A systems approach to sub-typing of rheumatoid arthritis

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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.

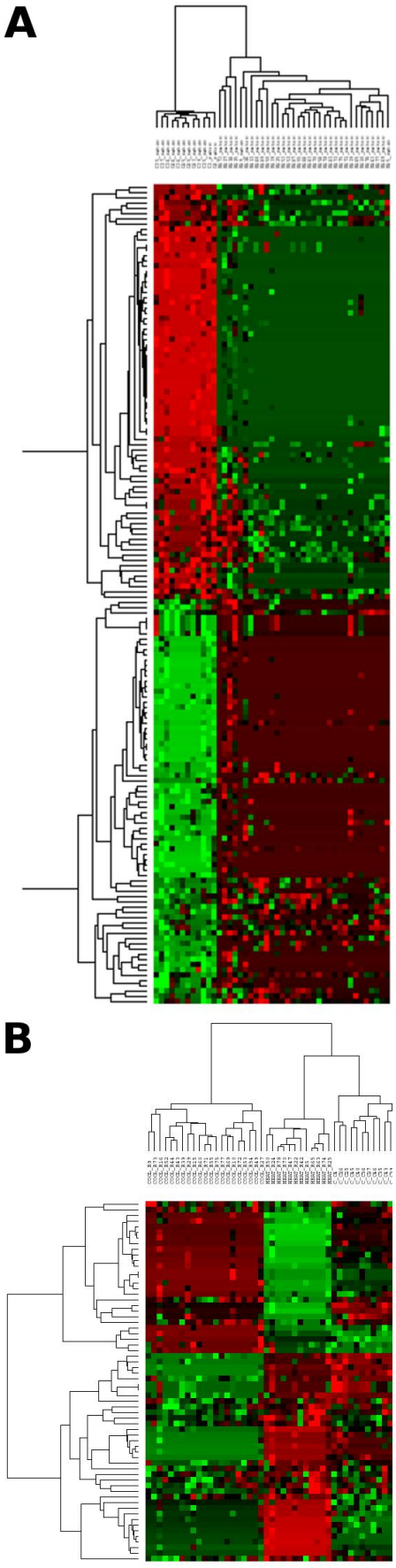

Figure 1. Panel A shows a hierarchical cluster analysis of the expression of all genes (146

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).

