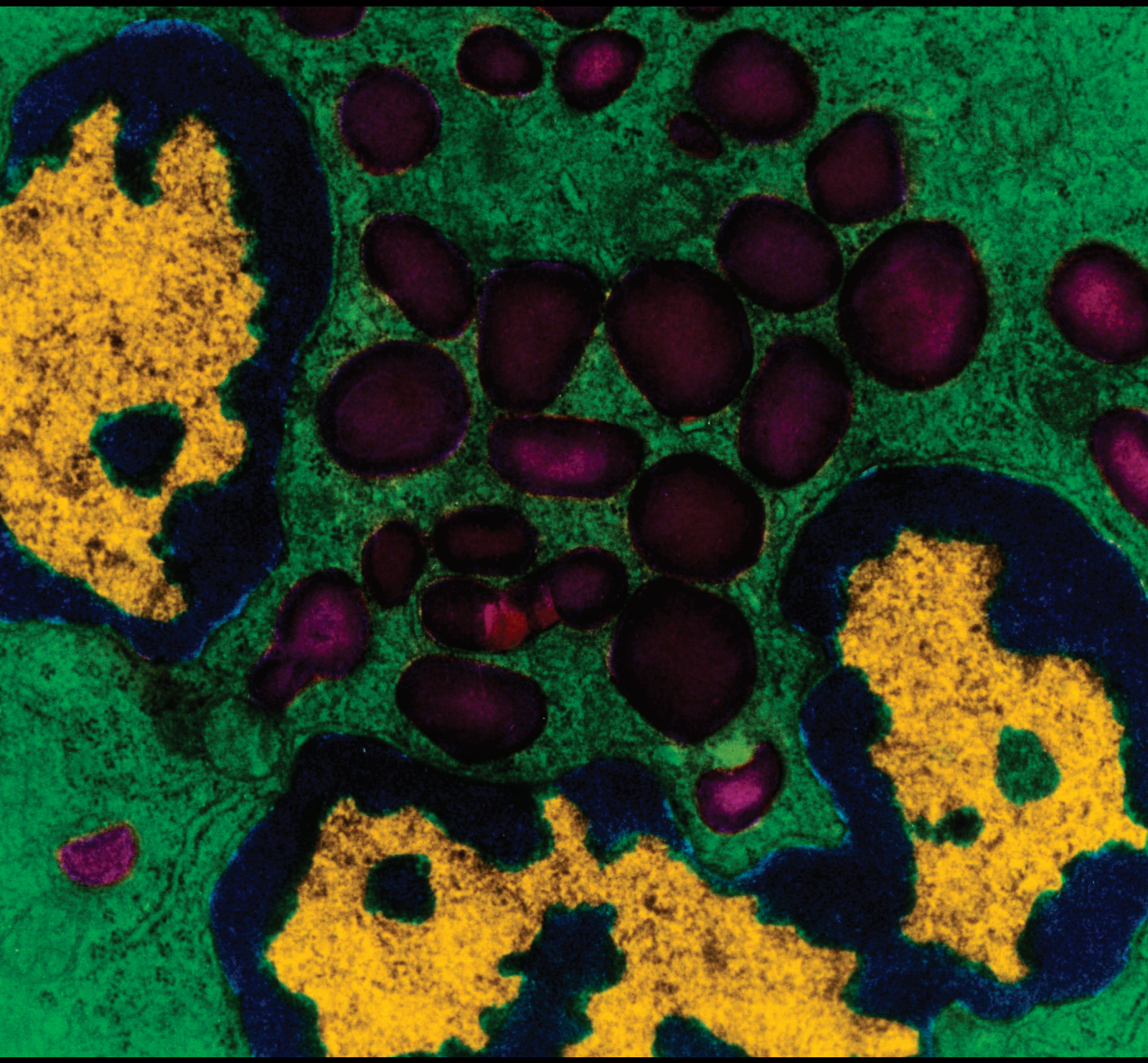
Mediators of Inflammation

Biomarkers in Rheumatoid Arthritis

Guest Editors: Vincent Goëb, Patrice Fardellone, Jean Sibilia, and Frédérique Ponchel



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Editorial Board

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Editorial
Biomarkers in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that leads to severe joint destruction with deformity and irremediable disability. Early diagnosis of RA and timely initiation of treatments (synthetic and/or biological disease modifying antirheumatic drugs) are both instrumental to limit joint damage and optimize the functional outcome of patients, according to the well-known concept of a "window of opportunity" to begin the treatments.

New diagnosis criteria are available since 2010, thanks to the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) [1], which include 2 different types of biomarkers: inflammation (CRP or ESR) and immunity (autoantibodies: rheumatoid factors (RF) and anticitrullinated peptides antibodies (ACPA)). These new criteria allow better management of patients with notably the early opportunity of treatment with methotrexate. However, a significant proportion of patients with early arthritis does not fulfil these criteria for RA and are then wrongly labelled "undifferentiated" arthritis [2]. Furthermore, among the autoantibodies family, ACPA are specific for the RA disease but lack sensitivity, unlike RF which have strong sensitivity but low specificity [3]. Thus there is still a need for new diagnosis biomarkers that would allow establishing a diagnosis of RA at the very beginning of the disease continuum, importantly before the occurrence of the first joint erosions.

Which type of biomarkers do we need? Several classes of markers are available: genetic polymorphisms, proteomic markers, gene-expression analysis [4], and autoantibodies. All may be used in clinical practice since their utility was demonstrated. In this special issue of "*Mediators of inflammation*," novel biomarkers in RA are described. Patrice Fardellone et al. from the University of Picardy will discuss bone remodelling markers in RA, notably for bone formation (osteocalcin, serum aminoterminal propeptide of type I collagen) as well as bone resorption (C-terminal telopeptide of type 1 collagen, pyridinoline). They discuss how such bone remodelling markers allow physicians to evaluate the effect of drugs, notably biologicals that are able to reduce inflammation and also exert a protecting effect on bones. Furthermore, bone remodelling is the result of a tilted balance towards resorption or formation and involves numerous regulatory factors such as hormones, growth factors, vitamins, and cytokines, notably osteoprotegerin (OPG) and receptor activator for nuclear factor-κB (RANK) ligand [5]. The signalling pathway OPG/RANK/RANKL maintains the balance between the activity of osteoblasts and osteoclasts. V. Milanova et al., from the Bulgarian Academy of Sciences, in "*TLR2 elicits IL-17-mediated RANKL expression, IL-17, and OPG production in neutrophils from arthritic mice,*" will show that the toll-like receptor 2 engagement increases IL-17 mediated RANKL expression and also inhibits OPG production in neutrophils from arthritic mice.

There is currently a great hope for biomarkers that would predict the response to treatment for individual patients in order to gain time and avoid irreversible damage, unnecessary risk of adverse events, as well as reduce long term disease associated cost, since uncontrolled inflammation over time leads to significant patient and health economic burden. I. Duroux-Richard et al. will thus explain how circulating

micro-RNA, and notably miRNA-125b, are potential valuable biomarkers in RA in "*Circulating miRNA-125b is a potential biomarker predicting response to rituximab in rheumatoid arthritis*." Circulating levels of miRNA-125 may predict the response to rituximab in RA patients and their interest must therefore be reassessed by other teams to be used in daily clinical practice.

Cytokine networks are well-recognized as a relevant source of contributive biomarkers in RA [6]. Adipokines are biological active substances synthesized by the white adipose tissue that regulate the energy homeostasis and metabolism and are soluble mediators involved in chronic inflammation and metabolic dysfunction. Their interest as biomarkers in RA is thus to be expected. Therefore, A. Burska et al. in "*Cytokines as biomarkers in rheumatoid arthritis*" and A. Del Prete et al. will explore the interests and limitations of using cytokines as biomarkers in RA with a special emphasis on adipokines for the latter.

Since the interest of autoantibodies in pathogenesis, diagnosis, and prognosis of different autoimmune diseases is obvious, the development of their knowledge is logical. Three main classes of posttranslational modifications are associated with RA: citrullination, of course, but also oxidation and carbamylation. A. Burska et al. will therefore review the relevance of autoantibodies to these 3 types of posttranslational modifications in RA in "*Cytokines as biomarkers in rheumatoid arthritis.*"

Remission with minimal use of drugs is now the goal of therapy for RA patients. Synovial (MMP3), cartilage (urinary CTX II, COMP), and bone biomarkers may be useful in managing drugs reduction when patients with RA achieved clinical remission. In this special issue, D. Dénarié et al. will try to shed light on the following issue: "*Could biomarkers of bone, cartilage, or synovium turnover be used for relapse prediction in RA patients?*" in this paper: "*Could biomarkers of bone, cartilage or synovium turnover be used for relapse prediction in rheumatoid arthritis patients?*"

We sincerely hope that this special issue of "*Mediators of inflammation on biomarkers in RA*" will be of interest to readers and deepen their knowledge of this subject. Kind regards.

Vincent Goëb
Patrice Fardellone
Jean Sibilia
Frédérique Ponchel

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Review Article

Bone Remodelling Markers in Rheumatoid Arthritis

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Bone loss in rheumatoid arthritis (RA) patients results from chronic inflammation and can lead to osteoporosis and fractures. A few bone remodeling markers have been studied in RA witnessing bone formation (osteocalcin), serum aminoterminal propeptide of type I collagen (PINP), serum carboxyterminal propeptide of type I collagen (ICTP), bone alkaline phosphatase (BAP), osteocalcin (OC), and bone resorption: C-terminal telopeptide of type 1 collagen (I-CTX), N-terminal telopeptide of type 1 collagen (I-NTX), pyridinolines (DPD and PYD), and tartrate-resistant acid phosphatase (TRAP). Bone resorption can be seen either in periarticular bone (demineralization and erosion) or in the total skeleton (osteoporosis). Whatever the location, bone resorption results from activation of osteoclasts when the ratio between osteoprotegerin and receptor activator of nuclear factor kappa-B ligand (OPG/RANKL) is decreased under influence of various proinflammatory cytokines. Bone remodeling markers also allow physicians to evaluate the effect of drugs used in RA like biologic agents, which reduce inflammation and exert a protecting effect on bone. We will discuss in this review changes in bone markers remodeling in patients with RA treated with biologics.

1. Inflammation, Joint Erosions, and Bone Mass

Rheumatoid arthritis (RA) is a chronic disease characterized by articular erosions, periarticular bone loss, and chronic inflammation leading to increased risk of osteoporosis [1]. Systemic bone loss associated with RA is multifactorial: glucocorticoids, decrease of physical activity, and the disease itself, particularly when uncontrolled. Bone loss, whether periarticular or systemic, shares, at least partially, similar mechanisms. From the very early stages of RA, bone loss in RA correlates with parameters of inflammation and functional status. Joint erosions measured with Larsen's score are correlated with bone mineral density (BMD) and vertebral deformities [1–5]. Relevant literature on bone remodelling markers in RA patients and the effect of biologic agents on bone remodelling were identified using PubMed database with bone remodelling markers, biologic agents, and rheumatoid arthritis as key words. Systematic reviews and randomized controlled studies were both analyzed.

2. Cytokines and Signaling Pathways

Among mechanisms involved in bone loss, proinflammatory cytokines play a major role in explaining hyper-osteoclastosis [6]. The nuclear factor-kappa B (NFkappaB) signaling pathway regulates the expression of hundreds of genes which are involved in diverse processes like inflammation. Receptor activator of NFkappaB Ligand (RANKL) is a membrane protein secreted by osteoblasts that binds to the RANK receptor on osteoclast precursors and provokes maturation of osteoclast cells (Figure 1). Its natural decoy receptor osteoprotegerin (OPG) produced by osteoblasts and stromal cells binds to and confines RANKL and prevents differentiation of osteoclasts [7, 8]. Various proinflammatory cytokines regulate expression of RANKL including tumor necrosis factor (TNF) and interleukin-1 (IL-1) [9–12]. RANKL values can predict the therapeutic response to anti-TNF therapy in RA patients [13], which is not the case for OPG [14], whereas OPG expression is increased in synovium of anti-TNF treated patients: with both infliximab and etanercept. In contrast,

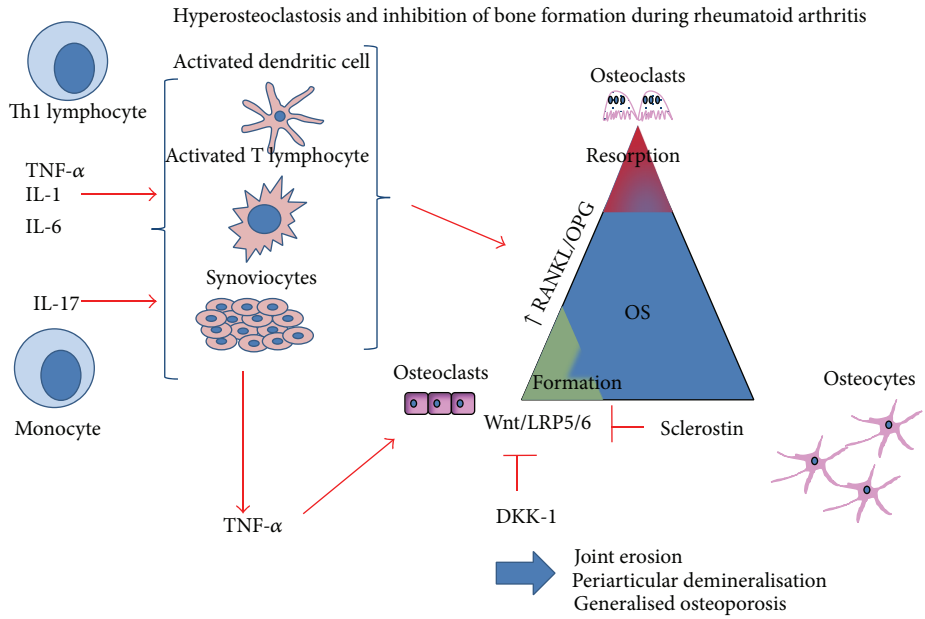

Figure 1

RANKL is not influenced by the treatment, showing that the ratio RANKL/OPG is of major importance in regulating bone resorption rather than each of the markers taken alone [15]. Then, it is not surprising that deleterious effects of RANKL on BMD can be prevented by denosumab which is an anti-RANKL monoclonal antibody, increasing BMD and reducing bone turnover in RA patients [16]. Bone formation is also decreased during inflammation as shown in mice. When Dkk-1, a protein that is a member of the dickkopf family, is increased by TNFalpha, it exerts its negative regulation on WNT pathway, blocking osteoblast differentiation and inducing expression of sclerostin (SCL), leading to the death of osteocytes [17]. Higher levels of Dkk-1 are associated with an increased risk of articular erosions independent of age, baseline radiologic features, C-reactive protein (CRP), or disease activity [18]. Interleukin-6 (IL-6) directly induces the production of RANKL by synoviocytes in RA patients through the pathway of janus kinase/STAT, phosphorylation of STAT3 and ERK1/2 [19, 20].

3. Bone Remodeling Markers

Bone matrix is mainly composed of type I collagen and type I collagen telopeptide fragments: I-CTX and ICTP can be measured in both serum and urine. They are very sensitive and specific markers of bone degradation [21, 22]. These two telopeptides are released from type I bone collagen by two different enzymatic systems: (1) ICTP, which is derived from matrix metalloprotease activity (MMP) and is very effective in bone erosions associated with RA, and (2) I-CTX, produced by cathepsin K which on the contrary is involved in systemic bone resorption [23]. In RA the ratio of synovial fluid to serum fluid is increased for ICTP but not for I-CTX. This suggests that ICTP is a sensitive marker of periarticular bone resorption linked to MMPs activity of various cells like synoviocytes [24].

II-CTX is not a bone remodeling marker but a marker of cartilage degradation, even if the two phenomena are closely related in RA. Both bone and cartilage markers are strong and independent predictors of articular erosions. This is illustrated by the COBRA study where high levels of I-CTX and II-CTX measured early in RA predicted an increased risk of further articular damage [25].

4. Effect of Biological Agents on Bone Metabolism in RA Patients

Randomized clinical trials have clearly demonstrated that biological agents are able to prevent partial or even total articular erosions in RA patients. This raises the question of their ability to prevent as well the generalized bone loss associated with inflammation encountered in RA patients. This preventive effect might be demonstrated by variation of bone remodeling markers or bone mineral density (BMD) during the course of treatment.

4.1. Anti-TNFalpha. In RA patients, BMD is inversely correlated with serum levels of TNFalpha. Bone formation rather than resorption markers better showed the bone response to anti-TNFalpha [26]. An open cohort study of 102 RA patients treated during one year with infliximab showed both the variations of bone loss at lumbar spine, hip, and hands and the variation of bone remodeling induced by the anti-TNFalpha. When BMD at lumbar spine and hip did not vary, it did incur a significant decrease of 0.8% at the hand ($P = 0.01$), giving evidence that metacarpal cortical bone loss is continuing. In

RA treated patients with good EULAR response, variation of BMD was favorable compared to other patients. Serum CTX and RANKL hugely decreased in comparison with baseline values at the same time as the decrease of DAS score and CRP [27].

Another multicentric and prospective cohort study included 48 women with an average age of 54.2 years (±2.1 SD) suffering from severe RA for 10 years (11.4 ± 7.8 SD) who initiated infliximab treatment after the failure of one nonbiologic agent (DMARD). None received bisphosphonates. 77% were under glucocorticosteroid treatment. BMD was not modified during the year of the study but serum I-CTX rapidly and significantly decreased by 30% at the 22nd week before going back to the baseline values. Inversely, PINP values remained stable with a P1NP/CTX ratio in favor of bone formation. The II-CTX, witnessing the cartilage degradation, was not modified in the study group but slightly decreased in patients with values above normal before the biologic agent [21].

In the "BeST" study, four different therapeutic strategies have been evaluated in 218 early RA patients: (1) sequential monotherapy, (2) combined treatment "step up," (3) combined treatment with glucocorticoids, and (4) treatment with infliximab. BMD was measured at lumbar spine, hip, and hands (from 2nd to 4th metacarpal) after 1 and 2 years. After 2 years for all treated groups there was a bone loss at each of these regions. It should be noted though that there was less bone loss in hands for groups treated with either prednisone or infliximab. Progression of erosions was correlated with the decrease of BMD at both hand and hip regions. The use of bisphosphonates protected only lumbar spine and hip from bone loss [28].

A search in PubMed database to identify studies analyzing the effects of anti-TNFalpha treatments on BMD and bone remodeling markers in RA patients has been able to identify four studies [29–32] in which BMD was either stabilized or increased at lumbar spine (up to 2.8%) or at hip (up to 13.1%). Only one study, concerning 48 patients, was negative [21]. Variations of bone remodeling markers were heterogeneous but showed a slight decrease of resorption and an increase of bone formation.

4.2. Anti-iL6 Agents. In vitro, iL-6 blockade reduces osteoclastic differentiation and bone resorption in monocytes cultures stimulated by RANKL or RANKL plus TNFalpha. In transgenic mice, formation of osteoclasts is also strongly inhibited by the anti-inflammatory effects of iL-6 blockade [33].

A pilot study compared 22 healthy nonosteopenic control women with 22 women suffering from active RA treated by perfusions of 8 mg/kg Tocilizumab (TCZ). At baseline, the OPG/RANKL ratio was 5 times lower in RA patients than in controls. Higher levels of Dkk-1, sclerostin, serum betaCTX, and osteocalcin were seen related to a hyper remodeling status and slowing down of bone formation in RA patients. In serum, OPG were negatively correlated with DAS28 score when RANKL levels correlated positively with CRP. After two months, OPG/RANKL ratio was increased when Dkk-1 decreased. Thanks to TCZ, OPG/RANKL increase was particularly significant in 10 patients who were in remission or in a low activity state in contrast with other 12 patients with still active RA. On the other hand, variations of Dkk-1 and sclerostin were similar in both groups. Thus, inflammation suppression by anti-IL-6 rapidly corrects bone homeostasis troubles due to RA [34].

The "OPTION" multicentric randomized pivotal study evaluated the effects of TCZ on bone and cartilage remodeling. They were 416 of 623 patients suffering from moderate suffering from moderate to severe RA who were selected because of an inadequate response to methotrexate. Methotrexate administration alone was compared to the administration of an association of methotrexate and TCZ (4 mg to 8 mg/kg every 4 weeks). TCZ reduced in a dose-dependent way the levels of procollagen type II N-terminal propeptide (PIINP), collagen helical peptide (HELIX-II), and matrix metalloproteinase-3 (MMP-3) after 4, 16, and 24 weeks. Among bone formation markers, only serum aminoterminal propeptide of type I collagen (PINP) significantly increased in comparison with placebo, when 1-CTX and ICTP, markers of bone resorption, decreased [35]. TCZ increases bone formation by increasing the expression of OPG when nonbiological agents have no effect. This is shown in a study of bone biopsies from subjects undergoing a prosthesis replacement of the knee [36]. Finally, TCZ also decreases the levels of dickkopf and normalizes the ratio OPG/RANKL [34]. In RADIATE study TCZ decreased C-reactive protein levels and significantly inhibited cathepsin K-mediated bone resorption, as measured by a decrease in CTX-I with a significant decrease in the CTX-I/OC ratio [37]. Furthermore, the SAMURAI study showed that Tocilizumab monotherapy is more effective at one year in reducing radiological progression in patients presenting with risk factors for rapid progression than in low-risk patients according to four independent predictive markers for progressive joint damage (urinary C-terminal crosslinking telopeptide (uCTX-II), urinary pyridinoline/deoxypyridinoline (uPYD/DPD) ratio, body mass index (BMI), and joint-space narrowing (JSN) score at baseline) [38].

5. Rituximab

B lymphocytes enhance bone resorption during RA by secreting RANKL [39]. B lymphocyte depletion obtained by using Rituximab results in a decrease of resorption bone markers [40] and inhibition of RA induced osteoclastosis; this effect is obtained by a reduction of the number of osteoclast precursors in synovium and thus increases the ratio OPG/RANKL in serum [41] and as such could protect BMD. In a prospective study with a follow-up of 3–15 months after Rituximab therapy there was no significant change of the bone formation markers (BAP) and ICTP. However, a nonsignificant tendency of decrease of RANKL (with no change of OPG) and a significant decrease of the bone degradation marker deoxypyridinoline crosslinked collagen I were observed. It appears thus that Rituximab lowered osteoclast activity [42].

6. Abatacept

CTLA4-Ig inhibits linking of CTLA-4 with the monocyte surface receptor CD80/CD86 [43] and could downregulate differentiation and maturation of osteoclasts acting directly on genes [44, 45]. CTLA-4 dose-dependently inhibits RANKL- as well as tumour necrosis factor- (TNF-) mediated osteoclastogenesis in vitro without the presence of T cells [44]. Furthermore, in mice, Abatacept protects against bone loss induced by PTH giving an explanation to the protective effect of Abatacept in RA [46].

7. Conclusion

Bone loss in RA is well documented and is a frequent comorbidity needing diagnosis and prevention. Bone remodeling markers are surrogates to evaluate bone formation, resorption, and further risk of fractures. So far, there is no consensus about their role in helping physicians in a clinical point of view. In addition to specific antiosteoporotic agents, when needed, biologic agents add their own nonspecific effect to protect RA patients against bone loss and osteoporotic fractures by reducing inflammatory-linked bone loss.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

A Polymorphism of *ORAI1* rs7135617, Is Associated with Susceptibility to Rheumatoid Arthritis

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Rheumatoid arthritis (RA), a chronic inflammatory disease usually occurring in synovial tissues and joints, is highly associated with genetic and environmental factors. *ORAI1*, a gene related to cellular immune system, has been shown to be involved in the pathogenesis of chronic inflammatory diseases and immune diseases. To identify whether *ORAI1* gene contributes to RA susceptibility, we enrolled 400 patients with RA and 621 healthy individuals for a case-control genetic association study. Five tagging single nucleotides polymorphisms (tSPNs) within *ORAI1* gene were selected for genotyping. An SNP, rs7135617, showed a significant correlation with the risk of RA. Our results indicated that genetic polymorphism of *ORAI1* gene is involved in the susceptibility of RA in a Taiwanese population.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects joints in the body. RA is also a chronic inflammatory disease that can lead to long-term joint damage, chronic pain, and loss of motor function in the hands. RA frequently affects smaller joints [1]. Symptoms caused by RA include joint stiffness, a low-grade fever, rheumatoid nodules, and lumps of tissue under the skin. The prevalence of RA is 0.5%~1%, which is relatively constant in many populations [1]. A high prevalence of RA was reported in Indians; in contrast, a low prevalence of RA was observed in Chinese and Japanese populations [1]. Differences of RA prevalence among populations reveal the importance of genetic factors in the risk of RA.

The cause of RA is still unclear. The immune system plays an important role in RA. Several genetic regions were

Table 1: Basal characteristics of patients with rheumatoid arthritis (RA) and of normal controls.

Characteristics	Patients with RA	Normal control
Number of subjects	400	621
Gender: female, no. (%)	329 (82.2%)	357 (57.5%)
Age (years)	62.4 ± 13.4	51.2 ± 16.2
Range (years)	22–90	11–88

reported to be associated with RA. The major histocompatibility complex (MHC) is a well-known region [2]. HLA DRB1 alleles were shown to be significant markers of RA in several populations [3–8]. In addition, using a genome-wide association study, Kochi et al. identified a polymorphism in a gene encoding chemokine (C-C motif) receptor 6 (CCR6) at 6q27, which was associated with RA [9]. The contribution of this region is estimated to be about 30% of the total genetic effects on RA susceptibility. This regulatory variant in *CCR6* was further confirmed in Taiwanese RA patients [10].

The store-operated calcium channel plays an important role in activation of T-lymphocytes. Orai1 is the pore-forming subunit of the store-operated calcium channel [11]. A loss of functional mutation of *ORAI1* was found to cause severe combined immunodeficiency (SCID) [12]. Genetic polymorphisms of ORAI1 were reported to be associated with a risk of HLA-B27-positive ankylosing spondylitis [13]. However, the role of ORAI1 in RA is still unclear. In this study, we assessed whether genetic variations in *ORAI1* contribute to RA susceptibility in the Taiwanese population.

2. Materials and Methods

2.1. Study Subjects. In total, 1021 Taiwanese individuals including 400 patients with rheumatoid arthritis (RA) and 621 healthy subjects were enrolled at Kaohsiung Medical University Hospital. Patients with RA were diagnosed to fulfill the revised criteria of the American Rheumatism Association for RA. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital. All participants were provided with sufficient information and a consent form for the study before clinical data and samples were collected.

2.2. DNA Extraction and Genotyping. Patients' genomic DNAs were isolated from whole blood samples using a Gentra extraction kit and ethanol precipitation as described in our previous study [14]. Genotyping for single-nucleotide polymorphisms (SNPs) of *Orai1* was conducted using a TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA). A polymerase chain reaction (PCR) was performed in a 96-well microplate with an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA). After the PCR, the fluorescence was measured and analyzed using system SDS software version 1.2.3 (Applied Biosystems, Foster City, CA).

2.3. Statistical Analysis. JMP 8.0 software for Windows (SAS Institute, Cary, NC) was used for the statistical analysis of genotyping results. Statistical differences in genotypes and allelic frequencies between cases and controls were assessed using a χ^2 test. A linkage disequilibrium (LD) map used to define the haplotype blocks was constructed using Haploview software (version 4.2; http://www.broad.mit.edu/mpg/haploview/). The haplotype analysis was performed to compare distributions of haplotype frequencies of *ORAI1* between cases (RA) and controls.

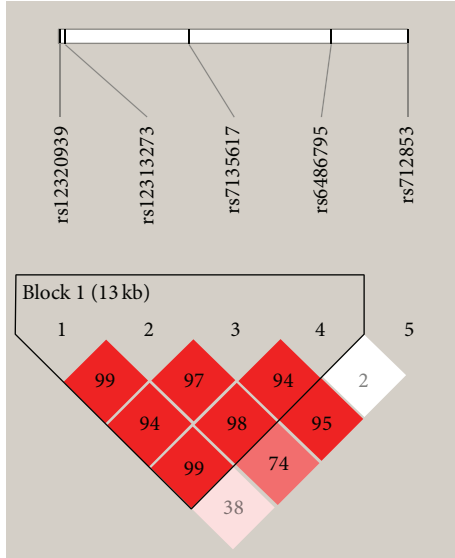

Figure 1: Five tSNPs on the LD map of *ORAI1* gene.

3. Results

3.1. Clinical Characteristics of Subjects. To investigate whether SNPs of *ORAI1* contribute to the susceptibility to RA, we performed a case and control association study. As shown in the Table 1, 400 rheumatoid arthritis patients and 621 healthy controls were recruited. Of the RA patients, 82.2% were female. The mean age was 62.4 years. In the healthy controls, 57.5% individuals were female and the overall mean age was 51.2 years.

3.2. A Significant Association between rs7135617 and Susceptibility of RA. In this study, five tagged SNPs (tSNPs) of *ORAI1* (rs12320939, rs12313273, rs7135617, rs6486795, and rs712853) with minor allele frequencies (MCFs) of >10% were selected from the HapMap Han Chinese database. Differences in genotypic and allelic frequencies of SNPs between cases and controls were compared. As shown in Table 2, rs7135617 revealed a significant association with RA in both the genotypic ($P = 0.004$) and recessive models (odds ratio (95% CI): 1.58 (1.14~2.19); $P = 0.006$).

3.3. Haplotype Analysis of ORAI1 Genetic Polymorphisms in the Susceptibility to RA. To further identify whether haplotypes of *ORAI1* were correlated with RA, we created an LD map (Figure 1) and analyzed haplotype frequency differences between RA patients and controls. The haplotype

Table 2: Genotyping and allele frequency of *ORAI1* gene in rheumatoid arthritis patients and normal controls.

	Genotype	Case (%) (n = 400)	Control subjects (%) (n = 621)	Allele	Case (%) (n = 400)	Control subjects (%) (n = 621)	Genotype P value	Recessive P value	Allelic P value
rs12320939	TT	95 (24.4)	144 (23.4)	T	382 (49.1)	602 (48.9)	0.868	0.715	0.945
	TG	192 (49.4)	314 (51.1)	G	396 (50.9)	628 (51.1)		1.06	1.01
	GG	102 (26.2)	157 (25.5)					(0.79–1.42)	(0.84–1.20)
rs12313273	CC	28 (7.8)	54 (8.8)	C	202 (28.0)	355 (28.9)	0.850	0.573	0.660
	CT	146 (40.4)	247 (40.2)	T	520 (72.0)	873 (71.1)		0.87	0.96
	TT	187 (51.8)	313 (51.0)					(0.54–1.40)	(0.78–1.17)
rs7135617	TT	83 (22.5)	96 (15.5)	T	318 (43.1)	505 (40.9)	**0.004***	**0.006***	0.331
	TG	152 (41.2)	313 (50.7)	G	420 (40.9)	731 (59.1)		1.58	1.10
	GG	134 (36.3)	209 (33.8)					(1.14–2.19)	(0.91–1.32)
rs6486795	CC	57 (14.9)	82 (13.3)	C	291 (38.1)	464 (37.7)	0.687	0.475	0.849
	CT	177 (46.3)	300 (48.7)	T	473 (61.9)	768 (62.3)		1.14	1.02
	TT	148 (38.7)	234 (38.0)					(0.79–1.65)	(0.85–1.23)
rs712853	CC	37 (9.7)	64 (10.6)	C	238 (31.1)	396 (32.7)	0.740	0.643	0.442
	CT	164 (42.8)	268 (44.3)	T	528 (68.9)	814 (67.3)		0.90	0.93
	TT	182 (47.5)	273 (45.1)					(1.38–0.59)	(1.13–0.76)

*Significant ($P < 0.05$) values are in bold.

Table 3: Haplotype frequencies of the *ORAI1* gene in rheumatoid arthritis patients and normal controls patients.

rs12313273/rs7135617	Case (%) (n = 400)	Control subjects (%) (n = 621)	OR (95% CI)	P value
T/T	304 (42.0)	501 (40.8)	1.09 (0.87–1.37)	0.4512
T/G	220 (30.4)	372 (30.3)	1.06 (0.84–1.35)	0.6210
C/G	197 (27.2)	354 (28.8)	Reference	

Haplotype frequency less than 1% was excluded.

analysis showed that no association was observed in pairwise allelic comparisons of rs12313273/rs7135617 (Table 3) or rs7135617/rs6486795 (Table 4).

4. Discussion

In this study, we screened SNPs of *ORAI1* and performed a case-control association study. In this study, 1021 subjects (400 cases and 621 controls) were recruited. Five genetic polymorphisms were selected for genotyping. Our results indicated a significant association between rs7135617 and susceptibility to RA. Previous studies reported significant associations between genetic polymorphisms of *ORAI1* and inflammatory diseases such as ankylosing spondylitis, calcium nephrolithiasis, and atopic dermatitis [13, 15, 16]. In this study, we found an SNP (rs7135617) located in the intron of *ORAI1* associated with a risk of RA in the Taiwanese population.

