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REVIEW

Transcript profiling towards personalised medicine in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that is heterogeneous in nature. The heterogeneity is reflected by the variation in responsiveness to virtually any treatment modality. Since our understanding of the molecular complexity is incomplete and criteria for categorisation are limited, we mainly consider the disease RA as group average. A powerful way to gain insight into the complexity of RA has arisen from DNA microarray technology, which allows an open-ended survey to comprehensively identify the genes and biological pathways that are associated with clinically defined conditions. During the last decade encouraging results have been generated towards the molecular description of complex diseases in general. Here, I describe developments in genomics research that provide a framework to increase our understanding of the pathogenesis and the identification of biomarkers for early diagnosis, prognosis and stratification, aimed at a personal medicine approach in RA.

KEYWORDS

Biomarkers, disease subtypes, DNA microarray, genomics, molecular profiling, personalised medicine, pharmacogenomics, rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease that primarily affects the joints. The aetiology of RA is unknown. Clinical and laboratory observations suggest an immune-mediated attack against self-antigens. This is featured by the connection HLA-DR loci, and the expression of autoantibodies, such as rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPA). The immune-mediated background is substantiated by the ameliorative role of immune-suppressive therapies.

Accordingly, initial alterations in the immune system are likely the basis for the development of RA. This is reflected by the finding that ACPA and/or RF are already present prior to the onset of RA.[1,2] Using serum samples stored in a blood bank, Rantapää-Dahlquist and colleagues showed that 34% of the RA patients were positive for ACPA up to nine years prior to diagnosis.[1] In analogy, Nielen and colleagues showed that 49% of the RA patients tested positive for IgM-RF and/or ACPA before onset of disease at a median of 4.5 years before symptom onset.[2] A recent prospective follow-up study of ACPA and/or IgM positive arthralgia patients has shown that ACPA positive patients are more likely to develop arthritis than ACPA negative, IgM-RF positive arthralgia patients (27 *vs* 6% after a median follow-up of two years).[3] Since not all ACPA and/or RF positive individuals ultimately develop RA the requirements to drive this process are likely to be different between the persons at risk.[4,5]

Once symptoms are present, RA manifests as a heterogeneous disease with a clinical spectrum ranging from mild to severe disease, and variability in secondary organ system involvement. The heterogeneous nature is reflected by variation in responsiveness to virtually any treatment modality. The heterogeneity most likely has its origin in its multifactorial nature, whereby specific combinations of environmental factor(s) and a varying polygenic background are likely to influence not only susceptibility but also the disease severity and prognosis. Unfortunately, our understanding of the preclinical phase and molecular complexity of RA is incomplete, and criteria for subtyping of patients, for example to select those patients who will benefit from a specific treatment, is currently lacking.

By definition, nearly every aspect of a disease phenotype should be represented by pathophysiological processes

driven by genes and proteins that are expressed in the patient. These genes and proteins typically represent a molecular signature that is associated with disease characteristics and subtypes and thus defines the samples unique biology. A very powerful way to gain insight into the molecular signature underlying pathophysiological processes has arisen from DNA microarray technology, which allows an open-ended survey to identify comprehensively the fraction of genes that are differentially expressed in blood and tissue samples among patients with clinically defined disease and could serve a role as clinically relevant biomarker.

Initially, several pitfalls were experienced using this multistage and relatively expensive technology, which highly depends on perfectly standardised conditions. Factors that could influence the sensitivity and reproducibility range from sample processing differences, variation in amount and quality of starting RNA, amplification and labelling strategies and dyes, to probe sequence and hybridisation conditions. In addition the lack of standardised approaches for normalisation and usage of data analysis algorithms could influence the outcome. Therefore, verification of results became an essential step in microarray studies. In order to set quality criteria for performing and publishing microarray studies, standards for microarray experiments and data analysis were created.[6]

Nowadays, after a decade of technical and analytical improvements, the technology and algorithms for data analysis have been shown to be robust and reproducible across properly designed and controlled experiments, and different research groups. The availability of the Paxgene whole blood isolation system, which directly lyses aspirated blood cells and stabilises the RNA in the aspiration tube, excludes *ex-vivo* processing artifacts. These developments make transcriptomic profiling superior to a proteomics approach for biomarker discovery. However, careful standardisation is still required for cell subsets and tissues that are obtained via *ex-vivo* manipulation.

