Review

Genomics, proteomics and metabolomics: their emerging roles in the discovery and validation of rheumatoid arthritis biomarkers

P. Castro-Santos[1], C.M. Laborde[2], R. Díaz-Peña[1,3]

[1]*Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile;*
[2]*Clinical Biochemistry Laboratory, SERALAB, Oviedo, Spain;*
[3]*Department of Immunology, Hospital Universitario Central de Asturias, Oviedo, Spain.*

Patricia Castro-Santos, PhD
Carlos M. Laborde, MD, PhD
Roberto Díaz-Peña, PhD

Please address correspondence to:
Roberto Díaz-Peña, PhD,
Facultad de Ciencias de la Salud,
Universidad Autónoma de Chile,
5 Poniente nº 1670
3460000 Talca, Chile.
E-mail: rdiazp@uautonoma.cl

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune inflammatory rheumatic disease which affects several organs and tissues, predominantly the synovial joints. Despite major advances, the aetiology of this disease is not completely understood. Although several biomarkers are routinely used in RA management and some of them can be detected even prior to the onset of the clinical disease, there is a high demand for novel biomarkers to further improve the early diagnosis of RA. The '-omics' techniques that have emerged and have been developed in recent years have allowed researchers to improve their knowledge of the aetiopathology of RA. At the same time, advances in screening technologies offer an excellent opportunity to find new biomarkers potentially useful for early diagnosis, stratification of patients, and even prediction of a better response to a specific therapy. This review describes what is known about the methodologies used in the discovery of novel biomarkers in RA, along with the findings of these methodologies, with specific attention to recent advances in the fields of genomics, proteomics and metabolomics.

Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory rheumatic disease that affects many tissues and organs, mainly synovial joints. This disease leads to progressive destruction of articular cartilage and ankylosis of the joints (1). It affects approximately 1% of the population worldwide (2). Although the disease can develop at any age, RA affects females more frequently than males and is mainly diagnosed between the ages of 40–60 years. RA is associated with reduced life expectancy and constitutes a major cause of chronic disability and handicapping. However, its aetiology has not been elucidated yet. In most cases, the symptoms develop gradually. This continuum can be staged in six phases (Fig. 1): genetic and epigenetic risk, asymptomatic autoimmunity and inflammation, symptomatic autoimmunity and inflammation, undifferentiated synovitis, classical RA, and evolution of chronic autoimmunity and inflammation (3, 4).

The preclinical phase of RA development includes changes in several immunologic markers observed in sera prior to the onset of RA. These markers include anti-citrullinated protein autoantibodies (ACPA), rheumatoid factor (RF), anti-carbamylated protein antibodies (anti-CarP) and changes in cytokine levels. ACPA show high specificity for RA, and have become a substantial component of the current ACR-European League Against Rheumatism (EULAR) classification criteria for RA (5). This great interest is due to the fact that their expression may precede the clinical appearance of RA by 10 years or more with an improved predictive capacity of both ACPAs and RF when they are used simultaneously. (6, 7). Anti-CarP are found in 37% of ACPA-positive and 16% of ACP-negative patients with RA and are additionally linked to more severe disease (8). Moreover, classic inflammatory markers such as C-reactive protein (CRP), interleukin (IL)-6, alpha tumour necrosis factor (TNF-α) and IL-10 are highly expressed in the synovial fluid and serum of RA patients and play an important role in its pathophysiology (9). Nevertheless, while all these observations are very promising, there is still a high demand for novel biomark-