ORAI1-mediated calcium signaling was reported to be involved in a variety of human diseases. Feske et al. identified a mutation in *ORAI1* from SCID patients [12]. A missense mutation resulted in the dysfunction of store-operated calcium entry that in turn attenuated immune responses [12]. Our previous studies indicated that ORAI1 was highly expressed in the spleen, an organ involved in immune system [16]. The rs7135617 within *ORAI1* was associated with an autoimmune disease, ankylosing spondylitis. Consistent with a previous report, this study also confirmed an important role of ORAI1 polymorphism rs7135617 in RA. However, functional role of "intronic splicing regulatory elements of *ORAI1*" underlying RA susceptibility is not clear. Therefore, we further applied Human Splicing Finder version 2.4.1 (HSF) [17] to analyze the possible functions of rs7135617G>T. Results indicated that rs7135617 was predicted as a potential target binding site of SR SC35 protein. SR SC35 protein is an important splicing factor which can influence selection of splice site [18]. The consensus value of rs7135617 wild-type (G) motif is 75.97 whereas the mutant-type (T) motif is 91.09. The variation of the consensus value (ΔCV) is +19.9%. A higher consensus value indicates higher strength and more possibility to be the splicing enhancer binding motif of SC35 protein. Combined with bioinformatics findings and genotyping data, our results imply that *ORAI1* polymorphism rs7135617 may influence splicing process which further affects calcium signaling.

This study has some limitations. First, the collection of samples did not contain clinical biochemical data of

Table 4: Haplotype frequencies of the *ORAI1* gene in rheumatoid arthritis patients and normal controls.

rs7135617/rs6486795	Case (%) (n = 400)	Control subjects (%) (n = 621)	OR (95% CI)	P value
T/T	295 (41.1)	501 (40.7)	1.00 (0.81–1.23)	0.9961
G/T	142 (19.8)	265 (21.5)	0.91 (0.71–1.17)	0.4629
G/C	271 (37.7)	460 (37.4)	Reference	

Haplotype frequency less than 1% was excluded.

RA patients. Therefore, this study was only able to detect associations between SNPs and the risk of RA. Second, rs7135617 is located in the intron. The T allele is a risk allele for RA. However, further functional role of *ORAI1* polymorphism rs7135617 requires experimental validation in order to clarify the mechanism underlying calcium signaling and susceptibility of RA.

Third, the study was limited by the modest sample size (1021 subjects); however, we believe this might be partly overcome by the fact that our samples are homogeneous and well defined in terms of phenotype assessment.

Given the polygenic nature of immune diseases such as RA, the susceptibility gene *ORAI1* could provide new clues to the pathogenesis of RA. Although a larger-scale population study is needed, our results, at least in part, indicated an important role of *ORAI1* gene in the susceptibility to RA. Further study of the relationship between *ORAI1* genotypes and the downstream functional relevance during chronic inflammation of the joints should be conducted in order to understand the etiology of RA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jeng-Hsien Yen, Che-Mai Chang, and Yu-Wen Hsu contributed equally to this work

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Review Article

Adipokines as Potential Biomarkers in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease characterized by severe joint injury. Recently, research has been focusing on the possible identification of predictor markers of disease onset and/or progression, of joint damage, and of therapeutic response. Recent findings have uncovered the role of white adipose tissue as a pleiotropic organ not only specialized in endocrine functions but also able to control multiple physiopathological processes, including inflammation. Adipokines are a family of soluble mediators secreted by white adipose tissue endowed with a wide spectrum of actions. This review will focus on the recent advances on the role of the adipokine network in the pathogenesis of RA. A particular attention will be devoted to the action of these proteins on RA effector cells, and on the possibility to use circulating levels of adipokines as potential biomarkers of disease activity and therapeutic response.

1. Introduction

An emerging body of evidence suggests that the white adipose tissue (WAT) plays more than just the role of energy storage compartment and thermal and mechanical insulator. WAT is now recognized as a pleiotropic organ specialized in endocrine functions being able to produce several hormones and other proteins involved in both physiological and pathological processes, including immunity and inflammation [1]. The biological active substances secreted by WAT contribute to the systemic "low-grade inflammatory state" associated with obesity [2, 3]. Indeed, increased circulating levels of several markers of inflammation occur in obese subjects, such as IL-6, TNF-α, C-reactive protein (CRP), and plasminogen activator inhibitor I (PAI-I) [4, 5]. It should be also considered that infiltrating macrophages represent an important source of inflammatory mediators which further promote and sustain inflammation [6]. The term "adipokines" is applied to all the biological active substances synthesized by WAT which function as regulators of energy homeostasis and metabolism; the same mediators are also involved in chronic inflammation and metabolic dysfunctions [7].

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder characterized by synovial inflammation, cartilage damage, and bone erosion, with 1% prevalence worldwide [8]. Although the pathogenesis of this disease is poorly understood, several observations indicate that adipokines affect tissues and cells involved in RA, including synovium, cartilage, bone, and immune cells [9]. In the present review we will describe the information available on the role of adipokines in RA pathogenesis, focusing on the role of adiponectin, leptin, chemerin, visfatin, resistin, lipocalin 2, SAA3, and a few others, in light of their possible consideration as new potential circulating biomarkers of disease activity and therapeutic response.

2. Adiponectin

Adiponectin (also called GBP28, AdipoQ, ApM1, and Acrp30) is a collagen-like protein with a structure similar to the complement factor C1q. Adiponectin is mainly produced by adipocytes and is present, in different molecular isoforms, at high levels (3–30 μg/mL) in the blood [10, 11].

Two adipokine receptors were recently identified, AdipoR1, mainly expressed in skeletal muscles, and AdipoR2 which is expressed in the liver [12]. The signaling transduction pathways of adiponectin receptors involve the activation of the adaptor protein APLL1 [13] and many signaling molecules, including AMPK, p38 MAP kinases, and PPAR-α and PPAR-γ [10, 14]. The main functions of adiponectin are, in the muscle, the increase of fatty acid oxidation and glucose uptake and, in the liver, the reduction of glucose synthesis.

Low levels of circulating adiponectin, as those observed in obesity, type 2 diabetes, atherosclerosis, vessel inflammation, and metabolic syndrome, suggest a protective function. Accumulating evidence supports a potential role of adiponectin in controlling inflammation. For instance, adiponectin was reported to inhibit the transformation of macrophages into foam cells [15], to stimulate the production of the anti-inflammatory cytokine IL-10 [16], to reduce the production of TNF-α [17], to induce tolerance in response to TLR ligands [18], and to promote the anti-inflammatory M2 macrophage polarization (Figure 1) [19]. The anti-inflammatory effects of adiponectin have been, to some extent, ascribed to its capacity to alter ceramide metabolism and to promote sphingosine-1-phosphate synthesis [20]. However, evidence that adiponectin may act as a proinflammatory mediator promoting extracellular matrix degradation and joint disruption is also available. Indeed, in cultured chondrocytes, adiponectin increases the expression of MMP-3 [21] and the secretion and activity of proinflammatory mediators, such as nitric oxide synthase type II (NOS2/iNOS), MMP-9, IL-6, MCP-1, and IL-8 [22, 23]. Similarly, adiponectin is able to stimulate the production of PGE2, IL-6, IL-8, vascular endothelial growth factor (VEGF), MMP-1 and MMP-13, cyclooxygenase 2 (COX-2), and microsomal prostaglandin E synthase 1 (mPGES-1) [24, 25] in RA synovial fibroblasts (Figure 1). In RA, the cellular targets of adiponectin may also include lymphocytes and endothelial cells, further supporting the role of adiponectin in this pathology [26].

In RA patients, the serum/plasma levels of adiponectin, as well as the levels in the synovial fluid, are associated with radiographic damage [27] and are increased compared to osteoarthritis patients (OA) and healthy donors [28, 29]. Increased adiponectin levels positively correlate with the disease activity score 28 (DAS28), the erythrocyte sedimentation rate (ESR), and the rheumatoid factor (RF) [30]. Recently, Klein-Wieringa et al. reported that the baseline levels of adiponectin can also predict radiographic progression over a four-year period independently of the presence of anticyclic citrullinated peptide (CCP) antibodies and body mass index (BMI) [31]. In addition, the elevation of total and high molecular weight adiponectin was described in patients with RA treated with anti-TNF agents (e.g., infliximab and etanercept) [32, 33] (Table 1). Finally, considering the detrimental effects of this adipokine in perpetuating joint inflammation, the use of adiponectin as a potential therapeutic target of blocking therapies has been proposed [34].

3. Leptin

Leptin, the product of *ob* gene, is a 16 kDa nonglycosylated hormone peptide [35] which binds the OB-Rb long form leptin receptor coupled to a JAK/STAT signaling pathway [36, 37]. Leptin is considered the major regulator of body weight, since it induces the decrease of food intake and increases energy consumption [38]. Leptin is mainly produced by WAT and the circulating levels of leptin correlate positively with the amount of adipose tissue and BMI [39]. However, leptin synthesis is also regulated by the action of inflammatory mediators [40]. Leptin is generally considered a proinflammatory adipokine. In fact, leptin stimulates the production of proinflammatory cytokines, such as TNF-α and IL-6, and reactive oxygen species in cultured monocytes. In addition, it induces the production of CC-chemokines by macrophages and alters the Th1/Th2 balance favoring the Th1 phenotype (Figure 1) [41–43]. Moreover, leptin null mice are protected in experimental models of T cell mediated hepatitis and experimental autoimmune encephalomyelitis [44, 45].

Leptin has been associated with autoimmune diseases, in particular with RA. However, there are conflicting observations concerning the circulating levels of leptin in RA patients, since some studies suggested a correlation between leptin levels and disease activity [28, 46, 47], while others failed to detect changes in circulating leptin levels [48]; interference of concomitant pharmacological treatments might be responsible for these apparently contrasting results. In experimental models of arthritis, leptin deficient mice showed a milder form of antigen-induced arthritis associated with the reduction of IFN-γ production and the increase in IL-10 secretion by in vitro reactivated lymph node cells [49]. In contrast, leptin-deficient and leptin receptor-deficient mice exhibited a delayed resolution of the disease [50]; the administration of leptin ameliorated disease activity [51]. These conflicting results do not allow coming to a clear conclusion on the role of leptin in RA. To note, leptin circulating levels apparently are not modulated in patients treated with anti-TNF-α therapy [52–54] (Table 1). Recently, the serum/synovial fluid ratios of leptin levels were associated with disease duration and erosion [55]. In addition, several in vitro studies sustained the pathogenic role of leptin in RA. In human and murine chondrocytes, leptin synergizes with IL-1β and IFNγ for the activation of type 2 nitric oxide synthase (NOS) and the induction of IL-8 and metalloproteinases via a JAK2, PI3K, and MAP kinase-dependent signaling pathway [23, 56–58]. Leptin also induced IL-8 in human synovial fibroblasts with a NFκB-dependent pathway [59]. Furthermore, leptin can also modulate the activities of several immune cells [60]. For instance, in murine dendritic cells, leptin increases CD40 expression and T cell priming (Figure 1) [61]. Matarese et al. showed that leptin-null and leptin receptor-null mice have increased levels of Treg cells and are protected in experimental models of autoimmune diseases [45]. In keeping with this observation, high leptin levels are associated with a reduction of Treg and with the activation of proinflammatory effector T cells [62–64]. Recently, it was shown that the leptin-induced state of overexpression of the mTOR pathway, in freshly isolated Treg cells, is

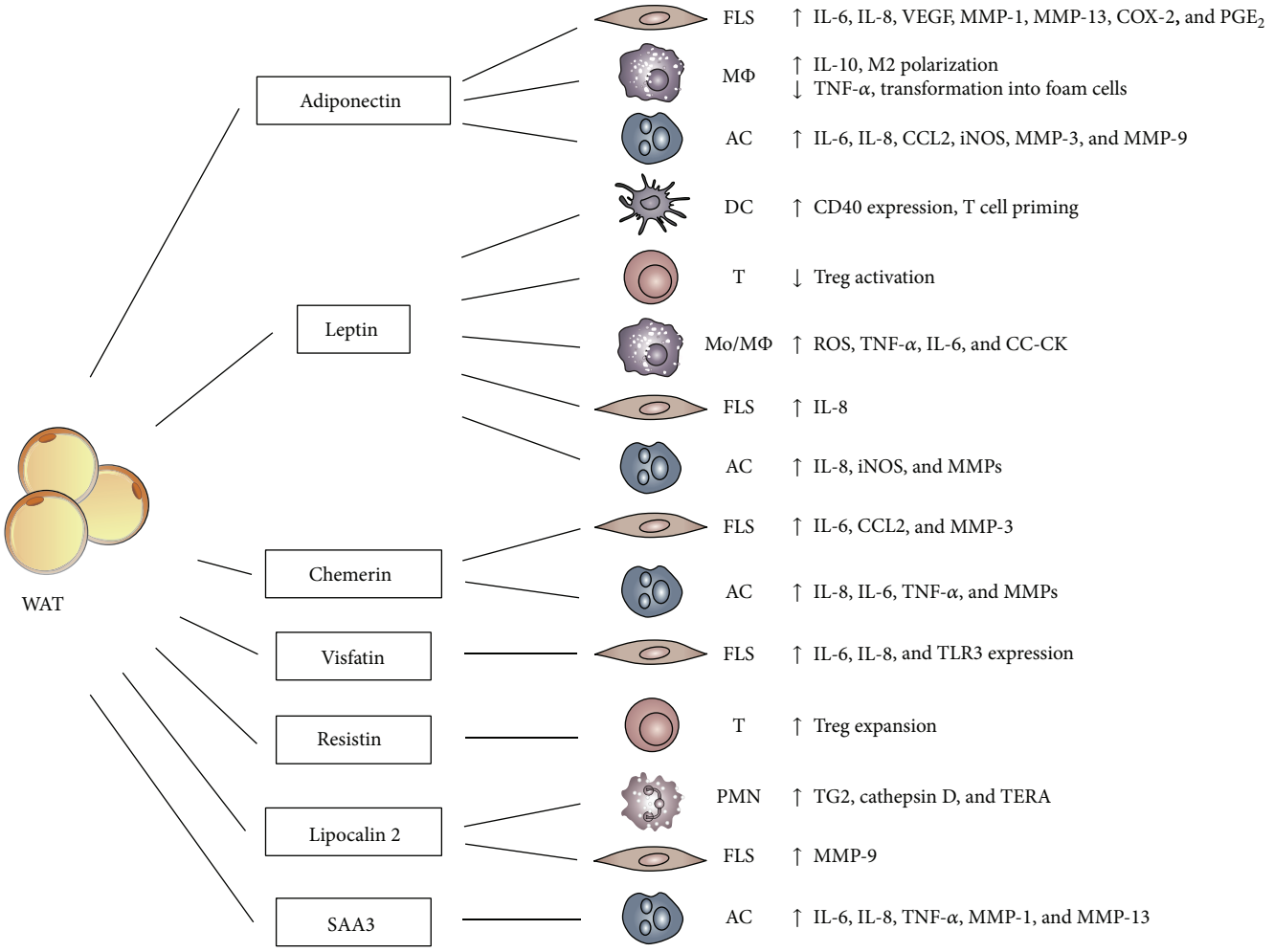

Figure 1: Role of adipokines on RA effector cells. The role of different adipokines on RA target cells is illustrated in the figure. WAT: white adipose tissue, SAA3: serum amyloid A3, FLS: fibroblast-like synoviocytes, AC: articular chondrocytes, PMN: neutrophils, MMP: metalloprotease, COX-2: cyclooxygenase 2, ROS: reactive oxygen species, iNOS: inducible nitric oxide synthase, CC-CK: CC-chemokines, TG2: transglutaminase 2, and TERA: transitional endoplasmic reticulum ATPase.

responsible for their state of hyporesponsiveness. Therefore, it is conceivable that Treg activation is dependent on the dynamic regulation of mTOR activity by the composition of the extracellular milieu, such as the concentrations of leptin and cell nutrients [65]. These results clearly depict leptin as a pleiotropic molecule placed at the crossroads of immune tolerance, metabolism, and autoimmunity. Further studies are needed in order to clarify whether leptin might represent a new disease activity biomarker and to explore its therapeutic potential in autoimmune diseases.

4. Chemerin

Chemerin is a 16 kDa protein, originally described as the product of the Tazarotene-induced gene 2 (Tig2) [66] and purified from ascitic fluids of ovarian cancer patients and synovial exudates of rheumatoid arthritis patients [67]. Chemerin is secreted as an inactive precursor protein which is subsequently converted into a bioactive protein following the proteolytic removal of the last six or seven amino acids from the C-terminal end [68]. Chemerin was first described as the functional ligand of the chemotactic receptor ChemR23. Dendritic cells, macrophages, and NK cells express ChemR23 and a role for chemerin in their recruitment into inflammatory sites was described in lupus erythematosus, oral lichen planus, and psoriasis [69–72]. More recently, the adipokine function of chemerin was proposed, since chemerin is mainly produced by WAT and plays important regulatory role in adipogenesis in vitro [73]. In addition, chemerin is considered a biomarker of adiposity, because chemerin levels strongly associate with BMI [74], markers of inflammation (e.g., TNF-α, IL-6, and CRP) [75], and metabolic syndrome [76]; chemerin circulating levels decrease with weight and fat loss [77]. Human articular chondrocytes express chemerin and its receptor ChemR23 and secrete proinflammatory cytokines, such as IL-6, IL-8, and TNF-a, and metalloproteases, in response to chemerin stimulation (Figure 1) [78].

Table 1: Correlation of adipokines with disease activity parameters and therapeutic response.

Adipokine	Correlation with					
	DAS28	BMI	IL-6/TNF/ESR	Anti-CCP	Radiographic progression	Therapeutic response
Adiponectin	pos [30]	neg [31]	pos [30]	neg [31]	pos [27, 31]	Anti-TNF: pos [32, 33]
Leptin	pos [46]	neg [46]	neg [31]	no [31]	neg [98]	Anti-TNF: neg [52]
Chemerin	pos [80]	neg, pos [80]	pos [78]	ND	ND	Anti-TNF: pos [81]
Visfatin	neg [102]	neg [149]	pos [31]	pos [31], neg [102]	pos [100]	Anti-TNF: neg [101], pos [91] Anti-CD20: pos [102]
Resistin	pos [30]	pos [31]	pos [31, 118]	No in serum but pos in SF [111]	ND	Anti-TNF: pos [118]
Lipocalin 2	ND	ND	ND	ND	ND	ND
SAA3	ND	ND	ND	ND	ND	ND
Vaspin	SF pos [150]	pos [147]	SF neg [150]	SF neg [150]	ND	ND
Omentin	SF neg [150]	neg [152]	SF neg [150]	SF pos [150]	ND	ND
Apelin	ND	ND	ND	ND	ND	DMARDs: neg [158]
Adipsin	ND	pos [31]	pos [31]	ND	neg [31]	DMARDs: pos [160]

Abbreviations: pos: positive; neg: negative; SF: synovial fluid; ND: not determined. Where not specified, the correlations are referred to serum levels. Positive correlation with therapeutic response is assumed when the adipokine levels are modified (either they increase or decrease) by the treatment.

In RA patients the expression of chemerin and ChemR23 in fibroblast-like synoviocytes (FLS) was found increased compared to OA patients. Chemerin was reported to mediate direct proinflammatory and stimulatory effects on the RA-FLS [79], suggesting a pivotal role of the chemerin/ChemR23 axis in the pathogenesis of RA. A recent study reported that RA patients have increased levels of circulating chemerin and chemerin levels positively correlated with disease activity (DAS28, ESR, and CRP) [80]. Circulating chemerin levels are negatively regulated by the anti-TNF therapy (adalimumab) in parallel with the reduction of disease activity markers, such as DAS28, ESR and CRP, and IL-6, and the macrophage migration inhibitory factor (MIF) levels [81] (Table 1). These results nominate chemerin serum levels as a biomarker for disease activity and therapeutic response.

5. Visfatin

Visfatin, also known as pre-B-cell colony-enhancing factor (PBEF) and nicotinamide phosphoribosyltransferase (Nampt), was originally described as a cytokine involved in early B-cell development and was later renamed visfatin since it is secreted mainly by visceral fat [82]. In addition, leukocytes, in particular granulocytes and monocytes/macrophages, from obese patients produce high levels of visfatin [83–85]. Visfatin is also produced by endotoxin-challenged neutrophils, where it functions as an antiapoptotic molecule acting at level of caspases 3 and 8 [86]. Visfatin was also suggested to have insulin-like functions [87, 88]. A specific receptor for visfatin has not been identified yet. Nevertheless, the proinflammatory action of visfatin was described to be mediated by the insulin signaling pathway through Akt phosphorylation [89].

Circulating levels of visfatin correlate with obesity and type 2 diabetes and are reduced after weight loss [90]. Visfatin was also proposed to promote atherosclerosis and to cause plaque destabilization through the induction of proinflammatory mediators and adhesion molecules in endothelial cells [91–93]. Several observations sustain the hypothesis that visfatin may play a major role in the pathogenesis of RA. Recent studies reported the upregulation of visfatin in activated RA-SFs in response to proinflammatory stimuli, such as IL-6 and the activation of TLR3 [94, 95] with visfatin acting as an autocrine positive feedback mechanism for IL-6 production [96]. In RA synovium, visfatin was predominantly expressed in the lining layer, lymphoid aggregates, and interstitial vessels. In RA-SFs, visfatin induced high amounts of chemokines such as IL-8 and CCL2, proinflammatory cytokines (i.e., IL-6), and matrix metalloproteinases (i.e., MMP-3) (Figure 1). Visfatin promoted fibroblast migration and induced phosphorylation of p38 MAPK; of note, inhibition of p38MAPK strongly reduced visfatin effects [97]. Finally, visfatin inhibition significantly reduced the severity of the disease and TNF-α circulating levels in the experimental model of collagen-induced arthritis [98, 99].

In RA, circulating levels of visfatin are increased [28], as well as its expression in synovial fluids and inflamed synovium [94–96]. Visfatin serum and synovial fluid levels correlated with the degree of inflammation, with the severity of the disease, and with joint damage [31, 95, 100]. Contradictory results are available on visfatin levels in patients undergoing anti-TNF-α therapy. In one study no significant changes were observed [101], while in others a negative correlation with therapy was found [91]. In general, visfatin serum levels better correlated with the number of circulating B cells rather than with the disease activity and were profoundly affected after B-cell depletion therapy with rituximab. The lack of change in

serum visfatin levels is suggested to predict worsening disease activity [102] (Table 1).

6. Resistin

Resistin is a cysteine-rich protein of 12.5 kDa also known as adipocyte-secreted factor (ASF) or "found in inflammatory zone 3" (FIZZ3) [103]. In RA experimental models, resistin promotes insulin resistance, while the function in humans is still unclear [104]. Even if resistin was originally described to be produced only by WAT, subsequent studies demonstrated that, in humans, resistin mainly derives from circulating monocytes and macrophages [105]. The resistin receptor is still unknown and recently TLR4 was proposed to mediate resistin proinflammatory functions in human cells [106]. Resistin has a strong impact on immune functions. It can enhance the expansion of Treg cells through an effect on dendritic cells (Figure 1) [107]. Proinflammatory mediators increase resistin expression; in turn, resistin induces TNF-α, IL-12, IL-6, and IL-1β production [108, 109]. These findings, together with the observation that the intra-articular injection of resistin in the knee joints induces arthritis, sustain the involvement of resistin in RA pathogenesis [110]. Several reports have demonstrated that serum resistin levels are significantly higher in RA than in OA patients or healthy controls [111–113]. The increased serum levels of resistin correlated with markers of inflammation, such as CRP, ESR, IL-1Ra, and total leukocyte count [47, 114–117], disease activity (DAS28), and joint destruction [112]. However, these results were not confirmed by other groups [111], and conflicting results were reported on the association between resistin and radiographic progression signs [27, 31, 100]. Recently, the anti-TNF-α therapy was reported to modulate resistin levels in RA patients [118, 119] (Table 1). Resistin levels in synovial fluids and in the sublining layer are higher in RA than in OA patients [29, 110, 112]. These results strongly suggest that resistin production is elevated at the site of inflammation and accumulates in the synovial fluid of RA patients. In anti-CCP positive patients, synovial fluid resistin levels, but not serum levels, correlated with disease progression suggesting resistin as a disease progression marker [111].

7. Lipocalin 2

Lipocalin 2 (LCN2), also known as siderocalin, 24p3, uterocalin, and neutrophil gelatinase-associated lipocalin (NGAL), is a recently identified glycoprotein stored in neutrophil granules [120] but mainly produced by WAT [121, 122]. LCN2 has been isolated in different isoforms and its functions are carried out by the activation of the cellular receptor megalin [123]. LCN2 binds and transports small lipophilic substances, such as retinoids, arachidonic acid, steroids, iron, and fatty acids [124–126]. Other functions that have been attributed to LCN2 are the induction of apoptosis in hematopoietic cells [127], the inhibition of bacterial growth [128, 129], regulation of iron metabolism [130], and insulin resistance [131]. LCN2 is induced by inflammatory stimuli through the activation of the NFkB pathway [132]; however dexamethasone promotes LCN2 production in chondrocytes [133, 134]. LCN2 is involved in the allosteric activation of MMP-9 [135] and levels of MMP-9 are higher in the serum and synovial fluid of RA patients [136]. Recently, LCN2 synovial fluid levels were found to be increased in RA compared to OA patients [137]. Through a proteomic approach, GM-CSF was found to induce LNC2 upregulation in neutrophils, which in turn can influence synoviocyte behavior through the release of several enzymes, such as transglutaminase 2 (TG2), cathepsin D, and transitional endoplasmic reticulum ATPase (TERA) (Figure 1), which contribute to both inflammation of synovium and proliferation of synovial cells, promoting the RA state [137].

8. SAA3

The serum amyloid A3 (SAA3) belongs to the family of acute phase serum amyloid A proteins produced by hepatocytes [138] and other cell types, including adipocytes [139, 140]. SAA3 was associated to altered metabolic and immunocompromised conditions [141, 142]. Several stimuli, such as TNF-α, IL-1β, dexamethasone, IL-6, and LPS, can increase SAA3 expression [139, 140, 143]. Recently, SAA3 was suggested to directly activate the MyD88-dependent TLR4/MD-2 pathway [144].

In a rabbit Ag-induced arthritis model, upregulation of SAA3 transcripts was detected in cells infiltrating into the inflamed joint, in the area where pannus formation starts and, most notably, also in chondrocytes. In vitro, recombinant human SAA induces matrix metalloproteinase transcription in human chondrocytes (Figure 1). Further, SAA is highly expressed in human RA synovium [145]. Recently, Geurts et al. proposed that a SAA3-promoter report may have a diagnostic value in the classification of RA molecularly distinct forms with different degree of synovial tissue inflammation [146].

9. Other Adipokines

Vaspin, visceral adipose tissue-derived serine protease inhibitor, is expressed predominantly in visceral adipose tissue [147]. Expression of the vaspin gene positively correlates with BMI and administration of the protein to obese mice improved glucose tolerance and insulin sensitivity [147, 148]. Vaspin levels are increased in the serum and synovial fluid of RA patients [149, 150] (Table 1).

Omentin, also known as intelectin, is a protein secreted by omental adipose tissue and highly abundant in human plasma [151]. Both circulating protein levels and mRNA levels in adipose tissue decrease in obese subjects and correlate negatively with markers of obesity, such as BMI, waist circumference, and circulating leptin [152] (Table 1). Expression of the omentin gene was reported in omental adipose tissue of patients with Crohn's disease, suggesting a role in chronic inflammatory diseases [151]. The levels of omentin were found significantly reduced in the synovial fluid of patients with RA compared to OA patients [150]. On the contrary, circulating

levels of omentin were significantly higher in patients with juvenile idiopathic arthritis compared to healthy controls [153].

Apelin is a bioactive peptide, originally identified as the endogenous ligand of the G-protein coupled receptor APJ [154]. Apelin is mainly produced by adipocytes, its expression is upregulated by insulin, and TNF-α and its levels are increased in obesity [155, 156]. Apelin has been implicated in the pathogenesis of OA, since high circulating levels are increased in the sera and synovial fluids of OA patients [157]. In early-stage RA patients serum apelin levels were found to be decreased but were insensitive to pharmacological treatment [158] (Table 1).

Adipsin, also known as complement factor D, is highly expressed in adipose tissue and in activated monocyte/macrophages [159]. Circulating levels of adipsin did not predict the radiographic progression of early-stage disease [31]; however, increased adipsin levels were found to be associated with a higher remission rate in early RA patients treated with DMARD [160] (Table 1).

10. Conclusions

The discovery of adipokines has profoundly changed our understanding of the functions of adipose tissue. The adipokine network is involved in the interplay between WAT, metabolic disorders, and immune-mediated diseases. Adipokines have shown to be able to modulate several aspects of inflammation as well as both innate and adaptive immune responses. Although in the past few years the implications of the adipokines in autoimmune diseases, including rheumatoid arthritis, have greatly increased, a clear picture of the role of these proteins in the pathogenesis and in the progression of this disease is still missing. Nevertheless, accumulating evidence on the modulation of serum and synovial fluid levels of many adipokines encourages their future exploitation as soluble biomarkers of disease activity and therapeutic response. Further studies are needed in order to translate the increasing number of experimental and clinical observations to the use of adipokines as clinical diagnostic markers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Autoantibodies to Posttranslational Modifications in Rheumatoid Arthritis

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Autoantibodies have been associated with human pathologies for a long time, particularly with autoimmune diseases (AIDs). Rheumatoid factor (RF) is known since the late 1930s to be associated with rheumatoid arthritis (RA). The discovery of anticitrullinated protein antibodies in the last century has changed this and other posttranslational modifications (PTM) relevant to RA have since been described. Such PTM introduce neoepitopes in proteins that can generate novel autoantibody specificities. The recent recognition of these novel specificities in RA provides a unique opportunity to understand human B-cell development *in vivo*. In this paper, we will review the three of the main classes of PTMs already associated with RA: citrullination, carbamylation, and oxidation. With the advancement of research methodologies it should be expected that other autoantibodies against PTM proteins could be discovered in patients with autoimmune diseases. Many of such autoantibodies may provide significant biomarker potential.

1. Introduction

Specificity and memory are the hallmarks of the adaptive immune system. Immunological memory is well recognised but still not fully understood. It was first observed in antiquity, during the plague infection in Athens. It is also the basis of vaccination, which was first attempted in India over a thousand years ago when smallpox inoculation to healthy people resulted in a milder epidemic, while protection lasted many years, particularly in the absence of reexposure to the antigen. Molecular immunology has now unravelled the early steps towards the establishment of immunological memory; however, several areas remain unexplained particularly the mechanism of plasma cell development and maintenance. The recent recognition of the specificity of novel autoantibodies in rheumatoid arthritis (RA) provides a unique opportunity to understand human IgG and B-cell memory development *in vivo*.

The early phases of B-cell development are well established. Initially, naive B-cells are released in the circulation, where they meet with their antigens, becoming activated B-cells. At this stage several models have been proposed and IgG can develop through different routes, in a T-cell dependent or independent manner. In a (classic) linear model [1], maturation occurs in the presence of T-cells in a germinal centre-like reaction (GCR); B-cells switch from secreting IgM to secreting IgG and undergo affinity maturation. Some of these B-cells then develop into memory B-cells and others into long-lived plasma cells (LL-PC). These LL-PC then move to a bone marrow niche where they can survive for years. They are dependent on CXCL12 expression. In a variation of this model, activated B-cells go through a short-live plasma

cell (SL-PC) stage before fully maturing into LL-PC [2]. In a third model both LL-PC and SL-PC secreting IgM and IgG were shown to develop directly from activated B-cells, independently of T-cell help. However this only occurs in the presence of antigen and alternative signals provided by innate immunity mechanisms such as direct TLR activation of B-cells [3]. It appears that these three models may actually coexist providing a first line of defense with rapid secretion of antibodies. However, further T-cell mediated maturation is necessary for a second line of defense involving long-term memory and LL-PC [4]. An inflamed environment such as the synovial membrane in RA (where CXCL12 is highly expressed in active disease [5]) is believed to provide an alternative niche for the survival of LL-PC.

Autoantibodies have been associated with human pathologies for a long time, particularly with autoimmune diseases (AIDs). Organ specific AIDs involve single or multiple autoantigens. In RA, autoantibodies have long been associated with the disease. Rheumatoid factor (RF), an autoantibody reacting against the Fc portion of IgG antibodies, was identified in the late 1930s. It was the most significant biomarker associated with RA until the discovery of anticitrullinated protein antibodies (ACPA). More recently, other posttranslational modifications (PTM) have been associated with the generation of specific autoantibodies that can be used as biomarkers [6–10]. While proteins are encoded by different sequences of amino acids, there are many ways to modify amino acids once introduced in protein sequences. Glycosylation, citrullination, methylation, acetylation, and ubiquitination are all types of physiological modifications. Other modifications can occur due to interaction with foreign substances (i.e., infections), environmental damage (such as UV exposure or chemical pollutants) leading to the formation of chemical adducts on the protein. Modifications including carbamylation, acetylation, ethylation, or methylation were sufficiently immunogenic to produce specific antibodies to these modified sequences of amino acids [11]. The analysis of autoantigen specific B-cell differentiation and maintenance, at the different stages of RA progression, provides a unique opportunity to understand disease and study immunological B-cell memory *in vivo* [12, 13].

