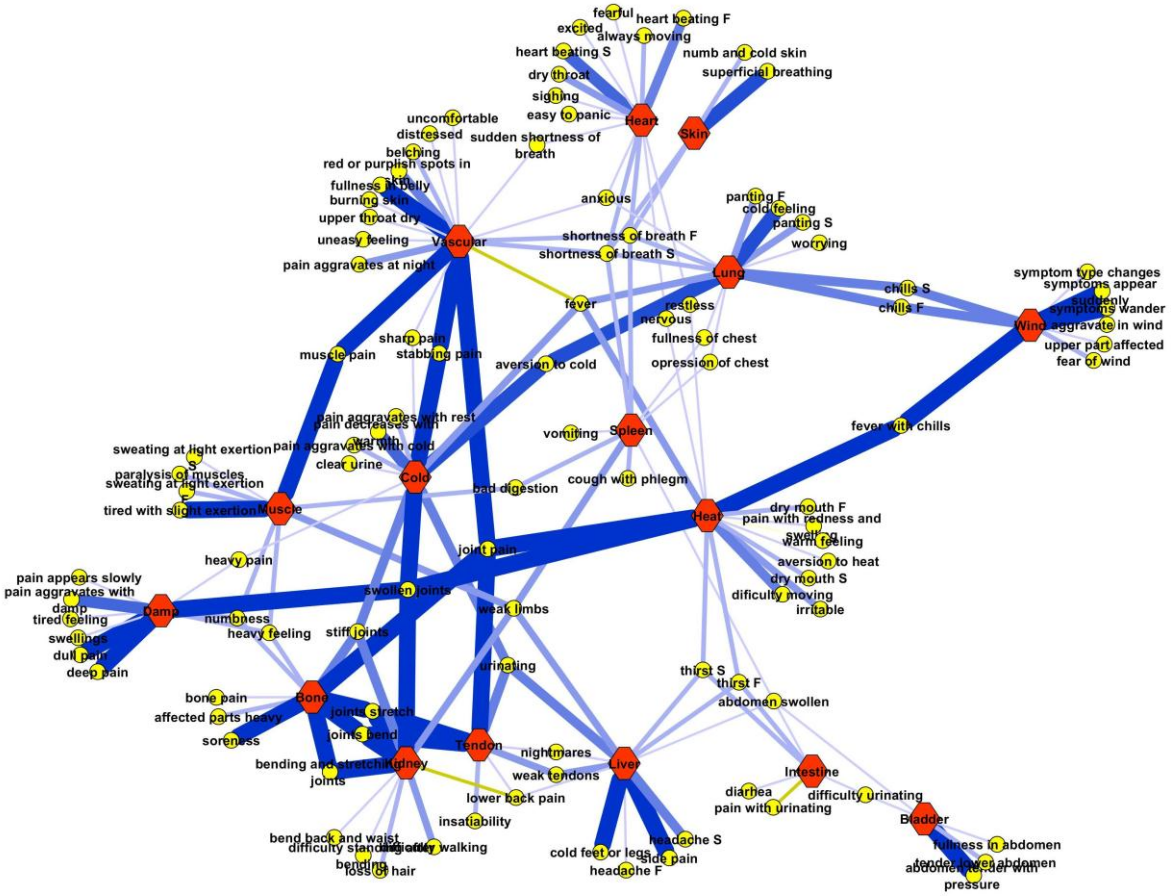

Figure 2. Example of a personalized Bi-syndromes network based on Vangermeersch 1994. The red hexagonal nodes represent the Bi-syndromes (Bi prefix was omitted for brevity), and the yellow circles represent the symptoms and signs related to the Bi-syndromes. The blue lines (edges) represent the relationships between symptoms and syndromes according to theory while the thickness of the lines represent the symptom scores of one patient. Thicker and darker colored edges denote a higher score and thinner, lighter edges denote a lower score.

The results of the CATPCA analyses are as follows. Parallel analysis showed four significant principal components for the model containing all the variables. Two sets of variables were selected, one set consisting of 44 variables with a total VAF > 50% and another set consisting of 30 variables with a VAF > 60% (see Supplementary information for the total VAF tables of both sets of variables). Parallel analysis revealed four significant components for the first set of variables and three significant components for the second set. After discussing both models with the Chinese medicine experts the model with fewer variables and components was retained for further analysis.

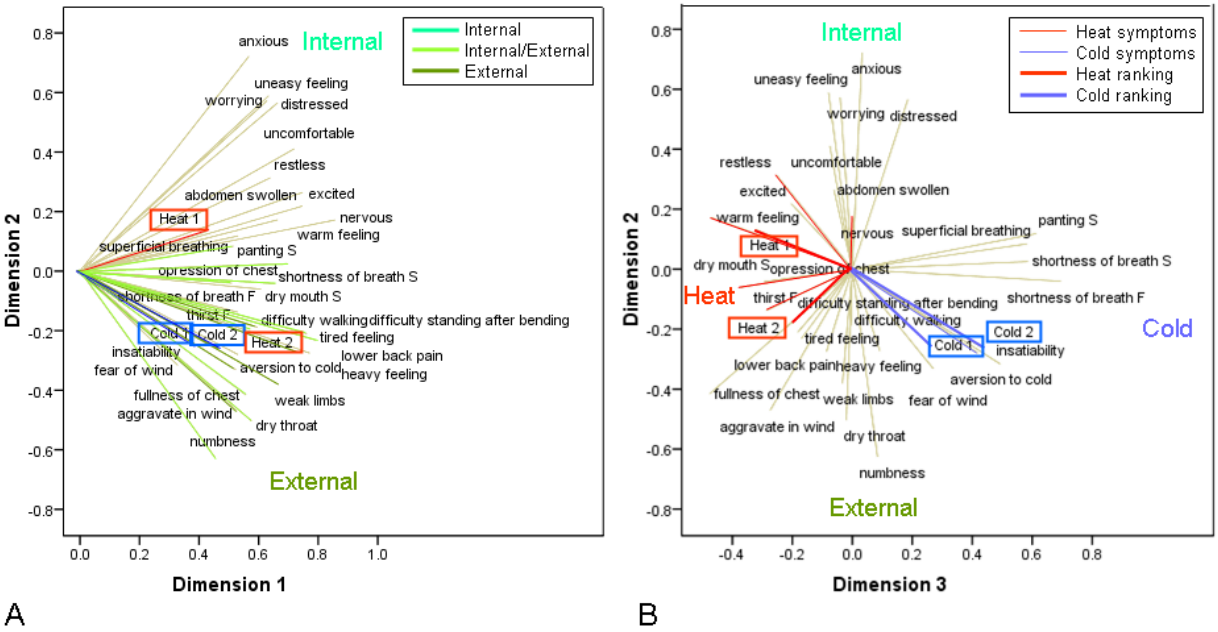

Figure 3. Loading plot of the first two dimensions (panel A) and the second and third dimension (panel B) of the CATPCA model for the 30-variables set. In Panel A, the loading vectors of the symptoms are colored according to their contribution (as indicated by one of the experts) to the Internal or External nature of the disease state. In Panel B, the loading vectors of symptoms which are theoretically related to Cold or Heat are pictured as thin blue or red lines. The supplementary Cold and Heat rankings of the two experts are plotted as thick blue or red lines.

In the component score plots (not shown) no clear groups of patients could be observed. The location of the objects in the score plots was compared with the type of arthritis the patients suffered from. No grouping of patients with a similar type of arthritis could be found. The loadings of the first two components of the CATPCA model are shown in Figure 3A. The length of a loading indicates the amount of variance explained by that loading and the

distance between two loadings indicates their similarity. All the loadings are positive in the first dimension (40.2% explained variance) indicating a general factor for which no clear interpretation was found. When the loadings in the second dimension (12.5% explained variance) are compared with the theoretical relationships between symptoms and the corresponding syndromes, two groups of loadings stand out. Five variables are theoretically related to the external pathogens Wind ('aggravate in wind', 'fear of wind'), Cold ('aversion to cold') and Heat ('thirst', 'dry mouth s'). Three of these symptoms, 'aggravate in wind', 'fear of wind' and 'aversion to cold', have a fairly high negative loading in the second dimension. Nine variables are related to Chinese Organ concepts such as Heart ('anxious', 'restless', 'excited', 'nervous'), Vascular ('anxious', 'uncomfortable', 'distressed', 'uneasy feeling') and Lung ('panting s', 'worrying'). Four of these symptoms, 'anxious', 'worrying', 'uneasy feeling' and 'distressed', have a fairly high positive loading in the second dimension. Two symptoms which are related to Damp, 'heavy feeling' and 'tired feeling', have a very low loading in the second dimension.

Looking at the distribution of symptoms from a Western medicine perspective reveals that the most common symptoms for rheumatoid arthritis (RA) 'joint pain', 'swollen joints' and 'stiff joints' do not contribute to the variation between the patients. On the other hand two other RA symptoms 'tired feeling' and 'weak limbs' are present but close together. The main osteoarthritis symptoms 'pain' and 'stiffness' are not present. Fibromyalgia is characterized by pain and stiffness as well, but also by 'anxious' and 'distressed', which have a high loading in the second dimension.

According to the Chinese medicine experts the second dimension is related to the Internal or External stage of the disease, one of the key diagnostic concepts used in Chinese medicine [21]. In the Internal stage the organs are affected while in the External stage the skin, muscles and channels are affected. The reaction of the body to the external pathogens Wind, Cold, and Damp that cause the arthritis is expressed by the symptoms that can be observed in the lower right part of Figure 3A. This indicates the first, external stage of the disease when the body defends itself against the invasion of the external pathogens. The appearance of Heat ('dry mouth s' and 'thirst') and Damp ('heavy feeling' and 'tired feeling') symptoms, indicates a transformation of Cold into Heat via Damp. Patients in this stage of the disease will have a low score in the second dimension. A high positive loading in the second dimension indicates a more chronic stage of the disease, in which patients will present Organ symptoms. If the position of the objects in the component score plots is compared to the loadings, it is possible

to get an indication of the stage of the disease according to Chinese medicine theory for each patient. The Internal versus External interpretation of the second dimension is in agreement with the distribution of the symptoms that are in theory related to the External or Internal stage of the disease as marked with different colors in Figure 3.

The Cold and Heat status of rheumatic patients

Figure 3B shows the second and third dimension of the CATPCA model. While the second dimension is mostly related to the Internal and External stage of the disease, the third dimension (10.4% explained variance) is related to the Cold or Heat status of the patients. Symptoms related to Heat and Cold according to theory are colored red and blue respectively in Figure 3 and are indicated in Figure 4. Five symptoms that are theoretically related to Heat have a negative loading in the third dimension ('restless', 'nervous', 'warm feeling', 'dry mouth s' and 'thirst'). The group of symptoms with the highest positive loadings in the third dimension ('panting s', 'superficial breathing', 'shortness of breath s' and 'shortness of breath f') are related to Qi deficiency. One symptom ('aversion to cold') theoretically related to Cold also has a fairly high positive loading in the third dimension.

Expert 1	Symptoms	Expert 2
Cold 3	cold feeling	Cold 3
Cold 3	pain aggravates with cold	Cold 3
Cold 3	pain decreases with warmth	Cold 3
Cold 3	aversion to cold	Cold 2
Cold 3	urinating	
Cold 3	clear urine	
Cold 3	sharp pain	
Cold 2	cold feet or legs	Cold 3
Cold 2	chills S	Cold 3
Cold 2	red or purplish spots in skin	Cold 2
Cold 2	numb and cold skin	Cold 2
Cold 2 & Heat 1	fever with chills	
Cold 2	stabbing pain	
Cold 1	stiff joints	Cold 1
Cold 1 & Heat 1	pain with urinating	
Cold 1	fear of wind	
Cold 1	joints stretch	
Cold 1	bending and stretching joints	
Cold 1	difficulty walking	
Heat 2	pain aggravates at night	Cold 2
	numbness	Cold 2
	pain aggravates with damp	Cold 2
	superficial breathing	Cold 1
	panting S	Cold 1
	shortness of breath S	Cold 1
	sudden shortness of breath	Cold 1
	sweating at light exertion S	Cold 1
	tired with slight exertion	Cold 1
	weak limbs	Cold 1
	weak tendons	Cold 1
	pain aggravates with rest	Cold 1
	diarhea	Cold 0.5

Heat 3	aversion to heat	Heat 3
Heat 3	pain with redness and swelling	Heat 3
Heat 2	burning skin	Heat 3
Heat 2	dry mouth S	
Heat 2	dry throat	Heat 2
Heat 2	upper throat dry	Heat 2
Heat 2	thirst S	
Heat 2	warm feeling	Heat 3
Heat 1	heart beating S	
Heat 1	insatiability	
Heat 1	always moving	
Heat 1	irritable	
Heat 1	uncomfortable	
Heat 1	distressed	
Heat 1	nervous	
Heat 1	restless	
Heat 1	nightmares	
Heat 1	excited	
Heat 1	difficulty urinating	
Cold 1 & Heat 3	fever	Heat 3
	abdomen swollen	Heat 1
	swollen joints	Heat 2
	swellings	Heat 2
	pain aggravates with rest	
	heavy pain	
	joint pain	
	thirst F	
	dry mouth F	
	difficulty moving	
	fullness of chest	
	fullness in belly	
	oppression of chest	
	difficulty standing after bending	

Figure 4. Cold and Heat related symptoms. In the first and third column the symptoms used by the experts for the Cold (blue) and Heat (red) ranking are marked. The importance is shown as a number behind the Cold and Heat label and by the color, dark blue or red is more important. The second column shows the names of the symptoms. Symptoms with a blue background are related to Cold according to theory, the red background ones are related to Heat according to theory. Symptoms with a brown background are related to both Cold and Heat. The table also shows which symptoms are important for the Cold and Heat ranking according to the forced classification model in bold type face and in red or blue.

