Review

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New laboratory markers for the management of rheumatoid arthritis patients

Abstract: Rheumatoid arthritis, the most prominent of systemic autoimmune rheumatic diseases, represents an important social health problem. Recent insights into the immunopathogenic mechanism of this complex and multiform illness might open new perspectives for a more appropriate laboratory approach. In this review we focus on the most relevant pathogenetic mechanism; indicating the laboratory biomarkers specifically linked to early diagnosis, prognosis, evolutive aspects of the disease, and therapeutic efficacy. Evidence based on laboratory medicine could provide the best outcome for patients.

Keywords: evidence-based laboratory medicine (EBLM); immunopathogenesis; laboratory assay; rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is the most prominent of systemic autoimmune rheumatic diseases that may affect different tissues and organs, although the synovial joints represent the major target. The onset of RA is mainly observed at the end of adolescence or between the 4th and 5th decades of life, while a second peak of incidence is reported between 60 and 70 years. In general, the number of cases increases with age, with a prevalence of 0.5%–1% in industrialized countries, and with rates of 5% in women over 55 years [1]. An early variant of RA is also described in childhood [2]. This disease represents an important social health problem because of its high incidence, invalidating progress and important disabilities. The involvement of the synovial joints, that results in severe dysfunction, leading to limitation of motion, may be accompanied and complicated by several extra-articular manifestations [3–5], such as pleural and pericardial diseases, rheumatoid nodules, Caplan’s syndrome, bronchiectasis [6, 7]. All these clinical conditions can lead to major reduction in functional and work abilities and health-related quality of life, while the RA-associated increased risk for cardiovascular disease may reduce life expectancy by 3–18 years [8]. Today, RA is considered a multifactorial disease, in which environmental factors, such as several subsequent viral infections, seem to play a role in the onset and persistence of the disease. Smoking is also considered a predisposing condition [9]. Genetic factors are, however, also implicated in the susceptibility and the evolution of RA. A genetic association with antigens of the major histocompatibility complex (MHC)-II, and in particular human leukocytes antigens (HLA)-DRB1, has been well established, although non-HLA associated loci have also been linked to the disease [10, 11]. Although the cause of RA is still unknown, autoimmunity plays an important role. It has become increasingly evident that autoantibodies mainly target proteins that undergo citrullination, a post-translational modification frequently occurring during inflammation, apoptosis, and keratinization. Pro-inflammatory substances released by immune cells are involved in the activation and maintenance of inflammation that causes swelling and subsequent damage of the cartilage and bone inside the joint. It is hypothesized that superantigens may be also implicated, while self-antigens (collagen, proteoglycans, rheumatoid factor and citrullinated proteins) probably play a role in the chronic evolution of the process. At present, there is no universal cure able to ultimately eradicate RA. The therapeutic approach in the management of RA has witnessed revolutionary changes over the last 15 years, thanks to the wider understanding of the molecular mechanisms involved in the pathogenesis of the disease. Glucocorticoids, non-steroidal
anti-inflammatory drugs, and few disease modifying anti-rheumatic drugs (DMARDs), including methotrexate, sulphasalazine, hydroxychloroquine, and gold have been the only treatment options until two decades ago. Since the discovery of new therapeutic targets, a significant breakthrough has been achieved in rheumatology with the development of biological agents. The first class of biologic agents include tumor necrosis factor (TNF) inhibitors, followed by many others with different mechanisms of action, such as rituximab, abatacept, and tocilizumab. These agents have changed the fate of patients with RA due to their good efficacy and safety features. Most importantly, the role of B cells in the pathophysiology of RA has been highlighted by depleting peripheral B cells using anti-CD20 agents or inducing apoptosis by the inhibition of B cell receptors and depleting this lymphocyte population from the germinal center [12]. This novel therapeutic approach can target intracellular B cell signaling and regulatory B cells. Moreover, new cytokine-directed biological drugs targeting important proinflammatory mediators, such as GM-CSF, interleukin (IL)-1, IL-6 and its receptor, IL-17, IL-20, IL-21, IL-23, have been or will be soon available, hopefully enhancing our therapeutic choices [13]. All recent knowledge on pathogenetic mechanisms has opened a new window upon the efficacious approach of laboratory performances both in the choice of biomarkers, and best practice regarding the patient outcome [14–16].