Many AIDs are characterized by chronic inflammation, which may play a major role when inflammation-associated events such as chemical or enzyme-mediated modification of protein provide a source of neoepitopes that can be recognised by antibodies as non-self. In situations of stress such as inflammation, all types of physiological responses can be used in an abnormal manner. Citrullination is an enzymatic PTM which has an important role in the normal function of the immune system, epidermis differentiation insulation of neurons and the plasticity of the central nervous system [14]. Chlorination of protein occurs via the conversion of hydrogen peroxide to reactive chlorine species, such as HOCl, by granulocytes notably during inflammation. Other forms of oxidation result from the formation of reactive species of oxygen, nitrogen, and sulphur as a cellular response to various stimulations by growth factors or cytokines [15]. Oxidation products of sugars and unsaturated lipids can also react with proteins to cause chemical modifications. Nonenzymatic glycation is a naturally occurring phenomenon leading to development of PTM of proteins, nucleic acid, or lipids; it occurs in presence of high blood glucose but is also associated with aging and other inflammatory or degenerative diseases [16] such as RA [17], osteoarthritis [18], and Alzheimer's disease [19–21]. Carbamylation is a nonenzymatic, irreversible PTM. Carbamylation of proteins, lipids, peptides, and amino acids is widespread in health in mammals and is a natural physiological phenomenon. However excessive carbamylation will appear once proteins are exposed to high concentrations of isocyanate derived from the increased dissociation of urea and this alters the function of proteins [22].

Important evidence that perturbations in protein structures introduced by PTM are important in RA was brought by studies of collagen II (CII) for which PTM were shown to dramatically alter immunogenicity [6, 23–25] rendering some of them arthritogenic [26–30]. CII is the predominant cartilage collagen and a known autoantigen [23, 31, 32]. The human joint contains abundant CII and collagen-induced arthritis is the common experimental animal model of RA [33, 34]. Thus, antibodies to CII should be of highest relevance in RA [32]. Nevertheless, antinative CII antibodies occur only in 3–27% of patients with RA [29, 35–37] and, as such, it has been difficult to substantiate the role of autoimmunity to CII in the pathogenesis of RA. However today autoimmunity to PTM CII has been clearly demonstrated (cit-CII [24], ROS-CII [6, 7, 10, 38], although specific anticarbamylated CII remains to be demonstrated in human sera). These findings support the possibility that chemical modification of self-antigens, in RA in particular and in inflammation in general, may be the cause of formation of neoepitopes leading to autoimmunity [16, 39].

2. Anticitrullinated Protein Antibodies (ACPA) in RA

ACPA were originally described using different names such as anti-keratin (AKA), antiperinuclear factor (APF) antibodies, antifilaggrin antibodies (AFA), or anti-Sa [40]. ACPA have been associated with human pathology [41] as well as preclinical disease since the early 90s [42] later confirmed [43, 44]. The importance of these antibodies was then recognised several years later when their presence was identified as a specific event associated with RA [45–50]. Many reports were published; however, their relevance was reduced to a few publications where appropriate controls and procedures had been followed, particularly with respect to the ELISA assays used to detect ACPA [51, 52]. In the early years, the use of ELISA for individual reactivities (citrullinated filagrin or keratin) or "first generation" commercially available ELISA kits (CCP1, Immunoscan RA, Euro-diagnostica [53]) showed equal reactivity between RA (22%), healthy control sera (27%), and all kinds of arthritis and inflammatory diseases [49] although clear differences in titres were observed (sensitivities 45–64% but specificity over 90%). Later, "second-generation" ELISAs, showed higher specificity (~98%) and sensitivity

(40–76% depending on disease stage) [54]; however, more recent work also showed potential association of ACPA with psoriatic arthritis [55], periodontitis [56], and osteoarthritis [38]. The main difference between these tests resided in the antigens used to detect ACPA. The diagnostic value of ACPA were therefore established by demonstrating the importance of using appropriate citrullinated peptide [40, 51, 57]. The development of a highly sensitive noncommercial ELISA, based on protein targets identified as reactive with ACPA in synovial tissue (i.e., alpha and beta fibrinogen) was therefore explored [58]. Importantly, positivity of ACPA for one or both to these two citrullinated peptides covered all reactivity in RA sera [59].

2.1. Citrullination. ACPA represent a family of autoantibodies. However, only IgG-isotype of ACPA is specifically associated with RA. The antigen which triggers the immune reaction recognized by ACPA lies in the modification of protein (i.e., citrullination). In summary, after years of research, it was shown that this modification converts an arginine into a citrulline amino acid residue (citrullination) and is performed by an enzyme, peptidylarginine deiminase (PAD), thereby producing the immunogenic epitopes (Figure 1(a)) [60]. A consensus sequence, present in a wide range of proteins, is required for the modification of the arginine residue by the PAD. Metabolic stress related citrullination has also been proposed to play a role in multiple sclerosis [61, 62], Alzheimer's disease [20, 21], and cancer [63, 64]. The modifications introduced by PAD enzymes have important physiological roles, especially during differentiation, development, and apoptosis. PAD enzymes are expressed in a wide range of tissues (epidermis, sweat glands, hair follicles, ovary, and testis). In the synovium, only the enzymes PAD 2 and 4 are expressed; however, their expression is not specific for RA as they are also found in other forms of inflammatory and noninflammatory arthritis [65]. How both intracellular and extracellular proteins are citrullinated remains at the centre of many debates. PAD enzymes are necessary to catalyse protein deimination. PADs are not actively secreted in the intercellular space, although quite a few of their targets are extracellular proteins. Two immune-mediated membranolytic pathways (mediated by perforin and the membrane attack complex MAC), which are active in the RA joint and of importance in RA pathogenesis, have been proposed as possible ways by which PADs may be released in the joint microenvironment [66]. Several human citrullinated proteins have now been identified as target antigens of ACPA in RA (collagen, fibrinogen, vimentin, enolase, etc.) [40, 58, 59, 67, 68]. ACPA recognise citrullinated cross-reactive proteins but it is the presence of ACPA that is specific for RA rather than their protein antigens. The local context in which the proteins targeted for citrullination are expressed does not seem to have much importance; for example, filaggrin, which is an epithelial target of ACPA in RA, is not expressed in synovial tissue [58].

2.2. Clinical Relevance of ACPA. In RA patients, the presence of ACPA was associated with progressive and destructive disease outcomes [69–71], X-rays demonstrating the presence of erosions earlier and at a greater frequency in ACPA+ patients [72]. ACPA positivity was also associated with the presence of RF and shared epitope (SE) [50]. Combination analysis showed independent additive effects of these three factors for high radiological risk [35, 50, 69, 73]. Furthermore, the extraarticular manifestations that often determine the severity and comorbidity of RA were also closely associated with ACPA positivity [74]. Therefore, although disease onset can follow a similar course, the erosive and destructive nature of ACPA+ RA has resulted in clinicians and scientists considering the diseases as two distinct entities [75]. The main clinical use of these antibodies is however their diagnostic value, now recognised for over 25 years [45–50, 76] but only more recently used as a diagnostic biomarker. Sensitivity (~40%) and specificity (over 95%) of ACPA as diagnostic biomarker are now recognised in early inflammatory arthritis patients with a suspicion of RA [54].

A study using matched serial serum samples (blood donations) from early RA patients with short disease duration highlighted the importance of ACPA in predicting disease severity [50, 77]. The results also showed that radiological damage was more apparent in the groups which had been ACPA+ even before diagnosis was achieved. Radiological progression was also more substantial in this group after 2 years of follow-up. Importantly, these associations were not observed with RF. In contrast, ACPA titres were reduced over the course of disease when patients had a good response to therapy and titres of ACPA at baseline were higher in patients with poorer response. Taking this a step further, van Gaalen and colleagues prospectively studied a cohort of patients at an earlier stage of the disease in order to determine which markers may predict disease progression and persistence [78]. Individuals with an inflammatory arthritis but who did not fulfill the American College of Rheumatology (ACR) classification criteria were recruited. Multivariate analysis confirmed ACPA as an important independent predictor of RA with 93% developing RA within 3 years if ACPA+ at baseline. Given the clinical relevance of ACPA, it is not surprising that the new ACR/European League Against Rheumatism (EULAR) 2010 RA classification criteria have included ACPA titre in order to improve the diagnosis of early RA [79].

Studies which have evaluated ACPA titres while treating RA are emerging with variable observations (recently reviewed in [80]). Conventional antirheumatic drugs (DMARDs, including methotrexate, hydroxychloroquine, minocycline, or sulfasalazine) induce a marginal reduction in ACPA titres (>25%) over the course of treatment in about 50% of patients and a more pronounced decrease (>50%) in less than 30% of patients [81]. Response to TNF blockade was associated with lower baseline titres for ACPA, other clinical parameters being similar [82]. Response was also associated with a sustained reduction in ACPA titres, other studies showing similar ~30% reduction of serum ACPA titres after anti-TNF treatment [81–92]. However, several other reports showed little or no effect on ACPA titre [93–97]. Therapeutic B-cell depletion (using Rituximab an anti CD20 antibody depleting naive, memory, and preplasma cells but not plasma

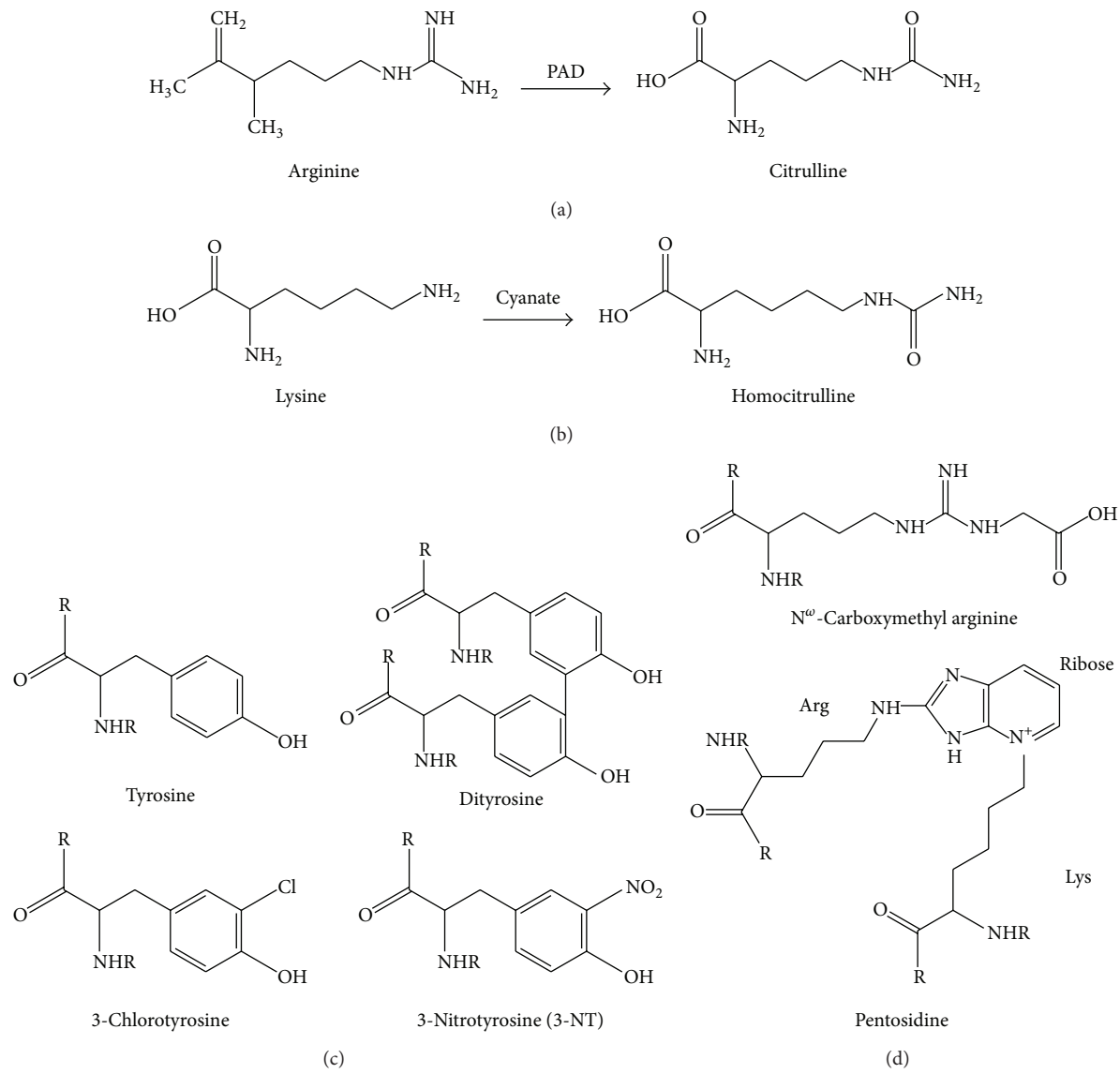

Figure 1: (a) Enzymatic generation of citrulline from arginine catalyzed by peptidylarginine deiminase (PAD); (b) non-enzymatic formation of homocitrulline by carbamylation of lysine by cyanate; (c) products of reactions between tyrosine with reactive oxygen species (forming dityrosine), reactive nitrogen species (forming 3-nitrotyrosine) and reactive chlorine species forming 3-chlorotyrosine; (d) examples of AGEs formed including carboxymethyl arginine and pentosidine (formed between an arginine and lysine residue).

cells) has marginal effect on ACPA titres [98–101] or not at all [102]. Significant reductions of ACPA titres were only observed in patients who responded to chemotherapy and higher titers of ACPA were associated with lack of clinical improvement [103].

3. Anticarbamylated Protein Antibodies (Anti-CarPA) in RA

The first demonstration of the deleterious effects of protein carbamylation in humans was made in the 1970s [104]. The quantification of carbamylation-derived products (CDPs) remains rarely used in clinical practice [105] and evaluation of antibodies against carbamylated proteins is just emerging. PTM through carbamylation has been implicated in vascular dysfunction in renal disease, atherosclerotic plaque formation [106], and antibiotic resistance [107].

3.1. Chemical Reaction. Unlike citrullination which is catalyzed enzymatically, carbamylation (often referred to as homocitrullination) is a chemical modification. It can occur ubiquitously in the presence of the reactive metabolite, cyanate. One of the cyanate sources is the spontaneous degradation of urea, which is constantly and ubiquitously generated in the body and always in equilibrium with cyanate. Therefore, wherever there is urea, there is cyanate and the potential for homocitrullination. However, under normal physiological conditions, concentrations of both are too low for any significant proteins modification.

Theoretically, any protein can be carbamylated *in vivo*. However, the susceptibility of each protein to such modification depends on various parameters, such as the number and accessibility of lysine and arginine amino groups, and the protein lifespan. As carbamylation is nearly irreversible, it is more likely to affect long-lived proteins as they may acquire homocitrulline residues over time [9]. Various CDPs can be formed, among them α-carbamyl-amino acids (or α-carbamyl-proteins) when α-amino groups are involved, and ε-carbamyl-lysine, also called homocitrulline, when ε-amino groups are involved (Figure 1(b)) [104]. The carbamylation of amine groups leads to a change in the charge of the molecule. Carbamylated derivatives may therefore acquire biological and antigenic properties that are different from those of the noncarbamylated molecules. On the other hand, carbamylation-induced conformational changes in proteins are also associated with partial or complete loss of protein functions [26], inhibition of enzymatic activities particularly relevant in RA such as matrix metalloproteinase-2 [108] or tissue inhibitor of metalloproteinase-2 [109], modification of hormonal activities (i.e., insulin [110], glucagon [111], adrenocorticotropic hormone [112], and erythropoietin [113]), and by affecting proteins such as haemoglobin [114], albumin [115], and collagen [116, 117].

3.2. Clinical Relevance. Carbamylated proteins may have a role in inflammation and as such in RA. They can modulate the functions of inflammatory cells, as evidenced by the inhibitory effect of carbamylated-albumin on the polymorphonuclear leukocyte respiratory burst [115, 118]. Carbamylation of low density lipoproteins (LDLs) by myeloperoxidase (MPO) seems to play a pivotal role inatherosclerosis [119–122] as well as in inflammation [106, 122–124]. Carbamylated collagen stimulates the production of active matrix metalloproteinase-9 (MMP-9) by monocytes, thus potentially enhancing extracellular matrix turnover [104, 125]. Therefore it is intriguing that homocitrulline also represents an immune target in RA.

In 2010, the presence of anti-CarPA (also called anti-homocitrullinated protein/peptide antibodies; AHPA) was demonstrated [26] in human sera and in an animal model of autoimmune arthritis expanding the set of known autoantibodies related to RA. Reactivity to carbamylated animal protein has been reported but the exact nature of the autoantigens recognised by anti-CarPA remains elusive. Fibrinogen is extensively accessible to homocitrullination and there are substantially more potential amino acid residues available for this type of modification in this molecule compared to citrullination [118]. The generation of antibodies to carbamylated regions of fibrinogen in RA patients was confirmed [118]. The RA specificity of anti-CarPA was suggested ($n = 84$) as these antibodies were not found in patients with other inflammatory rheumatic conditions SLE ($n = 37$, 5% weakly positive results) and psoriatic arthritis ($n = 37$, 3% weak reactivity) or normal healthy individuals ($n = 27$). The fact that some RA patients have reactivity to carbamylated but not citrullinated fibrinogen supports the concept that homocitrullination can generate unique structural antigens on proteins, that is, although cross-reactivity between ACPA and anti-CarPA was recently reported [118]. In another study carbamylated vimentin was used to detect anti-CarPA in RA patients [126]. Carbamylated vimentin was significantly more reactive than carbamylated enolase which suggests that the amino acids surrounding the modification (or even the whole molecule) are contributing to its immunogenicity [126]. The known association between ACPA and MHC class II SE expression [127, 128] was very recently supported for anti-CarPA with data showing that homocitrulline and homocitrullinated peptide could potentially bind to the SE [118].

Anti-CarPA IgG were found in the serum of 45% of RA patients and IgA anti-CarPA in 43% [9]. The presence of anti-CarPA partially overlapped with the presence of ACPA, but most interestingly was also found in 16% of RA ACPA− patients (30% were positive for anti-CarP IgA) [9]. The presence of anti-CarPA was detected in over 30% of such patients when ACPA− therefore offering an alternative biomarker to help the diagnostic of RA [9]. Furthermore, anti-CarPA positivity was related to clinical outcome [9]. Detection of anti-CarPA at disease presentation was predictive of a more destructive disease course (evaluated using Sharp-van der Heijde scores). Importantly, this was verified in both ACPA+ and ACPA− RA, notably offering a novel biomarker for the diagnostic of RA and, furthermore, a clinically useful prognostic biomarker for ACPA− disease.

In individuals with seropositive arthralgia (340 patients positive for rheumatoid factor (IgM-RF) and/or ACPA+), the prevalence of Anti-CarPA was 39% [129]. The presence of anti-CarPA did not correlate with RF. Anti-CarPA were associated with progression towards RA. Furthermore, established association indicated that anti-CarPA positive arthralgia patients were more likely to develop RA and notably within a shorter time frame compared to individual with only RF and/or ACPA positivity. Such increased risk of developing RA was maintained in double positive ACPA/anti-CarPA arthralgia patients even after correction for ACPA. Higher anti-CCP antibody levels were also observed in anti-CarPA positive patients. These observations suggest that alternative seropositivity in RA patients may each represent a different disease entity with its own genetic/environmental contributions [129, 130].

Despite these promising initial findings, further research is needed to clarify anti-CarPA responses and how they could contribute to the clinical management of RA. Additional studies using patients with a suspicion of RA as controls are needed to determine the specificity of anti-CarPA for RA diagnostics. Whether their presence predicts the development of (ACPA−) RA in patients suffering from unclassified joint complaints such as arthralgia or early signs of inflammatory arthritis remains to be established [9, 130]. Links with environmental factors (smoking, alcohol intake, body mass, hormonal status, periodontal disease, etc.) remains to be elucidated. Despite the association with SE, other genetic factors may be relevant. Early aggressive treatment in RA has been shown to prevent future damage [131, 132]. The clinical utility of a prognostic biomarker such as anti-CarPA in the management of ACPA− patients with respect to their risk of developing a more severe disease remains of great interest [9].

4. Antioxidized Protein Antibodies in RA

Oxidative stress is a term that is used to describe situations in which an organism's production of oxidants exceeds the capacity to neutralize them. The consequences are damages to cell membranes, lipids, nucleic acids, proteins, and constituents of the extracellular matrix such as proteoglycans and collagens. Several lines of evidence suggest a role for oxidative stress in the pathogenesis of RA [133–139]. Epidemiologic studies have shown an inverse association between dietary intake of antioxidants and RA incidence [140–143], and, reciprocally, an inverse association between antioxidant levels and inflammation [39, 144, 145]. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen (such as superoxide and peroxides), and a natural byproduct of the normal metabolism of oxygen. ROS are able to oxidize various amino acids, according to their oxidation potential. They have important physiological roles in cell signaling, apoptosis, ion transport systems, wound healing and blood homeostasis, and also the induction of host defense (respiratory burst), genes, and inflammatory responses. They can also be detrimental in situations of stress when their levels dramatically increase to the point of harming cells. This notably occurs when antioxidants normally protecting cells (superoxide dismutases, catalases, peroxidases, peroxiredoxins, and others) are unable to manage the amount of ROS produced [146].

Oxidative modifications by ROS are attractive candidates as instigators of autoimmunity and this might involve a process of "oxidative PTM intolerance" [10], resulting in a primary B-cell response against the posttranslationally modified self-antigen [10]. Oxidative stress-induced antibodies to carbonyl-modified protein have also been found to correlate with severity of chronic obstructive pulmonary disease [147] and SLE [148].

4.1. Chemical Reaction. Oxidative stress occurs during inflammation and causes proteins to become damaged by reactive species such as reactive oxygen, nitrogen, and chlorine species. NADPH oxidase is a major source of ROS in arthritic joints. This enzyme reduces O_2 generating large amounts of superoxide radical anion $^{\bullet}O_2^-$, which is considered the primary ROS and may be further reduced to H_2O_2, which in turn can be converted into highly reactive $^{\bullet}OH$ or react with Cl^- to generate HOCl (in a reaction catalyzed by the enzyme myeloperoxidase). iNOS also generates $^{\bullet}NO$ which is converted to $ONOO^-$ by reacting with $O_2^{\bullet-}$ [149, 150]. In addition, under conditions of oxidative stress, species such as peroxynitrite ($ONOO^-$) may be generated resulting in nitration of tyrosine residues to form 3-nitrotyrosine (3-NT) (Figure 1(c)) [138, 151, 152]. Indeed, antibodies recognizing 3-NT have been identified in the synovium of RA patients and correlate with disease activity [152].

In addition, these reactive species generate "secondary" reactive species such as lipid peroxidation products. Nonenzymatic oxidation by sugars can react directly or generate reactive products such as glyoxal and methylglyoxal; these reactive carbonyls are capable of undergoing Maillard reactions, first forming a Schiff base with the amine group of amino acids, such as lysine or arginine. This intermediate can then undergo an Amadori rearrangement to form stable advance glycation end product (AGE) such as carboxymethyl arginine or initiate peptide cross-linking to form pentosidine (Figure 1(d)) [153]. The presence of these PTM on protein increases as well as modifies their natural antigenicity and antibodies against the native and modified forms of these proteins are usually noncross-reacting and were detected in RA despite the absence of hyperglycemia [17, 154]. AGEs can have damaging effects on collagens by forming irreversible cross-links between the fibers in the triple helix [155–158].

Another potential reaction is chlorination of aromatic amino acids, in particular tyrosine residues, including 3-chlorotyrosine, within the polypeptide backbone (Figure 1(c)) [159]. Under conditions of oxidative stress, species such as peroxynitrite ($ONOO^-$) may be generated resulting in nitration of tyrosine residues to form 3-nitrotyrosine (3-NT) (Figure 1(c)) [138, 151, 152]. Indeed, antibodies recognizing 3-NT have been identified in the synovium of RA patients and correlate with disease activity [152]. Exposure of collagens to peroxynitrite results in nitration of tyrosine residues and formation of posttranslationally modified nitrotyrosine. These compounds are negatively charged and further disrupt the collagen structure. ROS levels are increased in autoimmune diseases such as RA and SLE. The overproduction of ROS may exceed the capacity for radical scavenging by antioxidant enzymes or small inhibitors. Exposure of proteins, nucleic acids, or cell membrane and free lipids to ROS modifies amino acids creating PTM proteins and lipids by initiation of peroxidation. There is no recognized specificity to the protein that can be modified and oxidation depends on steric and stochastic factors; however, enrichment for amino acid motif YXXK in the vicinity of chlorination has been observed [160]. Oxidized proteins identified in RA include collagens I, II, IX, and XI, proteoglycans, and hyaluronan. Increased oxidation of lipids is also a known feature of RA, with the appearance of foam cell-like structures within the rheumatoid synovium [39, 136, 161].

In the context of RA, immunoglobulins themselves can undergo glycation to generate AGE-IgG. Autoantibodies to such modified-IgG were also shown to be specifically associated with RA, whereas the actual formation of AGE-IgG was directly related to the intensity of the inflammatory response but was not specific to RA [162–164]. Similarly, modification of IgG by HOCl or peroxynitrite can induce a T-cell response against IgG HOCl and peroxynitrite in RA [165].

4.2. Clinical Relevance. The key ROS present in inflamed joints are superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$), which are involved in acute and chronic inflammation [6, 15]. Such ROS have been identified in synovial fluid of 90% of patients with RA, with a shift in the oxidant/antioxidant balance in favour of lipid peroxidation, which lead to the tissue damage observed in joints [166, 167].

Exposing CII to conditions which simulated those found in an inflamed joint, resulted in chemical modifications of native CII [6]. CII treated with hydroxyl radical ($^{\bullet}$OH-CII), hypochlorous acid (HOCl-CII), and peroxynitrite ($ONOO^{-}$-CII) demonstrated positivity for binding to autoantibody specifically recognizing these various CII-modified forms in sera from 93 of early RA patients in addition to glycation of CII (Gly-CII) carried out with ribose. No cross-reactivity with native CII was observed but reactivity to native CII was seen in <20% of sera [6]. Moreover, no anti-ROS-CII reactivity was detected in other inflammatory arthritis conditions (including psoriatic arthritis, SLE, ankylosing spondylitis, palindromic arthritis, scleroderma, Behçet's disease, primary Sjögren's syndrome, fibromyalgia, tendonitis and reactive arthritis [6].

In 2005 we showed that CII post-translationally modified by ROS (ROS-CII), present in the inflamed joints, is an autoantigen in RA [6]. In addition, cartilage damage as a result of collagen oxidation by glycation and formation of AGE-CII are evident despite the absence of hyperglycemia [168].

We have recently measured auto-reactivity to ROS-CII in synovial fluid (SF) and serum samples taken from various phases of RA [38] and demonstrated that anti ROS-CII reactivity is not related to markers of inflammation such as CRP and ACPA and has potential to serve as biomarker for several purposes. We observed high anti-ROS-CII reactivity in DMARD naïve early RA regardless of whether patients were ACPA+ or ACPA− and with no correlation with DAS28. The sensitivity and specificity of the binding of autoantibodies to ROS-CII in early RA compared with healthy controls (HC) was 92% and 98%, respectively. ROS-CII reactivity was lower in RA patients having received their first DMARDs treatment and achieving a good response. We also showed that anti-ROS-CII reactivity considerably vary over time in a mixed cohort of RA patients with established disease on several type of treatment [38]. This was in contrast to levels of ACPA which did not. We could not directly associate these changes with DAS28, however, patients in this cohort all had (very) active disease and it was impossible to fully ascertain longitudinal variation between active/remitting diseases.

Further pilot data showed that in a small cohort of ACPA+ arthralgia patients with no synovitis, only those within a few weeks (~12) of developing clinical evidence of synovitis were positive for anti-ROS-CII reactivity while those who developed symptoms after a much longer delay were negative. Interestingly, in a study conducted in type 1 diabetes, a condition associated with RA [169], anti-ROS-CII reactivity was restricted to SE-containing DRB1∗04 alleles (OR 3.62; 95% CI 1.12–11.74), known to confer the greatest risk for developing RA. Further work needs to establish whether patients with inflammatory synovitis but not yet RA (i.e., undifferentiated arthritis) would be positive however, 93% of early RA were, which altogether, strongly suggest a direct association with the development of synovitis, hence offer a measurable biomarker of disease development alongside the RA continuum [38].

Anti-TNF treatment showed reduction in oxidative stress, and these correlated with an improvement in disease activity [170–173]. However studies evaluating changes in anti-ROS autoantibody levels after RA anti-TNF treatment are still missing. Our own data however suggest variation [38] which will need to be confirmed before any biomarker value can be confirmed.

In addition to CII, studies of RA synovial fluid and tissue have demonstrated oxidative damage to hyaluronic acid [174], lipid peroxidation products [175, 176], oxidized low-density lipoproteins (ox-LDL) [136], and increased carbonyl groups reflective of oxidation of other proteins [136, 162, 177–179]. Evidence of oxidative damage to cartilage, extracellular collagen, and intracellular DNA has also been demonstrated. Protein chlorination occurs in RA at the disease site (i.e., synovial fluid and tissue) [159, 180] and it was proposed that this could be the link between arthritic inflammatory reactions and the initiation of autoimmune antibody responses. The risk associated with ox-LDL in RA is mostly related to cardiovascular risk hence not specific to RA. Ox-LDL are strong autoantigens, essential to the development and progression of the plaque in atherosclerosis as LDL molecules only become immunogenic due to the oxidative modification during early atherogenesis [181, 182]. Anti-oxLDL antibodies are extensively prevalent in patients with autoimmune diseases, including RA [183], SLE [184, 185], and antiphospholipid syndrome (APS) [186, 187], diabetes mellitus [188, 189], uremia [190]. Anti-ox-LDL antibodies bind ox-LDL and generate immune complexes. Circulating immune complexes are not in themselves harmful. They cause damage only if they are deposited in tissues (notably in the endothelium), resulting in inflammation [191, 192]. T-cells, primarily $CD4^{+}$ cells, have been found associated with these immune complex depositions [193–196]. Cardiolipin is also the target of oxidation (ox-CL). Anti-ox-CL antibodies are frequent in APS patients [197, 198] due to formation of neoepitope on cardiolipid, possibly with cross-reactivity with anti-oxLDL antibodies notably in patients with SLE [197, 199, 200].

Finally, autoantibodies targeting AGE-modified IgG are also present in serum of RA patients [154, 201]. Autoantibodies against AGE-IgG might be helpful in monitoring progress in the RA disease continuum and in combination with other clinical features of the RA might be a useful diagnostic tool [201].

5. Animal Model Testing of PTM-Targets and Antibody to PTM-Proteins

An important discovery in the association between anti PTM-protein and RA was the demonstration that these antibodies and their targets are both arthritogenic in animal models. The citrullinated forms of collagen II appeared more arthritogenic in rats than native collagen II [202, 203]. Within the human synovium, the immune reaction between citrullinated fibrin and ACPA results in the activation of effector mechanisms. Immune-complex containing ACPA and CII citrullinated peptide can activate blood macrophages via FcR resulting in the production of TNF-alpha in mice [204, 205]. A similar response by synovial macrophage would promote local inflammation which in turn will favour plasma

extravasation and fibrinogen polymerisation. These deposits then could get citrullinated by locally expressed PAD and therefore become new target for ACPA closing the circle for self-perpetuation. PAD 2 and 4 are expressed in the RA synovium (and in other inflamed tissues) but importantly in correlation with the intensity of inflammation [65]. The arthritogenicity of chlorinated-CII versus native CII (Cl-CII) was also demonstrated in a rat strain [159, 206, 207]. This might be caused by an increased immunogenicity of Cl-CII, resulting in a stronger antibody-inducing capacity. Hydroxyl radical modification of collagen type II (OH-CII) also increases its arthritogenicity and immunogenicity and resulted in an early and more severe arthritis compared to native CII [208].

Anti-CarPA are now extensively studied to clarify whether they are directly involved in the pathogenesis of RA. carLDL induce an IgG response in LDL-$R^{-/-}$ mice and autoantibodies also bind to humans plasma proteins [209]. The immunogenicity and an arthritogenic role of the anti-homocitrulline immune responses were confirmed using animal model of arthritis. Immunization of several mouse lines (NMRI, BALB/c, and C57bl/6) with carbamylated-peptides leaded to a Tcell dependent activation of B-cell and the production of autoantibody [26]. Direct intra-articular injection of the carbamylated-peptides in these mice induced a severe erosive arthritis [26]. This study was also the first to report the presence of anti-CarPA in RA patients, both in the joints and circulation, and importantly in relation to erosions. Rabbits immunized with carbamylated-proteins resulted in high-affinity antibodies to homocitrulline-containing collagen telopeptides and to less strong anticitrulline-containing telopeptides and mutated citrullinated vimentin [27].

The exact pathogenic potential of anti-CarPA therefore appears to be similar to that of ACPA [210]. The possibility of cross-reactivity between these two antibody types demands further investigation into the identification of true targets in RA. If antibody responses to citrulline and homocitrulline are indeed arthritogenic, important questions remain: which antibodies are pathogenic? Is it the specificity of the target antigen, the quantity, and diversity of the response, and/or merely the binding affinity to available targets in the arthritic joint, which are important in determining arthritogenicity and clinical disease progression [211]?