The differentially expressed genes may then be used to provide insight into biological pathways contributing to disease and to identify classifiers for early diagnosis, prognosis, and response prediction.[7,8] This review describes developments in transcriptomics research to identify novel pathways that contribute to disease and to uncover clinically relevant biomarkers (*figure 1*). Ultimately this information may help clinicians to improve disease management.

Figure 1. *Schematic outline for disease subclassification in RA*

RA patients reveal a striking heterogeneity based on clinical, biological and molecular criteria. Categorisation of patients is expected to be of utmost importance for decision making in clinical practice. Recent developments in high-throughput screening technologies have provided the opportunity to characterise patients based on their molecular profile. Application of transcript profiling using DNA micro-arrays allows us to determine the molecular profile (barcode) of an individual patient. When associated with clinical read-outs we could select the clinical useful molecular markers and apply these in day-to-day clinical practice. The procedure starts with collecting peripheral blood cells (using e.g. PAXgene tubes) from each patient. Eventually, synovial biopsies and fibroblast-like synoviocytes may be obtained. This material can be processed to isolate mRNA, and then further analysed by high-throughput techniques such as DNA micro-arrays. Subsequently, computational algorithms will be applied to select biomarkers that allow subtyping of patients. This approach helps to elucidate the distinct pathological mechanisms at play that can explain the inter-patient variation in clinical presentation, disease progression and treatment response. Knowledge of the molecular differences between patients and differential pathogenic mechanisms in relation to drug response helps us to identify biomarkers that predict the responder status of targeted therapies in RA.

MOLECULAR MARKERS FOR HETEROGENEITY BETWEEN SYNOVIAL TISSUES

The first study on gene expression profiling in RA concerned synovial tissue biopsies using a combination of subtractive hybridisation and high-density cDNA arrays.[9] This study highlighted the increased expression of genes involved in chronic inflammation such as immunoglobulins and HLA-DR in RA synovium when compared with normal synovium. Comparative analysis of synovial tissue specimen from RA and osteoarthritis (OA) patients revealed that these diseases were characterised by distinct synovial gene signatures.[10] In particular genes involved in B and T cell regulation were upregulated in RA tissues.[11] Histological analysis confirmed that in RA synovium was characterised by a higher amount of infiltrating T cells and B cells when compared with OA synovium.

A large-scale gene expression profiling study of 30 synovial tissue specimens from patients with erosive RA revealed considerable heterogeneity among patients.[12,13] Systematic characterisation of the differentially expressed genes highlighted the existence of at least two molecularly distinct forms of RA tissues. One group, referred to as the RA high inflammation group, was characterised by genes involved in inflammation and adaptive immune response. The genes involved in the high inflammation tissues consist of

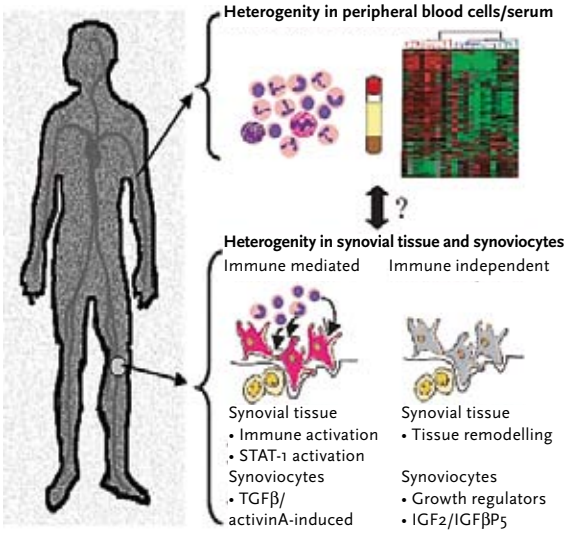
immunoglobulin genes and genes indicative for an activated IFN/STAT-1 pathway. Seven of these (TIMP2, PDGFRA, GBP1, Fos, CTSL, TUBB and BHLHB2) were also described by Devauchelle and colleagues.[10] Two of these (GBP1 and CTSL) are known to be regulated by type I IFN.