Pathogenesis

Presently, a three-step model has been proposed for ACPA-positive RA. In the first step, an environmental risk factor can induce protein citrullination, while the new epitopes may elicit the ACPA production in susceptible individuals, bearing a peculiar haplotype. HLA DRB1 – encoding the most widespread MHC class II HLA-DR β subunit paralog – is the genetic locus most specifically linked to the risk of developing RA in the Caucasian population. Several other genes have been considered as probands for the disease, such as protein tyrosine phosphatase non-receptor type 22 (PTPN22) Arg620→Trp, signal transducer and activator of transcription 4 (STAT4), TNF receptor-associated factors (TRAF)1/C5, all of which are involved in specific protein citrullination induced by environmental factors. The DRB1 allele is responsible for peptide binding specificities and codes for a conserved sequence of amino acids at residues 67–74 of the DR β chain, a region involved in the accommodation of the antigenic peptide and recognition of the T cell receptor [17]. Interestingly, DRB1*0404 or *0401 antigen binding grooves fit better citrullinated, rather than the corresponding arginine-bearing peptides. Genome-wide single nucleotide polymorphism (SNP) analyses of RA patients, compared with healthy controls, confirmed the genetic contribution of two of these residues, 71 and 74, and of three other amino acids, all of which are located in the peptide-binding groove, to RA susceptibility [18]. These data classify high-, intermediate-, low-risk and non-susceptibility alleles, depending upon the amino acid sequence between 70 and 74 residues within the groove. Instead, the variant PTPN22 is a gain of function mutation that enhances the ability of its gene product to inhibit T cell activation [19]. PTPN22, in fact, codifies an intracellular tyrosine phosphatase (LyP), which negatively regulates T lymphocyte activity, by modifying the intracellular signal of the T cell receptor (TCR). A study on the PTPN22 polymorphism 1858T, anti-cyclic citrullinated peptides (CCP) antibodies, rheumatoid factors of all different classes (IgM, IgA, IgG) and shared epitopes genes (HLA-DRB1*0404 or *0401) has established their strong relationship and the high positive predictive value for RA. The presence of PTPN22 1858T polymorphism and anti-CCP antibodies shows 100% specificity for RA [20]. In genetically predisposed individuals, several different environmental factors, depending on the studied population, can trigger autoimmunity response to citrullinated proteins, impairing tolerance. Among these, smoking and Porphyromonas gingivalis, a self-producing citrullinated protein bacterium involved in periodontitis, are attractive etiological agents playing a role in gene/environment/autoimmunity interactions [21].

In the second step, an inflammatory response can develop into the articular synovial layer, with the recruitment of ACPA and the deposition of immunocomplexes. In RA, ACPA are pathogenic, and specific for this disease, and have a strong association with HLA-DRB1 shared epitopes alleles. In rheumatoid synovium, peptidylarginine deiminase isoforms peptidylarginine deiminase (PAD)2 and PAD4 are abundant, thereby determining an active local citrullination of proteins, such as fibrin. Citrullinated epitopes appear longer before the clinical symptoms and they are associated with more severe joint erosions and an evolution of clinical course. Citrullination, a modification of arginine side chains catalyzed by PAD enzymes (deimination) increases the affinity of several peptides for shared epitopes, thus expressing HLA-DR enzymes and improving their presentation to T cells. The most well-characterized epitopes are filaggrin, fibrinogen, vimentin, type II collagen, and α-enolase. Cellular survival response can be triggered by misfolded proteins in the endoplasmic reticulum (ER). ER-stress associated genes are highly
expressed in the rheumatoid synovium and synovial cells. ER chaperone glucose-related protein 78 (GRP78), in vitro, is crucial for the proliferation of synoviocytes and angiogenesis, and strongly contributes to RA pathogenesis [22]. In summary, autoimmunity to citrullinated protein antigens has specificity for RA and defines a clinically and genetically distinct form of the disease.