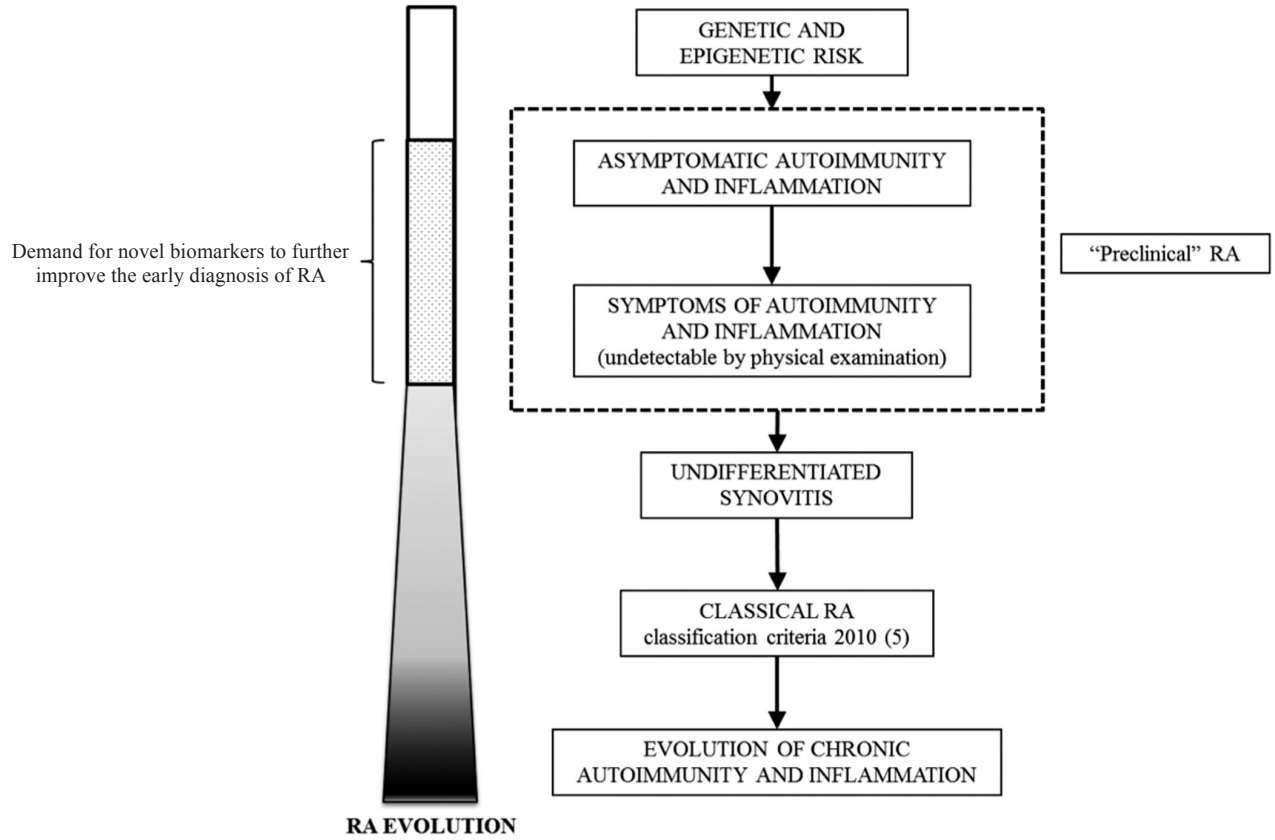


Fig. 1. Phases of development of RA.

ers to improve early RA diagnosis. Furthermore, although ACPA analysis has significantly improved the RA diagnosis, significant differences persist between ACPA assays (10), and it is unquestionable that novel biomarkers are required for a better diagnosis of ACPA-negative RA patients.

In this review, we focus on the various methodologies used in the discovery of novel biomarkers, with specific attention to recent advances in the fields of genomics, proteomics and metabolomics. While these techniques are steadily evolving and currently are investigational, they have the potential to open up a new era for the diagnosis and treatment of RA (11) (Fig. 2).

Genomics in RA

Like many autoimmune diseases, RA exhibits a multifactorial aetiology in which genetic and environmental factors are well established (3). Genetic susceptibility is evident in familial clustering and monozygotic twin studies (6). Besides, disease progression, outcome and RA phenotype have been