The second group of RA tissues was characterised by a low inflammation gene signature that was reminiscent of that of tissues from patients with OA. While inflammation and immune-related genes were decreased, these tissues showed an increased expression of genes involved in tissue remodelling activity, which is associated with fibroblast dedifferentiation. Remarkably, the high and low inflammation tissues revealed reciprocal expression of specific matrix metalloproteinases (MMP). Whereas levels of MMP11 and 13 were increased in low inflammation tissues, levels of MMP1 and 3 were increased in high inflammation tissues.[13]

Tsubaki and colleagues demonstrated that tissue heterogeneity within RA can already be observed in the early phase of RA (duration of less than one year after diagnosis).[14] Analogous to the previous study using biopsies from long-standing RA patients, the early RA patients could be divided in at least two different groups based on their gene expression profiles.

In approximately 5 to 10% of synovial tissues T cells, B cells, and follicular dendritic cells (FDCs) are spatially organised into structures resembling lymph nodes with germinal centres (GC). The remainder of the tissues lack FDCs and show either a diffuse or an aggregated T-cell and B-cell infiltrate. Histological analyses revealed that the differences observed in global gene expression among the rheumatoid synovia are related to differences in cell distribution. Tissues that contain ectopic GC-like structures were selectively present in the high inflammation tissues. The GC-containing tissues revealed increased Ig transcript expression in accordance with the presence of B cells and/or plasma cells, which may reflect local production of antibodies. These tissues also showed enhanced expression of the chemokines CXCL12 and CCL19 and the associated receptors CXCR4 and CXCR5, which are important for the attraction of T cells, B cells, and dendritic cells.[15] In addition genes involved in JAK/STAT signalling, T-cell and B-cell specific pathways, Fc-receptor type I signalling in mast cells, and IL-7 signal transduction (e.g. IL-7 receptor α (IL-7R α)/IL-2Rγ chains and IL-7) were elevated. These findings suggest a role for the IL-7 pathway in synovial lymphoid neogenesis in RA, analogous to its role in the development of normal lymphoid tissue.[14] Tissues with a diffuse type of infiltrate showed a profile that indicated repression of angiogenesis and increased extracellular matrix remodelling. Overall, the gene expression profiling of rheumatoid synovium has provided insight into the molecular basis of the heterogeneous nature of synovial disease pathogenesis in RA (*figure 2*). It remains to be determined if a specific molecular profile applies to all affected synovia in a single patient, and if the profile is stable during the course of disease.

Figure 2. *Schematic representation of RA subtypes*



Schematic overview of the discovery of RA subtypes based on differential gene expression in peripheral blood and affected target tissues. The exact relationship between the peripheral blood profile and tissue profile needs to be further investigated.

GENE EXPRESSION IN MESENCHYMAL CELLS DERIVED FROM AFFECTED TARGET TISSUES

Fibroblast-like synoviocytes (FLS) are major players in joint destruction in RA. These cells are considered to be sentinel cells that contribute to leucocyte migration and local immune response through the production of various immune modulators.[16-18]

Gene expression profiling analysis of rheumatoid FLS revealed the overexpression of genes responsible for tumour-like growth when compared with FLS derived from traumatic control patients.[19] Moreover, an increased expression was observed for PDGFRα, PAI-1 and SDF1A. Other investigators studied the influence of tumour necrosis factor-α (TNF) on FLS, since TNF showed to be of primary importance in the pathogenesis of RA.[20,21] These studies are instrumental to define TNFα response signatures that can be used to monitor the pharmacodynamics of TNF blockade.

Profiling studies of FLS derived from 19 RA patients revealed considerable heterogeneity. The distinct FLS subtypes were associated with a specific phenotypic characteristic of the synovial tissue from which they were

derived.[22] The FLS subtype that reveals similarity with 'myofibroblasts' was associated with high-inflammation tissues. The myofibroblast is a specialised fibroblast, which expresses α-smooth muscle actin (SMA), an actin isoform typical of vascular smooth muscle cells. These myofibroblast-like FLS showed a markedly increased expression of transforming growth factor β (TGFβ) response genes. Among these response genes were SMA, SERPINE1, COL4A1 (type IV collagen-α chain), IER3 (immediate early response 3), TAGLN (transgelin), and the gene for activin A as a potential agonist for the induction of the TGFβ response programme. Similar cells have recently been identified in the human $TNF^{+/-}$ transgenic mouse model of arthritis.[23] Studies from the field of oncology indicate that myofibroblasts present in tumours play a crucial role in angiogenesis and cell trafficking through the production of extracellular matrix proteins, chemokines and growth factors. Hence, it is hypothesised that the increased presence of this specific type of fibroblast, which is characterised by increased expression of SMA among other genes, is selectively associated with high-inflammation tissues and contributes to angiogenesis and cell trafficking in RA synovium. FLS that are characterised by increased expression of growth-related genes and Igf2 and IGFBP5, were associated with low-inflammation tissues.