In the final step, the presence of immunocomplexes induces the increasing presence of immunity cells and cytokine production, which can act together to perpetuate, in RA, the chronic inflammation at articular sites. Dysregulated expression of cytokines drives both inflammation and tissue destruction, thereby implying the evaluation of cytokine patterns for a complete and effective understanding of patient’s immunoresponse [23]. Recently, the role of IL-36a, which is up-regulated in RA synovium, has been pointed out. This interleukin, expressed by plasma cells, and leading to IL-6 and IL-8 production by synovial fibroblasts, may represent the biological link between autoimmunity and the gradual development of synovitis [24]. Hypoxia and Th1 lymphocytes-driven inflammation contribute to the pathogenesis of RA, although Th1 cytokines are not sufficient to induce angiogenesis, even together with hypoxia. Instead, Th2 cytokines induce angiogenic activity in normoxic and hypoxic conditions, suggesting a complex and not yet clarified interaction between hypoxia, angiogenesis and inflammation [25]. The presence of IL-17 in synovial fluid and tissue of RA patients may suggest an important pathogenic role of this cytokine, as supported by several mouse models. IL-17A induces angiogenesis, cell migration and cell invasion, all key processes in the pathogenesis of RA, by stimulating GROα and monocyte chemoattractant protein-1 (MCP-1) expression in RA synovial fibroblasts [26]. Interestingly, MCP-1 and regulated on activation normal T cell expressed and secreted, (RANTES) polymorphisms do not contribute to individual RA susceptibility or severity in the Caucasian population [27]. Involvement of MCP-1, IL-1β, IL-6, IL-8, TNF-α in RA synovial alterations has been described for many years. In each single patient, the integration of cytokine profiles with autoantibody response may be extremely important for prognosis, for the choice of the most appropriate therapeutic treatment, and the evaluation of its efficacy [28]. Moreover, there are more CD4+ CD25high Treg cells present in synovial fluid than in peripheral blood, where they have shown a functional defect impairing their ability to suppress CD4+ CD25 T cells. This defect can be reversed by anti-TNF-α therapy [29]. Furthermore, NK cells seems to play an exclusive role in the pathogenesis of autoimmune disease, and in the case of RA, these specialized cells represent a significant fraction of the lymphocytes (8%–25%) in the synovial fluid of RA patients and could be detected in the joint early during the disease course [30]. The majority of the NK cells in the synovial fluid of RA patients are CD56bright (approx. 60% of NK cells), a subphenotype also found in the blood of RA patients, but at much lower frequencies (approx. 10% of NK cells). The synovial NK show an elevated expression of CD94/NKG2A and decreased expression of killer immunoglobulin-like receptor (KIR)s and CD16. Due to an upregulated expression of several chemokine receptors and adhesion molecules, they may participate in the preferential recruitment into the synovium [31]. The synovial CD56bright NK cells express higher levels of activation markers (CD69 and NKP44) and produce more TNF-α and IFN-γ than those from the peripheral blood of the same patients. [32]. Synovial NK cells could induce monocytes to differentiate into DCs, and, as reported, produce IL-22, a cytokine that promotes proliferation of synovial fibroblasts, thereby representing a target for molecular therapy [33]. Aberrant expression of MHC class I polypeptide-related sequence A in the inflamed synovium may augment CD56bright NK cell activation, resulting in dysregulated production of proinflammatory cytokines rather than in immunoregulation. Taken together, these findings suggest that the enrichment of CD56bright NK cells may contribute to the initiation and/or perpetuation of dysregulated production of proinflammatory cytokines in the synovium of RA patients [34]. The genetic associations between inflammatory arthritis and KIR haplotypes support the hypothesis that dysregulation of cytokine production by CD56bright NK cells in the synovium and/or decreased cytotoxicity by peripheral CD56dim NK cells may contribute to the pathogenesis of RA. Distinct antibody profiles have been associated with subgroups of patients who exhibited high serum levels of TNF-α, IL-1β, IL-6, IL-13, IL-15 and GM-CSF. Significantly increased autoantibody reactivity against citrullinated epitopes was observed in patients within the cytokine “high” subgroup [35, 36]. Increased levels of TNF-α, IL-1α, IL-12p40 and IL-13, and the chemokines eotaxin/chemokine ligand (CCL)11, MCP-1 and interferon-inducible protein 10, were present in early RA as compared with the controls [37]. Between chemokines, only IL8/cysteine x cysteine (CXC)L8 concentrations were higher in patients with psoriatic arthritis and ankylosing spondylitis than in patients with RA [38, 39]. Increased blood levels of proinflammatory cytokines are associated with autoantibodies targeting citrullinated antigens and surrogate markers of disease activity in patients with early RA [40]. Protomic analysis of serum autoantibodies, cytokines and chemokines enables stratification of patients with RA into molecular subgroups [41] (Table 1).
The basis of international guidelines, their assay is still used as corollary procedure for determining a first diagnosis [42]. Evidence-based laboratory medicine (EBLM) predicts that a laboratory biomarker should have several properties: it should be present in a biosample which is representative of the disease localization; recorded from a patient, and thus specifically linked to his/her disease; and it has to be measured by a standardized and reproducible analytical methodology. The ideal biomarker should clearly indicate: health/disease condition; high/low exposure to risk factors; effectiveness/no response to therapy. Thus, each different laboratory biomarker can be used properly in a selected phase of clinical decision (predictivity, diagnosis, prognosis, monitoring therapeutic efficacy or adverse effects), and in all cases, their choice must be related to a real improvement of the patient management [43] (Figure 1).