6. Autoantibodies and B-Cell Development in RA

To date the overall development of the anti-PTM-protein antibody producing B-cell clones remains poorly understood. ACPA of the IgG are the immunoglobulin isotype specifically associated with RA [57]. This suggests that an immune reaction leading to the development of IgG ACPA is taking place at some point before the onset of RA. ACPA of the IgG subclasses 1 to 4, are detected; however a major bias is observed towards an IgG1 (86% alone) and IgG4 but with a very limited involvement of IgG2 and 3 [57]. Such bias correlates closely with an imbalance toward Th1 polarisation which is well described in RA.

The presence of B-cell reactivity to Cl-CII in RA patients was established [6, 38]. Spontaneous production of ACPA could only be obtained from B-cells isolated from the synovial fluid and bone marrow of IgG ACPA+ RA patients. The presence of IgG ACPA up to 15 years before symptoms has also been reported [44, 212]. A cross-sectional study also reported that titres of IgG ACPA appeared higher shortly before the onset of RA suggesting reactivation of the producing B-cells [44]. Finally, the strongest argument in favour of this immune reaction is the T-cell response to citrullinated peptide observed in RA patients but not in healthy controls [213, 214].

The hypothesis that each stage of the disease represents an evolution in ACPA specific B-cell maturation is therefore attractive. At this stage, however, it has not yet been either demonstrated or nullified. ACPA have been shown to be present at detectable levels years before the first manifestation of RA with high risk for these individuals to develop RA within 5 years [22, 124]. In the preclinical phase (ACPA positivity but no disease symptoms), ACPA-IgG circulate (sometimes for many years) suggesting that, at least, isotype-switched ACPA-specific B-cells are present. During this disease initiation phase, cross-sectional analysis also showed that ACPA titres are higher just before onset of symptoms [215–217].

In vivo, differences in ACPA levels [73], fine specificity or epitope spreading [218, 219], avidity [220–222], isotype usage [223], and glycosylation [224] may be associated with differences in the potential to activate effector mechanisms, thereby influencing their biological potency [220]. Epitope spreading is often a hallmark of progressive B-cell responses and was described for ACPA and was associated with an increase and/or shift in antigen recognition during the course of an autoimmune response [218, 225, 226]. Fine mapping analysis of preclinical sera compared to early and established RA showed subtle difference in either the identity or the numbers of epitope detected between the different phases of the disease [43, 215, 216, 227, 228]. Our own unpublished data using the same platform showed a particular epitope detected exclusively in synovial fluid which may represent a unique specificity with local retention of the ACPA (as not detected in sera) suggesting local B-cell reactivity. Despite the association between the presence of anti-CarPA and the broadening of ACPA's fine specificities, anti-CarPA are generated independently of ACPA and, to date, are largely noncross-reactive although the panel of currently available carbamylated antigens remains limited. The effect of anti-CarPA in arthralgia patients is notably independent of the effect of ACPA (after correction) [129]. It will be of great interest to expand the investigation for anti-CarPA and anti-ROS specificities, particularly among ACPA− patients and determine whether these antibodies could have pathological effects in RA patients [229].

A few studies already have shown that circulating ACPA-IgG differ in avidity but still relatively little is known about avidity maturation of ACPA before and during the RA continuum [220]. Lower ACPA avidity was reported in ACPA+ asymptomatic individuals compared to avidity in ACPA+ patients with joint symptoms (arthralgia), which was similar

to avidity observed in established RA patients [221, 222]. Following immunoablative therapy, ACPA-IgG of low avidity developed again which suggested a newly generated autoimmune response [103]. However, the development of high avidity ACPA-IgG remains speculative and their presence may be only characteristic for specific RA patients, refractory, or less responsive to immunosuppressive treatment [103]. As mentioned previously, all immunoglobulin isotypes (IgM, IgA, and IgE ACPA) contribute to overall ACPA activity in RA serum [223, 230, 231]. Although autoantibodies of IgG isotype are generally the most relevant, other studies have shown that IgA were also specific for RA [231, 232]. IgG are associated with radiographic progression in RA [73, 77], but patients positive for IgA-ACPA with recent onset RA were reported to suffer a more severe disease course over the first three years [233] and the higher the number of different isotypes, the greater long-term radiographic joint damage at 5-year follow-up [234]. This data suggests that the development of the anti-CCP isotype repertoire occurs early in the course of arthritis [217, 235].

Glycosylation of the Fc-part of antibodies affects their function with either a pro- or an anti-inflammatory outcome functionality [236]. The glycosylation profile of ACPA in RA is characterised by a low content of galactose (hypoglycosylation) and sialic acid residues [224]. Hypoglycosylation of ACPA was more pronounced than that of total IgG1, resulting in a proinflammatory Fc-glycosylation pattern of ACPA that could be one mechanism driving inflammation in RA [224, 237]. Fc-glycosylation of ACPA showed significant differences between SF and serum and, in contrast to ACPA in serum, ACPA isolated from SF were found to be highly agalactosylated [224]. IgG glycosylation showed association with RA activity [238]; however, this pattern was not useful to predict clinical response to MTX and anti-TNF treatment in RA [239]. Finally, the specific ACPA-Fc hypoglycosylation was detected already 6 months prior to RA onset [237].

TNF-alpha is an important factor in GCR. If disease initiation was to coincide with a time when B-cells are undergoing early TNF/GCR dependent maturation phases, TNF-blockade in early disease should result in definite ACPA titre reduction. Studies of the effect of TNF blockade in early disease are still lacking and are in progress. In established disease TNF-blockade is clinically efficient but may not be able to interfere with the course of B-cell differentiation anymore; hence studies analysis ACPA titres over the course of anti-TNF therapy in established disease showed variable results. In long lasting RA, B-cell ablation does not result in major ACPA titres reduction in contrast to total IgM titres (but not IgA and IgG) [98–102]. Plasma cells not being directly depleted by the therapy due to the fact that they do not express CD20, suggesting that, in established RA, ACPA-LL-PC are present. The small reduction in ACPA titres reported after B-cell depleting therapy (< than 20–30%) nevertheless suggests that a small pool of ACPA producing cells (memory and SL-PC) are affected by the therapy [240] notably as SL-PC were evidenced in the synovium of RA patients and were shown to secrete autoantibodies including ACPA [241, 242].

The direct analysis of ACPA producing B-cells has proven difficult. The classic molecular tools used to label antigen-specific B-cells have not been very successful to date in isolating ACPA-B-cells (MHC-tetramers, biotinylated-peptide specific for ACPA BCR). ACPA-specific T-cell clones were detected in established disease [213]. However, it remains to be determined whether they play a role in anticitrullination response in RA and most importantly when. The further elucidation of the B-cell maturation path will require serial samples from preclinical stages, then early and fully established disease and the examination of somatic hypermutation and affinity maturation.

Data generated to date therefore establish the presence of an immune reaction resulting in the secretion of ACPA. Yet, the primary stimulus leading to such production remains unknown. An environmental association between the presence of ACPA and smoking has been established [219, 243], and smoking is the most recognised environmental factor reproducibly associated with RA. Recently, silica exposure has also been linked to RA [244–246] and other immunologically mediated diseases [247]. A study looking at the link between genetics and environmental factors has shown that the presence of ACPA was associated with the shared epitope HLA-DRB1 in a dose dependent manner but that smoking was only important in patients positive for ACPA secretion [75, 248, 249]. This observation may suggest that physiological processes associated with smoking have a role in the initial generation of ACPA. A model has been proposed in which smoking (and other agents) triggers the production of IgG ACPA [250]. A second event leads to the citrullination of synovial proteins which would direct ACPA immunity towards the joints [218]. The disease would then be initiated, and, if uncontrolled, become chronic. The role of ACPA in the self-maintenance of RA, once it is established, is a more easily understandable model; however, the exact nature of the citrullinated protein target of ACPA remains elusive.

Therapeutic B-cell depletion results in disease improvement (by 6 to 10 weeks) but not in ACPA serum titres reduction. Synovial depletion of B-cells however is delayed (26 to 30 weeks) probably accounting for the time necessary to eliminate short-live plasma cells from the tissue [98]. Therefore some of the benefit of the therapy must be related directly to the removal of B-cell (not plasma cells) from the tissue. There has been speculation that synovial B-cells in RA may have some unusual lack of responsiveness abolishing their proliferative capability (anergic B-cells) leaving intact their antibody production [251]. Two-way interaction between B-cells and T-cells may be a great relevance here: B-cells provide signals to T-cells through antigen presentation and T-cells provide "help" to B-cells through the delivery of cytokines and cell mediated stimulation, creating a self-sustained feedback loop. Whether B-cells stimulate T-cells to stimulate B-cells, vice versa or, more simply, which cell makes the initial mistake and trigger autoimmunity has been a point of debate for years [252]. In favor of B-cell, an argument has been put forward for $CD4^+$ T-cell activation being dependent on B-cells in the synovium, in the context of a GCR and in a HLA-DRBI restricted manner where the antigen is harbored by the B-cells [253]. Breaking this loop should, in

itself, restore self-tolerance. Therefore, the removal of ACPA secreting B-cell may be more relevant to reestablishing self-tolerance in RA as it may remove ACPA themselves but also the source of activation for the two-way interaction between B- and T-cells.

7. Conclusion

The findings presented in this review support the hypothesis that PTM of self-antigens, in RA and in inflammation in general, are a cause of the formation of neoepitopes giving rise to autoantibodies. Whether the breakdown of tolerance occurs because antibodies against modified self-protein are promiscuous and bind both the modified and unmodified self-antigen or whether they are truly specific for modified proteins is unclear. Nevertheless, these processes contribute to the vicious circle of chronicity by providing novel immune reactivity, resulting in further stimulation of the immune response against self-antigens. With the advancement of research methodology it should be expected that novel specificities of autoantibodies against PTM proteins could be discovered in patients with autoimmune diseases. Many of these autoantibodies could have significant biomarker potential. Clearly, animal models suggested therapeutic advantages in preventing the generation or binding of potentially pathological autoantibodies to the extracellular matrix collagen and collagen-like structures. B-cell responses to native CII have long been known in RA but, as PTM-CII reactivity induces or worsens experimental arthritis, it is possible that blocking PTM could ameliorate arthritis [7, 206]. As such, antioxidants and inhibitors of oxidative enzymes have already been shown to ameliorate arthritis in animal models [254, 255]. The translation of antioxidant therapies to human clinical studies has produced disappointing results, but targeted approaches using novel inhibitors of oxidative enzymes offer new hope for the treatment of RA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Circulating miRNA-125b Is a Potential Biomarker Predicting Response to Rituximab in Rheumatoid Arthritis

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Although biologic therapies have changed the course of rheumatoid arthritis (RA), today's major challenge remains to identify biomarkers to target treatments to selected patient groups. Circulating micro(mi)RNAs represent a novel class of molecular biomarkers whose expression is altered in RA. Our study aimed at quantifying miR-125b in blood and serum samples from RA patients, comparing healthy controls and patients with other forms of rheumatic diseases and arthritis, and evaluating its predictive value as biomarker for response to rituximab. Detectable levels of miR-125b were measured in total blood and serum samples and were significantly elevated in RA patients compared to osteoarthritic and healthy donors. The increase was however also found in patients with other forms of chronic inflammatory arthritis. Importantly, high serum levels of miR-125b at disease flare were associated with good clinical response to treatment with rituximab three months later ($P = 0.002$). This predictive value was not limited to RA as it was also found in patients with B lymphomas. Our results identify circulating miR-125b as a novel miRNA over expressed in RA and suggest that serum level of miR-125b is potential predictive biomarker of response to rituximab treatment.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory autoimmune disorder that may affect many tissues and organs but principally attacks the joints. RA is a multifactorial disease of unknown aetiology and complex pathogenesis, consisting of at least two subtypes, with different causes and severity [1, 2]. The clinical course of RA fluctuates and prognosis is unpredictable. A major issue is that up to 70% of patients with recent onset of RA show evidence of radiological erosions within 3 years. In the long term, major outcomes include joint deformity and misalignment, need for joint replacement surgery, functional disability, and premature death due to accelerated atherosclerotic cardiovascular and coronary heart diseases [3]. Numerous studies have demonstrated that aggressive treatment of early RA results in better clinical outcome [4]. Nine biologics are available for RA treatment, often used in combination with methotrexate. Each type targets a specific inflammatory mechanism and has largely improved the outcome of RA in many patients [5, 6]. Biologics are however prescribed on a trial-and-error basis when methotrexate alone has failed, response is heterogeneous, and roughly one-third of patients are nonresponders. It may thus take some time to find the

Table 1: RA patient characteristics.

	Blood analysis	Sera analysis			
	RA patients ($n = 16$)	Responders ($n = 16$)		Nonresponders ($n = 16$)	
	Mean ± s.d.	Mean ± s.d.		Mean ± s.d.	
Age (years)	59.1 ± 3	59.8 ± 3		57.6 ± 15	
Gender (%F)	87.5	80.8		85.7	
Disease duration (years)	16 ± 2.2	13.7 ± 1.45		16.3 ± 2.6	
ACPA positive (%)	81.3	87.5		78.6	
DMARDs failed	3.5 ± 0.25	3.7 ± 0.25		3.5 ± 0.4	
		M0	**M3**	**M0**	**M3**
Baseline DAS 28	5.4 ± 0.25	6.2 ± 0.2	3.7 ± 0.2	4.8 ± 0.3	5.2 ± 0.3
Baseline CRP level (mg/L)	12.3 ± 4	29.4 ± 6	11.4 ± 4	20.2 ± 8	15.3 ± 5
Baseline HAQ level	1.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.2	1.75 ± 0.2	1.5 ± 0.2

DAS 28 score: a measure of RA activity score (28 joints were evaluated); DMARDs: disease modifying antirheumatic drugs; s.d.: standard deviation; ACPA: anticitrullinated protein/peptide antibodies; CRP: C-reactive protein l; HAQ: health assessment questionnaire.

best drug for a patient. Considering the high cost of biologics and the possibility of severe side effects, the identification of predictors of response to biologic therapies would improve patient care and medical cost-effectiveness.

Clinical and serological characteristics solely are insufficient to predict treatment outcome. Anticitrulline peptide antibodies (ACPA), elevated CRP, serum levels of EGF, MCP-1 or TNFR, and gene profiling have been proposed to identify responders to biologics [7–9]. More recently, micro(mi)RNAs have emerged as a new category of biomarkers and patients with RA have clear alterations of the expression of miRNAs [10]. Conserved throughout evolution, miRNAs are an abundant class of endogenous, short noncoding, regulatory RNA molecules that control gene expression in a sequence-specific manner by targeting mRNAs for degradation or translational repression. The potential value of miRNAs as molecular biomarkers for diagnosis, prediction of disease outcome, and prediction of therapeutic response is well documented in cancer [11], whereas in RA it remains poorly explored [12, 13]. Since 2008, the presence of miRNAs in human body fluids has been documented, and several studies reported the optimization of direct miRNAs detection in blood or sera [14, 15]. Recently, Murata et al. identified a signature of seven plasma miRNAs as diagnostic biomarkers specific for RA patients, even ACPA-negative [16]. Nevertheless, there are no reports so far about miRNA expression predicting treatment outcome in patients with RA.

Rituximab is the world's best-selling cancer drug and was originally developed to treat non-Hodgkin's lymphoma [17]. It is a chimeric monoclonal antibody directed against the CD20 surface antigen of B cells and FDA approved in 1997 to be used in combination with methotrexate to treat RA patients who have moderate-to-severe active disease and have failed one or more anti-TNF drugs [18]. Large randomized controlled trial has demonstrated efficacy in longstanding RA patients who failed to respond to methotrexate or anti-TNF drugs [19]. Despite effective depletion of circulating B cells in nearly all patients [20] and complete resolution of inflammation in some cases, only half of them however respond to rituximab treatment. Consequently, there is much interest in identifying molecular biomarkers that predict whether a patient will respond or not to rituximab. In addition to sharing a common treatment, RA and a substantial fraction of lymphomas share pathogenic inflammatory responses due to aberrant activation of NF-κB signals [21]. Since miR-125b is an evolutionary conserved miRNA that regulates signal pathways of inflammation [22], B cell differentiation [23, 24], TNF production, and apoptosis [25] that are biological pathways of importance for both lymphoma and RA, we assessed whether miR-125b is deregulated in RA and useful as potential biomarker predictive for rituximab response.

2. Materials and Methods

2.1. Patients and Healthy Controls. Fresh peripheral blood and serum samples were obtained from healthy donors ($n = 13$) with no history of autoimmune diseases or patients with osteoarthritis (OA, $n = 7$) and rheumatoid arthritis (RA, $n = 48$) fulfilling the 2010 ACR/EULAR criteria [26]. Among the 48 RA patients, we included 32 patients treated by rituximab. Samples were also obtained from patients with receptor-associated periodic syndrome (TRAPS, $n = 5$) and spondyloarthropathies (SpA, $n = 15$). Informed consents were provided in accordance with procedures approved by the local human ethics committee (Comité de Protection des Personnes Sud Méditerrannée IV: ID RCB 2008-A01087-48).

The characteristics of RA patients are summarized in Table 1. Patients were assessed for overall disease activity using the 28-joint-count Disease Activity Score (DAS28) as previously described [27]. The criteria for patient eligibility were combined methotrexate (MTX) treatment; DAS28 ≥ 4.5; and resistance to at least 2 Disease Modifying Antirheumatic Drugs (DMARDs) (MTX and anti-TNF included). For one month or more before the start of this study, every patient was given stable doses of oral corticosteroids and did not receive any intra-articular steroid injections. Patients were treated with rituximab (MabThera, Roche) as recommended by the manufacturer and the French Drug Agency ANSM (intravenously 1,000 mg one time at day 0 and day 15). RA patients were separated in two subgroups according to their clinical response to the rituximab after 3 months (M3) of treatment (DAS28 M3-M0), following the EULAR

Table 2: B lymphoma patient characteristics.

	Sera analysis	
	Responders ($n = 8$)	Nonresponders ($n = 5$)
Median age (years)	56 (52–79)	59
Gender (M/F)	7/1	2/3
Histology		
Diffuse large B cells	7	1
Indolent	1	4
Stage		
I/II	3	2
III/IV	5	3
Treatment		
Rituximab monotherapy	5	1
Rituximab chemotherapy	3	4

criteria: for nonresponders (NR), DAS28 > 5.1 and the ratio DAS28 M3-M0 ≤ 0.6; for good and intermediate responders (R), DAS28 < 3.2 or DAS28 > 3.2 and DAS28 M3-M0 > 1.2.

Staging procedures for lymphoma patients ($n = 13$) were in accordance with international recommendations [28]. Clinical characteristics are summarized in Table 2. None of these patients presented concurrent RA. Patients were treated either by rituximab alone for four weekly infusions or by rituximab-chemotherapy regimen when appropriate. Response was assessed 4–6 weeks later according to international recommendations [28, 29]. Patients in complete or partial response were classified as responder patients and those in stable disease or progressive disease were classified as nonresponder patients.

2.2. Blood RNA Isolation and miRNA Quantification Using RT-qPCR. Blood samples were collected using EDTA-coated tubes (BD Vacutainer 5 mL; BD Diagnostics, France) according to standard procedure. Aliquots of 0.5 mL of blood samples were immediately transferred to 1.2 mL of RNA later medium (Applied Biosystems) and stored at −20°C. Total RNA was extracted using a modified protocol from the Ribopure-Blood RNA isolation kit (Applied Biosystems). Briefly, 10 μL glacial acid (Sigma, France) was added to blood cell lysate (800 μL, steps 1 and 2 according to the manufacturer's instruction). The samples were extracted with acid phenol/chloroform, 1 mL of GuSCN lysis solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sodium N-lauroyl sarcosinate), and 0.1 M beta-mercaptoethanol and 1.25 volumes of ethanol were added to the aqueous phase. The samples were passed through a filter cartridge and washed, first with wash solution 1 (70% EtOH/30% GuSCN lysis solution) and second with wash solution 2 (80% EtOH/50 mM NaCl). The RNA was eluted in 100 μL elution solution preheated to 80°C and stored at −20°C. The concentration and integrity of RNA were determined by NanoDrop ND-1000 spectrophotometry (NanoDrop Tech, Rockland, Del) and by a Bioanalyser Agilent 1.

For miRNAs analysis, 10 ng of total RNA was reverse transcribed using 50 nM human microRNA specific stem-loop RT primers, 50 units/μL MultiScribe reverse transcriptase, 10XRT buffer, 100 mM each dNTPs, and 20 units/μL RNase inhibitor (Applied Biosystems). Reaction mixtures (15 μL) were incubated in a thermocycler Mastercycler (Eppendorf, France) for 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C and then maintained at 4°C. Real-time PCR was performed on the resulting complementary DNA using TaqMan microRNA specific primers and TaqMan Universal PCR Master Mix. All the experiments were performed according to the manufacturer's protocols, using a pipetting robotic platform epMotion 5070 (Eppendorf) and a LightCycler 480 Detection system (Roche, France). The expression of the U6B small nuclear RNA (RNU6B) was used as endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle (Ct) method.

2.3. MicroRNA Extraction from Serum and Quantification Using RT-qPCR. Whole blood was separated into serum and cellular fractions within 2 h following collection. Sera were stored at −20°C. RNA extraction of 400 μL serum was performed by acid phenol:chloroform extraction and precipitated with ethanol over night at −20°C [14]. After precipitation, 40 μL of sterile water was added to the RNA isolation.

Typically, a 15 μL reverse transcriptase reaction contained 6.7 μL of purified RNA and reverse transcription was performed according to the manufacturer's instruction. Real-time PCR was performed on the resulting complementary DNA using TaqMan microRNA specific primers and TaqMan Universal PCR Master Mix. Since U6 and 5S rRNA were degraded in serum samples [14, 15], results were normalized by subtracting the global miRNA levels in the sample (average Ct of 6 miRNAs, hsa-miR-142-3p (ID 000464), hsa-miR-142-5p (ID 002248), hsa-miR-24 (ID 000402), hsa-miR-181d (ID 001099), hsa-miR-15b (ID 000390), and hsa-miR-125b (ID 000449) for RA sera; average Ct of 4 miRNAs, hsa-miR-16 (ID 000391), hsa-miR-24, Let7-a (ID 000377), and hsa-miR-125b for B lymphoma sera) from the level Ct of miR-125b.

2.4. Statistical Analysis. Patients' parameters were analyzed with the nonparametric Wilcoxon signed-rank test. Correlations with miR-125b expression levels were quantified with the Spearman's correlation test and the Fisher transformation was applied. All other data were analysed statistically using the Mann-Whitney U test. P values less than 0.05 were considered statistically significant. The Power and Precision V3 Software (http://www.power-analysis.com/) was used to calculate the 1-β error (the probability of a $P = 2\alpha < 0.01$ not appearing at random) for the difference in sera levels of mir-125b between responders and nonresponders.

3. Results

3.1. Quantification of Mature miR-125b in RA Blood Samples. Using microarray technology, we identified miR-125b as

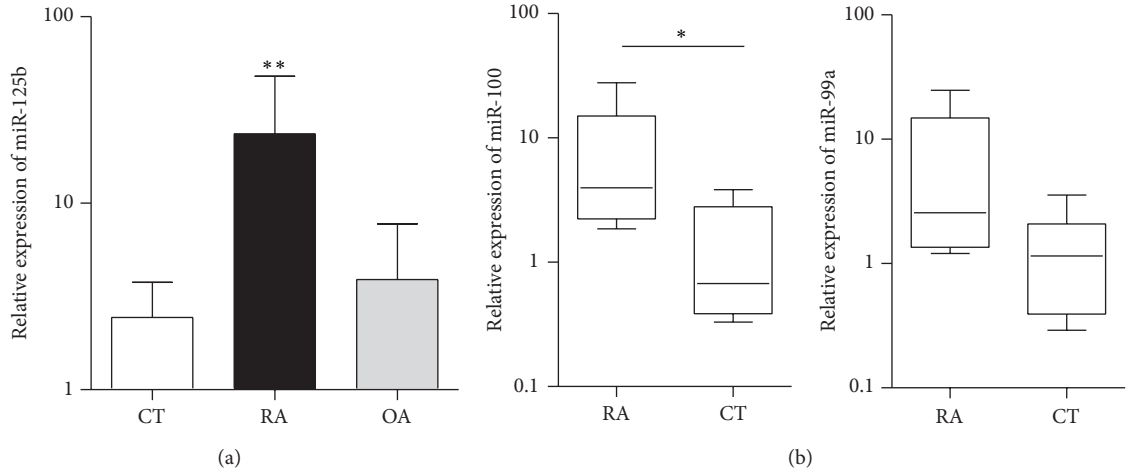

Figure 1: miR-125b is overexpressed in RA blood. (a) Expression levels of miR-125b in blood of healthy individuals (CT, $n = 13$) and patients with osteoarthritis (OA, $n = 5$) and rheumatoid arthritis (RA, $n = 16$). (b) Expression levels of miR-100 and miR-99a in blood samples from CT ($n = 7$) and RA patients ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ as determined by Mann-Whitney test.

deregulated in pooled blood samples from RA patients as compared with healthy donors (unpublished data). First, we validated these data analysing miR-125b expression on individual blood samples collected from sixteen RA patients having severe disease and similar clinical features (Table 1). Total RNAs were isolated from 0.5 mL of whole blood and the mature miR-125b form was quantified using RT-qPCR (Figure 1(a)). Expression levels were normalized with respect to U6 gene expression and expressed as $2^{-\Delta Ct}$. Mature miR-125b relative expression was significantly higher in patients with full-blown RA than in samples from healthy donors and OA patients ($P = 0.026$). More specifically, we observed that miR-125b was overexpressed in 12 of 16 (75%) blood samples (median of healthy donors = 2.22).

In humans, there are two paralogs coding for the same mature miR-125b sequence. They are located on two different polycistronic miRNA clusters on chromosomes 11 (hsa-miR-125b-1) and 21 (hsa-miR-125b-2), harbouring miR-100/let-7a-2/miR-125b-1 and miR-99a/let-7c/miR-125b-2, respectively [30]. To determine whether the increased mature miR-125b expression observed could be preferentially related to the upregulation of one of these 2 miRNA clusters, we quantified one miRNA encoded by each cluster (Figure 1(b)). Although only miR-100 reached statistical significance, both miR-99 and miR-100 were similarly overexpressed in blood from RA patients as compared with healthy donors, suggesting that both clusters are similarly deregulated in RA.

3.2. Mature miR-125b Overexpression in Serum Samples from RA Patients. Since miRNAs are also present in serum, we investigated whether miR-125b upregulation could also be measured in RA serum samples. Using real-time quantitative PCR [24, 31], detection of miR-125b was confirmed with serum from 32 patients with full-blown RA (Figure 2(a)). In addition, to determine whether this miRNA is specific for RA, its expression level was analysed in patients with OA. Analyses showed that serum miR-125b expression levels were significantly different between RA and OA patients ($P < 0.01$). To assess the potential of serum miR-125b as noninvasive biomarker of RA, serum samples from other rheumatic diseases including TRAPS and SpA were also analysed (Figure 2(b)). The expression levels of miR-125b measured in serum from RA patients were not different from other rheumatic disorders tested. Although further studies will be necessary, our data suggested that change in serum miR-125b is not specific for RA.

3.3. High Expression of miR-125b in RA Serum Predicts Good Response to Rituximab Therapy. We next determined whether the detection of miR-125b in serum of patients with active RA could be used as biomarker to predict clinical responses to rituximab (Figure 3). Serum samples were collected prior to treatment and miR-125b expression levels quantified by RT-qPCR. When the 32 RA patients were divided in two sub-groups according to their clinical response to rituximab after 3 months of treatment (Figure 3(a), $P < 0.001$), results showed that high expression of miR-125b was associated with a good response to anti-CD20 therapy ($P = 0.002$, Figure 3(b)). Indeed, serum levels of miR-125b before the initiation of treatment were higher in good responders compared with nonresponders, while two other miRNAs also detectable in serum, namely, miR-142-3p and miR-142-5p, were not expressed at significantly different levels in both groups of patients (Figures 3(c) and 3(d)). These data suggest that RA patients with low expression of miR-125b at the time of disease flare have significantly lower chance to improve clinically after 3 months of rituximab treatment and that serum abundance of miR-125b could be used as predictive biomarker. With mean value 0.36 ± 0.26 for responders ($n = 16$) and 0.19 ± 0.12 for nonresponders ($n = 16$), the power analysis yielded a 1-β value of 71%. Power calculations estimated that, keeping the difference between means and the SD-values constant, 40 patients in each treatment group would be the minimum sample size required so that 1-β value will be close to 100%. This signifies that an analysis of a single sera sample for mature miR-125b contents will serve as a very

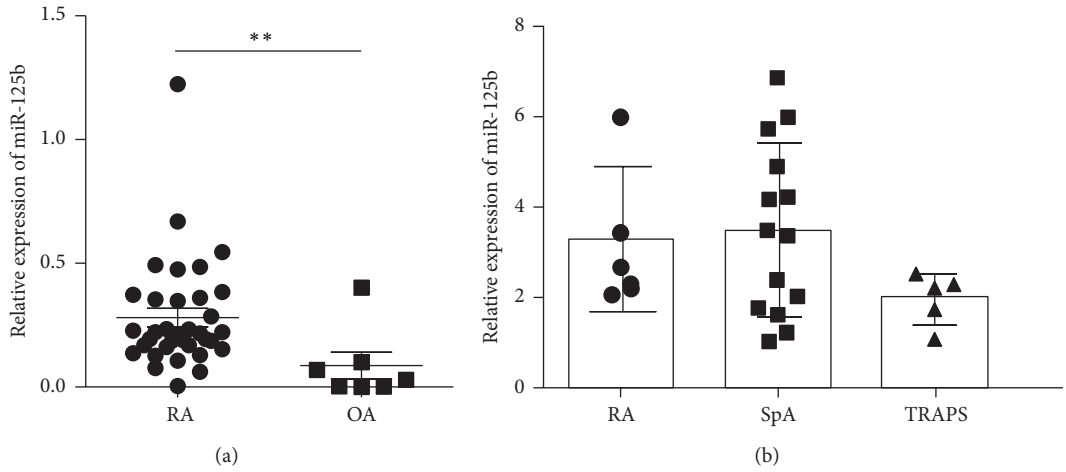

Figure 2: Serum levels of miR-125b in various rheumatic diseases. (a) Serum levels of mature miR-125b in patients with rheumatoid arthritis (RA, $n = 32$) or osteoarthritis (OA, $n = 7$). (b) Serum levels of miR-125b in patients with tumor necrosis factor receptor periodic syndrome (TRAPS), spondyloarthropathies (SpA), and rheumatoid arthritis (RA). miR-125b was quantified by RT-qPCR as described in M&M ($n = 5$–15/group). $^{**}P < 0.01$ as determined by Mann-Whitney test.

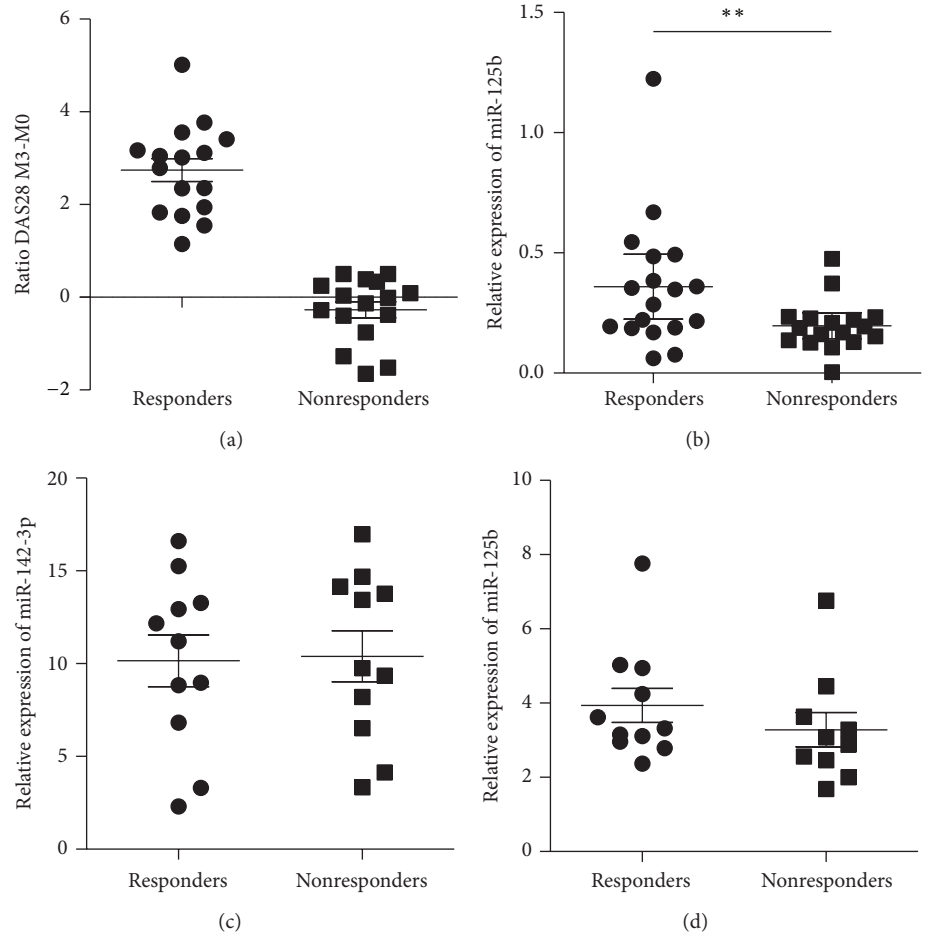

Figure 3: miR-125b as predictive biomarker for rituximab treatment outcome in RA. (a) DAS28 M3-M0 represents the difference at baseline versus 3 months after rituximab treatment. Patients were considered nonresponders (NR, $n = 16$) or responders (R, $n = 16$) according to EULAR criteria. Serum mature miR-125b (b), miR-142-3p (c), and miR-142-5p (d) are quantified in RA patients according to their clinical response. $^{*}P < 0.05$, $^{**}P < 0.01$ as determined by Mann-Whitney test.