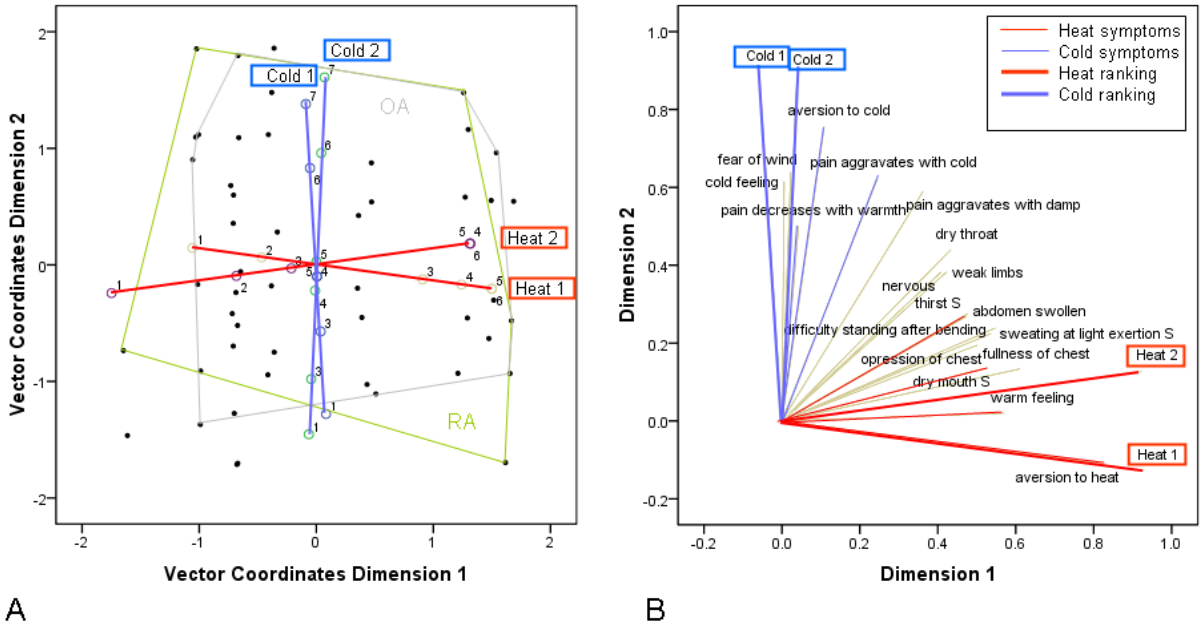

Figure 5. Panel A shows a bi-plot based on a semi-supervised CATPCA model including 19 variables and the 4 expert ranking variables. The four expert rankings are shown on which the categories after transformation are marked by circles. The outline of the group of rheumatoid arthritis (RA) patients is shown as well as the outline of the group of osteoarthritis patients (OA). In Panel B the loadings are shown. The loadings corresponding to symptoms which are related to Cold or Heat according to theory are represented by thin blue or red lines respectively. The Cold and Heat expert rankings are the thick blue en red lines respectively.

The Cold and Heat rankings of the experts, plotted as supplementary variables in Figure 3, have fairly high loadings in the third dimension indicating that this dimension is related to the Cold and Heat rankings. The Cold rankings are related to the other high positive loadings in the third dimension ('panting s', 'superficial breathing', 'shortness of breath s' and 'shortness of breath f'). One expert indicated to have used these symptoms to rank the Cold status of the patients (Figure 4). The Cold rankings are much closer together than the Heat rankings indicating that the two experts agree more on the Cold status of the patients than on the Heat status.

The experts were asked to indicate which symptoms they used for the ranking of the Cold and Heat status of the patients. In Figure 4 the symptoms reported by the two experts are given. Additionally, the symptoms theoretically related to Cold and Heat are indicated by the red and blue color in the center column. In the figure the overlap in symptom use between theory and the experts is visualized, as well as the overlap between the two experts. The Cold symptom 'aversion to cold' with a large loading in dimension three shown in Figure 3B was indeed indicated by both experts as important, although one expert assigned this symptom a lower status. Of the Heat symptoms with a large loading in dimension three 'warm feeling' was deemed important by both experts. 'Dry mouth s', 'nervous' and 'restless' were important for one expert, while 'thirst f' was mentioned by neither expert. Figure 4 also shows that both experts used a larger set of symptoms to rank Cold and Heat than indicated by theory. Furthermore, both experts indicated to have used symptoms that are not related to Cold and Heat according to theory. This might be due to differences between various Chinese medicine schools and to experience with using symptoms in daily practice.

In the following analysis the Cold and Heat rankings are introduced into the model with a large weight to find the symptoms that are most closely related to the expert rankings, based on the patients scores. Figure 5A is a bi-plot in which the patients scores and Cold and Heat ranking loadings are both plotted in the component space. The position of a patient point relative to the Cold and Heat loadings indicates the Cold and Heat ranking of the patient. Patients in the upper part of the figure for example have a high Cold ranking while patients in the right part of the figure have a high Heat ranking. Figure 5A shows that the distances between the various ranking categories is not equal. Additionally the distance between category 3 in Heat 1 and Heat 2 is large indicating that a Heat ranking of 3 for expert 1 should be interpreted very different from a Heat ranking of 3 for expert 2. To see whether the Cold or Heat ranking has any relationship with the type of arthritis the patients suffer from the outline of the largest groups of patients, the rheumatoid arthritis (RA) and osteoarthritis (OA) patients, are marked by lines. Clearly, the scores of the OA and RA patients are overlapping and is therefore unrelated to the Cold and Heat rankings.

In Figure 5B shows the loadings resulting from the forced classification analysis. The Heat rankings have a high positive loading in the first dimension and the Cold rankings have a high positive loading in the second dimension. This loadings plot reveals which symptoms, and related questions, are most related to the Cold and Heat rankings. 'Aversion to cold', 'fear

of wind' and 'pain aggravates with cold' are most related to the Cold rankings and 'aversion to heat', 'fullness of chest' and 'dry mouth' to the Heat rankings. 'Aversion to cold' and 'fear of wind' also have a fairly high positive loading in the third dimension (indicating Cold) of the unsupervised approach (Figure 3B). 'Fullness of chest' and 'dry mouth' have a fairly high negative loading in the third dimension (indicating Heat) of the unsupervised approach.

The results of this study are summarized in Figure 4. First of all the symptoms used in theory to determine the Cold and Heat status of patients are indicated with a blue and red background respectively. 'Fever' and 'swollen joints' have a brown background since they are bridge symptoms between Heat and Cold. Secondly, the figure shows that both experts reported they used most of the theoretical symptoms. Seven symptoms that were not used by either expert are placed at the bottom of the list. Thirdly, the figure shows the agreement and disagreement between the experts on symptom use. In the fourth place, the symptoms resulting from the forced classification analysis are marked by bold type face.

Of the Western symptoms for rheumatoid arthritis 'joint pain' is indicated as a Heat symptom in Chinese diagnosis, 'stiff joints' is indicated as a Cold symptom and 'swollen joints' is indicating both Cold and Heat. However neither expert used 'swollen joints' and 'joint pain' to rank the Cold or Heat status of the patients. 'Weak limbs', another RA symptom showed up in the forced classification as an important indicator for Heat. Some pain related symptoms which can be present in various rheumatic diseases appear to be relevant for Cold and Heat ranking. 'Pain with redness and swelling' is indicated by both experts and theory as an important Heat symptom. 'Stabbing pain' on the other hand is indicated by one expert as a Cold symptom. 'Heavy pain' was not mentioned by the experts, but according to theory it is a Cold symptom. Additionally the results of the analysis show that emotional symptoms more prevalent in fibromyalgia patients are more related to Heat, especially the symptom 'nervous' is mentioned in theory as a Heat symptom and is also an important Heat indicator in the forced classification results.

The combination of the patient questionnaire scores information with the analysis and expert rankings resulted in a set of symptoms that are most qualified to develop into a tool to determine the Cold and Heat status of patients in a clinical setting.

Conclusions

This study introduced a systems diagnosis approach, the collection of a large number of

symptoms that are usually not used in clinical diagnosis in Western medicine, as an additional dimension of looking at patients with a rheumatic disease. Individual patient scores on this questionnaire can be visually presented in a graph to help the interpretation of the relationships between the symptoms occurring in that patient (Figure 2). This new method of 'symptom fingerprinting' is comparable to other systems biology fingerprinting tools to determine disease state, risk for a disease, and chances of treatment effect [47-49].

The systems diagnosis questionnaire results of 49 patients with a rheumatic disease in this study reveal two interesting and significant dimensions of information. One dimension is related to the stage of the disease with the key symptoms 'anxious', 'worrying', 'uneasy feeling' and 'distressed' for the Internal stage, and 'aggravate in wind', 'fear of wind' and 'aversion to cold' for the External stage. The concept of Internal and External is widely used in Chinese medicine to choose the right treatment option. The fact that this concept explains 12.5% of the total variation in the data shows that it might be a relevant difference in other patients with rheumatic diseases as well.

The second interesting dimension is related to the Cold and Heat status of the patients, explaining 10.4% of the variation in the data. The key symptoms are 'panting s', 'superficial breathing', 'shortness of breath s', 'shortness of breath f' and 'aversion to cold' for the Cold status and 'restless', 'nervous', 'warm feeling', 'dry mouth s' and 'thirst' for the Heat status. A forced classification approach revealed that 'Aversion to cold', 'fear of wind' and 'pain aggravates with cold' are related to the Cold rankings and 'aversion to heat', 'fullness of chest' and 'dry mouth' are related to the Heat rankings by two Chinese medicine experts in these 49 patients. The characterization of the Cold and Heat status in this study is limited by the patient reported symptoms and might be improved by including observations of an expert of the patient.

We believe that the future of medicine lies in an integration of perspectives on disease, health, prevention and medicine. Looking in more detail and at the same time more comprehensively at patients is the way forward towards personalized medicine [5,50]. One step in that direction is finding new sub-groups of patients and optimizing treatment for these sub-groups. For Chinese medicine practitioners it is standard to take the Internal or External status as well as the Cold or Heat status into account for choosing the best treatment option. This study characterizes the symptoms related to these subgroups which might be used to develop a diagnostic tool to diagnose these subgroups in clinical practice. Further studies are needed to examine differences in response to medication by Cold and Heat sub-groups of

arthritis patients. In this way the combination of Chinese medicine concepts with Western therapeutic options offers exciting opportunities for more personalized treatment and eventually personalized health.

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S1. Onderzoek diagnose van patiënten met een reumatische aandoening

Hartelijk dank voor het meewerken aan dit onderzoek naar de diagnose van patiënten met een reumatische aandoening. Het invullen van de vragen zal ongeveer een kwartier duren.

Gelieve alle vragen in te vullen. Indien geen antwoord mogelijk is dit graag vermelden. Alle informatie zal vertrouwelijk worden behandeld.

Voor het beantwoorden van de vragen wordt regelmatig gevraagd naar de Mate en of Frequentie van een symptoom. Hiertoe dient u een waarde van 1 tot en met 7 in te vullen, waarbij 1 overeenkomt met nooit of niet, en waarbij 7 overeenkomt met zeer ernstig of zeer vaak.

Succes!