Common and uncommon laboratory biomarkers of RA are summarized in Table 2.

RF is the prototypic marker of RA for diagnostic and prognostic purposes, since it is associated with joint destruction, even if it is not clearly indicated as a mediator of these effects. Moreover, in seropositive RA patients, the levels of RF are not related to bone damage and status of the disease [44]. For several years, evaluation of RF Ig isotypes has been used to enhance the serological diagnosis of RA [45]. More recently, it has been proposed as a useful tool to predict patient response to biological therapy [46]. Several proteolytical enzymes can be involved in the pathogenesis of RA. Between those, metalloproteinases represent a family of important factors which cause the destruction of articular tissue. The role that proteolytic enzymes play in RA can be summarized as follows: matrix metalloproteinases (MMP 1,2,3,9); cystein proteinases (cathepsin B,H,L); serin proteinase (elastase, PA, cathepsin G); and aspartic acid proteinase (cathepsin D). MMP-3 can be a very useful marker for the prediction of joint destruction [47]. MMP-3 (stromelsyn-1) is expressed by synovial and articular cells, fibroblasts, chondroblasts,
and osteoclasts. It acts upon extracellular substrates of cartilages, such as fibronectin, collagen IV and V, elastin, proteoglycans, but also together with other MMPs in the disruption of cartilages [48]. It is present in the synovial fluid during the active phase of disease and its levels correlate with serum concentrations, independently from patient's age and severity of the disease. It is produced very early during the course of RA and elevated serum levels can be predictive of articular disruption, within 6–12 months from the event. Together with RF, it can be a laboratory marker of active phlogosis and predictive of invalidant evolution [49]. Polymorphisms in the vascular endothelial factor A (VEGF-A) gene and MMP-1,3 intergenic locus may influence the age of onset of RA [50]. Currently, a more specific immunological profile has been proposed to evaluate each patient at the onset of disease, or their relatives in order to predict the risk of disease. All anti-CCP and RF isotypes analyzed occurred more commonly in unaffected first-degree relatives from multicase families than in the controls, but had different isotype distribution from the patients with RA [51].