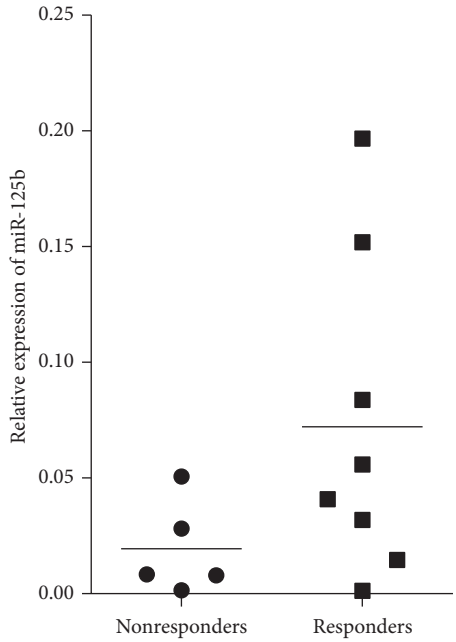

Figure 4: Serum miR-125b predicts rituximab response in B lymphoma patients. Expression levels of miR-125b were quantified by RT-qPCR as described in M&M in the serum of patients with B lymphoma before initiation of rituximab treatment. Clinical response was analyzed 4–6 weeks later and patients were considered nonresponders (NR, $n = 5$) or responders (R, $n = 8$) according to international recommendations.

good predictor or clinical marker for a patient's response to treatment.

3.4. Predictive Value of miR-125b in the Serum of Patients with B Lymphoma under Rituximab Treatment. To assess whether high serum expression levels of miR-125b could predict the therapeutic outcome for disorders other than RA treated with rituximab, we measured its expression levels in the serum of thirteen patients with B lymphomas (Figure 4). Samples were collected before initiation of the rituximab treatment and patients were divided into responders versus nonresponders according to their clinical response. Although it did not reach statistical significance, there was a tendency of higher expression levels of miR-125b in the group of responders to rituximab versus nonresponders. Consistent with results in RA samples, miR-125b was overexpressed in 6 out of 8 (75%) serum samples (median of nonresponders = 0.01) from responder patients, suggesting that serum concentration of miR-125b is a potential biomarker of rituximab response, predicting treatment outcome for patients with RA and B lymphomas.

4. Discussion

Rheumatoid arthritis (RA) is a heterogeneous disorder with fluctuating and unpredictable clinical course. Although a large panel of therapies is available to clinicians, they sometimes fail or produce partial responses, rarely achieve sustained remission, and are associated with systemic complications. Most importantly, prolonged delay in achieving adequate disease control impacts quality of life for RA patients. Current classification of patients based on the clinical phenotype and autoantibody production is not optimal and today's main challenge is to treat RA patients as early as possible with the most adequate treatment. Towards this goal, identification of biomarkers enabling to match therapies with specific subgroups of patients is of major interest. Recently, miRNAs emerged as an important class of new blood-based biomarkers that can associate their specific expression profile with disease development and severity, as well as response to treatment. This is particularly well documented across a large spectrum of cancers [32]. The possibility to detect miRNAs, not only in diseased tissues, but also in body fluids, opened great opportunities for these molecules in terms of clinical application [14, 15, 31, 33, 34]. As few publications suggested that miRNAs could be used as biomarkers with diagnosis implications in RA [16, 35], we thought to investigate whether miRNAs could also predict response to therapy. We found that mature miR-125b is overexpressed in both serum and blood samples from RA patients and well differentiated them from healthy donors or patients with OA. It is however not specific for RA as we also found elevated miR-125b levels in samples from patients with other rheumatic diseases including TRAPS and SpA. Furthermore, high expression levels of circulating miR-125b before initiation of treatment with rituximab were associated with good clinical response.

Although circulating miRNA still remains a new field in RA, one publication compared the quantification of 5 miRNAs in the plasma and synovial tissue of RA patients and shows that miRNAs released in the synovial fluid are similar to synovial tissue miRNAs, but distinct from plasma miRNAs [13]. The authors conclude that the detection of cell-free miRNAs in the serum of RA patients is more likely reflecting distinct composition and activation status of the haematopoietic compartment than of the joint space; suggesting that the systemic inflammatory aspect of the disease more than the rheumatic part might predominantly influence the blood miRNA pattern in RA. This might explain why we found that miR-125b well differentiated RA patients from OA individuals but not from patients with rheumatic disorders displaying a systemic inflammatory component such as TRAPS and SpA. Moreover, this is in agreement with the literature as miR-125b belongs to the miRNAs that are involved in haematopoiesis. It is highly expressed in normal haematopoietic stem cells (HSC) and is progressively down-regulated in committed myeloid and lymphoid progenitors. Its abnormal overexpression in these populations is associated with the development of lymphoproliferative diseases [36], myelodysplasia, and acute myeloid leukemia or B-cell acute lymphoid leukemia [23, 37, 38]. More recently, miR-125b overexpression has been correlated with the maintenance of the naive state of $CD4^+$ T cell, preventing $CD4^+$ T cell differentiation and acquisition of an effector/memory phenotype by $CD4^+$ T cells [39]. Interestingly, when analyzing the data published by Li and colleagues, miR-125b expression appears significantly up-regulated in $CD4^+$ T cells of RA patients compared with healthy controls [40]. Finally,

very high miR-125b levels have been proposed to inhibit early steps of differentiation induction of granulopoiesis [41]. Overall, these data suggest that high levels of miR-125b in the blood of RA patients might reflect defective lineage differentiation and enhanced blast proliferation, leading to abnormal abundance of haematopoietic progenitors blocked at early stage of their lineage differentiation program, similar to what is observed in leukemic malignancies.

A contribution from B lymphocyte-derived miR-125b was also possible as miR-125b is up-regulated in germinal center (GC) lymphocytes compared to memory B cells [24]. Finally, B cells play a critical role in the pathogenesis of RA as B-cell depletion shows positive results for the treatment of RA. However, the expression levels of miR-125b in RA serum were not altered after 3 months of rituximab treatment (data not shown), further suggesting that the over-expression of miR-125b in RA patients is more likely due to the contribution of the T lymphocyte and myeloid compartments than of the B lymphocytes. The detection of miRNAs in serum was quite unexpected as RNA molecules are unstable in the circulation. Studies showed that extracellular miRNAs exhibit high stability in body fluids as they circulate associated with proteins or within membrane vesicles such as exosomes or microparticles [15, 33]. In addition to the usefulness of circulating miRNAs as biomarkers, there are evidences regarding their possible function in distant cell-to-cell communication. Identification of the form and source of extracellular miR-125b will thus clarify its role in arthritis.

Although blood is easily accessible, noninvasive and of great interest for new biomarker discovery, very few studies report the detection of miRNAs in plasma or serum of RA patients [12, 31, 42]. Until now, only Murata and coworkers suggested miRNAs as potential biomarkers in RA [13, 16]. Authors showed that plasma miR-132 concentrations were significantly lower in RA than in healthy donors and that plasma levels of miR-16 were correlated with disease activity assessed by the DAS28 (28-joint Disease Activity Score), although not specific for RA since similarly altered in the plasma from OA patients. More recently, they identified a signature of seven miRNAs termed ePRAM (for "estimated probability of RA by plasma miRNAs"), elevated in RA plasma relative to healthy donors, and allowing RA diagnosis with high specificity and sensitivity, even in ACPA-negative patients [16]. Interestingly, the ePRAM signature includes miR-125a-5p that belongs to the miR-125 family, consisting of three homologs in humans (hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2), which are transcribed from three different loci that code for different mature sequences with the same seed region and therefore might have similar functions [43]. In the present study, we show that miR-125b is also detectable, both in blood and serum samples, and significantly elevated in RA patients as compared to healthy controls and OA patients, extending the observations of Murata and colleagues to the miR-125 family members as potential biomarkers in RA. However, we did not find significant correlation with DAS28, HAQ and CRP (data not shown). Circulating miR-125b has been reported as part of the blood-based miRNA signatures of ovarian and prostate cancers [15, 34]. Here, we add miR-125b to the short list of blood-based miRNAs as biomarkers in RA, and more importantly show for the first time that miR-125b can possibly predict disease biotherapy success in RA.

Previous reports in the cancer field have indicated that miRNAs could play an important role in predicting drug responses, but nothing was known until now in RA. Molecular prediction of treatment response for RA patients is still unmet medical need and is particularly important to help rheumatologists to select the optimal therapy for a given patient as they benefit from a large panel of biological drugs for which the benefice/risk ratio is not equal depending on patients and disease duration [44]. In case of rituximab, better clinical response was found associated with lower levels of IFN-γ and B-cell activating factor (BAFF), with the Fcγ receptor III (FcγRIII) genotype and the C/G-174 polymorphism in interleukin-6 (IL-6) gene [45]. In addition, an initial nonresponse to rituximab depends on circulating preplasma cell numbers at baseline and on incomplete depletion following treatment. Recently, a prospective study showed that good clinical response to rituximab is associated with the presence of B cell markers in the serum, more specifically with rheumatoid factor positivity or high anti-CCP antibody positivity and elevated IgG levels [46]. However, we found no correlation between the presence and/or levels of anti-CCP antibodies and miR-125b expression levels (data not shown). Our data suggest that miR-125b might be considered as an additional predictive biomarker for response to rituximab treatment as its expression level in the circulation, before the initiation of rituximab treatment, predicts therapy outcome. Importantly, this was not only observed for patients with RA but we found similar tendency for patients with B lymphoma, suggesting a broader application. Indeed, further determination is required including reproducibility experiment using other cohorts and comparison with other control groups such as patients with infection. In addition, RA patients used in our study have established disease and failure to anti-TNF drugs. It is thus not clear yet whether miR-125b can predict response to rituximab for patients with earlier RA and who had never received any biological therapy.

5. Conclusions

In conclusion, we have identified miR-125b as potential useful marker to predict successful outcome of rituximab treatment. This is the first time a miRNA is identified as potential biomarker for treatment efficacy and prediction of individual targeted therapy in RA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

TLR2 Elicits IL-17-Mediated RANKL Expression, IL-17, and OPG Production in Neutrophils from Arthritic Mice

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We investigated the ability of neutrophils to express receptor activator of nuclear factor kappa-B ligand (RANKL), to secrete osteoprotegerin (OPG), and to produce IL-17. Arthritis was induced by intra-articular injection of zymosan, a ligand for Toll-like receptor 2 (TLR2). Frequencies of neutrophils in bone marrow (BM), blood and synovial fluid (SF), receptor expression, and cytokine production were evaluated by flow cytometry. 1A8 antibody (1A8 Ab) was used to deplete neutrophils in zymosan-injected SCID mice. IL-17, RANKL, and OPG amounts in SF, serum, or cell cultures were determined by ELISA. The development of arthritis was associated with increased secretion of IL-17, RANKL, and OPG in serum and SF, elevated frequencies of $Ly6G^+CD11b^+$ cells in BM, blood, and SF and upregulated RANKL expression. Both IL-17 and OPG were absent in serum and SF after neutrophil depletion; therefore we assume that they were released by neutrophils. In vitro blood $Ly6G^+CD11b^+$ cells from arthritic mice produced spontaneously IL-17, IFN-γ, and OPG and expressed RANKL. This phenotype was sustained by IL-17. TLR2 engagement increased IL-17 and IFN-γ production, potentiated IL-17-mediated RANKL expression, and inhibited OPG secretion. We conclude that TLR2 regulates the destructive potential of neutrophils and its targeting might limit joint alterations in arthritis.

1. Introduction

Neutrophils are the most abundant cells in SF at the initial phase of rheumatoid arthritis (RA). They deliver signals or/and release factors regulating the functions of synovial fibroblasts, chondrocytes, osteoclasts, and other inflammatory cells like monocytes, T and B cells, dendritic cells, and NK cells. Neutrophils from RA patients are functionally different from those of healthy donors (reviewed by [1]). They have an active NF-κB signaling pathway and produce considerable amounts of reactive oxygen species and tumor necrosis factor (TNF)-α [1]. Their cytoplasm is enriched with granules containing proteases, phospholipases, defensins, and myeloperoxidase (just before being reviewed in [2]). The release of all these factors in SF induces collagen and proteoglycan depletion, receptor shedding, cytokines degrading, and activation of cytokine precursors. Neutrophils from RA patients have also delayed apoptosis and are susceptible to stimulation via TLRs and receptors for complement fragments, growth factors, and cytokines (reviewed by [3]).

Among the members of the Toll-like receptors, family is TLR2. The receptor interacts with microbial lipopeptides such as peptidoglycan from gram-positive bacteria, lipoarabinomannan from mycobacteria, and zymosan (ZY) from yeast cell wall. TLR2 has an extracellular domain with leucine-rich repeats and a conservative intracellular Toll/IL-1 receptor (TIR) domain. TLR2 forms homodimers or heterodimers with TLR1 or TLR6 [4]. Its downstream pathways involve myeloid differentiation factor 88 (MyD88), c-Jun N-terminal kinase, NF-κB, and phosphatidylinositol 3-kinase (PI3K), which promotes NF-κB-dependent transcription too (reviewed by [5]). Various studies have shown the role of TLR2 signaling for the development of arthritis (reviewed by [6]). Among them are investigations on TLR2-deficient mice describing a direct suppression of neutrophils function in these animals and better outcome from arthritis [7, 8].

IL-17 is characteristic for the early stage of arthritis and plays a role in various inflammatory and autoimmune pathologies [9–11]. Elevated IL-17 mRNA expression in SF can predict the progression of joint damage and occurs before the

disease onset [12, 13]. The overexpression of IL-17 promotes the development of collagen-induced arthritis, while IL-17 neutralization inhibits bone erosion and cartilage damage [14, 15]. IL-17 interferes with RANKL signaling pathway, osteoclastogenesis and maintains matrix turnover and cartilage destruction, especially in the presence of TNF-α [16, 17]. The cytokine promotes not only joint inflammation but also a bone-protective potential of neutrophils in periodontal disease [18].

Neutrophils from RA patients express RANKL and secrete a decoy RANKL receptor, OPG [19]. We have found abrogated RANKL expression on neutrophils that contributes to better outcome from collagen-antibody-induced arthritis in properdin-deficient mice [20]. In a model of collagenase-induced osteoarthritis glucosamine inhibits bone destruction and decreases the number of RANKL-bearing neutrophils in SF [21]. Our previous studies involving patients with osteoarthritis show altered TNF-α production in response to TLR2 stimulation and elevated TLR2 and RANKL expression on blood neutrophils [22, 23]. In the present work we investigate the bone-destructive activity of $Ly6G^+CD11b^+$ cells in TLR2 ligand driven arthritis. To confirm that neutrophils directly participate in bone resorption monoclonal 1A8 Ab recognizing that Ly6G was administrated to zymosan-injected SCID mice. $Ly6G^+CD11b^+$ cells were depleted in circulation and we measured the concentrations of IL-17, RANKL, and OPG in SF and serum. We examined IL-17 and IFN-γ production of blood neutrophils by flow cytometry and we evaluated the effect of IL-17 and TLR2 stimulation on cytokine production, RANKL expression, and OPG secretion in these cell cultures.

2. Materials and Methods

2.1. Animals. All experiments were approved by the Animal Care Committee at the Institute of Microbiology, Sofia, in accordance with the National and European Guidelines. BALB/c and SCID (CB17) mice were purchased from the Charles River Laboratories (USA), kept under standard conditions of a 12–12 hours light-dark cycle, and fed with a laboratory diet and water ad libitum. Mice (weigh 20–22 g) were anesthetized by intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg; Sigma-Aldrich, Munich, Germany) supplemented with buprenorphine hydrochloride analgetic (0.1 mg/kg; Sigma-Aldrich).

2.2. Arthritis. BALB/c mice were injected intra-articularly (i.a.) at ankles or knees with 10 μL of zymosan suspension (20 mg/mL; Sigma-Aldrich) or 10 μL of endotoxin-free phosphate-buffered saline (PBS; control group). To deplete neutrophils in SCID mice monoclonal 1A8, Ab (endotoxin free, 100 μg in 200 μL per mouse; Biolegend, London, UK) was administered i.p. at days −2, +2, and +4. At day 0 SCID mice were injected i.a. with PBS (PBS + Ab group) or zymosan (ZY + Ab group). The development of disease for 7 days was compared to untreated PBS- (PBS group) or zymosan- (ZY group) injected SCID mice. To monitor cell depletion $Ly6G^+CD11b^+$ cell, frequencies were assessed in BM at days −2, +2, and +4.

2.3. Histology. At day 7 of arthritis, ankle or knee joints were dissected, fixed in 10% paraformaldehyde/PBS, decalcified in 5% nitric acid for 1 week, dehydrated, embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E) or Safranin O [24]. The degree of injury was graded by a three score system applied for cell infiltration and proteoglycan loss (score 0—no abnormality; score 3—severe abnormalities) and determined by two independent observers using light microscopy (Leica Microsystems, Wetzlar, Germany). Cartilage erosion was expressed as the percentage of impaired cartilage from the total cartilage surface and was determined after photo capturing by a DS-Ril Nikon camera (Nikon Instruments Europe, Amstelveen, The Netherlands) and image analyses by ImageJ 1.42 software (Research Services Branch, NIH, Bethesda, MD, USA).

2.4. ELISA Assay. SF was harvested from ankles or knees by lavage with 25 μL of PBS containing 1 mM EDTA (Sigma-Aldrich). Serum was obtained after centrifugation of collected blood. RANKL, OPG, and IL-17 were quantified in SF, serum, or culture supernatants by ELISA kits from Abcam (Cambridge, UK; detection limit < 4 pg/mL and of < 1 pg/mL, resp.) and from Biolegend (London, UK; detection limit < 8 pg/mL). The samples were assayed in triplicate. The concentrations of RANKL, OPG, and IL-17 were calculated from a standard curve of the respective recombinant mouse protein using Gen5 Data Analysis Software (BioTek Instruments, Bad Friedrichshall, Germany).

2.5. Cell Isolation and Phenotype. Synovial cells were isolated by centrifugation of SFs. Peripheral cells were obtained from heparinized blood after Histopaque (Sigma-Aldrich) density gradient centrifugation. BM cells were collected from the tibia and femur. Exclusion dye staining with 0.05% Trypan blue showed more than 95% viable cells in isolated populations. After washing, cells were resuspended at 1×10^5/mL in 2% FCS/PBS and incubated with Abs against mouse Ly6G (clone 1A8; Biolegend), CD11b (clone M1-70; Biolegend), and CD69 (clone H1.2F3; BD Pharmingen, BD Biosciences, Heidelberg, Germany). RANKL expression was evaluated after incubation with biotinylated Ab against mouse RANKL (clone IK22/5; Biolegend) or biotinylated rat IgG2a (isotype control; Biolegend) followed by avidin-fluorescein isothiocyanate (FITC) staining (4 μg/sample, R&D Systems, Wiesbaden-Nordenstadt, Germany) [21]. The samples were analyzed with flow cytometer (BD LSR II) using BD FACS-Diva v6.1.2 Software (Becton Dickinson GmbH, San Jose, CA, USA).

2.6. Purification and Activation of Blood Neutrophils. Neutrophils were purified from heparinized blood as described previously [25]. Cell population consists of >95% viable cells and of 89-90% positive cells for Ly6G and CD11b. Neutrophils were resuspended at concentration of 1×10^6/mL in sterile complete RPMI-1640 medium (Biowhittaker; Lonza, Basel, Switzerland) containing 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Sigma-Aldrich), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; PeproTech EC,

London, UK). The cells were stimulated with zymosan (20 μg/mL; Sigma-Aldrich) in the absence or presence of IL-17 (40 ng/mL; Abcam). After 24 hours, 37°C, cells were harvested, washed, and analyzed for RANKL expression and intracellular cytokine production. OPG concentrations in culture supernatants were also measured.

2.7. Intracellular Flow Cytometry. Neutrophils or synovial cells (1×10^6/mL) were stimulated with phorbol myristate acetate (PMA; 10 ng/mL; Sigma-Aldrich) and ionomycin (2 μM; Sigma-Aldrich) in the presence of brefeldin (GolgiStop, BD Pharmingen) for 4 hours. Cells were harvested, washed, stained with antibody against Ly6G, then fixed, and permeabilized (BD Cytofix/Cytoperm kit, BD Biosciences). After incubation with Abs against IL-17 (clone TC11-18H10), IFN-γ (clone XMG1.2), and appropriate isotype controls (all from BD Pharmingen), cells were subjected to flow cytometry analysis.

2.8. Immunoblotting. Blood neutrophils (1×10^6/mL) were stimulated with zymosan (20 μg/mL) and GM-CSF (50 ng/mL) for 10 min, 37°C in the absence or presence of IL-17 (40 ng/mL). Cells were washed with ice-cold PBS and lysed for 15 min on ice with buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40, 0.5 mM phenylmethanesulfonylfluoride (PMSF), 1 mM Na_3VO_4, 5 mM NaF, and 1 μg/mL protein kinase inhibitor cocktail (all from Sigma-Aldrich). Cell lysates were centrifuged at 13 000 g, 4°C. Supernatants were discarded, and cell pellets were resuspended and incubated for 1 h on ice in buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM PMSF, and 1 μg/mL protein kinase inhibitor cocktail and centrifuged at 13 000 g, 4°C. Cell lysates (20 μg/line) were separated by 10% SDS/PAGE gel electrophoresis and transferred onto nitrocellulose membrane (Thermo Scientific, Rockford, IL, USA). After blocking with 5% BSA/PBS buffer, the membranes were probed overnight with Abs against methyl histone H3 (mono methyl K9, 1 : 500 diluted, Abcam) or lamin, nuclear lamina protein (clone C-20, Santa-Cruz Biotech, Heidelberg, Germany). After washing, immunoblots were incubated with peroxidase-conjugated anti-rabbit IgG (Fab_2) antibody (1 : 1000 diluted; Abcam) and then developed using a chemiluminescent substrate kit (Sigma-Aldrich). Protein band density was analyzed by ImageJ 1.42 software (Research Services Branch, NIH, Bethesda, MD, USA). In each sample H3K9 lines were normalized to that of lamin and presented in units.

2.9. Statistical Analysis. Statistical analysis was accomplished by InStat3.0 and GraphicPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± SEM. Kruskal-Wallis and Mann-Whitney U-tests were performed to compare the histological scores and the percentages of cartilage erosion between groups and to calculate statistical significance of the differences. For other data, the differences in the mean values between groups were analyzed with the two-tailed Student's t-test. Differences were considered significant when $P < 0.05$.

3. Results

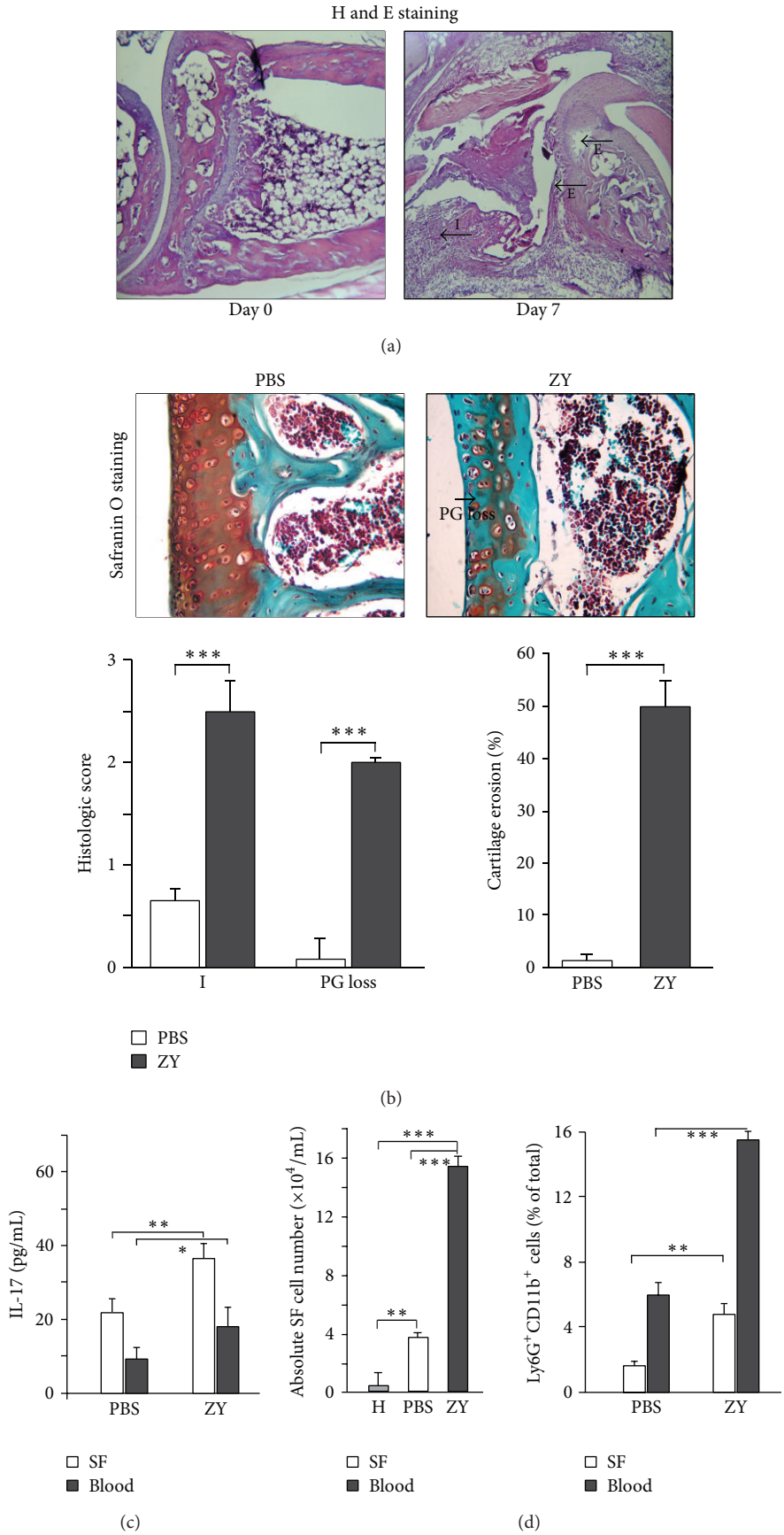
3.1. Neutrophils Depletion in Arthritic Mice Decreases IL-17 and OPG Amounts in Synovial Fluid and Serum. TLR2-driven arthritis was induced by i.a. injection of zymosan into BALB/c mice. Histological evaluation of H&E and Safranin O stained joint sections showed cell infiltration, cartilage erosion, and proteoglycan loss at day 7 of arthritis induction (Figures 1(a) and 1(b)). The amount of IL-17 raised in SF and serum of arthritic mice (Figure 1(c)). Cells accumulated in SF (Figure 1(d)). We observed increased frequencies of $Ly6G^+CD11b^+$ neutrophils in SF and blood (Figure 1(d)). CD69, characteristic for active and primed cell state, was upregulated on SF and blood $Ly6G^+$ cells from zymosan-injected mice (Figure 1(e)).

Next we designed an experiment for neutrophil depletion by specific 1A8 Ab. SCID mice were used in these settings because they lack mature T and B cells but have intact innate immunity. The mice were treated with monoclonal 1A8 Ab at days −2, +2, and +4 of zymosan injection (Figure 2(a)).

This schedule was chosen because TLR2 ligand induced granulopoiesis even after initial 1A8 Ab administration (Figure 2(a), day +2) and the loss of $Ly6G^+CD11b^+$ cells in BM granulocyte subset (Figure 2(a), day −2). Thus cell depletion was maintained by additional Ab treatments at days +2 and +4 (Figure 2(a)). At day 7 of arthritis and 3 days after the last administration of 1A8 Ab, the neutrophils partially recovered in BM but were still absent from the circulation and SF (Figure 2(b)). Histological evaluation of the joint sections at day 7 showed a considerable decrease in cell infiltration, PG loss, and cartilage erosion in 1A8 Ab-treated ZY group (Figure 2(c)). Disease improvement was associated with diminished amounts of RANKL in serum and SF (Figure 2(d)). The administration of 1A8 Ab completely inhibited TLR2 ligand-induced production of IL-17 (Figure 2(e)) and OPG (Figure 2(f)) suggesting that $Ly6G^+CD11b^+$ neutrophils might be the source of both mediators in circulation and SF. This notion, however, posed more questions about the ability of $Ly6G^+CD11b^+$ neutrophils to produce IL-17 and OPG in response to TLR2 stimulation.

3.2. Altered IL-17 and IFN-γ Production of Blood Neutrophils from Arthritic Mice in Response to TLR2 Stimulation In Vitro. Neutrophils in circulation of arthritic mice can be a source of proinflammatory cytokines as they were activated and/or primed and expressed the early activation marker CD69 (see Figure 1(e)). We purified neutrophils from blood of control (PBS-injected) and arthritic BALB/c mice. The cells were cultured in the presence of GM-CSF, a factor sustaining cell survival. $Ly6G^+CD11b^+$ cells were stimulated in vitro with TLR2 ligand and/or IL-17 for 24 hours. The intracellular production of IL-17 and IFN-γ was evaluated by flow cytometry (Figures 3(a) and 3(b)).

$Ly6G^+CD11b^+$ cells from nonarthritic mice were IFN-γ^-/IL-17$^-$, IL-17 induced autocrine IL-17 protein expression and IFN-γ production in control neutrophils (representative dot-plots and graphs, Figure 3(a)). Zymosan was able to prime the control neutrophils for IFN-γ synthesis but it failed



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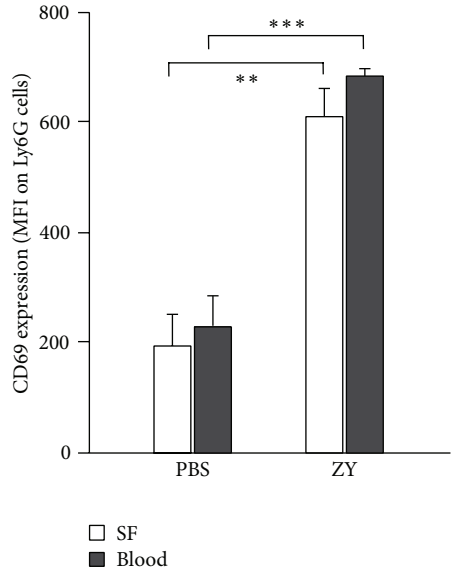


(e)

Figure 1: Arthritis induced by intra-articular TLR2 ligand (zymosan; ZY) injection into BALB/c mice. (a) Representative photomicrographs of hematoxylin and eosin (H&E) stained ankle sections show cell infiltration (arrow, I) and cartilage erosion (arrows, E) at day 7 of zymosan injection (200 μg/10 μL per ankle) (magnification 40x). (b) Photomicrographs of Safranin O stained sections (magnification 100x) and scores for cell infiltration (I), proteoglycan loss (PG loss), and cartilage erosion indicates severe joint injury in zymosan-injected mice (ZY) in comparison to PBS-injected group (PBS). Values are the mean ± SEM (10 sections/mouse; $n = 10$ mice/group; 5 experiments). $^{***}P < 0.001$, Kruskal-Wallis and Mann-Whitney U-test. (c) Increased IL-17 amounts in SF and serum of mice with arthritis (day 7). Bars show the mean ± SEM ($n = 5$ mice/group; 3 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$, Student's t-test. (d) Cells accumulate in SF and frequencies of $Ly6G^+CD11b^+$ cells increase in SF and blood at day 7 of arthritis. Values are the mean ± SEM ($n = 10$ mice/group; 5 experiments). $^{**}P < 0.01$; $^{***}P < 0.001$ versus healthy (H) or versus PBS-injected groups (PBS), Student's t-test. (e) Blood and SF $Ly6G^+$ cells upregulate surface CD69 at day 7 of arthritis. Values are the mean ± SEM ($n = 5$ mice/group; 3 experiments). $^{**}P < 0.01$; $^{***}P < 0.001$, Student's t-test. MFI: mean fluorescence intensity.