Datum	

1. Algemene informatie			
Leeftijd aanvang ziekte		C-reactive protein	
Reumafactor		Hb	
ESR		ACR klasse	

Reguliere medische diagnose
Wie heeft de diagnose gesteld?
Reguliere medicatie + bijwerkingen
Overige medicatie
Korte ziekte geschiedenis

2. Locatie van de symptomen	Antwoord
Ervaart U een vol gevoel in de borst? - is dit gevoel vooral gelocaliseerd in het midden boven deel van de buik?	Mate (1-7): [ ] Ja/Nee
Ervaart U een druk op de borst?	Mate (1-7): [ ]
Ervaart U hoofdpijnen?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Ervaart U een abnormaal bewustzijn van het kloppen van uw hart, stoort dit andere gedachten?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Ervaart U een brandend gevoel van de huid?	Mate (1-7): [ ]
Heeft U haaruitval?	Mate (1-7): [ ]
Heeft U rode of paarsachtige plekken in of onder de huid?	Mate (1-7): [ ]

3. Ademhaling	Antwoord
Is uw ademhaling oppervlakkig?	Mate (1-7): [ ]
Heeft U last van hijgen?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Ervaart U kortadmenigheid? - Vindt dit plotseling plaats?	Mate (1-7): [ ] Frequentie (1-7): [ ] Ja/Nee
Ervaart U een droge mond?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Ervaart U regelmatig een droge keel? - Is deze droogheid gelocaliseerd in het bovenste deel van de keel?	Frequentie (1-7): [ ] Ja/Nee
Geeft U slijm op bij het hoesten?	Frequentie (1-7): [ ]

4. Klimaat	Antwoord
Voelt U zich koud? - Is deze koude vooral in de voeten of benen aanwezig?	Mate (1-7): [ ] Ja/Nee
Ervaart U dorst?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Ervaart U rillingen?	Mate (1-7): [ ] Frequentie (1-7): [ ]
Voelt U zich warm?	Mate (1-7): [ ]
Heeft U soms koorts? - Gaat de koorts soms gepaard met rillingen?	Frequentie (1-7): [ ] Ja/Nee
Ervaart U moeilijkheden met bewegen?	Mate (1-7): [ ]

Heeft U afkeer van hitte?	Mate (1-7): [ ]
Heeft U afkeer van koude?	Mate (1-7): [ ]
Ervaart U spontaan zweten of zweten bij de geringste inspanning?	Mate (1-7): [ ] Frequentie (1-7): [ ]

5. Spijsvertering	**Antwoord**
Is uw onderbuik pijnlijk of erg gevoelig?	Mate (1-7): [ ]
- Wordt dit gevoel versterkt door er op te duwen?	Ja/Nee
Voelt uw buik gezwollen of uitgezet aan?	Mate (1-7): [ ]
Ervaart U een vol gevoel in de buik?	Mate (1-7): [ ]
Heeft U last van diarree?	Frequentie (1-7): [ ]
Hoe vaak moet U boeren laten?	Frequentie (1-7): [ ]
Hoe vaak moet U overgeven?	Frequentie (1-7): [ ]
Ervaart U een onverzadigbare drang om te eten?	Frequentie (1-7): [ ]
Ervaart U een slechte vertering?	Mate (1-7): [ ]

6. Emoties & Gedrag	**Antwoord**
Bent U altijd in beweging?	Mate (1-7): [ ]
Bent U gemakkelijk geïrriteerd?	Frequentie (1-7): [ ]
Voelt U zich ongemakkelijk?	Frequentie (1-7): [ ]
Vreest U wind of tocht?	Mate (1-7): [ ]
Bent U bang?	Mate (1-7): [ ]
Raakt U gemakkelijk in paniek?	Frequentie (1-7): [ ]
Voelt U zich gestrest?	Frequentie (1-7): [ ]
Maakt U zich zorgen?	Frequentie (1-7): [ ]
Heeft U een ongemakkelijk gevoel?	Frequentie (1-7): [ ]
Piekert U veel?	Frequentie (1-7): [ ]
Voelt U zich nerveus?	Frequentie (1-7): [ ]
Voelt U zich rusteloos?	Frequentie (1-7): [ ]
Ervaart U nachtmerries?	Frequentie (1-7): [ ]
Voelt U zich opgewonden?	Mate (1-7): [ ]
Moet U vaak zuchten?	Frequentie (1-7): [ ]

7. Kwaliteit van de symptomen	**Antwoord**
Kunt U de aangedane gewrichten buigen?	Ja/Nee
- Kunt U de aangedane gewrichten strekken?	Ja/Nee
Is het buigen en strekken van uw gewrichten beperkt?	Mate (1-7): [ ]
Ervaart U een zwaar gevoel in uw ledematen, lichaam en of hoofd?	Mate (1-7): [ ]

Voelen de aangedane delen van uw lichaam zwaar aan?	Mate (1-7): [ ]
Ervaart U een moe gevoel in uw ledematen, lichaam en of hoofd?	Mate (1-7): [ ]
Ervaart U gevoelloosheid in het lichaam en ledematen?	Mate (1-7): [ ]
Voelt uw huid gevoelloos en koud aan?	Mate (1-7): [ ]
Voelt U stijfheid in uw gewrichten?	Mate (1-7): [ ]
Heeft U gezwollen gewrichten?	Mate (1-7): [ ]
Heeft U zwellingen in uw lichaam?	Mate (1-7): [ ]
Voelt U zich moe na de geringste inspanning?	Ja/Nee
Voelen uw vier ledematen zwak?	Mate (1-7): [ ]
Voelen uw pezen zwak?	Mate (1-7): [ ]
Ervaart U moeilijkheden met het lopen?	Mate (1-7): [ ]
Heeft U een gebogen rug en middel? - Heeft U moeite met recht op staan na buigen?	Mate (1-7): [ ] Mate (1-7): [ ]

8. Veranderingen in de symptomen	**Antwoord**
Verplaatsen de symptomen zich?	Ja/Nee
Worden de symptomen erger wanneer het buiten winderig is?	Mate (1-7): [ ]
Is het bovenste deel van uw lichaam gemakkelijker aangedaan dan het onderste deel van uw lichaam?	Ja/Nee
Verschijnen de symptomen plotseling?	Ja/Nee
Verandert het type symptomen?	Mate (1-7): [ ]

9. Pijn	**Antwoord**
Heeft U last van lage rugpijn?	Mate (1-7): [ ]
Heeft U pijn?	Mate (1-7): [ ]
Waar ervaart U pijn?	[Spieren] [gewrichten] [botten] [onderrug] [zij]
Wat voor soort pijn ervaart U?	[Zeurend] [stekend] [scherp] [diep] [zwaar] [dof]
Komt de pijn langzaam, gradueel op?	Ja/Nee
Ervaart U verlamming in uw spieren?	Mate (1-7): [ ]
Wordt de pijn erger met vochtig, mistig weer?	Mate (1-7): [ ]
Wordt de pijn 's nachts erger?	Mate (1-7): [ ]
Wordt de pijn erger bij koude en koud weer?	Mate (1-7): [ ]
Wordt de pijn erger door te rusten?	Mate (1-7): [ ]
Gaat de pijn gepaard met roodheid en zwelling?	Ja/Nee
Wordt de pijn minder door warmte en beweging?	Mate (1-7): [ ]

10. Urineren	**Antwoord**
Hoe vaak moet U plassen?	Frequentie (1-7): [ ]
Is de urine helder van kleur?	Mate (1-7): [ ]
Ervaart U moeilijkheden bij het plassen?	Mate (1-7): [ ]
Ervaart U pijn bij het plassen?	Mate (1-7): [ ]

Hierbij verklaar ik deze vragenlijst naar waarheid te hebben ingevuld.

Handtekening patiënt:

Eventueel kunt u hieronder uw adresgegevens opgeven om op de hoogte gehouden te worden van het onderzoek.

Naam	
Adresgegevens	
E-mail	

Hartelijk dank voor uw medewerking!

Drs. Herman van Wietmarschen

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Table S2

VAF table CATPCA analysis Figure 3.

Variable	VAF
nervous	,798
aggravate in wind	,755
anxious	,745
fullness of chest	,744
warm feeling	,732
superficial breathing	,731
excited	,725
distressed	,724
shortness of breath S	,713
dry mouth S	,703
lower back pain	,695
worrying	,690
tired feeling	,682
thirst F	,663
numbness	,663
difficulty standing after bending	,654
uneasy feeling	,648
insatiability	,641
aversion to cold	,639
uncomfortable	,639
restless	,632
panting S	,627
weak limbs	,625
shortness of breath F	,621
opression of chest	,620
dry throat	,619
heavy feeling	,617
fear of wind	,616
abdomen swollen	,611
difficulty walking	,605
pain aggravates with damp	,576
cold feeling	,573
weak tendons	,560
heart beating S	,554
pain	,549
sweating at light exertion S	,534
thirst S	,530
pain aggravates with cold	,526
dry mouth F	,524
tender lower abdomen	,523
stiff joints	,515
symptom type changes	,512
red or purplish spots in skin	,512
swellings	,502

Table S3

VAF table CATPCA analysis Figure 5.

Variable	VAF Dim 1	VAF Dim 2
Heat 2	,845	,035
Heat 1	,697	,188
aversion to heat	,557	,138
fullness of chest	,396	,005
difficulty standing after bending	,377	,003
sweating at light exertion S	,352	,002
abdomen swollen	,318	,012
dry throat	,316	,075
warm feeling	,315	,026
dry mouth S	,312	,002
opression of chest	,303	,001
thirst S	,302	,010
pain aggravates with damp	,291	,192
weak limbs	,289	,054
nervous	,288	,061
fullness in belly	,283	,003
excited	,252	,007
Cold 1	,059	,752
Cold 2	,105	,693
aversion to cold	,129	,471
fear of wind	,058	,366
cold feeling	,043	,337
pain aggravates with cold	,196	,268
pain decreases with warmth	,043	,212

7. Characterization of rheumatoid arthritis subtypes using symptom profiles, clinical chemistry and metabolomics measurements

Abstract

Objective. The aim is to characterize subgroups or phenotypes of rheumatoid arthritis (RA) patients using a systems biology approach. The discovery of subtypes of rheumatoid arthritis patients is an essential research area for the improvement of response to therapy and the development of personalized medicine strategies.

Methods. In this study 39 RA patients are phenotyped using clinical chemistry measurements, urine and plasma metabolomics analysis and symptom profiles. In addition a Chinese medicine expert classified each RA patient as a Cold or Heat type according to Chinese medicine theory. Multivariate data analysis techniques are employed to detect and validate biochemical and symptom relationships with the classification.

Results. The questionnaire items 'Red joints', 'Swollen joints', 'Warm joints' suggest differences in the level of inflammation between the groups although c-reactive protein (CRP) and rheumatoid factor (RHF) levels were equal. Multivariate analysis of the urine metabolomics data revealed that the levels of 11 acylcarnitines were lower in the Cold RA than in the Heat RA patients, suggesting differences in muscle breakdown. Additionally, higher dehydroepiandrosterone sulfate (DHEAS) levels in Heat patients compared to Cold patients were found suggesting that the Cold RA group has a more suppressed hypothalamic-pituitary-adrenal (HPA) axis function.

Conclusion. Significant and relevant biochemical differences are found between Cold and Heat RA patients. Differences in immune function, HPA axis involvement and muscle breakdown point towards opportunities to tailor disease management strategies to each of the subgroups RA patient.

Based on: Herman A van Wietmarschen, Weidong Dai, Anita J van der Kooij, Theo H Reijmers, Yan Schroën, Mei Wang, Zhiliang Xu, Xinchang Wang, Hongwei Kong, et al. (2012) Characterization of rheumatoid arthritis subtypes using symptom profiles, clinical chemistry and metabolomics measurements. *PloS One* 7(9): e44331.

Introduction

Discovering subtypes of rheumatoid arthritis (RA) patients is considered a key research area for the improvement of response to therapy (1,2). RA is a heterogeneous disease which is illustrated by the very good response of some patients to a biological therapy, but a complete lack of response in a large number of other patients (3). Another striking observation is that in a large group of RA patients low disease activity or remission can be achieved using a single conventional disease-modifying anti-rheumatic drug (DMARD), which contrasts with the current viewpoint to offer aggressive therapy in an early stage of the disease to all patients (4). Personalized medicine aims to provide the information that allows targeting the right treatment option to the right patient (5). The first step in this approach is to find relevant subtypes of patients for which a different treatment strategy would clearly be beneficial.

Several subtypes of RA patients have been identified based on particular clinical and molecular features (6,7). Markers such as disease duration and age have been identified that predict response to treatment (8,9). Although some molecular markers have been found to predict functional and structural outcomes, these markers rarely find their way into clinical practice. One reason is the difficulty to translate markers found in trial populations to routinely measurable and cost-effective predictors for individuals (10). This indicates that there is a need to develop new robust and reliable clinically applicable tools to identify subtypes of patients.

Discovery of novel relevant subtypes of RA patients could be improved by using prior knowledge. In this study a Chinese perspective on subtypes of RA patients is used to focus the analysis of the data. According to this perspective RA patients can be divided in two groups (Cold RA and Heat RA) which are treated very differently in Chinese medical practice (11,12).

Cold and Heat are general concepts used in Chinese medicine to distinguish between two types of reactions of the body to some disturbance (13). A Cold reaction is characterized by pallor, intolerance of cold, absence of thirst, loose stools, clear profuse urine, a pale tongue and a slow pulse. A Heat reaction is characterized by flushed face, fever, thirst, irritability, restlessness, constipation, deep-colored urine, reddened tongue and a rapid pulse (14). These two types of reactions are expressed in any type of disease to a certain extend. However, Cold and Heat are especially important for rheumatoid arthritis because this disease is perceived in classical Chinese medicine as the result of an invasion of three out of the four existing

external pathogens: Wind, Cold, Heat and Damp (13).