These data support the notion that cellular variation between target tissues is reflected in the phenotypic characteristics of the stromal cells (*figure 2*).

GENE EXPRESSION IN PERIPHERAL BLOOD CELLS

Knowing the systemic nature of RA and the communication between the systemic and organ specific compartments, we and others also studied whole blood and/or peripheral blood mononuclear cells (PBMC) to obtain disease-related gene expression profiles. The peripheral blood may not directly have implications for our understanding of disease pathogenesis, but is especially suitable to analyse gene expression profiles that provide a framework to select clinically relevant biomarkers.

Accordingly, several investigators studied gene expression levels in peripheral blood cells to address the question whether disease characteristics are detectable from gene expression levels in peripheral blood cells. Bovin and colleagues identified 25 genes discriminating between PBMC of RA patients (n=14) and healthy controls (n=7).[24] These genes reflected changes in the immune/inflammatory responses in RA patients, such as the calcium-binding proteins S100A8 and S100A12. No significant differences between RF-positive and RF-negative RA were observed. Szodoray and colleagues studied gene expression differences in peripheral blood B cells from eight RA patients and eight healthy controls.[25] A total of 305 genes were upregulated, whereas 231 genes were downregulated in RA B cells. In a larger study with 29 RA patients and 21 healthy controls, Batliwalla and colleagues identified 81 differentially expressed genes, including glutaminyl cyclase, IL1RA, S100A12 and Grb2-associated binding protein (GAB2) as the main discriminators. This profile correlated with an increased monocyte count.[26] These findings indicate that there are clear differences in peripheral blood markers between RA patients and healthy controls that may have diagnostic potential.

Other investigators addressed the issue of heterogeneity in peripheral blood gene expression profiles among RA patients. Olsen and colleagues studied gene expression differences in PBMC between early (disease duration less than two years) and established RA (with an average disease duration of 10 years).[27] Out of 4300 genes analysed, nine genes showed a threefold increased expression in the early RA group. These genes included colony-stimulating factor 3 receptor, cleavage stimulation factor, and TGFβ receptor II, which affect B-cell function. A total of 44 genes, which are involved in immunity and cell cycle regulation, were expressed at threefold lower levels. The observation that a quarter of the early arthritis genes overlapped with an influenza-induced gene set led the authors to suggest that the early arthritis signature may partly reflect the response to an unknown infectious agent. We studied gene expression profiles of whole blood cells of 35 RA patients and 15 healthy individuals.[28] This analysis confirmed previous observations of increased expression of, for example, the calcium-binding proteins S100A8 and S100A12 by RA blood cells. The significantly differential expressed genes represent specific biological processes related to immune defence, including type I IFN-response genes, indicative that this pathway is also activated systemically in RA. This type I IFN signature may be a direct reflection of increased type I IFN activity or other ligands known to activate the IFN/STAT-1 pathway.

Upregulation of IFN-response genes has now been observed in peripheral blood cells and/or target tissue of (a subset of) patients with other autoimmune diseases such as SLE, scleroderma, Sjogren's syndrome, multiple sclerosis, and type 1 diabetes. These findings suggest that an activated IFN-response gene expression programme is a common denominator in rheumatic diseases, and autoimmune diseases in general. Type I IFNs (IFNα and IFNβ) are early mediators of the innate immune response that influence the adaptive immune response through direct and indirect actions on dendritic cells (DCs), T and B cells, and natural killer cells. A likely candidate in RA is IFNβ, which is highly produced in the

synovium and could serve a role as a secondary feedback mechanism aimed to dampen the inflammation.[29,30] The importance of IFNβ production in RA is highlighted by Treschow and colleagues, who showed that IFNβ deficiency prolonged experimental arthritis.[31] Moreover, transfer of IFNβ-competent FLS was able to ameliorate arthritis in IFNβ-deficient recipients. However, although treatment with recombinant IFNβ revealed promising results in experimental arthritis, treatment of RA patients with IFNβ has been unsuccessful.[32] Alternatively, type I IFNs could affect the initiation or amplification of autoimmunity, thereby contributing to disease. It is speculated that the IFN response activates immature myeloid DCs, which normally regulate deletion of autoreactive lymphocytes. Subsequently, IFN-matured DCs may activate autoreactive T cells leading to autoreactive B-cell development and autoantibody production.[33] In the case of SLE, autoantigen/autoantibody complexes may trigger pathogen recognition receptors (such as TLRs) that induce IFNα production and thereby perpetuates the IFN response programme.