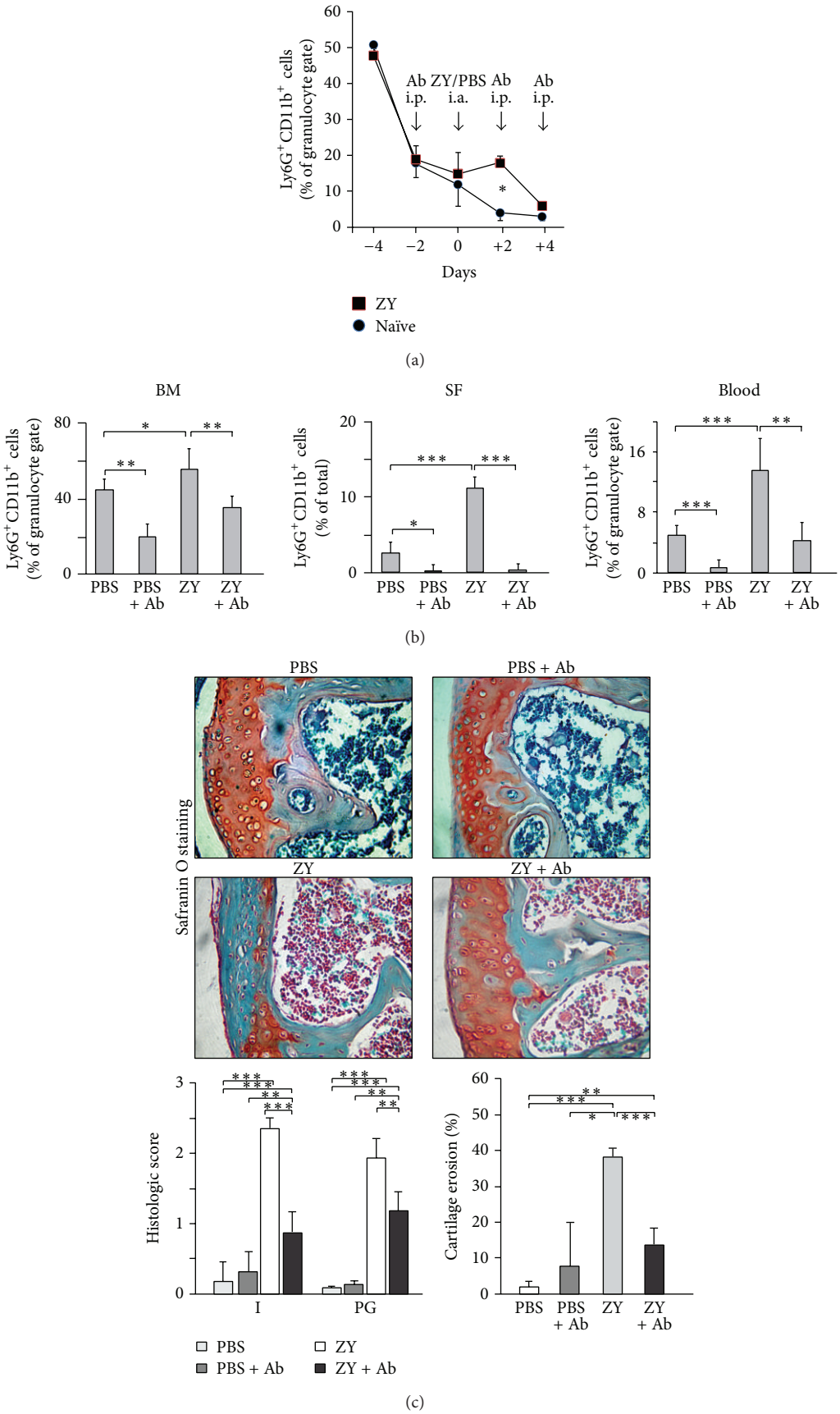
to initiate IL-17 production even in the presence of exogenous IL-17 (Figure 3(a)).

Neutrophils from arthritic mice produced IL-17 and IFN-γ spontaneously unlike the cells from the control group (Figure 3(b)). We detected around 4% $IL\text{-}17^+$ cells and near 2% IFN-γ^+ cells (dot-plot histograms and graphs, Figure 3(b)). $IL\text{-}17^+$ but not IFN-γ^+ neutrophils were influenced by exogenous IL-17 in the cultures (Figure 3(b)). Zymosan provided stronger signal for IL-17 synthesis and amplified the generation of $IL\text{-}17^+$ cells more efficiently (Figure 3(b)). However, TLR2 enhanced the frequencies of IFN-γ^+ neutrophils, but it failed to potentiate further IFN-γ synthesis in the presence of IL-17. Together our data demonstrated (i) that blood neutrophils from arthritic mice have an increased potential to produce IL-17 and IFN-γ in comparison with control cells and (ii) that TLR2 is necessary to be stimulus (signal) for enhanced generation of $IL\text{-}17^+$ and IFN-γ^+ neutrophils in cultures from arthritic group.

Neutrophil function can be regulated by epigenetic mechanisms involving methylation and acetylation of histones. We evaluated by immunoblot the levels of monomethylated H3K9 in blood neutrophils activated with zymosan and/or IL-17. Methylated H3K9 was undetectable in the nuclear extracts of nonstimulated $Ly6G^+CD11b^+$ cells from control mice and present in those from arthritic group (Figure 4).

IL-17 elevated the levels of the methylated protein in neutrophils from control but not from arthritic mice (Figure 4). These data suggest that TRL2 and IL-17 pathways might have an important impact in the epigenetic control of neutrophil functions and cytokine production. In the context of our studies they could crosstalk and compete to regulate neutrophils activities in health and disease.

3.3. TLR2 Ligand and IL-17 Regulate RANKL Expression and OPG Secretion by Neutrophils. At day 7 of arthritis induction, the level of IL-17 in serum was elevated (Figure 1(c)) and the frequencies of $IL\text{-}17^+$ neutrophils were enhanced in the circulation (Figure 3(b)). In order to study how this altered IL-17 production influences the destructive potential of neutrophils, we analyzed the expression of RANKL, a molecule directly involved in bone erosion and resorption. Blood $Ly6G^+CD11b^+$ cells from nonarthritic mice did not express RANKL (histograms, Figure 5(a)). Exogenous IL-17 induced RANKL expression (histograms, Figure 5(a)) and increased the frequencies of $RANKL^+$ neutrophils in the control group (graph, Figure 5(b)). However, TLR2 ligand failed to trigger RANKL expression on neutrophils from control mice even in the presence of IL-17 (histograms, Figure 5(a) and graph, Figure 5(b)).



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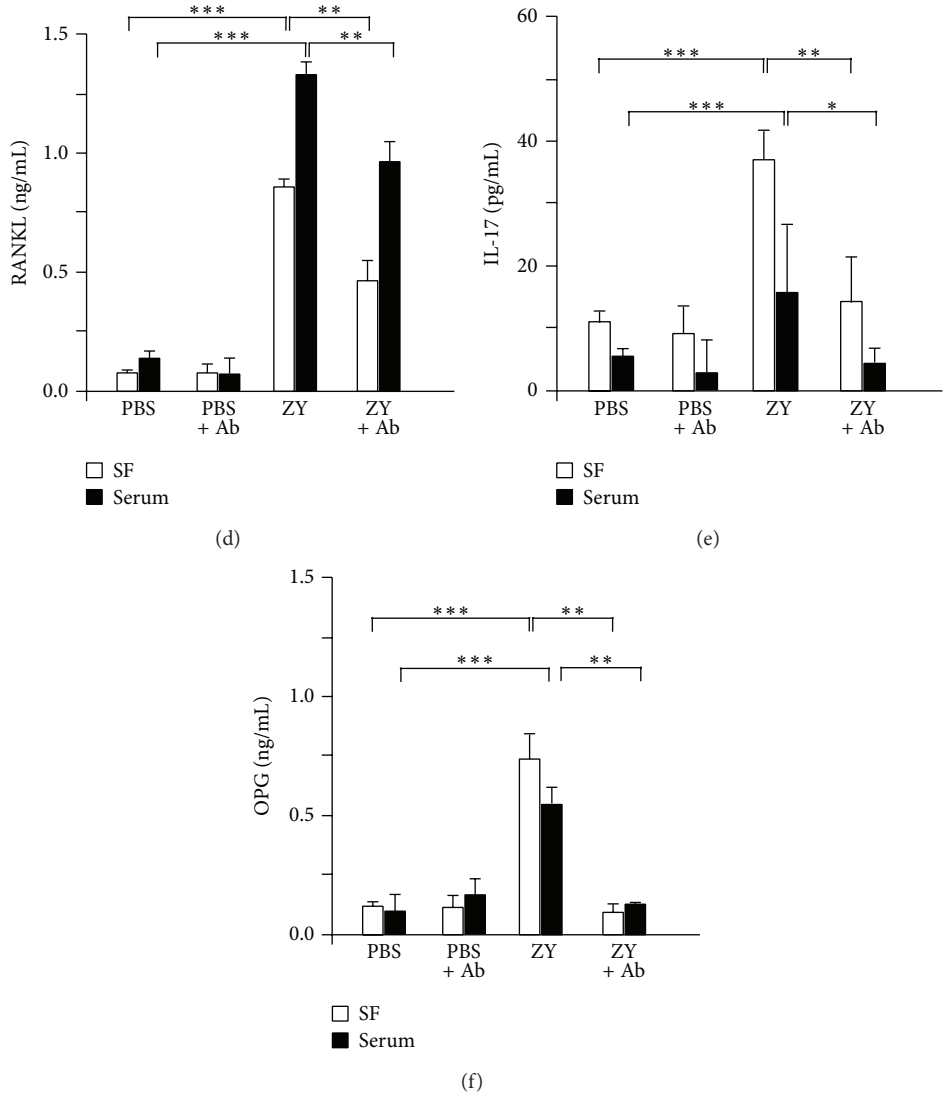

Figure 2: Depletion of $Ly6G^+CD11b^+$ cells with monoclonal 1A8 antibody (Ab). (a) SCID mice were injected intraperitoneally (i.p) with 1A8 Ab (100 μg/mouse) at days −2, +2, and +4 (arrows showing the injections). The mice received intra-articular (i.a.) knee injection of PBS (10 μL; naive) or zymosan (200 μg/10 μL; arrow ZY) at day 0. Flow cytometry analysis indicates the loss of $Ly6G^+CD11b^+$ cells in BM after Ab treatments. Values are the mean ± SEM ($n = 7$ mice/group), Student's t-test. (b) At day 7 of TLR2 ligand injection (or 3 days after the last 1A8 Ab administration) $Ly6G^+CD11b^+$ cells partially recover in BM but are completely lost in blood and SF of 1A8 Ab-treated mice. Bars indicate the mean ± SEM ($n = 7$ mice/group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's t-test. (c) The administration of 1A8 Ab attenuates joint damages as shown on the representative photomicrographs (magnification 100x) and by histological scores for cell infiltration (I), proteoglycan (PG) loss, and percentages of cartilage erosion. Values are the mean ± SEM ($n = 7$ mice/group). $^{**}P < 0.01$; $^{***}P < 0.001$, Kruskal-Wallis test and Mann-Whitney U-test. $Ly6G^+$ cell depletion decreases the amounts of RANKL (d), IL-17 (e), and OPG (f) in serum and SF of ZY mice. Values in (d), (e), and (f) are the mean ± SEM ($n = 7$ animals/group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's t-test.

Blood $Ly6G^+CD11b^+$ cells from arthritic group expressed RANKL unlike neutrophils from nonarthritic mice. Exogenous IL-17 increased the intensity of RANKL staining but not the frequencies of $RANKL^+$ neutrophils (Figures 5(a) and 5(b)). Zymosan failed to change RANKL expression but yet in combination with IL-17 enhanced substantially RANKL staining intensity and the frequencies of $RANKL^+$ cells (Figures 5(a) and 5(b)).

In the same experimental setting we evaluated the amount of secreted OPG in culture supernatants (Figure 5(c)). Blood neutrophils from nonarthritic mice produced OPG upon IL-17 and TLR2 ligand stimulation alone or in combination (Figure 5(c)). $Ly6G^+CD11b^+$ cells from arthritic mice secreted more OPG in cell cultures than the control cells (Figure 5(c)). By contrast to controls exogenous IL-17 failed to elevate OPG production by neutrophils from arthritic group. The engagement of TLR2 ligand diminished OPG secretion in cultures of $Ly6G^+CD11b^+$ cells from arthritic mice. This effect was amplified by IL-17 (Figure 5(c)), simultaneously to the

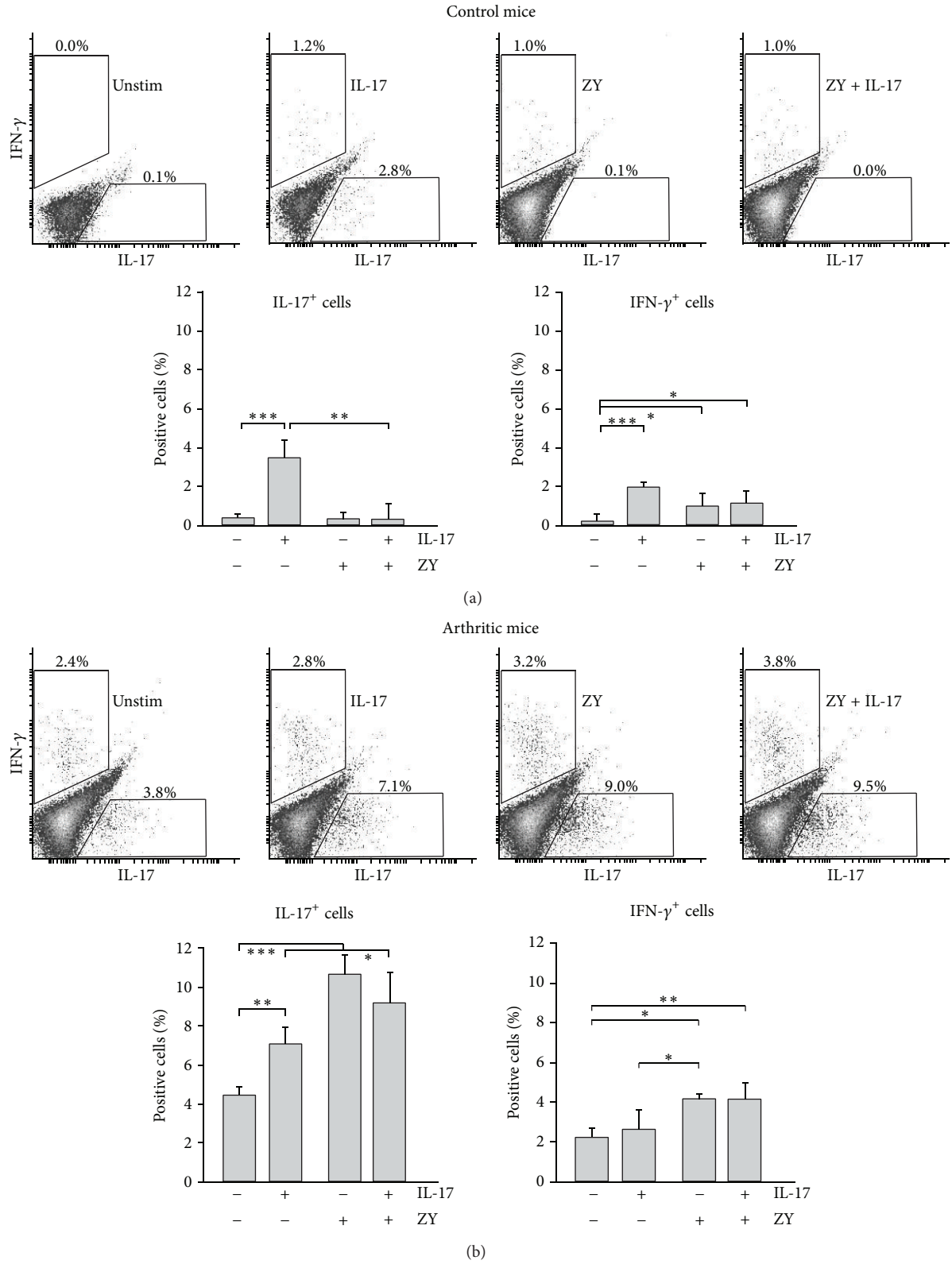

FIGURE 3: Ly6G$^+$CD11b$^+$ cells from arthritic mice produced IL-17 and IFN-γ. Purified blood neutrophils were cultured (1×10^6/mL) in medium with GM-CSF (50 ng/mL) and stimulated with zymosan (20 μg/mL) or/and IL-17 (40 ng/mL) for 24 hours. Intracellular IL-17 and IFN-γ production was evaluated by flow cytometry. (a) Ly6G$^+$CD11b$^+$ cells were IFN-γ^-/IL-17$^-$ as shown on dot-plot histograms and graphs. IL-17 induces autocrine IL-17 protein expression and IFN-γ synthesis in control cells. (b) Dot-plot histograms and graphs show spontaneous IL-17 and IFN-γ production by Ly6G$^+$CD11b$^+$ cells from arthritic group. Zymosan provides stronger signals for IL-17 synthesis than exogenous IL-17 and amplifies the generation of IL-17$^+$ cells. Bars on graphs (a) and (b) represent the mean ± SEM (n = 5 animals/group; 3 experiments). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's t-test.

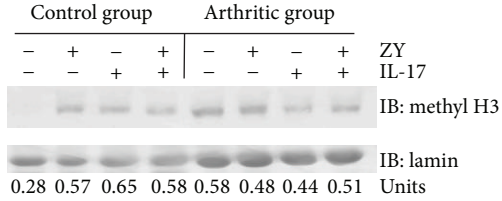

Figure 4: Methylated H3K9 (methyl H3) in nuclear extracts from neutrophils. $Ly6G^+CD11b^+$ cells were purified and stimulated with (20 μg/mL) or/and IL-17 (40 ng/mL) for 10 min. Nuclear extracts were obtained as described in Section 2. Methylated H3K9 in nuclear extracts from neutrophils was detected by immunoblot (IB). Lamin in nuclear extracts was used as a control for protein content. Densitometry analyses of IBs were performed by ImageJ 1.42 software (NIH, Bethesda, MD, USA). The density of methyl H3K9 lines was normalized to lamin expression in each sample and was presented in units.

upregulated RANKL expression on $Ly6G^+CD11b^+$ cells (Figures 5(a) and 5(b)).

3.4. RANKL Expression, IL-17, and IFN-γ Production of $Ly6G^+$ Cells in SF. In vivo $RANKL^+$ neutrophils in blood and SF of arthritic mice can originate from mature neutrophils in BM. We found more $RANKL^+$ cells in BM $Ly6G^+CD11b^+$ population from arthritic mice in comparison with control (Figure 6(a)). RANKL-bearing $Ly6G^+$ cells accumulated in SF of mice with arthritis (Figure 6(b)).

The cytokine production of synovial $Ly6G^+$ cells was also studied. The cell number yield from each mouse was low to run intracellular flow cytometry. Thus, we pooled the SF cells from five mice per group and stained them for Ly6G, IFN-γ, and IL-17. Flow cytometry analyses were performed on gated $Ly6G^+$ population. Synovial $Ly6G^+$ cells from arthritic mice expressed IFN-γ and IL-17 unlike the cells from control mice (Figure 6(c)). It appeared that most of the cells were IFN-γ^+IL-17^+ (Figure 6(c)).

4. Discussion

The role of neutrophils in joint diseases has been mainly associated with the secretion of proteolytic enzymes and reactive oxygen radicals. Our study showed that the bone destructive potential of neutrophils in arthritis was sustained by increased IL-17 production and RANKL expression and inhibited OPG secretion. In vitro exogenous IL-17 enhanced the functional activity (IL-17 and IFN-γ production and RANKL expression) of blood neutrophils from both control and arthritic mice. The effects of IL-17 were amplified by TLR2 ligation on $Ly6G^+CD11b^+$ cells from arthritic mice only. Therefore, we conclude that targeting TLR2 signaling on neutrophils may limit bone resorption and joint damage in arthritis.

Neutrophils arise from granulocyte precursors in BM. Mature BM $Ly6G^+CD11b^+$ cells maintain the replenishment pools in blood and spleen [25]. Various proinflammatory factors and mediators trigger granulopoiesis and enhance the mobilization of mature Ly6G cells from BM [2]. Among them is TLR2 which regulates neutrophil release via transcriptional upregulation of G-protein-coupled receptor kinase-2 and chemokine receptor CXCR2 downregulation [26]. In our model the stimulatory effects of TLR2 on granulopoiesis and neutrophils trafficking was confirmed by increased frequencies of $Ly6G^+CD11b^+$ cells in BM, blood, and SF and by CD69 expression on circulating neutrophils. In vitro neutrophils upregulate CD69 in response to various stimuli that induce cell activation or priming like TLRs, TNF-α, GM-CSF, IFN-γ, or IFN-α [27].

The depletion of neutrophils by specific antibody shows a crucial role of neutrophils for disease progression in a model of collagen-antibody induced arthritis [28]. Two clones of Abs are available for neutrophil elimination, clones RB6-8C5, and 1A8. While 1A8 Ab recognizes Ly6G, RB6-8C5 binds to two Ly6 isoforms, Ly6G, and Ly6C. Besides on neutrophils, Ly6C is found on dendritic cells, subpopulations of lymphocytes, and monocytes [28]. We used more a specific 1A8 clone to target the population of mature neutrophils. $Ly6G^+CD11b^+$ cells disappeared from blood and SFs of 1A8 Ab-treated mice. The administration of 1A8 Ab reduced the degree of joint damages as shown by decreased scores for cell infiltration, cartilage erosion, and PG loss and diminished amount of bone-erosion accelerating marker RANKL in SF and serum. 1A8 Ab completely abrogated OPG and IL-17 production indicating that $Ly6G^+CD11b^+$ cells were the source of both factors in serum and SF. We think that neutrophils may provide a certain level of IL-17 that later on during the development of arthritis can be amplified by mast cells, monocytes, or T cells secreting also IL-17 [29, 30]. In RA patients as well as in our study IL-17 appears at initial stage of disease and before the disease onset [9–11]. Moreover, the level of IL-17 in SF can predict the progression of joint damage in RA patients [12].

Three recent studies have shown that neutrophils produced IL-17 under infectious and allergic conditions [31–33]. Intracellular IL-17 production was detected in synovial and blood $Ly6G^+CD11b^+$ cells from arthritic BALB/c mice. IL-17 protein synthesis is regulated by IL-17 gene as well as of genes for key transcription factors. Transcription is fine-tuned by epigenetic histone modifications such as methylation and acetylation. We observed the expression of monomethylated H3K9 in neutrophils from arthritic mice but not in control cells. The methylation of H3K9 induces gene silencing and is associated with human and mouse granulocytes differentiation and abnormalities in myeloid leukemia [34, 35]. In our study IL-17 triggered its own production and H3K9 methylation in nonarthritic neutrophils proposing that certain genes are silenced in order to acquire IL-17 expression. Epigenetic modification of IL-17 gene expression is mainly studied in T cells. Considerable amounts of IL-17 can be maintained in T cells by increasing histone H3 acetylation and methylation at the IL-17 gene promoter [36]. Our data are too preliminary, but they outline the interest to study IL-17 transcription and epigenetic modification of IL-17-dependent gene expression in neutrophils and granulocytes and in neutrophils in particular.

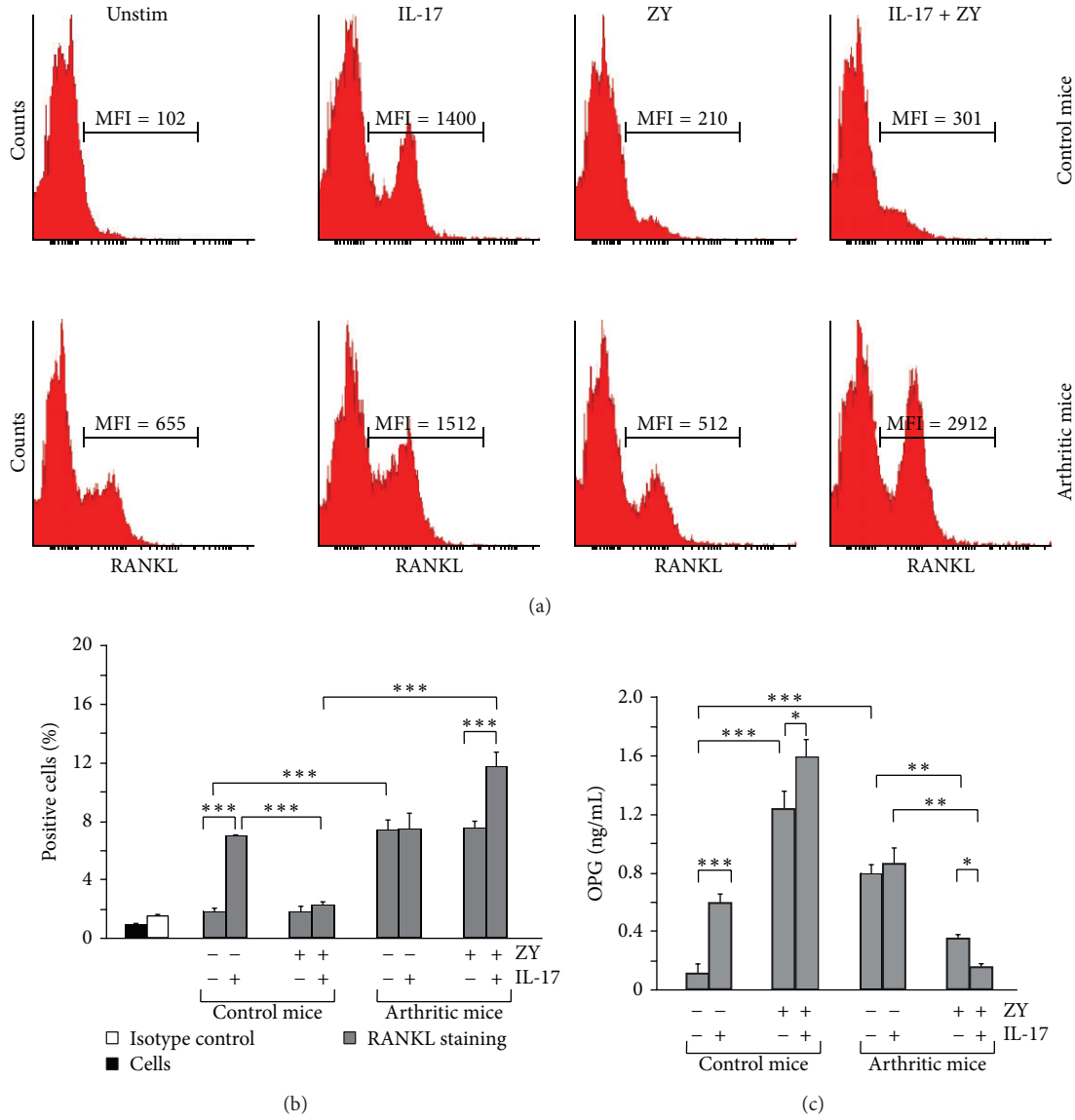

FIGURE 5: RANKL expression and OPG secretion by neutrophils were regulated by TLR2 ligand and IL-17. Purified neutrophils were stimulated as in Figure 3. (a) Representative histograms show RANKL expression on blood neutrophils from nonarthritic and arthritic mice. MFI: mean fluorescence intensity. (b) Graphs indicate that IL-17 increases the frequencies of RANKL$^+$ neutrophils in the control group. More RANKL$^+$ cells are found after stimulation with TLR2 and IL-17 in arthritic group. (c) IL-17 and zymosan induce OPG secretion by control neutrophils and inhibit OPG production by cells from arthritic mice. Values in (b) and (c) are the mean ± SEM ($n = 5$ animals/group; 3 experiments). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's t-test.

Ly6G$^+$CD11b$^+$ cells from nonarthritic and arthritic mice were sensitive to IL-17 stimulation, but they responded differently to simultaneous IL-17R and TLR2 ligation in vitro. While zymosan blocked the effects of exogenous IL-17 on neutrophils from nonarthritic mice, it amplified IL-17 production in Ly6G$^+$CD11b$^+$ cells from arthritic group. TLR2 and IL-17 signaling pathways can interfere at various levels. In particular, a conserved motif in the cytoplasmic domain of receptor for IL-17 with homology to the TIR domain has been identified [37]. TIR domain has a specific docking site for adaptor protein MyD88 that allows the involvement of TLR2 signaling pathway. Common transduction proteins can sustain the assembly of IL-17R and can regulate the strength of receptor expression [38]. At transcriptional level both pathways can interfere through NF-κB activation and pathway (reviewed by [39]). We speculated that TLR2 and IL-17R pathways directly compete for intracellular kinases, adaptor proteins, or transcription factors. In neutrophils from nonarthritic mice activated molecules or factors were limited and less available for both signaling. By contrast Ly6G$^+$CD11b$^+$ cells from arthritic group were primed or activated (as shown by the expression of CD69) and in turn they have in disposal high numbers of common transduction molecules and transcription factors. Thus both pathways via their crosstalk can provide a mechanism for regulation of neutrophils activities in health and disease.

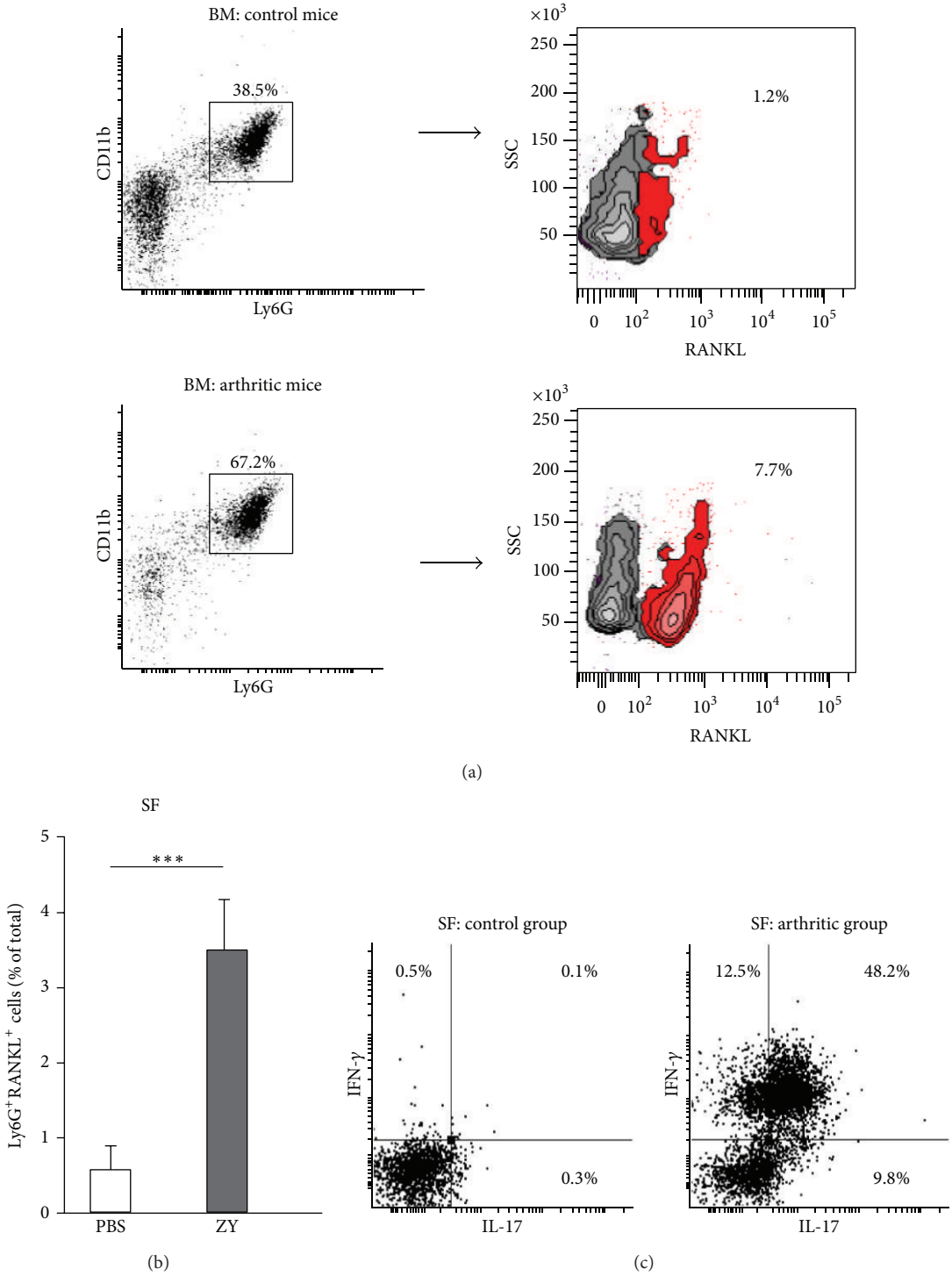

Figure 6: RANKL expression, IL-17, and IFN-γ production of Ly6G$^+$ cells in BM or SF. (a) Dot-plot histograms show increased frequencies of Ly6G$^+$CD11b$^+$ cells in BM of mice with arthritis than in control group. The density plots indicate a higher distribution of RANKL$^+$ cells in Ly6G$^+$CD11b$^+$ population from arthritic group (SSC: side scatter). (b) RANKL-bearing Ly6G$^+$ cells accumulated in SF of mice with arthritis. Bars represent the mean ± SEM ($n = 5$ animals/group; 3 experiments). $^{***}P < 0.001$, Student's t-test. (c) Synovial cells from 5 animals per group were pooled and intracellular cytokine production was performed on gated Ly6G$^+$ cells. Representative dot-plot histograms show IFN-γ and IL-17 production in Ly6G$^+$ cells from SF of mice with arthritis (day 7) ($n = 5$ animals/group; representative from 3 experiments).