Some work has been done to elucidate biological mechanisms related to Cold and Heat types of RA patients. In 2009 we measured 64 differently expressed genes in CD4 positive T-cells of RA patients. This set of genes was enriched for the immune system functions and especially for apoptosis regulation. In Heat RA patients apoptosis related genes were upregulated while in Cold patients apoptosis resitance genes were upregulated (11). Additionally, a number of plasma metabolite concentrations was significantly different between Cold and Heat RA. Later, genes related to calcium signaling, cell adhesion, PPAR signaling and fatty acid metabolism were found in CD4 positive T-cells of Heat RA patients (15). Toll-like receptor signaling related genes were found in the T-cells of Cold RA patients. In a similar study, Cold RA was related to Alanine, aspartate and tyosine metabolism and Heat RA to the MAPK pathway, Wnt signaling and insulin signaling, also found by measuring gene expression in CD4 positive T-cells (16). A GC-MS analysis of Cold and Heat RA plasma revealed elevated plasma levels of glycochenodeoxycholate, proline, saturated and mono-unsaturated phosphatidylcholine (PC) but decreased levels of urea, free fatty acid (FFA) and polyunsaturated PC in Heat RA compared to Cold RA (17).

Recently it was shown that Cold RA patients respond much better to a combination therapy with diclofenac, methotrexate and sulfasalazine than Heat RA patients (18). However, in clinical studies and clinical practice the features of Cold and Heat might be valued differently by each Chinese medicine practitioner . To improve acceptance of this classification in Western clinical practice it is important to standardize the classification. Further characterization of the physiological, constitutional and biological differences between the Cold and Heat subtypes of RA patients is valuable for increasing our understanding of the disease mechanism.

In this study a systems biology strategy is employed to study the differences between Cold and Heat types of RA patients, classified by a Chinese medicine expert. Data were collected on symptoms, clinical blood parameters and urine metabolites. Multivariate statistics were employed to discover the most discriminating features for the two groups. In particular, a categorical principal component analysis (19,20) was used for the questionnaire and clinical chemistry data and partial least squares discriminant analysis is used for the metabolomics data (21). The questionnaire was used to determine symptom patterns related to Cold and Heat RA that could be used in clinical practice to determine these subtypes in an objective manner. The metabolomics and clinical results were related to biological processes and

related to current understanding of the heterogeneity of rheumatoid arthritis.

Subjects and methods

Subjects

Female subjects were recruited in the Zhejiang Xinhua Hospital in 2010. The institutional review board of the hospital gave written approval and judged that the study was conducted according to the ethical guidelines. A doctor explained the details of the study to the subjects and ensured written informed consent of each subject. All eligible subjects were diagnosed by a rheumatologist as rheumatoid arthritis patients according to the ACR criteria (22). Inclusion criteria were a female gender due to the much higher prevalence of RA in women and age > 18 years. Each patient was classified by the same Chinese medicine expert as either a Heat subtype or a Cold subtype. When the subtype was unclear the patient was not included in the study. General information such as age, disease duration, medication, etc. was collected using a standard form. Student's t-Test (two-tailed) was used to evaluate the differences between the two groups. Furthermore, a symptom questionnaire was completed by all the patients. Blood and urine samples were taken from each patient after fasting overnight for routine clinical measurements and metabolomics analysis.

Symptom Questionnaire

A short version of a recently developed systems diagnosis questionnaire was used in this study (23). The 106 item questionnaire was shortened to 57 items by two Chinese medicine experts to target it more directly to Cold and Heat related symptoms. The final version of the questionnaire contained items related to five categories of symptoms: breathing, digestion, climate, quality of the symptoms and pain. The questionnaire was translated into Chinese. An English language version of the questionnaire is added as supplementary information (Text S1). The consistency of the data was checked and the data were recoded (23).

Clinical measurements

Routine clinical chemistry measurements were conducted on fasting blood samples by the

Zhejiang Xinhua Hospital. These measurements consisted of the complete blood count and the following serum measurements: uric acid (URIC), creatinine (CREA), blood urea nitrogen (BUN), triglycerides (TG), cholesterol (CHOL), total protein (TP), albumin (ALB), globulines (BIB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), creatine kinase-MB (CKMB), lactate dehydrogenase (LDH), immunoglobulin A (IGA), Immunoglobulin G (IGG), immunoglobulin M (IGM), complement 3 (C3), complement 4 (C4), antistreptolysin O (ASO), rheumatoid factor (RHF), c-reactive protein (CRP), anti-cyclic citrullinated protein antibodies (CCP). The results of each clinical parameter were uniformly binned into 19 categories to increase the robustness (19).

Metabolomics measurements

Urine samples were prepared for Ultra Fast LC/MS-IT-TOF (Shimadzu, Japan) analysis (24). The technical variation of the analysis over time was monitored by a pooled quality control (QC) sample strategy (25). The analysis was performed on a Ultra Fast LC/MS-IT-TOF with gradient elution using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. For the LC a T3 column (ACQUITY UPLC HSS T3 1.8um, 2.1x100 mm, Waters) was used. Ion mode was switched between positive and negative mode between each scan (24).

Sample preparation for the plasma metabolomics analysis was conducted as follows: 200 μL plasma was mixed with 800 μL acetonitrile, then the mixture was centrifuged at 14000 rpm (Biofuge Stratos, Thermo Scientific, USA) for 10 min at 4℃. The supernatant was freeze-dried and redissolved with 200 μL of 20% acetonitrile.

Chromatographic separation was performed on a BEH C8 column (10 cm×2.1mm, 1.7 μm, Waters, USA) using a Thermo Fisher Accela LC system. The gradient duration was 31 min at a flow rate of 300 μL/min with mobile phase (A) 0.1% formic acid solution and (B) acetonitrile. The injection volume was 6μL. The samples were analyzed randomly and the same QC strategy was used as in the urine analysis.

An LC-MS method was used to measure the plasma metabolites. The LTQ-Orbitrap XL (Thermo-Fisher Scientific) settings were as follows: capillary temperature 325 ℃, source voltage 4.5 kV, capillary voltage 49 V for ESI+ analysis; capillary temperature 325 ℃, source voltage -3.5 kV, capillary voltage -40 V for ESI- analysis. The mass range was set at 100-1000 m/z. The resolution of the Orbitrap was set at 30000.

The urine metabolomics measurements resulted in a list of features after preprocessing the data using Profiling Solution (Shimadzu, Japan) (24). The plasma metabolomics data were processed by SIEVE software (V1.2, Thermo-Fisher Scientific) for peak picking and peak alignment. The parameter of MZ width was set at 0.01 Da and RT width was set at 0.6 min. Gradient peaks were removed. Features not present in at least 80% of the samples in one class were removed. Zeros were replaced by the smallest measured value divided by 2, the urine data were mean centered and the plasma data was autoscaled. Principal component analysis was then used to screen the data for outliers (objects with scores deviating from the scores of the bulk of the samples) (26), to determine the stability of the analysisover time (by comparing the scores of the QC samples) and monitor possible batch effects (by checking possible time trends in the scores of the objects).

Statistical analysis

Questionnaire and clinical chemistry data. The focus of the data analysis is to identify questionnaire items and clinical parameters that distinguish between the subgroups of RA patients classified by the CM expert. A forced classification approach (27) using Nonlinear Principal Component Analysis (NLPCA) was chosen. NLPCA is a Principal Component Analysis method that is suitable for variables with mixed measurement level and variables that may have nonlinear relationships to each other (19). The possibility to capture nonlinear relationships is important because the human body is a nonlinear system expressing complex behavior (28). Such complex behavior has been observed in the immune system that plays a key role in rheumatoid arthritis. For instance cytokine dose response curves show an initial threshold and stable attractors have been found in cytokine systems (29). More important is the ability of NLPCA to deal with the questionnaire data that are measured on an ordinal scale or as binary data. The questionnaire data analysis approach using NLPCA is described in detail in a previous paper (23).

NLPCA uses an 'optimal scaling' approach to find quantifications for categorical variables and optimal transformations for interval variables, which means that the percentage of variance of the transformed variables accounted for by the principal components is maximal (30). The questionnaire items and clinical parameters were all included in a single NLPCA analysis allowing the interpretation of relationships between questionnaire items and clinical parameters.

The use of a the classification variable with a very large weight, here 1000 will cause the objects to cluster together into subclouds in the object space. The weight was chosen such that the objects of both classes were separated completely. As the classification variable is a binary variable, one principal component is sufficient to describe the class differences. In this study we used CATPCA in SPSS version 17.0 (31).

An important consideration in NLPCA is the analysis level chosen for the variables (20). For the questionnaire variables the analysis level was kept the same as the measurement level (39 ordinal and 3 nominal). Subsequently the presence of nonlinear relationships between the clinical chemistry variables and the classification variable was examined by comparing numerical, spline ordinal and spline nominal transformations of the clinical chemistry variables (i.e., from most restricted to least restricted transformations). A substantial increase in the total variance accounted for (total VAF) due to an analysis level with more degrees of freedom then verifies that a less restrictive model is more appropriate.

After choosing the appropriate analysis level, variables with a proportion variance accounted for (VAF) > 0.20 were selected for further analysis to increase the stability of the model and to focus on the most important variables. Leave two out cross validation was used to estimate the classification error of the model. In this approach two objects are reserved as a validation set while a NLPCA model is built using the other 37 objects. The class of the two left out objects is then predicted using the NLPCA model. This procedure is repeated until each of the objects is left out once and the class of each object is predicted. An overall classification error is subsequently calculated and a final model is built using all the available objects. Permutation testing was used to check whether this final model is different from a model based on a random classification.

Metabolomics data. A partial least squares discriminant analysis (PLS-DA) model was built for both the urine and plasma data set to find the most discriminating features between the Cold RA and Heat RA groups , a standard, well accepted technique used in the metabolomics field to discover characteristic differences between groups (32). A separate model for the urine and plasma data was built to examine the performance of a metabolite profile measured in one compartment in a single analysis. Such a profile would be much easier to develop into a diagnostic tool that can be applied in a clinical setting than a combination of two metabolomics profiles.

A tenfold double cross-validation scheme was followed by permutation testing (250 times) (33). The inner loop of the cross-validation scheme is used to estimate the optimal number of

principal components. In this inner loop two objects are left out to validate the number of principal components. The outer loop is used to estimate the error rate of the model. Again, in the outer loop two objects are left out to independently estimate the error rate. Variables were selected using a jack-knifing procedure in which variables with a standard error in the regression vector above a threshold were removed from the dataset. This threshold is decreased stepwise until the error rate of the model starts to increase. The model with the lowest error rate is then chosen as the optimal model and a final model is then built for the corresponding set of variables and including all the objects. Subsequently, the significance of the resulting model is determined by permutation testing. The same double cross-validation procedure is used to build a model of the same data set but with a permuted classification. After 250 times permuting the classification, the classification error of the 250 models resulting models is compared with the classification error of the model based on the correct classification. The error rate of the correct classification model should be lower than 95% of the distribution of error rates resulting from the permuted classifications.

Identification of the most discriminating features indicated by the PLS-DA model was then attempted for both the urine and plasma model (34). Evidence for the identity of features was accumulated by integrating information on the following: accurate mass of the ions and some fragments, retention time, HMDB database and in house human urine compound lists. The identification of some compounds was then verified by authentic standards. Finally, a biological interpretation was sought for the most discriminating features.

Results

Subjects

In total 50 patients were enrolled in the study. Eleven of these patients did not fulfill the inclusion criteria and were removed from the analysis. Seven of those eleven patients were male and therefore excluded, of two persons there was no RA diagnosis available, one patient was in the hospital for other reasons and therefore excluded and finally one person was excluded from this analysis because the symptom questionnaire was not completed. Finally, 20 RA patients of the Cold subtype and 19 of the Heat subtype were retained in the study.

Table 1 shows the patient characteristics per group expressed as the means of the age, disease duration, height and weight. None of these characteristics were significantly different (p-

value < 0.05) between the Cold RA and Heat RA patient groups. 34 patients received western medication such as methotrexate, sulfasalazine. other DMARD's and NSAID's. 36 patients received Chinese herbal medicine.