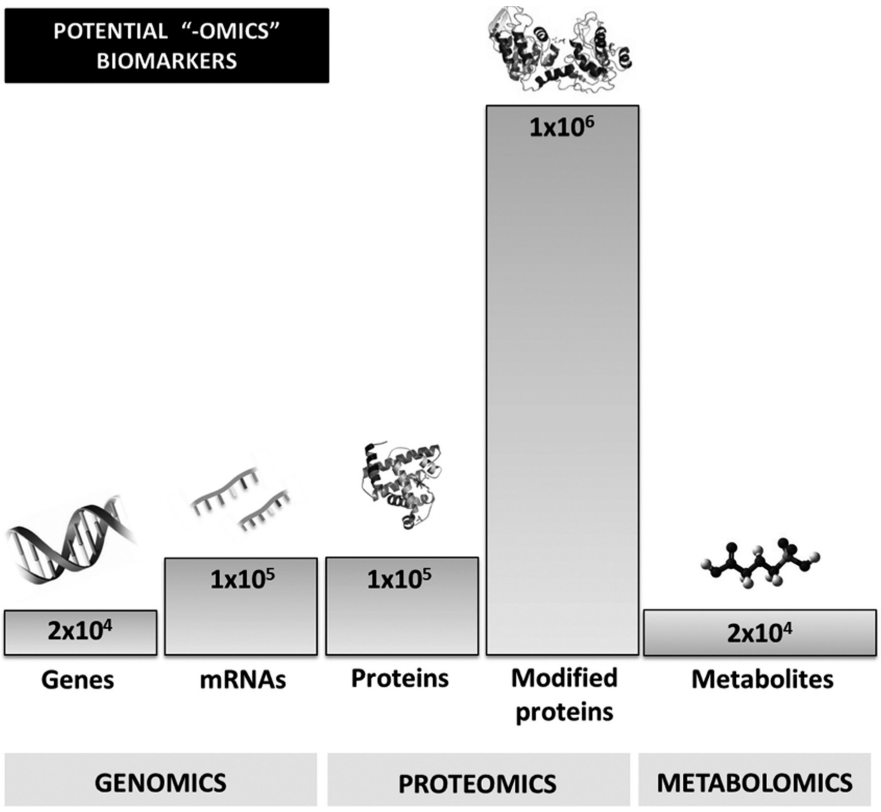


Fig. 2. The great potential of "omics" techniques lies in the vast number of genes, proteins and metabolites that could be used as novel biomarkers of RA (11).

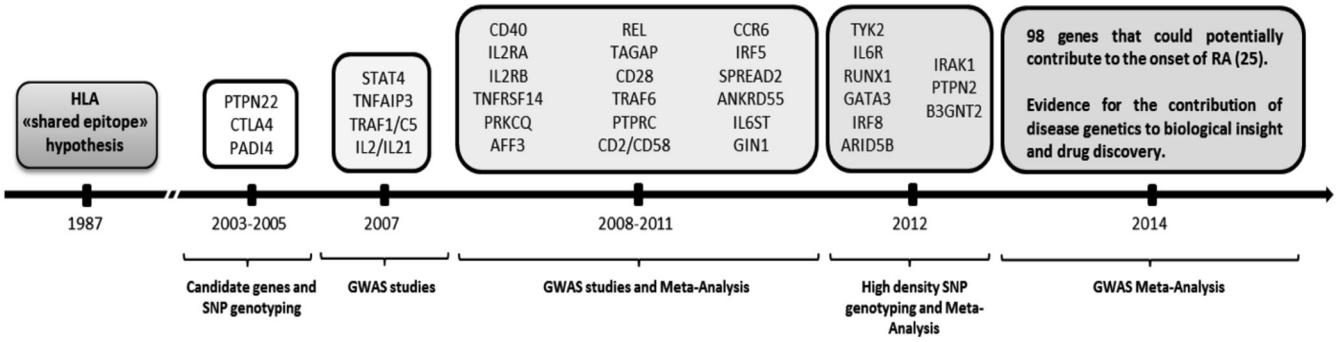


Fig. 3. Timeline of discovery of genes associated with RA.

associated with genetic factors (12, 13). The pathogenesis of RA has a polygenic basis and genetic risk factors can be classified into two groups: 1) major histocompatibility complex (MHC) genes and 2) non-MHC regions.