IL-17 increases the recruitment of neutrophils at the site of inflammation and influences the production of various proinflammatory mediators [11]. We observed that IL-17 initiated IFN-γ production in Ly6G$^+$CD11b$^+$ cells from nonarthritic mice but failed to increase the frequencies of IFN-γ^+ cells in arthritic group. IL-17 can reduce degradation of mRNA for certain cytokines and can enhance cell responsiveness to second stimuli [40]. Indeed IL-17-producing neutrophils act proximally and are required for IFN-γ production [33]. In vivo TLRs, various proinflammatory cytokines, cell environment, and disease stage can elicit the action of IL-17 on neutrophils. In arthritic synovium IL-17 activates fibroblasts and synoviocytes to produce IL-6, IL-8, TNF-α, and GM-CSF and to express TLR2 favoring cytokine production, activation, and survival of neutrophils [41, 42]. In such environment most of Ly6G$^+$ cells in SF were double positive for IFN-γ and IL-17. The specific factors and cell populations in blood created the conditions that likely generated single IFN-γ^+ or IL-17$^+$ neutrophils. In cell cultures from arthritic group TLR2 restimulation sustained this phenotype and enhanced the generation of single IFN-γ^+ and IL-17$^+$ cells.

Bone destructive processes such as bone erosion and bone resorption depend on the activation of osteoclasts. These cells are sensitive to the action of IFN-γ and IL-17, but the RANKL/OPG system is crucial for their differentiation and maturation. Various studies show a correlation between severity of bone diseases and RANKL/OPG ratio in serum and SF (reviewed by [43]). OPG is a soluble protein from the TNF receptor superfamily and it inhibits osteoclast differentiation and activity. We assume that neutrophils at the initial stage of disease secreted OPG because the molecule was absent in serum and SF after cell depletion by 1A8 Ab. Experiments in vitro confirmed that OPG was released by blood neutrophils from nonarthritic mice upon TLR2 stimulation or spontaneously by neutrophils from TLR2 ligand-injected group. Ly6G$^+$CD11b$^+$ cells from arthritic mice failed to produce OPG after zymosan restimulation. This altered responsiveness to repeated stimulation might maintain low OPG levels during the development of arthritis that in turn can sustain osteoclastogenesis and osteoclast activation. We think that such mechanism may dominate at late stages of disease when more neutrophils are accumulated in SF.

OPG decoy receptor, RANKL, exists in two isoforms, a soluble protein, and a membrane bound protein. The latter is sensitive to the cleavage by proteases. Thus, the neutrophils producing proteases may affect the amount of soluble RANKL in biological fluids. Indeed we found that after neutrophil depletion the concentrations of RANKL decreased in serum and SF of zymosan-injected mice.

The membrane bound RANKL is expressed by BM cells, precursors of osteoclasts, and stromal cells [43]. In vivo TLR2 ligand induced RANKL expression on Ly6G$^+$CD11b$^+$ cells in BM. Despite that RANKL$^+$ pool of Ly6G$^+$ cells was small, it probably maintained the frequencies of RANKL$^+$ neutrophils in circulation and even contributed to the accumulation of RANKL-bearing cells in SF at early stage of disease (day 7). The study describing RANKL expression on blood neutrophils from RA patients supported our data [19]. The same authors indicated the role of the environmental factors since neutrophils from healthy donors upregulated RANKL after incubation with SF from RA patients [19]. We found that exogenous IL-17 induced RANKL expression on RANKL-negative blood neutrophils (control) and increased the density of surface RANKL on RANKL-bearing Ly6G$^+$CD11b$^+$ cells (arthritic group). TLR2 engagement potentiated the IL-17-mediated RANKL expression only on neutrophils from arthritic mice and inhibited OPG secretion in cell cultures at the same time. Thus we built the hypothesis that TLR2 signaling sustained the bone destructive potential of neutrophils by an interference with IL-17-dependent RANKL/OPG system.

In summary, our study showed that (i) exogenous IL-17 induced autocrine IL-17 production, IFN-γ synthesis, and RANKL expression on blood neutrophils from nonarthritic mice and these effects were inhibited upon simultaneous TLR2 stimulation; (ii) Ly6G$^+$CD11b$^+$ cells from arthritic group produced IL-17, IFN-γ, and OPG spontaneously and expressed RANKL; (iii) TLR2 increased IL-17-mediated RANKL expression and inhibited OPG secretion by neutrophils from arthritic mice. Together these data suggest that TLR2 signaling might be a good target to limit the bone destructive potential of neutrophils in joint diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Could Biomarkers of Bone, Cartilage or Synovium Turnover Be Used for Relapse Prediction in Rheumatoid Arthritis Patients?

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Objective. The aim of this review is to clarify the usefulness of bone, cartilage, and synovial biomarker in the management of rheumatoid arthritis (RA) therapy in remission. *Synovial Biomarkers*. High MMP-3 levels are associated with joint progression in RA patients, but there is no data about their utility in clinical remission. IIINys and Glc-Gal-PYD seem to be more specific to synovium, but more studies are required. *Cartilage Biomarkers*. Unbalance between cartilage break-down biomarkers (urinary CTX II and COMP) and cartilage formation biomarker (PIIANP) was described. This unbalance is also associated with joint destruction and prognosis of destruction. No data are available on patients in remission. *Bone Biomarkers*. RA activity is correlated with an increase of bone resorption markers such as CTX I, PYD, and TRACP 5b and a decrease of bone formation markers such as OC and BALP. RA therapies seem to improve bone turnover in limiting bone resorption. There is no study about bone marker utility in remission. *Conclusion*. Biomarkers seem to correlate with RA activity and progression. They also could be used to manage RA therapies, but we need more data on RA remission to predict relapse.

1. Introduction

Rheumatoid arthritis (RA) is the most frequent chronic autoimmune inflammatory rheumatism, with a worldwide prevalence around 1% [1]. RA severity is related to joint destruction characterised by erosion and space narrowing that is responsible for joint functional disability [2–4]. Early diagnosis and treatment are crucial in order to prevent joint destruction and preserve joint function defining the "window of opportunity" concept [5, 6]. Since few years, the concept "Outside-Inside" suggested a beginning of RA disease also in the subchondral bone marrow [7]. In fact, a subchondral bone loss at the metacarpal phalangeal head starts since the early phase of RA disease [8]. Furthermore, joint inflammation due to synovitis is one of the most powerful predictors of new bone erosion [9]. So, the synovial membrane was the first actor mainly described by production of some mediators induced by inflammatory cytokines such as TNF or others. These mediators induced cartilage matrix degradation and subchondral bone loss [10, 11]. These data support a strong interaction between synovial membrane, cartilage, and subchondral bone. Inflammatory joint induced the release of specific protein fragments from its various compartments into the serum and the urine, which may be used as tissue specific biomarkers [12]. By this way, biomarkers of each component of the joint could be useful to manage RA patients.

TNF inhibitors and other biologics reduce synovitis, biomarkers of inflammation, and bone destruction. However, dissociation between clinical and radiological effect of TNF inhibitors has been reported. These TNF inhibitors are able to block joint destruction, even if RA disease is still active [13–15]. In 2014, in front of early RA patient, the goal of early RA therapy is to obtain remission according to the new criteria for remission ACR/EULAR [16]. However, though clinical remission was obtained, in some patients a structural progression can occur [17] probably due to persistence of joint inflammation [18, 19]. Exploration with specific biomarkers of each component of the joint could be helpful to investigate this paradigm [20].

In daily practice in 2014, only DAS28 combining clinical parameters with erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) is used. ESR and CRP are inflammatory biomarkers, but not specific to the joint. So, they are not strongly correlated with joint involvement. Despite its large usefulness in daily practice, DAS28 fails to strongly predict the joint progression or a real remission. At the time of "personalized medicine," which aims to individually improve treatment management [21], biomarkers of the joint will be useful in RA especially at the early stage. The aim of this paper is to review some biomarkers of synovial, cartilage, and bone turnover in RA, clarify their utility in RA management, and analyze data in remission.

2. Synovial Biomarkers

Here, we focused our review on three major synovial biomarkers. Their interests to manage RA are summarized in Table 1.

Matrix metalloproteinase-3 (MMP-3 or stromelysin 1) is a proteinase secreted by synovial fibroblasts and chondrocytes. Its activity results in degradation of aggrecan core protein, cartilage link protein, fibronectin, and collagen types IV, VII, IX, and XI [22]. MMP-3 is present in RA synovial fluid and overexpressed in rheumatoid synovium [23, 24]. One MMP-3 polymorphism was described to be associated with higher joint damage in RA [25, 26]. Otherwise, serum MMP-3 level was suggested as a predictor for joint destruction in early RA [27, 28] or established RA [29, 30]. In fact, circulating MMP-3 level seems to be genetically determined [26]. Correlation between serum MMP-3 level and joint damage progression appeared to be independent of rheumatoid factor (RF) or ACPA status [31]. The next step was to assess MMP-3 variation induced by RA therapy and particularly during biological therapies. Anti-TNF therapy decreased MMP-3 expression in RA patients [32, 33]. Similar results were observed with tocilizumab (IL-6 blocker) [34] or abatacept (inhibitor of costimulation) [35]. Then, MMP-3 monitoring was investigated to improve therapeutic strategy. This was the purpose of the T-4 study [36]. The best outcome was observed in the group combining DAS28 and MMP-3 monitoring [36]. Finally, MMP-3 was also investigated in RA remission situation. Its level was similar in RA patients in remission or not induced by anti-TNF therapy [37]. However, normal MMP-3 level in RA patients treated with tocilizumab was predictive to absence of relapse after tocilizumab cessation [38]. To summarize, high MMP-3 level was associated with disease activity and joint progression in RA patients and should be used in association with usual inflammatory markers to follow therapy efficiency. However, this biomarker was never tested in patients in clinical remission to predict structural remission.

Another synovial biomarker considered in RA is the glycosylated form of pyridinoline (PYD) [39]. PYD is mainly a bone resorption biomarker but is also related to remodeling of cartilage and synovium [40]. The glycosylated analogue of PYD, glucosyl-galactosyl-PYD (Glc-Gal-PYD), can be assessed in urine and appeared to be specific to synovial tissue [39]. Urinary Glc-Gal-PYD level was higher in patients with early RA than controls and its high level is associated with higher risk for the progression of joint damage [28]. In established RA, urinary Glc-Gal-PYD was associated with changes of the erosion, joint space narrowing (JSN), and the total Sharp score [41]. After one year of anti-TNF therapy, the levels of urinary Glc-Gal-PYD was similar in RA patients with or without progressive joint damage over one year of anti-TNF therapy, but its reduction over one year was higher in patients with progressive joint damage [41]. These results suggested that, in some patients, other mechanisms were possibly involved than TNF related inflammation.

The last synovium biomarker recently developed is the nitrated type III collagen (IIINys), which was explored in both osteoarthritis (OA) and RA patients. In patients with joint disorder, the synovial membrane contains nitrated proteins [42]. IIINys was increased in serum from OA patients [43] and RA patients [44]. Its level was the highest in RA patients which suggests that it is related to synovial tissue inflammation [44]. However, no more data are currently available for this biomarker.

We attempted to describe synovial biomarkers and put out their interest in RA management. Despite many studies reviewed, no data are currently available to predict relapse in RA patients in remission. So, these biomarkers need to be tested in this situation.

3. Cartilage Biomarkers

Then, we focused on three main cartilage biomarkers with a summary of their characteristics in Table 2. Two are biomarkers of cartilage breakdown, whereas the third one is a biomarker of cartilage formation. Cartilage homeostasis consists in balance between degradation and formation. In RA, there is an imbalance in favour of destruction [45].

Cartilage is mainly composed of collagen type II (70%) and proteoglycans including aggrecan which is the most abundant one. MMPs and aggrecanases are mediators of cartilage degradation. Several cartilage degradation fragments can be measured. Collagen type II C-telopeptide (CTX-II) is a neoepitope generated from MMPs, derived from the carboxy-terminal part of type II collagen [46]. In early RA, urinary CTX-II level was higher than in controls, and patients with high CTX-II level have a higher risk for the progression of joint damage over 1 year, independent of the extent of joint destruction at baseline and of clinical indices of disease activity [28]. In established RA, urinary CTX-II level was associated with rapid radiologic progression [47] or changes of the JSN Sharp score over one year [41]. Then, CTX-II was assessed during anti-TNF therapy in RA patients. After one year of anti-TNF therapy, the levels of urinary CTX-II were similar in RA patients with or without progressive joint damage over one year of anti-TNF therapy. In patients with progressive joint damage, reduction of urinary CTX-II was higher than in others [41]. No data on RA remission are available at this time.

Cartilage oligomeric matrix protein (COMP) is a non-collagenous extracellular matrix protein mainly found in cartilage maintaining the integrity of the collagen network [48]. Serum COMP was reduced in RA patients in remission

Table 1: Synovium biomarkers and their interests in RA management.

Synovial biomarker	Expressed in RA	Treatment response	Joint destruction	Effects on monitoring in clinical response and progression
MMP-3	[23, 24]	[32–35]	[25, 27, 29–31]	[36]
Glc-Gal-PYD	[28, 39, 40]	No data available	[28, 39, 41]	No data available
IIINys	[44]	No data available	No data available	No data available

MMP-3: matrix metalloproteinase-3; Glc-Gal-PYD: glucosyl-galactosyl pyridinoline; IIINys: nitrated type III collagen.

Table 2: More studied cartilage biomarkers and their interests in RA management.

Cartilage biomarker	Expressed in RA	Treatment response	Joint destruction
CTX-II	[28, 47]	[41]	[28, 41, 47]
COMP	[49]	[37]	[49]
PIIANP	[50]	No data available	No data available

CTX-II: collagen type II C-telopeptide; COMP: cartilage oligomeric matrix protein; PIIANP: propeptide of type IIA procollagen.

induced by anti-TNF therapy compared to other patients [37]. In early RA, early changes in serum COMP levels were related to radiological outcome over the first 5 years [49]. This biomarker was not yet analyzed during biologic therapy or in RA remission.

Serum propeptide of type IIA procollagen (PIIANP) arises from the maturation of type IIA procollagen. Thus, PIIANP is a biomarker of cartilage formation. Its level was decreased in patients with OA or RA. In RA patients treated with low-dose corticosteroids, serum PIIANP is significantly higher than in untreated patients [50]. No more data are currently available on biomarker of cartilage formation.

So unbalance between cartilage formation and breakdown is described in RA disease. No data are at this time available to describe their interest to predict relapse in RA patient in remission. More data are required in this situation to improve their utilities.

4. Bone Biomarkers

Bone homeostasis is highly regulated by balance between new bone formation and removing old bone. Activated osteoclasts degrade bone matrix while osteoblasts form new matrix [51]. Type I collagen constitutes 90% of bone matrix. Bone formation markers included the serum bone formation markers total osteocalcin (OC), the alkaline phosphatase bone isoenzyme (BALP), and the C- and N-propeptide of type I collagen (PICP and PINP). Bone degradation is driven by osteoclasts and results in stimulation by RANKL induced by IL-1β, IL-6, or TNF. Osteoclasts secrete cathepsin K, which degrades the collagen type I and releases C-terminal crosslinked telopeptide of type I collagen (CTX-I), or N-terminal crosslinked telopeptide of type I collagen (NTX) neoepitope. The crosslinked carboxyterminal telopeptide of type I collagen (ICTP) is another fragment of C-telopeptide end, which is not released with cathepsin K action but probably MMPs [52, 53]. Other type I collagen crosslinks are pyridinoline (PYD) and deoxypyridinoline (DPD) [54].

In established RA, uncoupling with low level of bone formation markers and high bone resorption markers was described in 1999 [55]. OC, a bone formation marker, was reduced in RA without destruction compared to controls. On the contrary, CTX-I, a catabolic bone marker, is higher in RA patients with destruction compared to other RA patients [55]. This uncoupling was recently confirmed by using an innovative way to assess bone damage in RA by high-resolution peripheral quantitative computed tomography (HR-pQCT) [56]. TRAP 5b level, a catabolic bone marker, was associated with bone erosions, whereas bone alkaline phosphatase (BAP) was associated with osteophytes [57]. Furthermore, in longitudinal studies, catabolic bone markers (CTX-I or PYD) are also good predictors for radiologic progression in RA [47, 58, 59].

Like cartilage and synovium turnover markers, bone biomarkers were assessed during various biological therapies. During anti-TNF therapy, ratio between bone formation markers and bone resorption markers increased during one year of treatment, suggesting improvement of the bone remodeling balance, mainly due to a decrease in bone resorption [60]. A differential effect was observed at one year of anti-TNF therapy between ICTP and CTX-I. ICTP, which is related to MMPs activity, remained decreased at one year, whereas CTX-I level, which is related to cathepsin K, returned to its baseline level at one year [60]. This suggests a strong effect of anti-TNF on local subchondral bone related to joint inflammation. Since TNF blockers already showed a reduction of the bone biomarker unbalance, TNF blockers also demonstrated a positive effect on bone mineral density in RA patients with or without a clinical response as observed at the joint level [61]. Serum RANKL was decreased during anti-TNF therapy [62]. All these data support that anti-TNF therapy is not only able to prevent joint destruction, but it is also able to prevent bone loss in RA patients. Similarly, with tocilizumab, bone formation biomarker PINP increased whereas bone resorption markers, ICTP and CTX-I, decreased [63]. So TNF or IL-6 inhibitors increased bone formation/bone resorption ration. This suggests a nonspecific effect of a pathway but an effect on suppression of joint inflammation. Denosumab is also a biotherapy targeting RANKL [64], but not a proinflammatory

cytokine. Denosumab reduced both serum PINP and CTX-I levels over one year [65], whereas urinary CTX-II decreased only at 3 months. Since denosumab targets RANKL, but not a proinflammatory cytokine, RA disease was not improved, but it reduced erosion progression. So according to the target, drugs have different effects. Blocking inflammation reduces bone loss, but blocking pathway induced in bone loss reduced it without effect on RA activity.

Among all these biomarkers, only CTX-I has demonstrated its ability to be associated with joint destruction, sensitivity to treatment, and prediction of joint progression. However, no data are available for relapse prediction in RA remission.

5. Discussion

We showed that synovium, cartilage, and bone turnover biomarkers are correlated with RA activity. To summarize, resorption markers increase with RA activity in the three components of the joint. Furthermore, these biomarkers could be useful to identify RA patients with high risk of rapid disease progression. This suggests that these selected RA patients require a rapid active therapy. Since these biomarkers reflected different compartments involved in RA, they will be useful to define structural remission in RA. Some of these reviewed biomarkers compose the multibiomarker disease activity (MBDA) test developed to quantify RA disease activity [66]. Recent data suggested that low MBDA was associated with clinical remission criteria [67, 68]. However, no study currently explored MBDA to predict relapse in RA remission. Treat-to-target strategy emerged since few years to manage early RA patients. This strategy aims to achieve clinical remission and appears to be a realistic today [69]. Only one study combining clinical and biomarkers demonstrated its utility in the treat-to- target strategy [36]. This study is the typical example of the "personalized medicine" [70]. The only biomarker with enough promising results is MMP-3. However, we need more studies to generate more data to define the place of these biomarkers in RA remission. At this time, we failed to have the "perfect" biomarker which could be used in RA management such as HbA1c in diabetes [71].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Cytokines as Biomarkers in Rheumatoid Arthritis

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RA is a complex disease that develops as a series of events often referred to as disease continuum. RA would benefit from novel biomarker development for diagnosis where new biomarkers are still needed (even if progresses have been made with the inclusion of ACPA into the ACR/EULAR 2010 diagnostic criteria) and for prognostic notably in at risk of evolution patients with autoantibody-positive arthralgia. Risk biomarkers for rapid evolution or cardiovascular complications are also highly desirable. Monitoring biomarkers would be useful in predicting relapse. Finally, predictive biomarkers for therapy outcome would allow tailoring therapy to the individual. Increasing numbers of cytokines have been involved in RA pathology. Many have the potential as biomarkers in RA especially as their clinical utility is already established in other diseases and could be easily transferable to rheumatology. We will review the current knowledge's relation to cytokine used as biomarker in RA. However, given the complexity and heterogeneous nature of RA, it is unlikely that a single cytokine may provide sufficient discrimination; therefore multiple biomarker signatures may represent more realistic approach for the future of personalised medicine in RA.

1. Biomarker Research

1.1. General Features of Biomarkers. Biomarkers are defined as anatomical, physiological, biochemical, molecular parameters or imaging features that can be used to refine diagnosis, measure the progress of diseases, or predict and monitor the effects of treatment. They can also be associated with the severity of specific disease states.

Biomarkers can be detected and measured by a variety of methods including physical examination, laboratory assays, and medical imaging. Some biomarkers arepresent in particular groups of patients but not others, and as a result they are defined as *qualitative biomarkers* in contrast to *quantitative biomarkers* that are present at various degrees/levels in all patients. The accessibility of a biological biomarker, which is defined by the methods that are used to access the biomaterial necessary to measure it, is an important factor in relation to its adoption in clinical practice. If a biomarker can be obtained in a minimally invasive manner (typically from blood, saliva, or urine) or use tissue imaging as opposed to tissue sampling (biopsy), it will obviously be more attractive.

In the context of rheumatic diseases, typical biological biomarkers could encompass genetic markers, products of gene expression, autoantibodies, cytokine/growth factors, acute phase reactants, tissue abnormalities visualized by immunohistochemistry in synovial biopsy, a product of tissue degradation, or a cell subset that can be phenotyped and enumerated. The sources of these biomarkers could be the serum/plasma, urine, synovial fluid, tissue biopsy, or cells from blood, fluid, lymph node, or tissue. In contrast, a clinical biomarker (i.e., clinical surrogate) would constitute a physical variable (sign or symptom), a clinical judgment, or an outcome measurement that emerges as a sequel of the underlying disease process. In rheumatology, this variable may be not only joint counts, global assessment, pain score, duration of morning stiffness, and other clinical variables but also composite indices or functional, radiographic scores.

1.2. Specificity and Sensitivity. *Sensitivity* and *specificity* are statistical measures of the performance of biomarker using a binary classification test. This measures use used a categorical classification of patients with respect to true and false positive/negative results.

Sensitivity relates to the biomarker's ability to identify positive results. It measures the proportion of individuals which are correctly identified by the biomarker. Sensitivity is different from positive predictive value (PPV, also called precision), representing the proportion of actual positives in the population being tested.

On the other hand, specificity relates to the ability of the test to identify negative results. It measures the proportion of people without the biomarker that are correctly not assigned to the condition. Sensitivity may be affected in case of a number of indeterminate test results. It is possible to exclude these cases from analysis or, alternatively, to treat them as false negatives (which gives the worst-case value for sensitivity but also underestimates it), but such exclusions should be stated when quoting sensitivity.

An optimal biomarker would aim to achieve 100% sensitivity (i.e., predict all people with the condition) and 100% specificity (i.e., not predict anyone from the control group). For any biomarker, there is usually a trade-off between the measures and their impact, setting acceptable limits and allowing detection of false positive (lowering specificity), but limiting false negative (increasing sensitivity).

Taking the example of anticitrullinated peptide antibodies (ACPA) in RA, sensitivity is usually reported around 68% and specificity is reported at 95% [1]. However, sensitivity is highly dependent on the group of individuals tested and values observed in established diseases that do not reflect the general RA patients' population or early disease. Indeed, in patient with recent onset of symptoms, studies have shown that sensitivity is much lower (ranging from 35% to 50%) even if specificity remains closer to 95% [2].

Multivariate markers are as follows: the concept of biomarker algorithm or multivariate biomarkers has recently been developed based on the observation that a single biomarker is often insufficient to predict the outcome of interest, when a combination of biomarkers is better at achieving the prediction. It is usually observed that multivariate biomarkers perform better in replicate studies than univariate biomarkers.

1.3. Need for Biomarkers in Rheumatoid Arthritis (RA). RA is a complex disease that develops as a series of events often referred to as disease continuum. Research into the preclinical and early phases of RA recently reviewed these events and categorised groups of individuals based on risk factors [3]. According to this new terminology, healthy individuals without RA are described as having potentially two main types of risks: (i) a genetic risk, for example, if they carry the shared epitope allele and (ii) an environmental risk if they smoke. They, however, do not present any laboratory evidence of symptoms or any signs of inflammatory arthritis. The first phase of RA disease progression would then be a state in which individuals develop features of systemic autoimmunity that can be measured by laboratory investigations and are known to be associated with RA (such as ACPA) [3] and more recently with carbamylated protein [4, 5]. These individuals still do not present any symptoms or signs of inflammatory arthritis. A further stage is then defined by the appearance of symptoms (such as arthralgia/morning stiffness), still with no evidence of any clinical synovitis. These individuals can come from both the genetic and environmental risk groups, from the systemic immunity group, or from the general healthy population. Finally, the last progression stage is represented by the development of clinically apparent inflammatory arthritis that may not yet fulfil the criteria for RA diagnosis [6], and hence it is being termed undifferentiated arthritis but is likely to evolve towards RA.

There are many situations in RA, which would benefit from biomarker discovery, considering that biomarkers may be broadly classified as diagnostic (detected when disease is present), prognostic (associated with disease outcome), or predictive markers (associated with drug response). Diagnosis is obviously an area where new biomarkers are still essential as RA is a condition where diagnosis relies on signs and symptoms even if recent progress has been made with the inclusion of ACPA to the recently updated criteria [6]. However, in RA diagnosis, the performance of biomarkers may greatly depend on the duration of symptoms at the time of test, the current level of inflammation, and the amount of destructive processes already undergone, as well as on the type of tissue tested. Prognostic biomarkers which predict the future course of the disease and provide information regarding the outcome irrespective of therapy would be very important in foreseeing the evolution of undifferentiated arthritis towards RA or with respect to the severity of RA which can be quite variable. Prognostic biomarker validation is therefore relatively straightforward, as it is associated with the disease and the patient and can be established (at least in theory) using data from a series of patients treated with standard treatment. The discovery of specific biomarkers for poor prognosis would, for example, enable early intervention and intensive treatment. Risk biomarkers for predicting rapid evolution or cardiovascular complications, for example, remain highly desirable. Monitoring biomarkers would be useful in predicting relapse and candidates are available using flow cytometry based cell subset phenotyping [7–9]. Predictive biomarkers would separate an RA patients' population with respect to their outcome in response to a particular event taking place (i.e., particular therapy). They are therefore present/absent prior to the outcome occurring and have obvious applications with the greatest potential to affect clinical practice by targeting drugs to relevant patient subgroups. Biomarkers allowing the selection of an optimal drug for a particular patient (acknowledging that certain subset of patients respond better to certain drug than others) may represent another essential step in patients screening that would notably allow personalised medicine models to be developed, tailoring therapy to the individual, shortening time from onset to effective treatment, improving cost and risk-benefit ratios of drugs, and ultimately achieving high response rate with minimal toxicity [10]; however, in patients with long-standing RA heterogeneity in disease presentation,

there remains a major obstacle even when using biomaterial as close to the disease site as synovial tissue [11].

There are several sources of tissue and body fluid that can be considered for biomarker discovery programs in RA. The suitability between the levels of invasiveness and the benefit provided by the biomarkers is however to be considered as well as the level of investigation patients would be likely to accept. Diagnostic biomarkers, considering the prevalence of the disease (1-2%), would need to use biological material which is easily accessible and a method of collection which would not impact on the progression of the disease. Blood and urine therefore appear more suitable compared to synovial tissue or fluid particularly at this early stage of the disease where mostly small joints are involved. Later in the disease continuum, tolerability for more invasive procedure such as fluid aspiration or biopsy collection would provide material reflecting the disease site more closely allowing for individual variability to be taken into account for a personalised medicine approach.

2. Cytokines as Biomarkers

2.1. Cytokine Classification. Cytokines are small proteins which play important roles in cell signalling. They are secreted by a variety of cellular sources acting either on the cell producing them (autocrine) or on the surrounding cells (paracrine). They are classified as proteins and sometimes peptides and can also be glycosylated. Cytokines usually circulate in very small amounts (picomolar 10^{-12} M) and, nonetheless, their concentration can increase up to 1,000-fold when required. Cytokines have originally been identified in the context of the immune system; however, it has now been shown that they are produced by and influence the behaviour of a variety of nonimmune cells. Cytokines are often referred to as "growth factors" by association with one of their most common effects, the induction of cell proliferation, despite a wide spectrum of roles in survival, apoptosis, differentiation, and functional activation (contribution to the immune response).

Over the years, cytokines have been categorized into various classes, families, or superfamilies. It has been done using either their numerical order of discovery (notably, in the interleukin family, currently up to IL-38) or a given functional activity (e.g., the larger tumour necrosis factor family). In that case, they are further divided between cytokines which enhance cellular immune responses (type 1) as opposed to those which favour antibody responses (type 2). This subclassification is performed using their function (early or late, innate or adaptive, pro- or anti-inflammatory, mitogenic, regulatory, survival functions) or, sometimes, using their primary cell of origin (monokine, lymphokine). More recently, classification has been achieved using structural homologies shared between related molecules. Nevertheless, despite sharing sequence homology and some promiscuity between their receptor systems, cytokines demonstrate specificity in their function and even opposing functions within members of the same family (best illustrated in the TNF superfamily).

Methods of detection for cytokines also vary considerably. Enzyme-linked immunosorbent assays (ELISAs) have long been considered the "gold standard," but, nowadays, the development of multiplexing technology has allowed biomarker programs to investigate whole cytokine networks as opposed to individual candidates notably enabling large data sets to be generated from small body fluid volumes. Several multiplexing technologies are now available, including the bead-based immunoassay (often referred to as Luminex assay), membrane-based ELISAs, and Mosaic ELISAs, as well as cytometric bead arrays (CBAs). Concerns have been raised related to the sensitivity of some multiplex solid-phase assays [12] as well as interference from heterophilic antibodies [12–19]. This is of particular relevance in autoimmune disease where rheumatoid factor (RF), a heterophilic autoantibody directed against the Fc portion of IgG is present notably in RA [12, 20–25].

2.2. Variability and Limitations of Cytokine Measurements

2.2.1. Patient Related Variability. There are a number of features and conditions that can influence cytokine production which are related to donor variability in both health and disease. Some of these characteristics are unlikely to change during treatment (genetic/ethnic background, gender, and age); however, others may greatly limit the ability to use cytokines as biomarkers in everyday practice. These factors such as diurnal rhythmicity and sample handling factors (collection methods, storage, and plasma versus serum) may influence the measurement of cytokines and are also likely to change with not only therapy but also stress and cachexia. Such factors are likely to contribute considerably to the disparities seen among similar types of clinical studies [53–55].

(1) Age and Gender Effects. Comprehensive analysis of 30 different biomarkers in ≈400 healthy donors, ranging in age from 40 to 80 years, showed an increase in serum interferon-inducible chemokines (MIG and IP-10), eotaxin, and soluble TNFR-II with advancing age [56]. Multiple studies discussed differences in cytokine production associated with donor age, and several reports have demonstrated that chronic, low-grade inflammation is linked with the aging process [57–59]. An age-related increase in IL-6 concentration has been reported in serum, plasma, and supernatants of mononuclear cell cultures obtained from elderly subjects [60, 61]. Some studies demonstrated that plasma levels of tumor necrosis factor (TNF) are elevated in elderly populations [59, 62–64]. Conversely, other cytokines regulating T cell functions, such as IL-2, may be decreased with aging. The suppressed production of IL-2 leads to a small clonal expansion of T cells thus decreasing the ability to develop specific immune responses [61]. Modifications of the immune system are globally evaluated as a form of deterioration called immunosenescence. However, ageing is also accompanied by a chronic low-grade inflammation state, showed by a 2 to 4-fold increase in serum levels of inflammatory mediators which act as predictors of mortality independently of preexisting morbidity. This proinflammatory status underlies biological

mechanisms responsible for decline in physical function, and inflammatory age-related diseases are initiated or worsened by systemic inflammation [65]. The term "inflammaging" has been coined to explain the underlying changes common to the most age-associated conditions [66, 67].

Longitudinal cytokine production in paediatric and adult patients identified multiple differences in terms of proinflammatory cytokines such as IL-6, IL-8, IL-1alpha, IL-1beta, MCP-1, MIP-1alpha, IL-15, IL-5, IL-17, IL-18, and IP-10 and of anti-inflammatory cytokines such as IL-10, G-CSF, IL-13, IFN-gamma, and IL-4 between the two groups [68]. Altogether, the age of onset in RA patients is to be taken into consideration as it may reflect the cytokine production profile. Men and women also present with gender related differences in the way their immune system responds to challenge [69]. Females demonstrate better B cell-mediated immunity than age-matched males (with higher immunoglobulin levels, stronger antibody responses, and increased resistance to certain infections). Gender also influences T cell immunity, females having greater resistance to induced tolerance, an increased risk to reject grafts, and higher levels of IL-1, IL-4, and IFN-gamma in contrast to men who produce more IL-2, -4, and -13 and whose monocytes secrete more IL-1beta and TNF-alpha [70]. Differences in cytokine production profile have also been suggested to play an important role in the gender bias with regards to the ratio of relapsing remitting and secondary progressive multiple sclerosis [71] as well as susceptibility to urinary infection [72]. Aging has also been associated with alterations of the musculoskeletal system and a decline in sex hormone levels, which have a central role in the regulation of bone turnover. The effect of age combined with gender on cytokines and markers of bone metabolism production showed an increased proportion of T cells producing IFN-gamma and IL-2, IL-4, IL-10, and IL-13 particularly in elderly women after menopause [73].