Table 1. Patient characteristics

	Cold (n=20)	Heat (n=19)	p
Age (years)	51 ± 13 (24-74)	54 ± 11 (34-77)	0,44
Disease duration (months)	90 ± 90 (4-240)	100 ± 100 (24-348)	0,44
Height (cm)	159 ± 4 (150-167)	160 ± 4 (155-170)	0,50
Weight (kg)	58 ± 18 (40-120)	58 ± 9 (37-75)	0,97

* Range is given in parenthesis

Clinical and symptom differences between RA subtypes

After merging scoring categories containing less than 7 observations, 15 variables were left with only a single category. There is no variation between the objects for these variables which were therefore removed from the analysis: 'Sudden shortness of breath', 'Amount of phlegm', 'Sticky phlegm', 'Colored phlegm', 'Tender lower abdomen', 'Aggravated with pressure', 'Smelly diarrhea', 'Chills S', 'Chills F', 'Fever with Chills', 'Pain aggravates at night', 'Stabbing pain', 'Sharp pain', 'Deep pain', 'Heavy pain'. After recoding, the data set used for further analysis contained 42 variables.

In the forced classification model the use of a 2nd degree (quadratic) nonmonotonic spline (nominal analysis level) with 2 interior knots for the clinical measurements shows substantially larger variance accounted for (11.8%) than a monotonic spline (ordinal analysis level) (8.2%), indicating a nonmonotonic relationship between the measurements. Also, a two interior knot spline shows a larger variance (11.8%) than a one interior knot spline (9.8%) (more knots allow for more freedom in the transformation). Therefore a 2nd degree spline nominal analysis level with two interior knots was chosen for the clinical chemistry variables. A new model was then built using this analysis level and used to select variables with a proportion of VAF > 0.20. A model was subsequently built including the 18 selected variables. The model using splines with 2 interior knots for the clinical chemistry variables was then compared once more with a model using one interior knot. The results showed that the increase in total VAF in the model with 2 interior knots was mostly due to the variable MCHC. Therefore the final analysis level of the clinical variables was adjusted to a 2nd degree spline with 1 interior knot, except for MCHC which was set to 2 interior knots. The final model had a total VAF of approximately 21% (see Table 2 for the loadings of the 18

variables). The transformation plots of the clinical chemistry variables of this model are included as supplementary information (Figure S1). Leave two out cross validation resulted in a classification error of 15%, which is substantially lower than the expected error rate based on random classification (which is 50%).

Table 2. Discriminating symptoms and clinical measurements

Variable name	*Loading*
b17 MCHC	,580
v40 Red joints	,544
v39 Swollen joints	,540
b37 IGG	,504
v25 Warm feeling	,495
b8 LY#	,490
v41 Warm joints	,490
v56 Dull pain	,438
v50 Pain worsens by warmth and movement	,399
b27 CHOL	,370
b38 IGM	,359
b19 PLT	,285
b25 BUN	-,374
b32 ALT	-,419
v19 Cold feeling	-,457
v29 Aversion to cold	-,472
b18 RDW	-,475
b44 CCP	-,495

RA patients that are classified as Heat by the Chinese medicine expert are characterized with higher optimal quantifications on average on the symptoms 'red joints', 'swollen joints', 'warm feeling', 'warm joints', 'dull pain' and 'pain that worsens with warmth and movement', and with higher levels of the optimally scaled clinical parameters mean corpuscular hemoglobin concentration (MCHC), IgG, lymphocyte number (LY#), IgM, platelet count (PLT) and cholesterol. Cold RA patients are best characterized with higher optimal quantifications on average on the symptoms 'cold feeling' and 'aversion to cold', and higher levels of the optimally scaled clinical variables blood urea nitrogen (BUN), alanine aminotransferase (ALT), red blood cell distribution width (RDW) and anti-cyclic citrullinated protein antibodies (CCP).

Both groups of patients have a similar average score on the symptoms 'pain' and 'stiff joints' as well as on the clinical parameters rheumatoid factor and c-reactive protein.

Table 3. Top discriminating urine metabolites

Importance	*Compound*	*2Log Cold/Heat[c]*
1	C8:1 acylcarnitine[b]	-0,73
3	C10:3 acylcarnitine[b]	-0,70
11	C8+OH acylcarnitine[b]	-0,89
13	acetylcarnitine[a]	-0,75
15	riboflavin[a]	-2,40
16	C11:1 acylcarnitine[b]	-0,98
21	pantothenic acid[a]	-0,92
27	C6:DC acylcarnitine[b]	-0,55
31	C5 acylcarnitine[b]	-1,21
32	C10:2 acylcarnitine[b]	-0,53
38	C8:2 acylcarnitine[b]	-1,19
41	C10:1 acylcarnitine[b]	-0,23
45	C9:3 acylcarnitine[b]	-2,26
49	C10:3 acylcarnitine isotope[b]	-0,66
50	C6:2:DC acylcarnitine[b]	0,42

[a] verified with authentic standard

[b] verified with characteristic 59 neutral loss, retention time order, accurate mass

[c] Calculated 2Log of the ratio between average levels in Cold and Heat patients

Urine and plasma metabolite differences between RA subtypes

Urine samples of 14 of the 19 Heat RA and 14 of the 20 Cold RA patients included in the analysis described above were collected successfully for the LC-MS analysis. In total 11 urine samples were either not collected at the hospital or the samples were not send to the laboratory for metabolomics analysis. PLS-DA analysis resulted in a final model containing 793 features. A classification error of 14% was obtained by a double cross-validation procedure. Permutation testing indicated that the error rate of this model is significantly ($p<0.05$) different from the distribution of models based on permuted classifications.

The tentative identification of the top 50 most discriminating metabolites resulted in three metabolites that were verified by comparing authentic standards with the samples: acetylcarnitine, riboflavin and pantothenic acid. Thirteen of the top features measured in positive ion mode as well as the acetylcarnitine standard showed the same neutral loss of mass 59.0747 by fragmentation, which is highly indicative of acylcarnitine compounds (35,36). The retention times of the features showing this loss was increasing with the mass,

indicating higher mass features were less polar. This agrees with the fact that larger mass acylcarnitines have longer fatty acid chains and are therefore less polar. Furthermore, several glucuronides and soy isoflavones are suspected, but the identity was not further determined (Table 3).

An LC-MS analysis of the plasma samples of 11 Cold RA and 14 Heat RA patients from the same set of patients that are used in the urine metabolomics and questionnaire analysis was performed. Three plasma samples were not available for analysis. Positive ion mode features were not included in the multivariate analysis because the QC samples were not stable. The PLS-DA analysis resulted in a model containing 214 features (classification error of 28%). Permutation testing indicated that the error rate of this model is significantly ($p<0.05$) different from the distribution of models based on permuted classifications. Some of the top 50 features resulting from the PLS-DA model could be identified with authentic standards (Table 4).

Table 4. Top discriminating plasma metabolites

Importance	*Compound*[a]	*2Log Cold/Heat*[b]
2	Dehydroepiandrosterone sulfate (DHEA sulfate)	-0,96
4	4-Methyl-2-oxovaleric acid	-0,25
6	Indoxyl sulfate	-0,44
10	Uric acid	-0,25
11	Cholesterol sulfate	-0,65
13	3-Methyl-2-oxovaleric acid	-0,26
47	Tryptophan	-0,23
50	Alpha-ketoisovaleric acid	-0,13

[a] all compounds are verified with an authentic standard

[b] Calculated 2Log of the ratio between average levels in Cold and Heat patients

Discussion

The subtypes of RA patients are characterized by four sets of features: clinical symptoms (questionnaire items), clinical chemistry measurements in blood, metabolite measurements in urine and in plasma. One hypothesis is that the Cold RA and Heat RA groups have a different inflammatory status. Of the most discriminating symptoms in the analysis, 'warm joints', 'swollen joints' and 'red joints' indicate a difference in inflammatory status. However, c-reactive protein (CRP), an important inflammation marker, was not retained in the final model because of a low proportion of total variance accounted for, based on the two groups.

Rheumafactor (RF), an important marker for RA disease activity (37), was not retained in the model either. On the other hand, the lymphocyte numbers are higher in Heat RA patients, although this level is not considered elevated and indicative of inflammation in standard evaluation (38). A comparable average disease duration of 90 and 100 months for the two groups suggests that not one of the groups can be considered an early arthritis or early aggressive arthritis group. These observations indicate that the severity of inflammation might be different between the groups when the joint symptoms are considered despite similar levels of rheumatoid factor and c-reactive protein.

Table 5. Warm, swollen & red joint scores

# Symptoms	*Cold**	*Heat**
0	60	26
≥1	40	74
≥2	10	74
3	5	63

* Percentages of subjects showing a positive score on a minimum of 0, 1, 2 or 3 of the symptoms.

Swollen joint counts are routinely used to establish disease activity in rheumatoid arthritis (39). In Table 5 the percentages of Cold and Heat patients with a positive score on zero to three of the symptoms 'warm joints', 'swollen joints' or 'red joints' are given. Even though high scores on the three symptoms are indicative for the Heat group of RA patients, 40% of the Cold patients also have a positive score on at least one of the three symptoms (frequencies of the scored categories per class are included as supplementary information Figure S2 and the raw symptom score data for 'swollen joints', 'warm joints' and 'red joints' is included as supplementary Table S1). The data indicate that the same number of patients in both groups are in pain and score a similar severity of pain (data not shown). These symptom observations indicate that the Heat RA group has much more joint problems than the Cold RA group. It would be interesting to further study the role these joint symptoms could play in sub-typing RA.

Our study shows that the important symptoms for the sub-typing are 'warm feeling', 'pain worsens by warmth and movement', 'cold feeling' and 'aversion to cold'. This is in agreement with a previous study in which a systems diagnosis of 49 patients with rheumatic diseases revealed 'aversion to cold', 'aversion to heat' and 'cold feeling' as highly relevant for the Cold and Heat ranking of the subjects (23). The symptom 'aversion to cold' and coldness in general

was found by others as well (12,40). Our study and other studies of the biology of Cold and Heat types of RA patients lead to the hypothesis that the currently unclear effectiveness of cold and heat therapy might be improved by targeting it to the right sub-type (41-43).

Anti-citrullinated protein antibody (CCP) is extensively studied as a predictor of disease progression in RA (44). In our multivariate model, CCP levels were higher in RA Heat patients suggesting that there might be a difference in disease progression between the two subtypes. The clinical parameters with the highest positive loadings were MCHC and IgG, while RDW showed the highest negative loading next to CCP. Some relationships between the Cold and Heat subtype and clinical measurements are nonlinear (see supplementary Figure S1 for the transformations of the variables). High as well as low serum levels off MCHC result in a positively transformed value, which is related to the Heat class. The highest levels of IgG are related to the Cold RA type, while lower levels are not so clearly Cold or Heat related. Blood urea nitrogen (BUN) levels increase in plasma with serious kidney damage (45). Higher or lower levels of BUN are related to the Heat RA group, average levels are related to the Cold RA group. Alanine aminotranferase (ALT) is an important parameter to distinguish Cold and Heat patients, which has been found to be related to insuline resistance and atherosclerosis risk in RA patients (46).

The metabolomics analysis in this study revealed higher urine levels of 11 acylcarnitines in the Heat group of RA patients. The enzyme carnitine palmitoyltransferase I (CTP I), located in the outer mitochondrial membrane, transfers acyl groups from coenzyme A to carnitines allowing the transport of fatty acids into the mitochondria for β-oxidation. Plasma carnitine levels are maintained by a combination of dietary intake, endogenous synthesis by the liver and kidneys and active renal reabsorption. Acylcarnitines can leave the mitochondria, enter the blood stream and can be excreted in urine (47).

Significantly lower total carnitine and acylcarnitine excretion in urine of RA patients compared to healthy controls has been reported , while free carnitine levels in urine and plasma are equal in both groups (48, 49). Because dietary intake of carnitines was equal in both groups, an impaired carnitine synthesis was suggested which is controlled by skeletal muscle breakdown. Decreased muscle mass and increased muscle turnover are observed in RA patients, which might be explained by a reduced CTP I activity. This was confirmed by a decreased creatinine and 3-methylhistidine excretion (49). In this study we find that the levels of a range of acylcarnitines are lower in the Cold RA group than in the Heat RA group. These findings suggest that Cold RA patients have less muscle mass and/or a more pronounced

muscle breakdown than RA Heat patients. A limitation of this study is that the plasma acylcarnitine levels are not known. Further studies should therefore be performed to validate the hypothesis that Cold and Heat subtypes of RA patients are related to muscle mass or muscle breakdown.

Decreased urine pantothenic acid and acylcarnitine levels have been shown to indicate an increase in beta-oxidation in patients using PPARα agonist drugs (50). Peroxisome proliferator-activated receptors (PPAR) activate fatty acid and cholesterol catabolism in the liver, activate gluconeogenesis and regulate amino acid metabolism. In addition, PPAR activates CTP I and CTP II activity. We report lower levels of pantothenic acid and acylcarnitine levels in Cold RA patients which might therefore indicate a difference in the activation of PPAR between the groups.