Anti-CCP-2 antibodies of both the IgG and IgA isotypes pre-dated the onset of RA by years; also, both IgG and IgA anti-CCP-2 antibodies predicted the development of RA, with the highest predictive value for IgG anti-CCP-2 antibodies; individuals who subsequently developed RA had increased concentrations of several cytokines and chemokines years before the onset of symptoms of joint disease [52].

The immunological profile consists in the definition of haplotype, evaluation of ACPA, and ANA autoantibodies (cytoplasmic pattern), measurement of plasma levels of Th1 and Th2 cytokine networks. A seminal paper has settled down the importance to identify a complete profile of RA-associated antibodies (AAB) to improve early diagnosis of the disease and provide prognostic and therapeutic indications. Anti-CCP antibodies demonstrate the best diagnostic performances for profiling, thus they must be used as first-line screening and identification of subgroups of patients. The use of multiplex assays may facilitate a wider implementation of profiling [53]. Moreover, a positive result of the immunoenzymatic evaluation of anti-citrullinated peptides (ACPA) against several structural proteins has to be considered the highest specific biomarker for RA. Even if in some patients IgM anti-second generation CCP-2 autoantibodies appear at the initial phase of the disease and seem to persist more frequently, both the IgG and IgA isotypes of anti-CCP-2 antibodies predate the onset of RA by years; the highest positive predictive value is associated with IgG anti-CCP-2 autoantibodies. However, ACPA is not an appropriate marker to monitor the disease [54, 55]. Autoantibodies against citrullinated proteins can be an important factor of osteoclastogenesis. Plasma cells produce ACPA with specificity for citrullinated vimentin, which binds to osteoclast precursor cells and stimulates the release of TNF, in turn enhancing the differentiation of these cells into mature osteoclasts. During the osteoclast differentiation process, the production of PAD2 enzyme is induced by calcium. The activity of PAD2 leads to the citrullination of vimentin, which is abundantly expressed on the surface of osteoclast-lineage cells [56]. Discrimination between RA and non-RA patients could be performed using CCP antibodies evaluation, especially in RF-negative patients [57]. Evidence to date suggests that the positivity of anti-CCP antibodies may be a predictor of RA onset. This analytical data can have a higher positive predictive value in a population at risk, rather than in a general one, thus the evaluation of haplotype profile can improve the early diagnostic outcome. A multi-biomarker test for the assessment of disease activity in RA patients has been performed using CAMERA study. This test may be complementary to the available disease activity laboratory evaluation to improve patient care and outcomes [58]. Four protein biomarkers, whose mass/charge (m/z) values have been well established, were identified as novel biomarkers related to RA, adding great value for laboratory diagnosis of RA [59]. In a recent study, a Multiple Biomarker Disease Activity (MBDA) score algorithm has been tested. It uses the same equation of the Disease Activity Score DAS28-CRP, which takes into account the C-reactive protein (CRP), and biomarkers useful to predict the Swollen Joint Count (SJC28), Tender Joint Count (TJC28), and general health (VAS-GH) as components of the equation (PTJC=predicted TJC;
PSJC=predicted SJC; PVAS-GH=predicted VAS-GH). By using a Venn diagram to predict potential markers, Curtis et al. evaluated and validated this multiple biomarker test to assess RA disease activity with a score, expressed from 1 to 100 [60]. More recently, using a multi-stage approach, the same group tested 130 candidate biomarkers, and ultimately set up an algorithm with 12 protein biomarkers to obtain an MBDA score for RA patients, with no effects from common comorbidities [61]. A study from a Japanese research group has demonstrated that MMP-3 ELISA method works well in the assessment of MMP-3 serum levels in routinely evaluated RA patients and the test may be important to better characterize the disease outcome in the follow-up [62]. The results of this study suggest that serum MMP-3 can be considered as a predictor of joint destruction in RA, treated with a non-biological disease modifying anti-rheumatic drug. Variations of the MMP-1 and MMP-3 genes affect the serum levels of MMP1 and -3 and disease activity. MMP-3 is significantly associated with disease activity, inflammatory mediators and cartilage breakdown, making it a potential biomarker of disease severity, but seemingly less useful than CRP and SAA as a biomarker of disease activity in early RA. A conclusive remark is that several closely linked polymorphisms in the MMP-1-MMP-3 loci have an important role in determining the circulating levels of these MMPs in RA, and that MMP-3 polymorphism is associated with the level of disease activity over time [63].