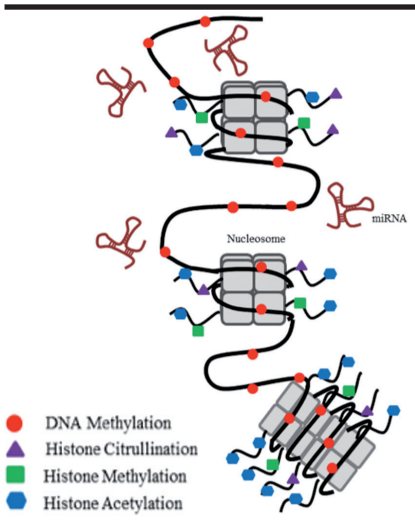
MHC region

About 50% of RA risk is thought to be genetic and one-third of this risk is associated with the HLA locus (6). MHC makes up 12.7% of total genetic variance (14). Among HLA genes, the HLA-DRB1 shared epitope (SE) alleles, which encode for a common amino acid sequence, are the most important risk factor described for RA susceptibility and progression (15). HLA-DRB1 SE alleles are present in 64–70% of RA patients, frequencies significantly higher than those observed in control populations (35.8%) (16, 17). In ACPA-positive RA patients, 80% have at least one SE, while in ACPA-negatives SE are present in 49%. Despite this finding, it has been reported that SE alleles at the HLA-DRB1 locus do not completely explain the association of the MHC region with the disease (18-20).

Non-MHC genetic associations

Outside of the HLA region, until 2007 candidate gene and single nucleotide polymorphism (SNP) genotyping studies had identified few loci involved in RA susceptibility, including PTPN22, PADI4 and CTL4 (21, 22) (Fig. 3). Since 2007 there has been a huge increase in the number of genes associated with RA. Specifically, 46 non-HLA RA risk loci have emerged from genome-wide association studies (GWAS) and subsequent meta-analysis of GWAS datasets (23, 24). A recent study has discovered 42 novel RA risk loci at a genome-wide level of significance, bringing the total to 101 (25). This study genotyped around 10 million SNPs in a total of over 100.000 subjects of European and Asian ancestry, 29.880 RA patients and 73.758 controls. The researchers were able to identify 98 genes that could potentially contribute to the onset of RA. Many of them also play a role in other diseases, including human primary immunodeficiency disorders and blood cancers. Among these genes, several were already targeted by existing drugs, although this was not known when the drugs were developed. This finding provides evidence for the contribution of disease genetics to biological insight and drug discovery.

Some transcriptome analyses (RNA-seq) have been performed in RA (26), showing differently expressed genes in RA synovial fibroblasts (RASFs), although the heterogeneous nature of RA and the different types of tissues used in microarray studies lead to variations between the studies (27-29). It is likely that relatively small changes in gene regulation can lead to an imbalance in tolerance or activation of immune cells. Also, these genes are intertwined with other pathways and systems to provide a complex fabric controlling the level of immune responsiveness. An important task in the near future will be the elucidation of the regulatory mechanisms involved in the regulation of inflammatory responses by the use of techniques such as chromatin immunoprecipitation (ChIP), ChIP-seq, and FAIRE-seq.



Differential epigenetics modifications in RA:

Methylation:
- IL-6 (33)
- Global hypomethylation (34, 35)
- MHC (36)
- COL1A1, MEFV, TNF, STAT3, TGFBR2 (32)

Acetylation
- Control of the immune response through FOXP3 (43), TNF (44), IL-6, IL-1β, Toll like receptor agonists or TNF (42, 45)

Cytrullination:
- Global hypercitrullination (47)

miRNA
- miRNA, miR-16 (49)
- miR-223, miR-132, miR-155 (50, 51)

Fig. 4. Epigenetic modifications.
The epigenome is the set of chemical compounds added to DNA that modify gene expression. It includes DNA methylation on CpG islands and histone modifications such as citrullination, methylation or acetylation. All these modifications change the DNA packaging, improving or preventing the access of RNA-polymerase. Additionally, small non-coding RNAs (miRNA), bind to mRNA and promote its destruction or prevent its translation, in other form of epigenetic control.

Epigenetics in RA

Epigenetics includes DNA methylation, post-translational histone modifications and miRNA, and could partially explain the low RA concordance in monozygotic twins (12–35%) (6) (Fig. 4), since these modifications are inheritable but very dynamic and usually modified by environmental factors. Longitudinal studies in twins showed an increment in the differences in their epigenomes with age (30). In fact, correlations among DNA methylation, histone modifications and environmental factors such as ageing, gender or exposure to tobacco have been described (31). DNA methylation is catalysed by DNA methyltransferase (DNMT) enzymes in CpG island regions surrounding the promoter region of the gene, causing the silencing of the latter.