Interestingly, CTP I activity is inhibited by omega-6 fatty acids while an increased ratio of omega-3 versus omega-6 fatty acids consumption stimulates fatty acid oxidation regulated by CTP I activity (51). A decrease in CTP I activity also seems to play a role in Chronic Fatigue Syndrome patients. In these patients supplementation with carnitine and acylcarnitine was found to decrease fatigue symptoms (52). These findings suggest that fatigue in rheumatoid arthritis might be related to a decreased CTP I activity and a changed carnitine homeostasis. Fatigue is one of the most important factors in the disease experience of RA patients (53). Our findings indicate that urine acylcarnitine levels are an important discriminator between two subtypes of RA patients, which might explain differences in the experience of fatigue. These findings also suggest that carnitine and acylcarnitine supplementation might be beneficial for Cold RA patients and less so for Heat RA patients. Further confirmatory studies should be conducted to prove this hypothesis and develop this targeted treatment option.

Significantly lower DHEAS levels have been reported in premenopausal RA patients and are correlated to low morning cortisol levels and high IL-6 levels indicating a suppression of HPA-axis function (54). Decreased HPA-axis function is associated with a decreased stress response which results in an inadequate response to stress factors. It is hypothesized that this inadequate response can lead to autoimmune and inflammatory disorders (54). This study shows a higher DHEAS levels in Heat RA patients compared to Cold RA patients suggesting that the Cold RA group has a more suppressed HPA-axis function. A text mining study (55) has shown that diseases related to Cold according to Chinese medicine theory are more related to hormone function disturbances and Heat related diseases to immune function disturbances, which is in agreement with our findings.

This study provides metabolite, symptom and clinical chemistry profiles for two subtypes of rheumatoid arthritis. The profiles indicate a number of biological processes that seem to be regulated differently in the two groups. RA therapy could be optimized for the two groups in various ways. This study suggests differences in the effects of carnitine and acylcarnitine supplementation for the two groups as well as differences in the effects of hormone treatments such as prednison. We think that the better characterization and biological understanding of Cold and Heat RA offered in this study can be used to tailor therapy to each subgroup. Additionally, this study offers a new subtype to include in studies aiming for an improvement of response to treatment. Finally, the mechanism and effect of specific Cold RA and Heat RA treatment options used in Chinese medicine should be studied and might be integrated in standard disease management strategies by using a standardized Cold RA and Heat RA diagnostic profile.

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Supplementary information

Figure S1. Transformation plots of the clinical chemistry variables. On the x-axis the categories of the original discretized variables are represented while on the y-axis the optimally scaled quantifications are shown. A negative quantification corresponds to the Cold classification and a positive quantification to the Heat classification.

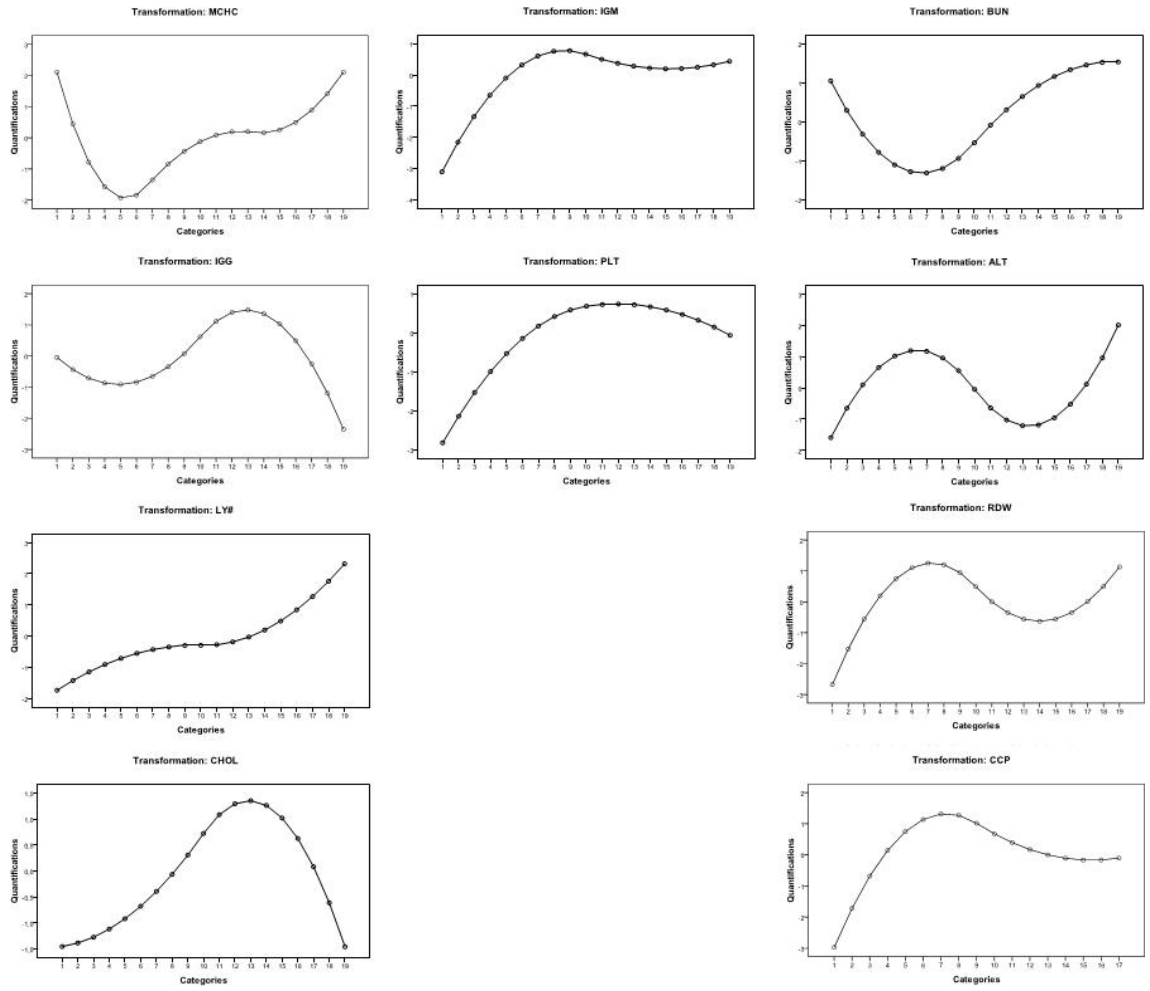

Figure S2. Frequencies of scored categories per class

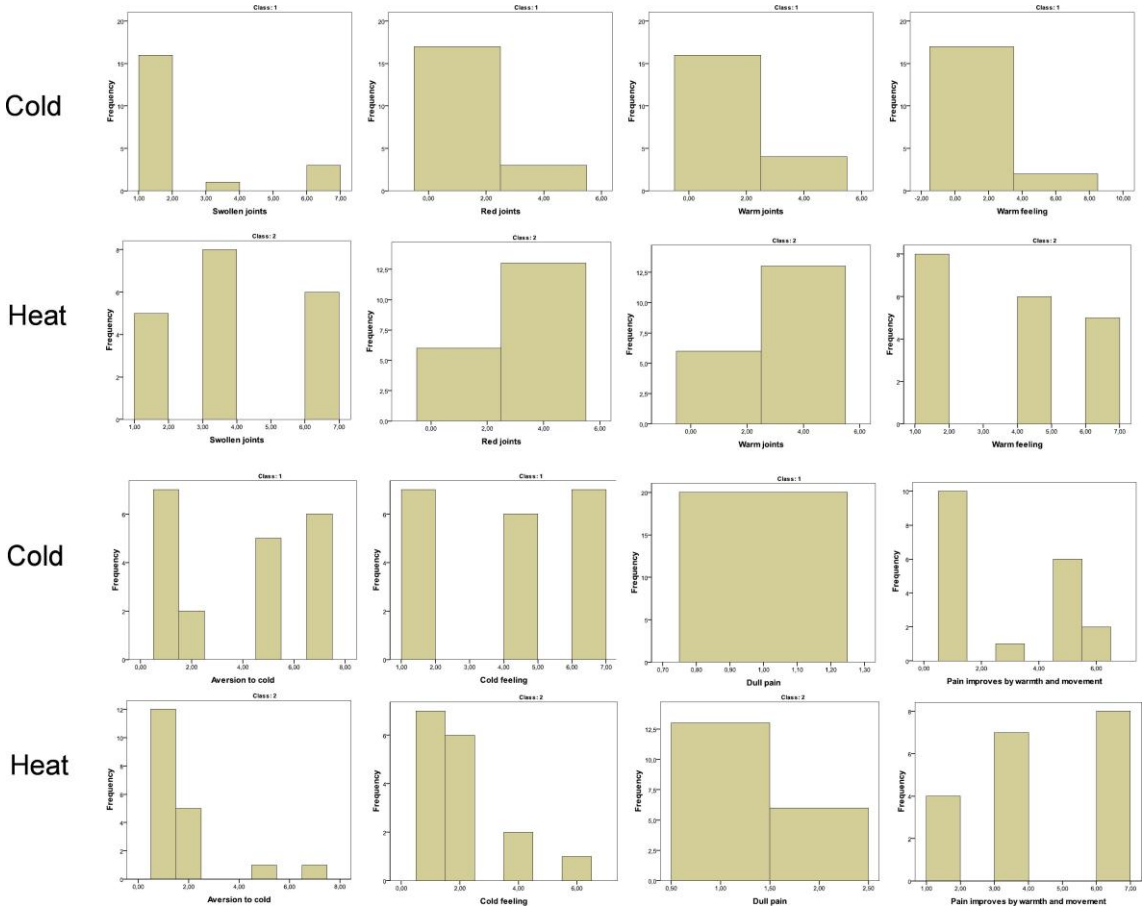

Table S1. Raw data of 'swollen joints', 'warm joints' and 'red joints' scores acquired by the symptom questionnaire.

Table S1. Raw data of warm, swollen & red joint scores			
Class	*Swollen joints*	*Red joints*	*Warm joints*
Cold	1	1	1
Cold	1	1	1
Cold	2	1	1
Cold	1	1	3
Cold	1	1	1
Cold	1	7	7
Cold	1	1	1
Cold	1	1	1
Cold	1	1	1
Cold	1	1	1
Cold	1	1	1
Cold	1	4	1
Cold	1	1	1
Cold	6	1	1
Cold	1	1	1
Cold	1	1	4
Cold	1	1	1
Cold	5	4	4
Cold	5	1	1
Cold	1	1	1
Heat	1	1	1
Heat	4	4	1
Heat	1	1	1
Heat	1	1	1
Heat	1	1	1
Heat	3	3	3
Heat	1	1	1
Heat	2	4	4
Heat	3	2	4
Heat	6	6	6
Heat	6	5	5
Heat	2	5	4
Heat	7	4	4
Heat	7	5	5
Heat	3	1	4
Heat	3	3	3
Heat	5	4	4
Heat	3	2	4
Heat	3	4	4
Mean rank Cold	15,05	15,25	15,40
Mean rank Heat	25,21	25,00	24,84
Mann-Whitney U	,002	,002	,004

Patients were asked to give a score between 1 and 7. For the construction of Table 5 the positive scores, scores higher than 1, were counted for the Cold RA and Heat RA group. In Table S1 mean ranks for the Cold RA and Heat RA groups are given for each symptom and

the differences between the groups are evaluated with the Mann-Whitney U test. The scoring of the three symptoms is significantly different between the Cold RA and Heat RA group.

Text S1:

Questionnaire diagnosis of Rheumatoid Arthritis patients Patient Nr: _____

This questionnaire is meant to be filled in under the supervision of your caretaker. Please answer all the questions. When an answer is not possible, please note this as well. All information will be handled with care and treated with respect.

The questions often require an estimation of the Severity and Frequency of the symptoms. Select a number between 1 and 7 for this, 1 meaning never or not and 7 meaning very severe or very often.