Recently, using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tools which is a random-effects method, Zhang et al. were able to summarize sensitivities, specificities, positive and negative likelihood ratio (LR+ and LR-, respectively), and diagnostic odds ratio from 17 studies. They concluded that based upon their high specificity and moderate sensitivity, anti-CCP-2 and anti-CCP-3 played an important role in confirming the diagnosis of RA. Anti-CCP-3 did not have better diagnostic performance than anti-CCP-2, but anti-CCP-2 had evident heterogeneity compared to anti-CCP-3, especially in American patients [64].

From pathogenic and clinical evidence, we may suggest a new approach of laboratory medicine to evaluate patients in all different evolutive phases of RA. First, the haplotype of the proband should be characterized, and in case of positivity, the relatives must be invited to take the test. This can be useful to predict the risk level of developing the disease. As mentioned before, internationally adopted clinical criteria imply that the laboratory data are important to make the diagnosis. Together with basic laboratory analytical evaluation, in our opinion it is appropriate to measure RF and ACPA. If both tests are positive, in order to completely and correctly evaluate the patient, a cytokine profile and MMP-3 measurements should be undertaken. The first can be obtained by multiparametric microarray and allows the patient immunological response, the prevalence of Th1 or Th2, cytokine network and the production of Th17 to be evaluated. The latter can be considered the biomarker of disease aggressiveness and progression. This complete laboratory evaluation can produce a correct and personalized therapeutic treatment and prognostic evaluation [65]. In the future, a significant improvement can be obtained by the evaluation of genomics and proteomics arrays, in order to characterize the individual patient profile at diagnosis and its response to therapeutic treatment.

**Conclusions**

The evaluation of multiple biomarker profiles is fundamental in order to enrol the patient in a specific target therapy treatment. This is most likely to be effective and safe based on similarities between patient’s disease characteristics and the cohort that responds to therapy. In this prospective, analysis of multiple biomarkers, and especially post-translational biomarkers in RA, can help to stratify the patient’s population in different subsets that exhibit different outcomes and respond to specific therapeutic treatment.

In summary:
1. RA is a complex disease with different clinically aspects and evolutionary forms.
2. HLA-DRB1 shared epitope and PTPN22 (gain-of-function of Lyp which inhibits T-lymphocytes activation) are two genes strongly associated with the risk of RA.
3. IL-6 and TNF-α play a central role in RA. IL-17 can be measured either in synovial fluid either in compromised tissues.
4. Treg CD4+CD25hi lymphocytes are predominant in synovial fluid, rather than in blood. Their levels can be normalized as effect of anti-TNF therapy.
5. NKp46 cytofluorimetric assay can account for NK activation.
6. Anti-CCP antibodies are specifically present during RA and support distinction between clinically and genetically different forms of RA.
7. All environmental factors which can alter antigenic tolerance against anti-CCP antibodies are not yet defined.
8. Anti-CCP-antibodies are present in serum several years before the early phase of disease (pre-clinical
window); anti-CCP antibodies and high levels of MMP-3 are associated with bone erosion and with a worse clinical evolution of the disease, if present in the synovial fluid.

9. The time-course measurement of the cytokine network can be effective for setting prognosis and therapeutic efficacy.

10. Multiparametric laboratory evaluation can be the correct approach in order to define different subgroups of patients and tailor a personalized therapy.

Conflict of interest statement

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