Global methylation studies comparing stromal fibroblast-like synoviocytes (FLS) from RA and osteoarthritis (OA) patients identify a distinguishing signature of nearly 2000 loci that are differentially methylated, including genes involved in cellular adhesion and migration and immune cell recruitment (32). In another study, a lower methylation of the IL-6 promoter and a subsequent higher expression in peripheral blood mononuclear cells (PBMC) from RA patients compared with controls was found (33). Also a global hypomethylation in RA compared with OA patients and controls was found in T cells and FLS (34, 35). Recently, a relevant study in ACPA+ patients and controls has identified two methylation clusters in the MHC region associated with RA (36). Very relevant are the studies of the effect of generally used therapies such as methotrexate in DNA methylation. Studies in PBMCs from RA, juvenile idiopathic arthritis or psoriatic arthritis showed that methotrexate therapy reverses the global DNA hypomethylation usually found in these patients (37, 38). It suggests that patients with lower global methylation may have a stronger response to methotrexate and global methylation might be used as a biomarker of response to the therapy.

Regarding the anti-TNF agents, a clear association between methylation of TNF gene promoter and its levels of expression has been described (39). Studies of the implication of previous TNF promoter methylation status in anti-TNF agent treatment response are now under development.

Histone modifications involve acetylation, methylation, citrullination, serine or threonine phosphorylation, sumoylation, ribosylation, butyrylation, propionylation, ubiquitylation and glycosylation of lysine residues, all of them with a significant effect in gene expression (40). The most studied is acetylation, controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs). Both HATs and HDACs also have a wide range of non-histone protein substrates involved in processes such as transcription, nuclear translocation or cytoskeletal changes (41). Remarkably, HATs and HDACs are involved in inflammation and HDACs inhibitors have been successfully tested in cancer, in animal models of systemic lupus erythematosus or multiple sclerosis (41) and, to a lesser extent, in clinical trials (42).

Specifically, in RA patients changes in the balance between HDACs and HTAs in synovial tissue and FLS has been described. The implication of HDACs in RA seems to be associated with their capacity to change the immune response. Remarkably, the deacetylation and subsequent degradation of FOXP3, controls the development of regulatory T cells (43), and correlation between HDAC activity and local TNF concentration has been described (44). Additionally, treatment of RA FLS and macrophages with HDAC inhibitors blocked the induction of IL-6 by IL-1β, Toll-like receptor agonists or TNF (42, 45).

A clinical trial in systemic onset juvenile idiopathic arthritis (SOJIA) with givinostat described an improvement in nearly 70% of the patients, meeting the Rheumatology Paediatric criteria (42).

Therefore, the involvement of acetylation in RA seems to be more associated with HDAC effects on inflammation and immune response than in histone modification, and acetylation emerges as a very common way of controlling protein expression.

Very relevant among the histone modifications is citrullination. This process is not specific for histones and is very important in the generation of ACPA. Citrullination consists in an enzymatic conversion of peptidyl arginine residues to citrulline and is mediated by the family of calcium-dependent peptidylarginine deaminases (PADs). It is a process involved in gene regulation, formation of neutrophil extracellular traps and chemokine regulation (46). Remarkably, enhanced neutrophil extracellular trap generation in RA has been recently described in patients. This process, named NETosis, consists in the extrusion of chromosomal DNA associated antimicrobial peptides and proteases. The process includes the generation of reactive oxygen species (ROS), nuclear delobulation and chromatin modification by PAD4 (47). ACPA development could be caused by this enhanced process. However, results published by other authors suggest a different mechanism, according to which hypercitrullination could be induced by two immune-mediated membranolytic pathways, mediated by perforin and the membrane attack complex (MAC), both active and previously involved in RA pathogenesis (46).