1. Breathing	**Answer**
Do you experience shortness of breath? - Does this occur suddenly?	Severity (1-7): Frequency (1-7): yes/no
Do you experience dryness of the mouth?	Severity (1-7): Frequency (1-7):
Do you experience dryness of the throat frequently? - Is this dryness located in the upper throat?	Frequency (1-7): yes/no
Do you cough with expectoration of phlegm? - How much phlegm? - How sticky is the phlegm? - How colored is the phlegm?	Frequency (1-7): Amount (1-7): Severity (1-7): Severity (1-7):

2. Digestion	**Answer**
Does your lower abdomen feel tender or very sensitive? - Is this feeling aggravated by pressure?	Severity (1-7): yes/no
Does your abdomen feel swollen or distended?	Severity (1-7):
Do you have a feeling of fullness in your abdomen?	Severity (1-7):
Do you have diarrhea?	Frequency (1-7):

- Is the diarrhea smelly?	Yes/No:
Do you have loose stools?	Severity (1-7):

3. Climate	**Answer**
Do you feel cold? - Is this cold located especially in the feet or lower limbs?	Severity (1-7): yes/no
Do you experience thirst?	Severity (1-7): Frequency (1-7):
Do you experience chills?	Severity (1-7): Frequency (1-7):
Do you feel warm?	Severity (1-7):
Do you experience fever occasionally? - Is the fever accompanied by slight chills sometimes?	Frequency (1-7): yes/no
Do you dislike heat?	Severity (1-7):
Do you dislike cold?	Severity (1-7):
Do you experience spontaneous sweating or sweating at the lightest exertion?	Severity (1-7): Frequency (1-7):
Is there a preference for cold drinks?	Severity (1-7):
Is there a preference for warm drinks?	Severity (1-7):
Is there a preference for cold food?	Severity (1-7):
Is there a preference for warm food?	Severity (1-7):

4. Quality of the symptoms	**Answer**
Do the affected parts of your body feel heavy?	Severity (1-7):
Does your skin feel numb and cold?	Severity (1-7):
Do you feel stiffness in your joints?	Severity (1-7):
Do you have swollen joints?	Severity (1-7):
Is the joint red?	Severity (1-7):
Is the joint warm?	Severity (1-7):
Do you have swellings in your body?	Severity (1-7):
Do your four limbs feel weak?	Severity (1-7):

Do your tendons feel weak?	Severity (1-7):

5. Pain	**Answer**
Do you experience pain?	Severity (1-7):
What kind of pain do you experience?	soreness, stabbing, sharp, deep, heavy, dull, tingling feeling
Does the pain aggravate with foggy, humid weather?	Severity (1-7):
Does the pain aggravate at night?	Severity (1-7):
Does the pain aggravate by coldness and cold weather?	Severity (1-7):
Is the pain accompanied with redness and swelling?	yes/no
Does the pain improve by warmth and movement?	Severity (1-7):

I declare to have filled in this questionnaire truthfully.

Signature patient:

Signature caretaker:

8. Conclusions and perspectives

Rheumatoid arthritis sub-types

The main aim of the research presented in this thesis was to find and characterize relevant sub-types of rheumatoid arthritis patients. Instead of an unsupervised approach for finding such sub-types knowledge from Chinese medicine was used to direct the research. In Chinese medicine diagnosis Cold and Heat are key concepts consisting of specific symptom patterns that are used to characterize patients. Consequently, the Cold and or Heat status of patients is very important for choosing the right therapy. In this thesis a systems biology approach was applied to characterize Cold and Heat types of rheumatoid arthritis patients. Multivariate statistical analysis was used to validate the classification of the two patient sub-types.

In Chapter 5 the first biochemical characterization of Cold and Heat RA patients has been presented. From the gene expression profiles measured in CD4+ T-cells, apoptosis turned out to be a key mechanism that is differently regulated in Cold and Heat RA patients. In Heat RA patients apoptosis was found to be stimulated compared to Cold RA patients, in which more apoptosis resistance genes were expressed in the particular subset of T-cells. In Chapter 7 a second study is presented in which Cold and Heat RA patients were characterized, this time with a more extensive LC-MS analysis of urine and plasma samples. This study reveals higher levels of 11 acylcarnitines in the urine of Heat RA patients compared to Cold RA patients. These findings suggest that Cold RA patients have less muscle mass and/or a more pronounced muscle breakdown than RA Heat patients. Carnitine homeostasis has been related to fatigue in chronic fatigue patients and might also be related to fatigue experience in RA, which is a major issue for RA patients. It would be interesting to study whether fatigue is particularly related to one of the Cold or Heat RA sub-types.

Additionally, it was found that levels of DHEAs in Heat RA patients were higher than in Cold RA patients suggesting that the Cold RA group has a more suppressed HPA-axis function. Decreased HPA-axis function is associated with an inadequate response to stress factors. It is hypothesized (Cutolo 2000) that this inadequate response can lead to autoimmune and inflammatory disorders.

In Chinese medicine Cold and Heat RA patients are treated very differently. The results described in Chapter 5 and 7 indicate substantial biochemical differences between the two

types of patients. A text mining study (Li 2007) shows that diseases related to Cold according to Chinese medicine theory are more related to hormone function disturbances and Heat related diseases to immune function disturbances, which is in agreement with our findings. It is hypothesized that Cold RA patients might benefit more from hormone based therapies (such as prednisone) and Heat RA patients from immune based therapies (such as anti-TNF agents).

Recently a new study was started but not yet completed to study the response of Cold and Heat RA patients to anti-TNF treatment. Currently 40-50% of the RA patients fail to respond adequately to these drugs, resulting in side effects but no beneficial effects for the patients. The RA patients have been classified in Cold and Heat based on a questionnaire and response is measured at several time points. The ability to predict these differences in response to treatment with one of the most expensive drugs on the market today would have an enormous impact on health care costs.

This unique approach of targeting therapy to sub-groups of patients based on Chinese diagnostic information could be extended in future studies. In the presented work only two general sub-types of RA are explored, which could be expanded with more sub-types. The study presented in Chapter 6 suggests that the key Chinese medicine concepts Internal and External constitute another important source of variation between RA patients. In addition, the more chronic stages of RA could be studied by exploring disturbances on the level of the Chinese concepts for Organ functions. Such studies might reveal certain clearly characterized sub-types of RA patients that can be treated differently.

Systems biology as a bridge between Western and Chinese medicine

The gene expression and metabolomics profiles of rheumatoid arthritis sub-types provide a biochemical view of some Chinese diagnostic principles. This biochemical view firmly grounds Chinese diagnosis in biology and makes acceptance easier. Recently this approach was discussed in a Nature publication (van der Greef 2011). The scientific community is clearly opening up for the integration of different views on health and science. The foundation of this integration is systems thinking and systems biology. Systems thinking is typical of Chinese medicine in which diagnosis and treatment is based on changing patterns of symptoms. Western science is recently rediscovering the principles and usefulness of systems thinking by developing comprehensive measurement platforms, and data analysis

approaches for studying relationships between measurements.

Besides an open mind, analytical platforms are needed that are able to capture a wide variety of types of information of the system. In this thesis, a gene expression study is described in which the expression of all the genes of CD4+ T-cell were measured. Additionally, several untargeted liquid chromatography and gas chromatography mass spectrometry methods were used to measure metabolite levels in urine and plasma. Although the results are promising, the options for measuring the system could be greatly improved. For instance, the interactions between 'compartments' such as the liver, urine, blood, muscle, brain, could reveal compensation mechanisms. Additionally, analytical platforms could be developed to measure less abundant metabolites. Especially the identification of unknown metabolites proves to be a challenge (Kind 2010).

Systems biology science will reveal much more information about a system when measurements in time become a more viable option. Rheumatoid arthritis as an example of a chronic disease, daily rhythms in the symptoms (Cutolo 2005) are well known, and relationships with disturbances of hormonal rhythms are extensively studied (Cutolo 2008). This has for instance resulted in glucocorticoid chronotherapy with optimal medication at 3 am (Cutolo 2012). More understanding of the rhythmicity of biological systems and disturbances of the system will result in new options for optimizing treatment. Several studies in this area of chronobiology are showing the dynamics in metabolite levels during the day (Eckel-Mahan 2012, Bass 2010, Froy 2010). However, it is a challenge to develop stable analytical platforms to measure large series of samples and analyze the huge amount of data resulting from such measurements.

The systems biology bridge can be strengthened by including the rich Chinese theoretical development and experience with symptom patterns. Symptom patterns seem to behave like attractors with stable states that can change to other states when certain bridge symptoms express themselves. It would be interesting to study changes in symptom patterns and changes in metabolomics and other -omics patterns simultaneously. A challenge here is to design an expert system that is able to easily capture relevant symptom information to prevent the use of extensive questionnaires.

Another approach to strengthen the bridge lies in an integration of nonlinear mathematics and complexity theory with biology to improve the analysis of patterns of relationships. Living systems are inherently nonlinear, where the output is usually not proportional to input, and which cannot be described by linear differential equations. Such systems consist of a large

and variable numbers of components, showing a large degree of connectivity (Higgins 2002). Living systems are also energetically open and organizationally closed, but in a state far from equilibrium. There is a constant uptake and incorporation of substances in the body while other substances are excreted. Systems biology needs to move away from only studying linear relationships and should include nonlinear possibilities.

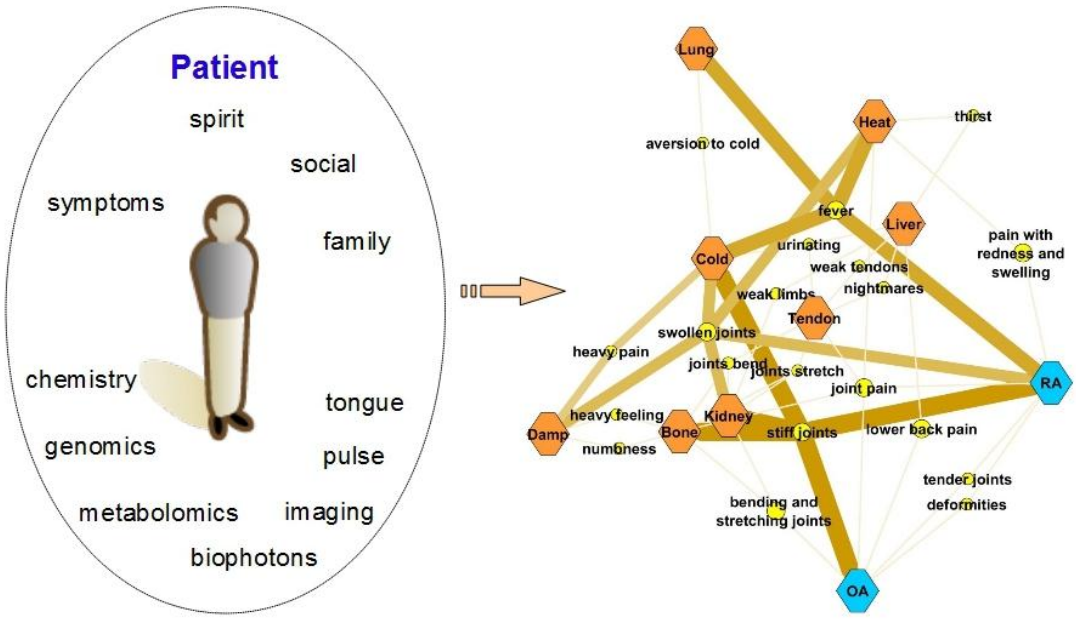

Figure 1. Integration of Chinese and Western concepts of arthritis. Patient information is collected on several levels (left panel). Symptoms which relate Chinese concepts (orange hexagons) with Western concepts (blue hexagons) are visualized in a network (right panel).

Finally, systems biology thrives by good visualization methods that can convey clear messages extracted from the usually huge amount of data. In several studies presented in this thesis, concepts from network theory were applied. In Chapter 2 the relationships between Western and Chinese concepts of arthritis were visually explored (Figure 1). Chapter 5 contains a network view of the genes expressed differently between the Cold RA and Heat RA patients. The relationships between the symptoms used in the questionnaire described in Chapter 6 were also visualized in a network view. Several tools for static network visualization such as Cytoscape (www.cytoscape.org), Pajek (pajek.imfm.si/doku.php?id=pajek) and Grephi (grephi.org) are developing rapidly, however dynamic visualization is much more challenging. New options for visualizing dynamic data were explored in the artist project Aqua Vita (www.theaquavitaproject.com), which used a combination of metabolomics and Chinese diagnosis data. This effort has resulted in an

appealing interactive visualization, novel ideas, and an exhibition with a large amount of public outreach. Bringing disciplines together enhances the creative process and can result in a better embedding of research in the society.

Personalized medicine and health promotion

The introduction of this thesis started out with describing some of the challenges the health care system is facing today. Slowly, scientists and clinicians start to realize that every patient is different and that therefore medicine needs to be developed that is personalized. In this thesis a step was taken in the direction of personalized medicine by characterizing two groups of rheumatoid arthritis patients (Figure 2). Instead of treating all patients in the same manner, a distinction can now be made between the Cold and Heat RA patients. Similarly, two pre-diabetes sub-types based on Chinese diagnosis have been characterized by metabolomics measurements (Wei 2012). A next step might be to find even more subtle sub-types of patients for which treatment can be optimized.