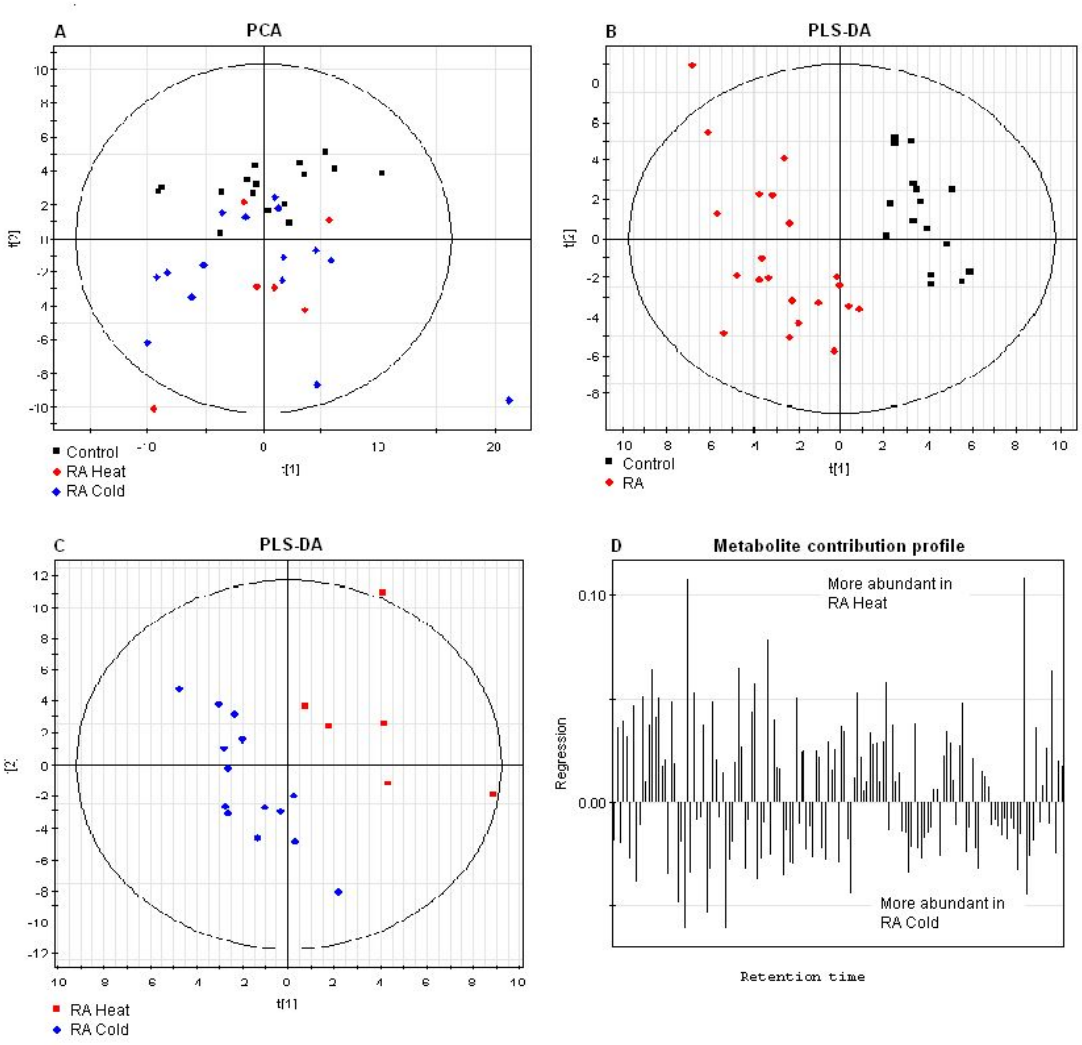

Figure 2. Panel A shows a PCA score plot in which all the RA patients and control participants are represented by a point. Different colors and symbols are used to visualize the control, RA Cold and RA Heat participants. Panel B shows a PLS-DA score plot for the first two principal components. The RA 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.