Finally, microRNAs (miRNA) bind to complementary sequences within the mRNAs and inhibit their translation or stability affecting protein expression. Several alterations in miRNA expression in RA patients have been described, both in the circulation and in synovial tissue (48). miR-146a has been found to be clearly overexpressed in synovial tissue, FLS, macrophages, B cells and T cells from the synovium of RA patients compared with normal donors and OA patients. miR-146a has also been described as a biomarker of RA disease activity since its expression levels correlate with C-reactive protein and erythrocyte sedimentation rate values (49). Similar results were found for miR-16 (49).

Also, miR-223 expression was found to be elevated in different studies, both systemically and in inflamed joints compared with normal donors, and its levels have been inversely associated with the tender joint count (50, 51).

However, miRNA was not found useful for early diagnoses. Only miR-132 can differentiate in plasma samples between healthy controls and RA patients, but it did not discriminate between RA and OA

patients (51). In the same study, plasma levels of miR-16, miR-146a, miR-155 and miR-223 were found inversely correlated with the DAS28 score and the tender joint count, being therefore useful for the follow-up of the disease and its response to treatment (51).

Proteomics in RA

The early diagnosis of RA is usually impeded by the broad heterogeneity of the disease and the insufficient accuracy and sensitivity of current clinical biomarkers in its early stages. In the present scenario, proteomic technologies appear to be a promising tool to facilitate a better understanding of RA pathophysiology, its development, and to provide novel and more sensitive biomarkers.

2-DE is the most common technique for the analysis and separation of complex mixtures of proteins thanks to its high resolution. Through two consecutive electrophoreses, the separation of hundreds or thousands of proteins contained in the sample can be achieved in a single gel (52, 53). Then, mass spectrometry (MS) allows the identification of the proteins of interest thanks to its high sensitivity, with detection limits between femtomoles (10^{-15} moles) and attomoles (10^{-18} moles), and its high versatility that allows the structures of many different types of compounds to be determined.

However, the increasing need to analyse more complex biological samples has led in the last years to develop new techniques that have facilitated the description of previously unknown proteomes not accessible to classical proteomic techniques (54).

Difference in gel electrophoresis (DIGE)

Classic staining (Coomassie blue or silver) requires individual staining of every single gel in order to undertake a comparative study, resulting in a large number of gels whose reproducibility is difficult to achieve. In addition, the linear dynamic range or sensitivity of these approaches is not always suited to the requirements of the samples. These two drawbacks have been solved by the development of difference in gel electrophoresis (DIGE). This technique is based on the labelling of the proteins using three fluorescent dyes (*e.g.* Cy2, Cy3 and Cy5). In this case, the images obtained from scanned gels permits the identification of significant changes in protein abundance by using principal component analysis (PCA). One of the main advantages of DIGE compared with classical 2-DE is that, by running an internal standard as part of each separation, more accurate and reliable results are obtained, reducing the differences due to experimental variations, and ensuring the superimposition of proteins.

Multidimensional liquid chromatography (i-TRAQ)

iTRAQ labelling (isobaric Tags for Relative and Absolute Quantification) is a novel methodology to identify and quantify protein modifications by chemical labelling. Unlike in DIGE, iTRAQ protein samples are digested with peptidase prior to labelling and the resulting peptides are separated and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). iTRAQ employs isobaric labelling of molecules consisting of three parts: 1) an active binding peptide capable of reacting with the N-terminal amino groups, 2) a reporter region for subsequent sample identification and 3) a balance group for adjusting the total mass to 145 Da (55).

Using tandem mass spectrometry, the analysis of the relative intensities of the reporter ions can be correlated with the variation in the levels in their original proteins.

Proteomics in blood and synovial fluid

In healthy joints, the physiological function of synovial fluid (SF) is to provide nutrition and lubrication to the articular cartilage. Under pathological circumstances such as inflammation observed in RA, larger amounts of SF are accumulated in the joint with an increased amount of cells, proteins and metabolites that can provide particularly valuable information for a correct diagnosis. For the identification of these potential biomarkers, Noh *et al*. combined 2-DE and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) analysis to undertake a comparative proteomic approach using SFs and serum samples (56). They identified 32 differentially expressed spots in SF and 34 spots in serum and classified them depending on the stage of RA. For example, zinc finger protein 658 was identified only in active RA, suggesting its potential use as a prognostic marker. Zheng *et al*. also found that several plasma proteins not previously described in RA were altered, including thymosin beta4, actin, tubulin, and vimentin (57).