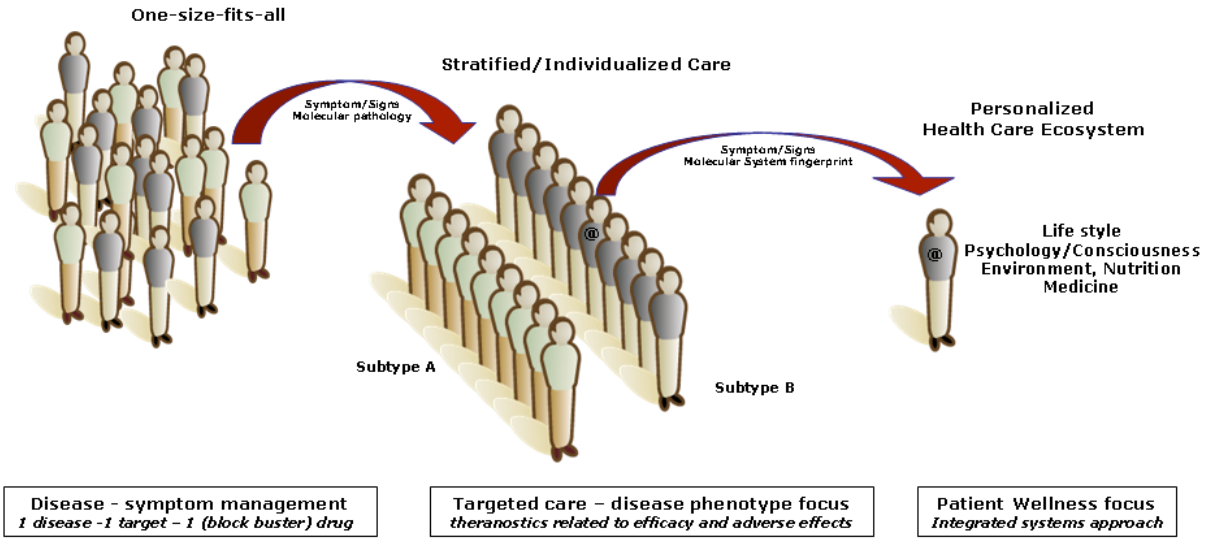

Figure 2. Steps from the current Western 'one-size-fits-all' concept to targeted care and eventually to a personalized health care system.

The development of health promotion strategies will have a major impact on the structure of the health care system and the well-being of people. Chinese medicine is especially developed as a health promotion strategy and contains the knowledge to monitor changes in the healthy state of the body. This knowledge of diagnosis, which includes the development of symptom patterns, is essentially lacking in Western medicine. In this thesis, first steps are

taken to integrate this knowledge with Western systems biology information. Symptom patterns can further direct biomarker research in the area of disease prevention and health promotion. In addition, Chinese medicine includes life-style advice and herbal medicine that are aiming for health promotion and can be used in very early stages of shifting towards an unhealthy state.

A movement towards health promotion invites the question of who is responsible for which part of the health care options. A shift towards a more equal patient-practitioner relationship is already happening and will place the doctor more in the role of a health coach. Health promotion strategies will be much more integrated in daily life by, for example, health apps on mobile phones, courses in stress reduction techniques that people can take, personalized nutrition advice, etc.

As health care will become patient-centered, integrated, preventive and personalized, the role of scientists also needs to change. Science conducted in multidisciplinary teams, including patients, consumers and health care organizations, allows the development of health care products that are actually desired by patients and consumers. The scientist will act as a knowledge organizer and integrator. Discoveries will more easily lead to strategies that can enter clinical practice and reach the consumer. The future health of the society and of the participants therein will emerge from the many interconnected relationships between various actors and the inspiration these relationships will bring about.

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Samenvatting

De huidige geneeskunde richt zich vooral op het bestrijden en managen van ziekten. Ontwikkelingen op dit vlak hebben geleid tot een enorme verlenging van de levensduur van mensen met bijvoorbeeld kanker, hart- en vaatziekten en diabetes. Echter, zoals de vicepresident van GlaxoSmithKline in 2003 al opmerkte, werken 90% van de medicijnen in slechts 30-50% van de patiënten. Er is dus veel ruimte voor verbetering.

Een recente ontwikkeling is de gedachte dat medicijnen meer op het individu toegesneden moeten worden. Het is van belang wie welk medicijn krijgt, op welk moment en in welke dosis. De effectiviteit van medicijnen kan enorm verhoogd worden door de juiste categorie patiënten te vinden voor een bepaald medicijn. Voor de ontwikkeling van deze geïndividualiseerde zorg is goede diagnose essentieel. In dit proefschrift staat diagnose centraal.

In China heeft zich de afgelopen millennia een geneeswijze ontwikkeld met een meer beschrijvend diagnostisch systeem. Patronen van symptomen hebben een betekenis gekregen en kunnen vertaald worden in een behandeling. In dit proefschrift wordt getracht Chinese kennis van diagnostiek te vertalen naar manieren om de Westerse zorg te optimaliseren.

Systeem biologie is bij uitstek een geschikte werkwijze om deze vertaling te bewerkstelligen. Deze benadering berust op het meten van informatie op verschillende niveaus van een systeem, waarbij het meten van metabolieten in bloed en urine in dit proefschrift de nadruk heeft. Deze benadering kan gebruikt worden om biologische verschillen tussen patiënten groepen te vinden en te karakteriseren. In dit proefschrift wordt een systeem biologische benadering gepresenteerd waarbij reumatoïde artritis patiënten kunnen worden ingedeeld in twee groepen op basis van Chinese diagnostische eigenschappen. Metabolomics en gen-expressie analyses werden uitgevoerd om mogelijke biologische verschillen tussen deze twee groepen te ontdekken. Hieronder volgt een overzicht van de gepresenteerde hoofdstukken van het proefschrift.

Hoofdstuk 2 is een zoektocht naar een visie op geïndividualiseerde geneeskunde en de mogelijkheden om deze visie vorm te geven. Diagnose wordt beschouwd als een sleutelbegrip. Een betere integratie van systeemdenken in de levenswetenschappen en medische wetenschappen wordt voorgesteld, wat uit kan groeien tot een systeemwetenschap. Daarnaast wordt de waarde van het systeemdenken in de Chinese geneeskunde voor de

ontwikkeling van geïndividualiseerde geneeskunde uitvoerig belicht en worden methoden beschreven voor het integreren van het Chinese denken in de Westerse wetenschap.

Metabolomics is naast andere -omics technologieën zoals genomics en proteomics één van de hoekstenen van systeembiologie. In **hoofdstuk 3** wordt een overzicht gegeven van het metabolomicsveld vanuit het perspectief van 30 jaar metabolomics ontwikkeling bij TNO. De omslag van een door technologie gedreven ontwikkeling van metabolomics naar een door biologie getrokken ontwikkeling wordt beschreven. Dit alles geïllustreerd met een verscheidenheid aan metabolomics toepassingen en afgesloten met een visie op de rol van metabolomics in het veranderende landschap van de zorg.

In **hoofdstuk 4** wordt dieper ingegaan op metabole processen die betrokken zijn bij reumatoïde artritis. Een gedetailleerd overzicht wordt gepresenteerd van metabole processen die de afgelopen tientallen jaren beschreven zijn. Een geïntegreerde analyse van de informatie resulteerde in een belangrijke rol voor celdood en vetmetabolisme in reumatoïde artritis. Een andere conclusie van de literatuurstudie was het belang van de dynamiek van bepaalde metabolieten voor het ziekte proces evenals voor de behandeling.

Hoofdstuk 5 beschrijft de eerste klinische studie waarin twee subtypen van reumatoïde artritis patiënten worden beschreven die gebaseerd zijn op een Chinese diagnose. De twee subtypen worden gekenmerkt door verschillende genexpressie profielen gemeten in CD4 positieve T-cellen en verschillen in een GC-MS metabolomics analyse van bloed plasma. De studie beschreven in dit hoofdstuk is het eerste bewijs voor biologische verschillen tussen deze twee subgroepen van RA patiënten, gebaseerd op een combinatie van Westerse en Chinese diagnose.

Hoofdstuk 6 beschrijft het logische vervolg op de resultaten van hoofdstuk 5, het standaardiseren van de Chinese diagnose waarmee de subtypen van RA patiënten kunnen worden vastgesteld. Hiertoe is een systeemdiagnose-vragenlijst ontwikkeld en toegepast waarmee uitgebreide symptoomprofielen van reumapatiënten zijn verzameld. De grootste variatie in symptoomprofielen tussen de patiënten bleek te corresponderen met de Chinese basis concepten Intern en Extern en de concepten Koude en Hitte. Deze twee algemene indelingen die gebruikt worden in de Chinese diagnose en behandeling bleken dus duidelijk terug te vinden te zijn met deze systeem diagnose vragenlijst.

In **hoofdstuk 7** wordt een klinische studie beschreven vergelijkbaar met die van hoofdstuk 5. In deze studie werden wederom RA-patiënten ingedeeld in Koude en Hitte typen patiënten op basis van een Chinese diagnose. Daarnaast werd een systeemdiagnose-vragenlijst ingevuld

door de patiënten. In deze studie werden standaard klinisch chemische bepalingen gedaan op bloedmonsters. Plasma en urine werd geanalyseerd met een globale LC-MS methode. De meest opvallende bevinding was de lagere hoeveelheid van 11 acylcarnitines in de urine van Hitte RA patiënten, in vergelijking met de Koude RA patiënten. Daarnaast werd een lagere hoeveelheid dehydroepiandrosterone sulfate (DHEAS) gevonden in Koude RA patiënten, wat kan duiden op een meer onderdrukte HPA-as functie in deze patiënten.

In dit proefschrift wordt laten zien hoe relevante subtypen van reumatoïde artritis patiënten kunnen worden gevonden door te kijken naar Chinese diagnostische kenmerken. Systeemdenken en systeem biologie blijkt een goede methode te zijn om biologische verschillen tussen deze subtypen te vinden en beschrijven. Het indelen van patiëntengroepen in kleinere subgroepen met specifieke kenmerken is een eerste stap richting geïndividualiseerde geneeskunde. In de toekomst kan de response van deze subgroepen op medicatie worden onderzocht en zo nodig de medicatie worden aangepast aan de subgroep. Bovendien kan deze methode helpen met het beschrijven en in kaart brengen van meer subgroepen met ieder een toegesneden behandelstrategie. In een verdere toekomst kan deze methode bijdragen aan het ontwikkelen van een persoonlijk zorg ecosysteem waarin de nadruk ligt op preventie, participatie, en het bevorderen van gezondheid.

Curriculum vitae

Herman van Wietmarschen was born in 1974 in Oss. In 1997 he received a degree in Medical Biology at the University of Utrecht. His major was in biophysics at the BBL Institute on the subject: the interaction of depth cues. In between he studied philosophy at the University of Utrecht for a year with a focus on bioethics. After his studies he joined the foundation Discussion about Biotechnology as a research journalist where he worked for nearly nine years. Meanwhile in 2003 he joined a branch committee of the Taoist Tai Chi Society of the Netherlands which has inspired him to improve his health by practicing Taoist Tai Chi. Later he also became a board member of the organisation. In 2006 he started as an associated researcher at the group Critical Technology Construction from the Wageningen University, developing and writing grant proposals in the area of science and technology studies. At the same time he also accepted the function of researcher at the Osteo- and Rheumatoid Arthritis Foundation in Amsterdam where he was responsible for scientific evaluation of complementary treatments in the area of arthritis as well as the funding of scientific research in this area. Inspired by the variety of cultural perspectives on health and health promotion he started a PhD project in 2008 from which this thesis is the result. He also joined the Scientific Advisory Board of the Osteo- and Rheumatoid Arthritis Foundation in which function he evaluated grant proposals to the Foundation. In 2008 Herman also became a board member of the CAM researchers network. In 2010 he was asked to join as an editor of the Journal of Integral Medicine. In October 2012 he started as a scientist innovator at TNO in Zeist. His topic will be systems biology of health, measuring health and developing tools for health promotion.

List of publications

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Nawoord

Dan opeens is het proefschrift klaar, een stuk onderzoek is afgerond. Althans zo lijkt het. Inmiddels hebben drie nieuwe aio's het stokje overgenomen. Zij zullen het thema opnieuw vormgeven en uiteraard spannende nieuwe ontdekkingen doen. Dit kan alleen met ondersteuning van vele anderen in de afdeling. Veel dank voor iedereen die dit mogelijk heeft gemaakt en gaat maken.

Inmiddels ben ik bij TNO aan de slag gegaan met onderwerpen die erg in het verlengde liggen van het werk beschreven in dit proefschrift. Naast nieuwe TNO uitdagingen kan ik deel blijven uitmaken van het SDPPM team en mijn steentje bijdragen aan het werk in Leiden.

Zonder mensen bij naam te noemen wil ik uiteraard iedereen bedanken die ik op mijn pad ben tegengekomen. Ik heb vele interessante, soms provocerende, ook treurige, inspirerende momenten meegemaakt. Sommige koffiepauzes zijn onvergetelijk waarbij alle politieke kleuren uit de samenleving verstrengeld waren in soms hevige discussies.

Het SDPPM team wil ik in het bijzonder bedanken. De stijl van werken en de culturele uitwisseling met Chinese partners in het onderzoek heeft mij enorm aangesproken en geïnspireerd. Het palet van verschillende talenten aan boord is uniek, een waar genoegen om met jullie allemaal samen te werken.

Tenslotte heeft dit proefschrift niet tot stand kunnen komen zonder support van mijn lieve partner Klaartje. Tijdens deze jaren hebben wij een zoontje en dochtertje mogen krijgen en ook zij hebben bijgedragen aan de vorm waarin het onderzoek gegoten werd.