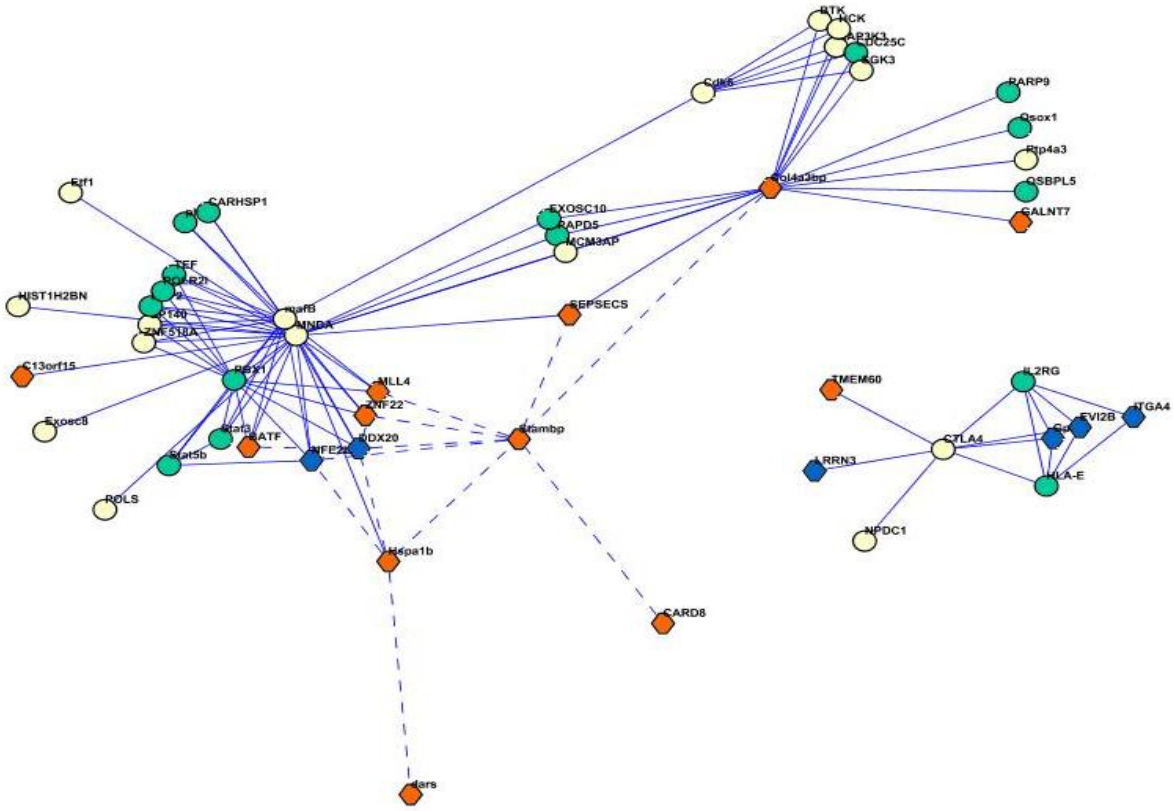

Figure 3. A network illustrating the functional relationships between genes involved in processes of the immune system (solid lines) and apoptosis (dashed lines). Genes colored dark green are upregulated in RA patients, light green colored genes are downregulated in RA patients. Octahedrons denote genes 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.

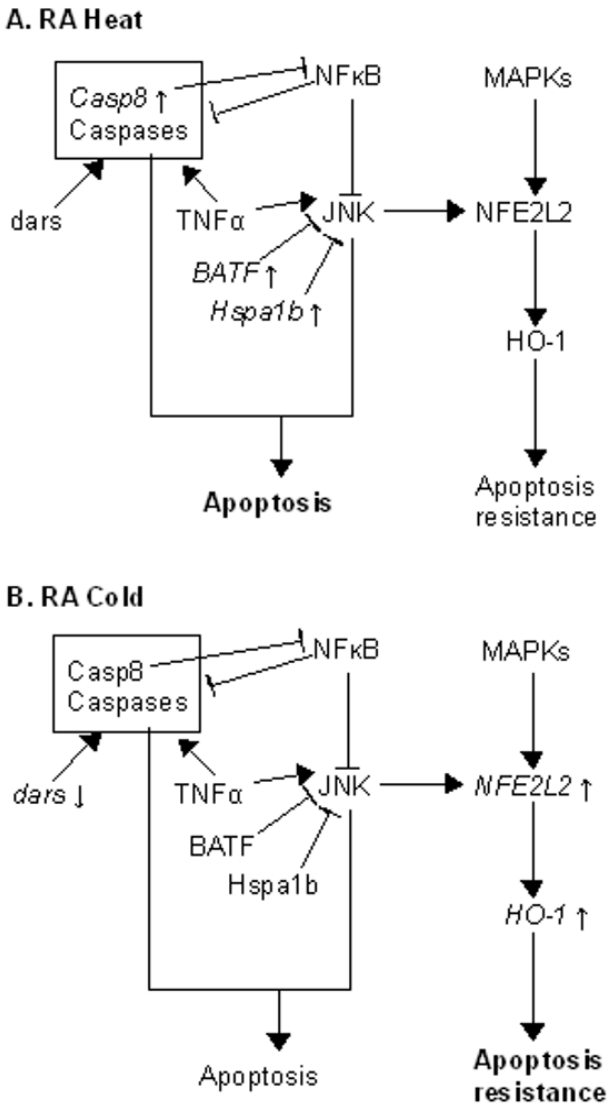

Figure 4. Apoptosis regulation in RA Heat and RA Cold patients. In RA Heat patients the expression of Caspase 8 was increased which resulted in a stimulation of apoptosic processes. In RA Cold the combination of no enhanced expression of Caspase 8 and the increased expression of NFE2L2 (Nrf2) and HO-1 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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