Using a nano-liquid chromatography system and MALDI-TOF/TOF for peptide identification Mateo *et al*. have recently described 136 different proteins in SF with greater relative abundance of several of them related to complement activation, inflammation and the immune response, such as the major matrix metalloproteinases and neutrophil-related proteins (58). Balakrishnan *et al*. found 135 differentially expressed proteins using i-TRAQ. The most interesting result of this study was the description of three upregulated proteins not previously reported to be associated with RA: coronin-1A, fibrinogen like-2 and macrophage capping protein (59).

Compared with metabolomics, proteomics studies of RA are definitely one step ahead. An example of this difference is the existence of proteomics studies aimed not only at SF but also at particular cells of synovial tissue such as fibroblast-like synoviocytes (FLSs). Li *et al*. have recently used 2-DE and MALDI-TOF-MS to discover the statistically altered levels of 33 proteins including enzymatic and structural proteins (*e.g.* α-enolase, ERp60 and lamin-A/C), signal transduction proteins (*e.g.* annexin 11, peroxiredoxin 1 and TrpRS) and heat-shock/chaperone proteins (*e.g.* TCP-1, GRP75 and HspB5) that might be potential biomarkers for RA diagnosis in the future (56).

Metabolomics in RA

Along with proteomics, metabolomics offers the major potential for biomarker discovery. Metabolomics can be defined as the science that studies the set of final products and by-products of the metabolic pathways. These products,

called metabolites, include every molecule involved in the metabolism of any living system (61). The great diversity of their chemical properties (organic acids, amino acids, amines, sugars, steroids, etc.) and the broad ranges of concentration found in biological samples, require the combined use of different separation techniques (62, 63).

Thus, the continuous improvement of these analytical techniques is necessary due to the great variability of existing metabolites in terms of polarity, solubility and volatility with a wide dynamic concentration range in biological samples. In many cases, it is also necessary to combine different chromatographic platforms to cover the broadest possible range of metabolites. In this sense, the two main platforms used in metabolomics analyses are nuclear magnetic resonance (NMR) and MS, usually coupled with a separation method such as liquid chromatography (LC) or gas chromatography (GC) (64-66). Other possible techniques include Fourier transformation infrared spectroscopy (FT-IR) (67, 68), and capillary electrophoresis (CE).

The combination of these different analytical techniques offers important advantages when analysing the complete metabolome. For example, NMR is one of the preferred platforms for urine and plasma analysis since it is a nondestructive technique that does not require prior separation of the metabolites and provides detailed information about the contained molecular structures (69). However, one of its main limitations is its poor sensitivity, although this can be greatly improved in combination with MS.

In any case, the physicochemical characteristics of the metabolites of interest will be the criteria that will determine which is the most suitable platform for their metabolomic analysis. Due to each of these platforms having inherent advantages and disadvantages only their combined use will lead to the best possible results.

Metabolomics in blood, urine and synovial fluid

Using two different chromatography-mass spectrometry approaches, Madsen *et al*. have found three increased metabolites (glyceric acid, D-ribofuranose and hypoxanthine) and six decreased metabolites (histidine, threonic acid, methionine, cholesterol, asparagine and threonine) in plasma samples of RA patients compared with healthy controls (70).

Looking further ahead, Young *et al*. conducted a serum metabolic fingerprint in established RA patients. They found that some metabolites including 3-hydroxybutyrate, lactate, alanine, taurine, acetylglycine and methylguanidine contributed to the differentiation between groups of patients with different stages of RA and healthy controls. One of the most important findings pointed out by the authors was the increased level of 3-hydroxybutyrate. This ketone body seems to reflect the importance of metabolism as a source of energy in the hypoxic inflamed joint and is consistent with previous studies which observed decreased levels of lipids in SF (71). Moreover, 3-hydroxybutyrate has also been found increased in the serum of acute coronary syndrome patients and has been recently described as a good biomarker of cardiac muscle hypoxia (72).