Remarkably, the increased expression of the type I IFN response genes was characteristic of not all, but approximately half of the patients. Moreover, the immune defence gene programme that was activated in a subgroup of RA patients was reminiscent to that of virus-infected macaques.[34] We found that an activated immune response, characterised by a viral response signature, defines a subgroup of RA patients with significantly increased titres of ACPA.

PHARMACOGENOMICS IN RA TOWARDS PERSONALISED MEDICINE

Therapies to target the proinflammatory mediator TNF-α, B and T lymphocytes are approved worldwide for the treatment of RA. Clinical experience showed that the targeted therapies with biologicals are effective for most but not all of the RA patients, reflecting that there are 'responders' and 'nonresponders'.[35] Given the destructive nature of RA, the risk of adverse effects, and considerable costs for therapy, there is a strong need to make predictions on success before the start of therapy. If we rely solely on clinical or radiographic manifestations we will probably be responding too late to maximise protection. However, clear criteria for such classification are still lacking.

Ideally, a molecular biomarker signature as a predictor for therapy responsiveness should be obtained prior to the start of therapy in a readily available biosample, such as peripheral blood. Ultimately, this may lead to a personalised form of medicine, whereby a specific therapy will be applied that is best suited to an individual patient. I will present the results of pharmacogenomic studies to provide insight into the pharmacology of TNF blockade by soluble antagonists such as etanercept, infliximab or adalimumab, which are effective for approximately two thirds of the patients, and to predict the response to therapy. In essence similar studies can be carried out for therapies directed against T and B lymphocytes. The term *pharmacogenomics* emerged in the late 1990s and is associated with the application of genomics in drug development. *Pharmacogenomics* is defined as: 'The investigation of variations of DNA (genetics) and RNA (transcriptomics) characteristics as related to drug response'.

The concept of a personalised form of medicine has attracted interest in the search for molecular criteria to dissect TNF responders from nonresponders in RA. Initial pharmacogenomics approaches aimed to understand the pharmacological effects of TNF blockade in the peripheral blood compartment in order to gain a comprehensive understanding of the mode of action. Pharmacogenomics studies revealed a similar change in the expression of a pharmacogenomic response gene set in the peripheral blood compartment of all RA patients treated with infliximab, irrespective of clinical response. This result suggests the presence of bioactive TNF in the circulation irrespective of clinical response.[36,37]

Detailed analyses in search of (subtle) differences in the pharmacogenomic response profiles between responders and nonresponders identified informative sets of genes whose expression changes during therapy and were associated with clinical response.

Koczan and colleagues determined the pharmacogenomic differences after 72 hours in 19 RA patients (12 responders and 7 nonresponders) following administration of etanercept.[38] They report on an informative set of genes including NFKBIA, CCLA4, IL8, IL1B, TNFAIP3, PDE4B, PP1R15 and ADM involved in NF-κB and cAMP signalling whose expression changes after 72 hours that is associated with good clinical responses (disease activity score (DAS)28 >1.2). We showed that patients who developed an increased type I IFN response after one month of treatment had a worse clinical response to treatment.[39] This was reflected by less improvement in DAS and higher tender joint counts and higher health assessment questionnaire-disability scores after treatment. Likewise, all patients without an anti-TNF induced increase in type I IFN gene activity had a good or moderate response to treatment as assessed by the EULAR response criteria. Comparative analysis did not reveal an overlap between the three gene sets.

No significant gene expression differences between responders and non-responders were found at baseline.

Lequerre and colleagues studied in 13 patients (6 responders and 7 nonresponders) who started with an infliximab/methotrexate combination.[40] Treatment response was determined after three months based on a difference in disease activity score (DAS)28 score ≥1.2 to define responders. In a validation study with 20 patients (10

responders and 10 nonresponders) a set of 20 transcripts in PBMC, which covered a diverse set of proteins and functions, was selected as classifiers.