Similarly, van Wietmarschen *et al*. have published a very interesting study in which they characterise subtypes of RA patients by their clinical symptoms, clinical chemistry measurements in blood and metabolite measurements in urine and plasma (73). These metabolomic measurements in urine samples by Ultra Fast LC/MS-IT-TOF analysis showed that the most discriminating metabolites were riboflavin, pantothenic acid and several acylcarnitine compounds. In plasma, the top discriminating metabolites were dehydroepiandrosterone sulfate, 4-methyl-2-oxovaleric acid, indoxyl sulfate, uric acid, cholesterol sulfate, 3-methyl-2-oxovaleric acid, tryptophan and alpha-ketoisovaleric acid. Overall, one of the most interesting results of this study was that currently used clinical markers for diagnosis and prognosis of RA, C-reactive protein (CRP) and rheumatoid factor, were not included among the most discriminating metabolites, highlighting the importance of finding new biomarkers.

Most metabolomic studies in humans are restricted to plasma and urine. However, the use of extravascular fluids (cerebrospinal, synovial, ascitic and pericardial fluid) may be indicated in cases of particular diseases. Among them, the study of synovial fluid has attracted much interest since the particular pathogenesis of RA makes SF one of the most relevant biological samples. However, there is only a limited number of published metabolomics studies focused on SF. Among the wide range of metabolites that can be found, many of the studies conducted to date have focused on the lipid fraction. Although those studies focused on the fatty acid, cholesterol and triglyceride fractions (74, 75), more detailed profiling of the lipid species remain necessary. The specific application of metabolomics to the lipid fraction, called lipidomics, has allowed Giera *et al*. to identify potential RA biomarkers such as maresin 1, lipoxin A4 and resolvin D5 (76). Meanwhile, Hügle *et al*. used ^{1}H-NMR to define a characteristic metabolic profile permitting RA and other related diseases such as crystal arthropathy, spondylarthritis and osteoarthritis (OA) to be differentiated (77).

Discussion

Current clinical practice requires more sensitive and more specific biomarkers to ensure the early diagnosis and correct stratification of RA. In this context, the development of '-omics' techniques will bring considerable progress in our knowledge of the disease and make a determinant impact in the discovery of new potential biomarkers suitable for their introduction into clinical practice.

The potential of this technology is already being reflected in several milestones. Genetic analyses undertaken in recent years have revealed a new picture for RA pathogenesis and made us aware of heterogeneity among individuals and populations. However, probably due to the high complexity of RA, we are far from finding an ideal biomarker with total precision. The final goal in the coming years will be to identify genetic variants involved in different clinical manifestations and

RA-associated features, which will allow us to predict the evolution of the disease, and finally, to establish new treatments for RA based on individual prognosis, enabling the development of personalised therapies for RA. Next genome sequencing will facilitate the identification of rare genetic variants (<0.5%) that may be functionally important, penetrant, and with the power to explain a certain proportion of the missing heritability of RA (78). Integrating sequencing data with large-scale genotyping will serve to discover RA risk alleles and to better understand RA pathogenesis.

Studies involving expression and, even more, epigenetic modifications present an additional concern to be considered: the type of sample. Epigenetic studies in RA should be focused on cells involved in hyperplasia of the synovium and the infiltration and inflammatory response. Proportions and activation status of the cells involved should be taken into account in order to avoid confusing elements. Another important issue is the differentiation between causative and consequent features. Epigenetic changes found may play a role in the development of the disease or may be consequences of inflammation and its evolution. SF is definitely a biological sample of great interest in the diagnosis of RA. However, only a small number of studies have been carried out in SF with the aim of finding new biomarkers of disease. Compared with the metabolomic analysis of plasma, our knowledge of the composition of SF in the inflammatory process inherent to RA is still in its early days. With this goal in mind, the easy accessibility of SF should be one of the reasons to encourage researchers to use it in the coming years.

Because genomics, proteomics and metabolomics complement each other, full integration of their data will lead to personalised diagnosis. Moreover, '-omics' techniques will contribute to the discovery of new therapeutic targets and the subsequent development of new drugs. Also, some biomarkers may allow us to predict the patient's response to a particular treatment and may help us to develop a more personalised management of the disease.

To sum up, the continuous development of these techniques combined with new bioinformatic tools that have yet to appear will hopefully contribute to overcome the limitations that currently impede the study of RA.

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