At the synovial tissue level Lindberg and colleagues found 279 genes that were significantly differently expressed between the good responding and nonresponding patients.[41] Among the identified genes was MMP-3. We found that a number of genes involved in biological processes related to inflammation were upregulated in patients who responded to infliximab therapy, compared to those who did not show clinical improvement were identified. These results indicate that patients with a high level of tissue inflammation are more likely to benefit from anti-TNF treatment.[42]

Overall, the data reveal the presence of TNF bioactivity in all patients treated with TNF antagonists irrespective of the clinical response. The results suggest subtle pharmacological differences between responders and non-responders. However, the identification of biomarkers before the start of therapy in order to predict the response to anti-TNF treatment in RA has not revealed consistent results, yet. Therefore, additional studies using large cohorts of patients and more stringent response criteria are necessary.

PRECLINICAL DIAGNOSIS OF RA

In order to induce remission and thereby prevent irreversible joint damage in RA, early diagnosis and a timely start of effective treatment is of high importance. Ideally, early diagnosis in the asymptomatic/preclinical phase is required. Several studies have documented the appearance of ACPA and RF prior to the onset of RA.[1,2] Since not all ACPA and/or RF positive individuals ultimately develop RA other processes are involved. Hence, either additional factors are needed to result in a chronic inflammatory response ultimately leading to RA or some individuals may have a protective immune profile which suppresses disease development despite the presence of autoantibodies. To understand the differences between persons at risk who do and who do not develop RA, we analysed the gene expression profiles of blood samples of a unique cohort of ACPA/RF positive arthralgia patients at risk for RA (n=109) who were clinically followed for progression to arthritis. We demonstrated the heterogeneous nature of ACPA and/or RF positive arthralgia patients at risk for development of RA and identified sets of genes whose expression profiles segregate arthralgia patients at risk for RA into different subgroups.[43] Subgroups that are characterised by a gene signature of IFN-mediated immunity, cytokine activity, or haematopoiesis all contain at-risk persons who have developed arthritis. These gene expression characteristics increase the risk for arthritis development approximately fourfold, independent of ACPA status. Interestingly, the group of patients characterised by increased expression of genes involved in humoral immunity is devoid of patients who have developed arthritis in the follow-up period. These results indicate that predisposition for the development of arthritis can be used to predict the diagnosis of arthritis in ACPA and/or RF positive individuals at risk.

On the basis of our data, we propose three levels involved in susceptibility to RA. First, some genes predispose the individual to autoimmunity. Second, this altered immunoreactivity is directed to particular antigens, i.e. citrullinated antigens, which affect B- and T-cell recognition of epitopes. Third, other genes act on the progression of autoimmunity to target tissues. Our results imply that, among others, IFN-mediated immunity and cell trafficking specify the processes relevant to progression to arthritis besides autoantibody positivity.

These results suggest that higher-order combinatorial searches may improve the predictive performance of autoantibody status towards diagnosis of preclinical RA.

CONCLUSIONS

Gene expression profiling approaches have fuelled insight into the complexity of RA pathogenesis and provide a framework to identify biomarkers as a promising tool for future clinical applications. Molecular profiling of blood cells and synovial tissues has already revealed important pathways contributing to the spectrum of diversity in RA. Until now, studies have been carried out using cohorts of relatively limited size. For the future the clinical implications of these observations require further definition and independent validation in large well-powered cohorts.

Pharmacogenomics studies are just emerging. The results of these studies look promising, but full confirmation of the biomarker profiles in independent uniform cohorts is of the utmost importance to create value for prediction of therapy response in RA to pave the way to more individualised treatment strategies. However, caution must be taken in the interpretation of these results because of small sample size and differences in treatment response measurements. To increase the sample size collaborative efforts from different groups are essential. To maximise the usage of information from different sources standardised procedures for sample processing, technology and data analysis and the algorithms used are needed. Moreover, full and open access to genomics data is important. This will ultimately allow a multidisciplinary approach, whereby clinometric, cytometric, metabonomic, genomic, proteomic, and imaging data from different laboratories are integrated to assign and validate clinically relevant markers

that reflect disease pathogenesis (diagnosis), prognosis, heterogeneity, and facilitate selection of patients with a high likelihood to respond to therapy.

Gene profiling in persons at risk to develop RA has revealed gene signatures in the peripheral blood that pose an increased risk above ACPA and RF. This finding forms the basis to envision predictive models based on preclinical expression profiling as an 'evolving' evidence-based process for determining the risk of developing RA.

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