(2) Circadian Rhythm. Cytokines present a circadian pattern. For example, IFN-gamma, TNF-alpha, IL-1, and IL-12 production exhibits distinct diurnal rhythms that peak in the early morning [74] and are related to the rhythm of plasma cortisol and melatonin [75–77]. Taking IL-6 as an example, notably with respect to RA, IL-6 demonstrates important variation in serum or plasma levels in healthy subjects over a day period with a particular biphasic rhythm [78] altogether amounting up to a CV >23%. After correction for analytical variation, a rise in serum IL-6 in the late evening and the early morning has been reported in RA [78–82] as well as high variations between and within days not necessarily indicating rhythmicity [54]. Therefore, only IL-6 changes over twice the biological variation (>50% difference) should be considered significant [78]; however, in order to obtain comparable and meaningful results, the time of sample collection should be synchronized, with a morning sample collection time being ideal. This does not affect all cytokines but is not particularly well described for many and should be considered if/when validating a biomarker for clinical use.

(3) Food Intake. Long-term food intake patterns (i.e., obesity or weight loss) have been shown to affect circulating cytokine levels, notably TNF-alpha [83]. Postprandial cytokine levels are also affected by feeding; notably circulating IL-6 levels are increased, while TNF-alpha levels are decreased [84–87]. Food supplements (in particular, antioxidants such as glutathione and vitamins E and C) can attenuate the feeding-induced rise in plasma cytokines [88, 89]. Hence, patients should be instructed to maintain normal dietary habits and avoid food supplements prior to sample collection if the cytokine of interest is sensitive to such regulation [90, 91].

(4) Exercise. Physical exercise can affect cytokine levels in the circulation [54, 92]. While plasma cytokines are produced by many cell types, muscle cells are a major source of secreted cytokines during exercise [93, 94]. However, these particular responses are highly specific to the exercise protocol and physiological strain (duration, nature of the exercise, and intensity) [95, 96]. Several studies reported elevation of plasma IL-6 in healthy subjects, which peaked at the end of exercise. The magnitude of the IL-6 response was related to the duration and intensity of the muscle work, the mass of muscle recruited, and the subject's endurance capacity [78, 97–99]. In patients with RA, no changes in serum IL-6 were found after cycling. This could be due to the less strenuous exercise performed by the RA patients because of their widespread joint pain [78, 100]. In contrast, evidence suggests that the prophylactic effect of prolonged, endurance type exercise protocols may be mediated via the induction of an anti-inflammatory environment (increases in circulating levels of IL-1RA and IL-10) [101]; however, how/whether both are linked remains poorly defined. There is nevertheless consensus that exercise training protects against some types of cancers by enhancing antitumour immunity and reducing inflammatory mediators. Altogether, any unconventional strenuous activity prior to blood collection for cytokine measurements should be avoided.

(5) Stress. Stress and emotional problems were also shown to influence cytokines levels; however, studies yielded contradictory data with decrease, increase, and no change in proinflammatory cytokine production being reported [102–104]. Nevertheless, lower self-rated health was associated with higher levels of inflammatory cytokines IL-1 and TNF-alpha (controlling for age, education, and physical health) [104].

2.2.2. Preanalytical Related Variability. There are several specific problems posed by sampling conditions (i.e., preanalytical issues) in addition to those described above. Cytokines act either in a paracrine or an autocrine manner as they are released and consumed locally, close to the site where the immune reaction occurs. Therefore, they are rarely detectable in peripheral blood and then only at low levels [105]. Blood may thus only partly reflect pathologies, including RA, and therefore not be the material of choice. The half-life of many cytokines is also measured in minutes; hence, the time lapse between the collection and processing of the samples may be a significant factor limiting the use of cytokines as biomarkers.

Data reproducibility can be affected by normal human variability, which is relatively easy to control in model systems (i.e., in cell culture or even animal models) but is much

harder to control in real subjects. Designing and testing the sample collection (i.e., anticoagulants, stabilizing agents) and handling (temperature, elapsed time from collection to initial processing, and endogenous degrading properties of the analyte) and processing protocol/method will represent key elements in the successful development of any biomarkers [106].

(1) Serum or Plasma? In body fluids, cytokines can exist under multiple molecular forms related to posttranslational modifications (i.e., glycosylation), monomers/polymers, precursors, and degradation products or complexed with other proteins [107]. Such molecular forms can behave differently in assays used to determine their levels; therefore, choice of different analytical techniques will be determinant in selecting blood preparation. Serum and plasma are not interchangeable, and the use of one or the other will determine which technique should be used for analyte quantification (see Table 1). Therefore, a lack of consensus exists with respect to the optimal type of specimen to measure cytokines, and the question remains open as to whether plasma or serum should be used. It is important to determine if the method used to collect and prepare the sample may introduce alterations to the cytokine to be tested (i.e., cytokines, either individually or on all proteins in the sample) or whether certain preparation methods are desirable or not for certain cytokines [108].

Serum represents the soluble fraction of clotted blood. Serum preparation involves the removal of fibrinogen, platelets, and other circulating proteins. Clotting takes a minimum of 30 minutes but no longer than 60 minutes. Blood should then be centrifuged for 10 minutes and serum should be separated from the clot. Blood cells may get activated during the clot formation and cytokines may be released as a result (such as IL-1, IL-6, and CXCL8) [27, 90, 109, 110]. Rapid sample processing is therefore essential to accurately measure cytokines due to platelet release (i.e., IL-1, IL-6, sCD40L, and others) [21]. For this reason, in order to have correct estimates of specific cytokine levels, it may be preferable to measure them in plasma rather than in serum [34, 111]. This notably raised issues when comparing serum and plasma levels for TGF, IL-1, IL-6, IL-7, and so forth [38].

Plasma is the soluble fraction of anticoagulated blood. To obtain plasma, various anticoagulants can be used (ethylenediaminetetraacetic acid (EDTA), lithium/sodium heparin, and sodium citrate). Cytokine measurements were shown to be affected by the anticoagulant used [78] and, notably, lithium heparin and sodium citrate were shown to affect levels of IL-6 and TNF-alpha compared to EDTA plasma [35, 112, 113]. Citrate plasma collection also results in the reduction of total protein concentration due to the volume of citrate anticoagulant diluting the blood, in addition to an osmotic withdrawal of water from blood cells [114]. Endotoxin present in lithium heparin tubes when sterility is broken [113] can also induce cytokine release from cells, whereas EDTA inhibits endotoxin [26, 31]. Variation in cytokine levels could be attributed to anticoagulant-induced release of cytokines by blood cells notably in heparin plasma but not in EDTA plasma, [115]. Altogether, plasma collection with use of EDTA seems to bring the most consistent results [34, 35, 116] and more closely resembles data obtained in serum [31, 35, 39, 78, 90, 117]. Cytokine stability also appears increased in EDTA plasma [26, 118] perhaps through EDTA's role as a protease inhibitor. Further mechanisms can explain differences in stability such as change in degradation rate or modification of cytokine's structure due to the differential presence of other proteins in EDTA plasma compared to citrate plasma or serum (i.e., soluble forms of receptors) leading to a lack of recognition of the antibodies used in the ELISA. The limitation in using plasma remains the need for rapid separation after collection with changes occurring as soon as 30 minutes after sample collection [34].

Over the recent years, improvements in the collection tubes have been made, notably with the use of serum separator tubes, which include a gel that serves as a barrier between serum and the clot [106], or the substitution of plastic for glass allowing direct centrifugation [119].

Altogether, no single type of sample is optimal for every analyte; therefore, the development of assays for individual cytokines should require optimisation on a case-by-case basis, although it would be recommended to collect both serum and EDTA plasma.

(2) Time to Processing. Time is an important factor that needs to be accounted for when measuring circulating cytokines which have a relatively short half-life and an important risk of degradation notably when comparing them to other proteins such as antibodies [26, 34, 120]. Changes in the amount of cytokine detected depend on the delay and duration of sample processing and are likely due to altered production by cells after blood collection [31, 54, 120], or their binding by other proteins (i.e., soluble receptors or cells surface receptor) [42, 120, 121], or, finally, due to enzymatic activities (proteases) leading to cytokine digestion (see also Table 1). Rapid processing of samples is therefore essential, notably as samples obtained from patients often present with higher concentrations or increased activity of proteases or other factors which render specimens even more unstable than those obtained from healthy controls [111]. Ideally, samples destined for cytokine detection should be collected in sterile (endotoxin-free) tubes and processed quickly with a minimum of 30 minutes of clotting time but no longer than 60 minutes after blood draw, independently of the type of tube used (plasma or serum). Processed plasma or serum should be frozen at −80°C as soon as possible in small aliquots to avoid repeated freeze-thaw cycles [107, 122]. Some reports proposed to keep samples refrigerated at 4–8°C (but not on ice) after clotting for the duration of processing as room temperature favours proinflammatory cytokine degradation such as IL-6 but conversely stabilises TNF-alpha [26, 34, 120, 123, 124]. Most cytokines are relatively stable with the well-known exception of TNF-alpha and IL-6 [42, 125, 126]; therefore, the interval between blood draw and separation should not exceed 3–24 hours, even when the tubes are stored at 4–8°C and only when EDTA tubes are used (TNF-alpha however cannot be reliably measured any longer), although many cytokines have not been sufficiently tested [26, 35, 37, 78].

Table 1: Summary of reported preanalytical precautions to be enforced to measure some of the main cytokines. A large amount of the literature is contradictory, most likely due to different analytical discrepancies in the evaluation of the effect of preanalytical conditions. This table aims to provide a review of the literature available; however, there is no thoroughly enough conducted study allowing us to suggest definitive guidance as to the best condition to process samples universally (i.e., allowing for any cytokines to be tested). Furthermore, the inflammatory nature of RA further complicates such issues.

	Serum or plasma	Delays in separation (whole blood pending processing)	Storage condition (after separation)	Sensitivity to freeze-thawing (F/T) cycles
IL-1 (alpha and beta)	(i) Both are used [26] (ii) Higher heparin plasma concentrations compared to serum [27] (iii) Higher levels in EDTA plasma than in heparin plasma [28]	(i) Increased levels with delays in processing when kept at RT (ii) No significant change for up to 4 days of delayed processing in samples from healthy people; however, there is significant decrease in samples from trauma patients [29] (iii) Significant increase in serum, after delay of 48 h at 4°C, with RA patients, but in plasma there is an increase only if kept at RT [30] (iv) Prolonged delays before separation result in increased endotoxin-induced cytokine release in contaminated tubes [31, 32]	(i) Storage at 4°C results in an increase (ii) Heparin plasma showed time-dependent increases in concentration [31]	No significant change in stability in plasma/serum for up to 6 F/T cycles [26]
IL-2	(i) Heparin plasma concentrations are higher than in serum [27] (ii) Comparable or higher levels in EDTA plasma compared to heparin [28]	No significant change for up to 4 days of delayed processing in samples from healthy people; however, there is a significant decrease when processing samples for trauma patients [29]		
IL-4	(i) Heparin plasma concentrations are higher than in serum [27] (ii) Higher levels of IL-4 in EDTA plasma than in heparin [28] and higher concentration in serum than in heparin plasma [33]	(i) No significant change for up to 4 days of delayed processing [29] (ii) No significant change for serum or EDTA plasma stored before centrifugation at 4°C, RT, and 35°C [30]		
IL-5	Slightly higher levels in EDTA plasma than in serum [30]	(i) No significant change for up to 4 days of delayed processing [29] (ii) Plasma levels significantly increased if separation delayed by 4 h stored at 4°C. Further increase if stored at RT [30] (iii) Serum levels increased with delayed processing for 24 h at 4°C or 4 h at RT [30]		

Table 1: Continued.

	Serum or plasma	Delays in separation (whole blood pending processing)	Storage condition (after separation)	Sensitivity to freeze-thawing (F/T) cycles
IL-6	(i) Serum and EDTA plasma samples are comparable while levels in heparin and citrate plasma are lower [34] (ii) Serum levels are higher than EDTA plasma [30] (iii) Serum and EDTA, citrate, or heparin plasma gave comparable results [28, 35] (iv) Heparin plasma levels are higher than those of serum [27] and this anticoagulant is not recommended due to *ex vivo* Il-6 release prior to assay [31] (v) Endotoxin contamination (LPS) triggers release in heparin compared to EDTA plasma [32, 36]	(i) Reduced levels when samples are left unseparated for 24 h at 4°C or RT [26] or 4 h at RT [34] (ii) Significant reduction in stability and recovery with time at RT [26] (iii) Increased levels with delays in processing when left at RT (iv) No change in samples stored at 4°C for 24 h before centrifugation [35] (v) No change when left at 4°C or 20°C for up to 4 days before centrifugation [37] (vi) No significant change for up to 4 days of delayed processing in samples from healthy people; however, there is a significant decrease when processing samples from trauma patients [29] (vii) Plasma levels unchanged when stored for up to 3 h at 37°C but afterwards, an increase is observed [31] (viii) Increased endotoxin-induced cytokine release in contaminated tubes with delays in processing [32]	No change in levels in serum stored at 4°C, −20°C, and −30°C [37]	(i) No significant change for up to 6 F/T cycles (ii) No significanceobserved after 2, 3, and 4 times of repeated F/T cycles [37] (iii) No significant effect for up to 3 F/T cycles in EDTA plasma and serum but inconsistent stability in heparin plasma [26, 34]
IL-7	(i) No significant difference between plasma and serum IL-7 levels [38] (ii) Serum levels are significantly higher than in plasma [33] (iii) Heparin plasma concentrations are higher than in serum [27]	(i) 2 to 4 hours of delayed processing decrease IL-7 plasma levels [38] (ii) With 2 to 4 hours of delayed processing, serum levels are stable [38] (iii) No significant change for up to 4 days of delayed processing [29]		Stable for up to 3 F/T cycles
IL-8	(i) Comparable levels in heparin plasma and in serum [27] (ii) Higher serum levels than in heparin plasma [33] (iii) Lower levels in EDTA plasma than in heparin [28] (iv) LPS induced release in whole blood is up to 100 times higher in heparin versus EDTA plasma [36]	(i) Increased levels with delays in processing if left at RT [29] (ii) Stable levels if stored at 4°C		
IL-9		No significant change for up to 4 days of delayed processing [29]		

Table 1: Continued.

	Serum or plasma	Delays in separation (whole blood pending processing)	Storage condition (after separation)	Sensitivity to freeze-thawing (F/T) cycles
IL-10	(i) Higher levels in serum than in plasma [39] (ii) Lower levels in EDTA plasma than in heparin [28]	(i) Increased levels with delays in processing if left at RT (ii) The longer the delay, the less stable the levels (iii) No significant change for up to 4 days of delayed processing in samples from healthy people; however, there was a significant decrease in samples from trauma patients [29]	Storage temperature affects stability: the higher the temperature, the faster the decline [37]	No significant decline in levels observed after 2, 3, or 4 times of repeated F/T cycles [37]
IL-12 (p70 & p40)	Heparin and EDTA plasma levels are higher than in serum [27, 28, 30, 33]	(i) Levels decrease with delayed processing [29] (ii) No significant change for up to 4 days of delayed processing [29] (iii) Increase in serum after 48 h of delayed processing at 4°C and 4 h at RT [30] (iv) Stable in plasma for over 48 h at 4°C and for up to 48 h at RT [30]		
IL-13	(i) Heparin plasma levels are higher than those of serum [27] (ii) Comparable levels in EDTA and heparin plasma [28]	No significant change for up to 4 days of delayed processing [29]		
IL-15		No significant change for up to 4 days of delayed processing [29]		
IL-16				Decrease after the 5th F/T cycle [40]
IL-17	(i) Lower levels in EDTA plasma than in heparin [28, 33] (ii) Higher levels in serum than in any plasma (EDTA, citrate, and heparin) [33] (iii) Higher levels in EDTA plasma than in serum [30]	(i) No significant change for up to 4 days of delayed processing in samples from healthy people; however, there was a significant decrease in samples from trauma patients [29] (ii) Plasma levels increased if separation delayed by 4 h at 4°C with further increase with time (up to 24 h) [30]		

Table 1: Continued.

	Serum or plasma	Delays in separation (whole blood pending processing)	Storage condition (after separation)	Sensitivity to freeze-thawing (F/T) cycles
IL-18	Similar levels in serum and EDTA plasma [30]	No changes in EDTA levels over 48 h at 4°C, and significant increase after 24 h at RT [30]		
TNF-alpha	(i) Comparable results in serum and EDTA plasma [39] (ii) Lower levels in sodium citrate plasma (iii) Higher heparin and EDTA plasma levels than in serum [26, 27, 30] (iv) LPS induced release of TNF-alpha 20 times higher when in heparin compared to EDTA plasma [36] (v) Endotoxin induces high release [32, 41]	Contradictory data: (i) Reduced levels with delays in processing when kept at 4°C and RT [26, 42] (ii) Increased levels with delays in processing if left at RT [34, 43] (iii) No significant change for up to 4 days of delayed processing [29] (iv) Time-dependent increases in levels with delays at 37°C in heparin plasma [31]	(i) Reduction in samples kept at RT for 20 days (ii) Relatively stable in samples stored at 4°C [39] (iii) Stable at −70°Cfor over 9 months [42]	Contradicting data: (i) Levels increased with successive F/T cycles [26, 34] (ii) No differences reported in plasma and serum for up to 10 F/T cycles [39]
TGF-beta 1	(i) Higher levels in serum than plasma (citrate, EDTA) due to platelet degranulation during the clotting process [30, 44–46] (ii) EDTA plasma is not recommended because of the extreme interindividual variation of PLT activation and concurrent *in vitro* GF release [44] (iii) Sodium citrate can be used but is not as effective or reliable [44] (iv) CTAD (citrate theophylline dipyridamole adenosine) is recommended as it blocks the *in vitro* release of growth factors from PLTs (v) Plasma concentrations should be corrected by simultaneous measurement of markers of platelet degranulation [47]	(i) Increased levels with delay when plasma is left at RT or 37°C [48] due to platelet degranulation and release [45] (ii) Lower level in serum if left at 4°C than at RT [49] (iii) Speed of centrifugation affects recovery in plasma (2,500 ×g for 30 min yields lower levels than 1,200 ×g for 10 min) [49]		<5% deviation from baseline value in serum upon successive F/T cycles (for up to 100 F/T cycles) [48]
sCD40-ligand	(i) Use of platelet poor/free plasma is recommended as it is [50] higher in serum than in plasma (EDTA, citrate, and heparin) due to clot retraction and sCD40L shedding from the platelet surface [33, 50] (ii) EDTA anticoagulated plasma samples are not appropriate for sCD40L measurements [51]	(i) Increased levels after 3 h of delay in processing [50] (ii) Serum levels increase with time in delayed processing [50] (iii) No significant changes in serum or plasma levels detected after storage at 4°C for up to 48 h (iv) Significant loss observed in serum and plasma, left at RT [52] (v) Decreased levels with increasing centrifugation g values (200–13 000 g), which gradually deplete plasma of platelets [52]	(i) Loss in serum and plasma kept for over 4 h at RT [40] (ii) No change while stored at 4°C (iii) Significant decrease after 24 h at 37°C [40]	(i) Stable for up to 3 F/T cycles [52] (ii) Increased after 5 or 10 F/T cycles [40]

Table 1: Continued.

	Serum or plasma	Delays in separation (whole blood pending processing)	Storage condition (after separation)	Sensitivity to freeze-thawing (F/T) cycles
IFN-gamma	(i) Collection in sterile pyrogen free tubes is very essential (ii) Serum levels are higher than in plasma (EDTA, citrate, and heparin) [33] (iii) Heparin plasma levels are higher than in serum [27] (iv) Levels in heparin plasma are higher than in EDTA plasma [33] (v) Levels in EDTA plasma are higher than in heparin plasma [28]	(i) Significant reduction with time at both 4°C and RT in serum and EDTA tubes [26] (ii) IFN-gamma decreases if processing is delayed [29]		Stable for up to six F/T cycles [26]

The effects of centrifugation speed are more difficult to evaluate. Gradual increase in *g* values (from 200 to 13,000 g) is necessary to achieve graded depletion of platelets and leucocytes from plasma; however, it reduces the levels of certain cytokines (i.e., sCD40L) [52]. Of note, the use of blood tubes with gel separator imposes a certain centrifugation speed to allow separation of serum and cells but does not allow tubes to be chilled before or during centrifugation [127].

(3) Storage Temperature and Freeze-Thaw Cycles. By and large, most cytokines and soluble markers are quite stable if frozen (see also Table 1). Storage conditions, however, vary with a choice of temperatures from short-term storage at room temperature (RT) or 4–8°C (days) to medium term (a few months) more often between −20/−30°C and long term (years) at −70°C. Direct comparison of several cytokines in plasma stored for 20 days at RT, 4°C or −70°C, showed remarkably stable levels (IL-10) except for TNF-alpha particularly at room temperature [128]. In contrast, a more recent study of reliability and reproducibility of cytokine measurements in healthy donors [122] showed that, while most cytokine measurements are stable for up to 2 or 3 years when stored at −80°C (see details in Table 1), they do not all remain stable after repeated freeze-thaw cycles. After 4 years, most cytokines were degraded. Importantly in RA, levels of certain cytokines such as TNF-alpha increase with each successive freeze-thaw cycle [54, 90, 122]. Therefore, it remained difficult to compare studies from different centres even when using the same assay for cytokine measurements (i.e., commercial kit) [39]. Altogether, the consensus would recommend storing specimens at −80°C in as many small aliquots as possible to limit freeze-thaw cycles [129].

2.2.3. Analytical Variability

(1) Assay Type. Numerous immunoassays exist to measure cytokines both in their protein form: ELISA, nitrocellulose, or other solid phase assays, immunohistochemistry, and bead-based flow cytometry multiplex immunoassays, and in their molecular form: reverse transcriptase PCR, microarrays, and *in situ* hybridisation (Table 2). Immunoassays use antibody to immobilise cytokines on a solid surface and then identify them with different methods for quantification using colorimetric enzymatic reactions, fluorescence, luminescence, or even, in the past, radioactivity. There are two types of assays using either one or two antibodies: one being for cytokine capture adding more specificity compared to total protein plastic binding and the second one being for detection. The major benefit to using antibodies is that assays are more specific and reproducible. Several platforms for the detection and quantification of cytokines exist. There is no universal best method for cytokine measurements; however, the oldest technique (ELISA) is often used as gold standard despite the fact that direct comparison between many commercially available kits has not been performed. Cytokines show complex protein structures (monomers/polymers, precursors, various degrees of glycosylation, and degradation products) and their activity often depends on the integrity of such structure. Minor changes that may not be detected by physicochemical measurements, immunoassays, or biophysical methods may have dramatic effects on biological activity (e.g., cytokines may lose most of their biological activity but will remain detectable if measured as mass) [130]. The presence of soluble forms of the cytokine receptors (i.e., sIL-2R, sIL-7R, and sTNF-R) in biological samples and the existence of autoantibodies to cytokines (i.e., anti-TNF-alpha, IL-6, and IL-1) [131] may or may not interfere with the recognition of cytokines by either capture or detection of antibodies [39, 132–134]. Each method has advantages and limitations and should be carefully selected with respect to the research purpose. To date, most cytokine measurements in large studies essentially used ELISA, which is widely accepted as the "gold standard" method. The main limitation of ELISA remains that it allows the characterization of a single cytokine at a time, hence the development of multiplex technologies. One of the most commonly used methods for this is the multiple target based assay [135], which can measure up to 100 different analytes per sample from a small volume of body fluid [136], or more recently the cytometry bead assay (CBA) which relies on bead as solid phase and uses flow cytometry to discriminate between analytes [137]. Multiplex measurement of inflammatory cytokines in human serum by electrochemiluminescence assay was recently developed [138]. These multiplex assays are in concept close to ELISAs and dependent upon the careful choice of the capture/detection antibody pairs and proper buffering to minimize differences in assay performances [135].

Several studies have compared cytokine levels determined by ELISA and multiplex immunoassays with results showing either good or poor correlations between the methods. Therefore, it is not surprising that discrepancies in data comparing measurements of cytokines were observed when different commercial/manufacturers' kits were used, even if preanalytical conditions of samples collection, separation, and storage were identical [85, 136, 139]. The use of different antibody clones to capture and detect cytokines is also likely to affect results and change the level of sensitivity of such assays. Furthermore, some monoclonal antibodies recognise different molecular complexes (monomers/polymers, precursors, glycosylation, degradation products, or total bioactive or inactive forms) [140]. In summary, comparison of the same samples (eliminating preanalytical bias) using several commercial ELISAs demonstrated that variability was mostly attributable to each assay (measuring TNF-alpha, IL-1 alpha and IL-1beta, IL-6, IL-2, IFN-gamma, and the soluble receptors of IL-2 and TNF) but yielded comparable results when the same ELISA was used at different centres [85, 139]. The nature of the different pairs of monoclonal antibodies employed in each ELISA is most likely the major source of variability, but these findings also highlight the necessity of establishing international standards for all immunoassays as ranges are also widely variable between these commercial assays. If cytokines are to be employed as clinical biomarkers for diagnosis, prognosis, and prediction, accurate and reproducible assays need to be adopted internationally.

(2) Interferences. Interferences within immunoassays are numerous, complex, and usually difficult to resolve. Proteins

Table 2: Description and characteristics of assays measuring cytokines.

Cytokine assay technique	Description	Characteristics
Bioassays	*Bioassays* (commonly used shorthand for *biological assays*) are typically assays by which the potency or the nature of a substance is estimated by studying its effects on living organisms They can be conducted to measure the concentration/effects of a cytokine on a living cell Example: IL-2 bioassay using an IL-2 dependent cell line that will undergo apoptosis in the absence of IL-2 in a dose dependent manner They require tissue culture facility	Low specificity Semiquantitative detection Highly sensitive with detection limit < 1 pg/mL Narrow analytical range Time consuming (24–96 h) Low precision (CV = 20–100%) Drug interference Laborious protocol with high staff cost
ELISA	Quantitative detection of a molecule (bioactive and inactive) based on its capture by an antibody followed by its detection by another antibody coupled with a detection (commonly named ELISA) It requires specialised equipment	Less sensitive than bioassays <10 pg/mL Relatively large sample volume Wide analytical range High reagent cost Excellent precision (CV = 5–10%) No drug interference Simple and relative rapid protocol
Solid phase assay (Luminex)	Technology based on the detection of dyed microbeads capturing a cytokine with a first antibody and quantifying it with a second antibody coupled with fluorescence and lasers detection It allows multiplex detection	Small sample volume Lower sensitivity than ELISA Large range of analytes Sensitive to interferences from heterophilic antibodies (i.e., naturally occurring anti-antibodies), anti-cytokine antibodies, and presence of soluble receptors
Other solid phase assays	Mosaic ELISA ELISA like technology allowing multiple detection of cytokines in a 96-well plate format by spotting capture antibodies	Small sample volume Lower sensitivity than ELISA Only 8 analytes per test
Molecular techniques	All techniques allowing mRNA quantification Earlier detection of cytokines at transcriptional level however may not represent cytokine production and release They require specialised equipment	Highly specific Highly sensitive as they can detect changes at the single-cell level Complete analytical range (from single cytokine to as many as needed) Excellent precision No drug interference Simple and relative rapid protocol Relatively high cost

can show an altered expression pattern in more than one disease. The presence of lipids, complement factors, and other complex molecules in the blood was also shown to interfere with a number of assays. Human anti-animal antibodies present in biological samples (especially human anti-mouse antibodies) may cause problems; however, these may be blocked by the use of multiple species serums as blocking agents [141]. Haemolysis interference occurs rarely; however, it can affect some analytes. Lipaemia interferences were confined when using immunonephelometric and immunoturbidimetric assays, and, ideally, grossly lipaemic samples should be cleared (using ultracentrifugation of lipaemic samples with correction for volume displacement errors) or discarded. Antigen excess may, in some cases, result in false low values [142]. Complement factors and paraproteins are capable of binding to assay antibodies (capture and detection) causing interferences [142]. In addition, biological fluids may also contain naturally occurring antibodies to a variety of proteins, including cytokines themselves. Such antibodies, although at variable levels notably between normal donor and patient populations, can interfere with assays particularly if they share the same epitope on the cytokine [143]. The existence of autoantibodies against cytokines has been documented for TNF, IL-1 (alpha and beta), IL-2, IL-6, IL-8, IL-10, and IL-18 [144–148]. Autoantibodies against IL-1 are the best studied. Their prevalence is high with an affinity which can reach up to 10^{-11} M that is very similar to the affinity of antibodies developed for immunoassays [140]. However, the main issue remains heterophilic antibodies. These antibodies are naturally produced polyclonal autoantibodies with low specificity directed against multiple poorly defined antigenic immunogens. Most often, they are present in individuals exposed to foreign proteins (e.g., domestic animals and household pets). The occurrence of false positives in immunoassays [13–16] is often the result of heterophilic antibodies nonspecifically bridging the assay antibodies [18,

19]. As a result, studies have often overestimated cytokine levels notably when using the Luminex technology [12].

Blood samples from patients with autoimmune diseases, such as RA, may be problematic due to the presence of additional disease related autoantibodies [149]. RF is an autoantibody directed against the Fc portion of IgG and is found in 75% of patients presenting with RA as well as other diseases such as Sjögren's syndrome, infective endocarditis, systemic sclerosis, and systemic lupus erythematous (SLE) [24]. RF was shown to exhibit most of the heterophilic antibody properties with several antigen cross-reactions [25] and hence immunoassay in RA is particularly sensitive to this issue and needs careful evaluation for RF interference [12, 150–153]. Heterophilic immunoglobulin may further develop as a result of treatment with drugs attached to mouse (or humanised) monoclonal antibodies.

Several methods for removing heterophilic antibody (notably RF) from patients sera have been developed [21, 154–156]: (i) initial serial dilutions may be recommended, particularly when results demonstrate nonlinearity suggesting the presence of heterophilic antibodies, (ii) the use of blocking reagents such as nonimmune serum from the same species as the assay antibodies, species-specific polyclonal IgG, and multispecies mixture (20% normal mouse serum, 10% goat serum, and 10% rabbit serum), as well as commercial reagents such as HeteroBlock [155], and (iii) the specific removal of immunoglobulin G using sepharose-L or polyethylene glycol precipitation (PEG 6000) has also been used. These methods act by physical removal of the immunocomplexes [155], which are then separated by centrifugation. Several reports have been published investigating interference by heterophilic antibodies in RA sera using solid phase multiplexing technology including Luminex [23, 155, 157, 158], a glass chip/chemiluminescence platform, or a multiplex sandwich ELISA. They showed clear interference (i.e., false positive) in RF-positive sera but not in negative samples [157]. In our lab, all methods were efficient at blocking/removing relatively low RF quantities in serum samples from RA patients [12]; however, none of these methods were effective when high levels of RF were present (>100 U/L) and residual RF still generated false positive results particularly when using certain types of assays (Luminex) but not others (ELISA, membrane-based ELISA, Mosaic ELISA, or CBA).

(3) Standardisation and Quality Control. Commercially available immunoassays in the form of "kits" are now extensively used. Considerable variability can arise from the use of these assays. Differences in measured levels of cytokines in identical samples using different standards ranged from 10- to 100-fold [130, 159–161]. Some issues are related to the use of different epitope specificity of the antibodies, while others arise due to the use of various reference preparations (standards) for calibrating the assays [55]. Comparison of cytokine levels requires unit definition by a standard that is assay independent, which, once defined, should be used by any laboratory, thus providing a means of ensuring uniformity worldwide [130]. Variations as a result of differences in standards account for as much variability as sample collection, processing, or storage issues [31, 42, 125, 159–168]. All cytokine assays should therefore be calibrated against such standards, regardless of assurances provided by the kit manufacturers. Notably, results of cytokine assays should be reported in picograms or nanograms per milliliter instead of arbitrary units. Major international efforts to organise standardisation of cytokine measurements have been conducted by the World Health Organisation, (see details at http://www.nibsc.ac.uk/products/biological_reference_materials.aspx), The National Institute for Biological Standards and Control (NIBSC), and the Biologics Evaluation and Research (The National Institutes of Health (NIH), Bethesda, MD 20205, USA) (http://www.who.int/biologicals/) [130, 131, 169]. Nonetheless, baseline values for a lot of cytokines have not yet been reliably established in healthy controls (despite a range suggested by most manufacturers), making it difficult to interpret the biological significance of minor variations in cytokine levels in patients [170]. Furthermore, some cytokine assays are sensitive at relatively high concentrations that may not always cover the physiological range even in diseases [12]. Quality control (QC) measure is also an essential step of biomarker development. Therefore, during the analytical phase, QC should be considered to document analytical performance during any studies to determine the acceptance or rejection of an analytical run during postanalytical sample analysis [136, 171]. QC samples could be prepared to evaluate the lower, middle, and upper performance limits of an assay. A number of validation samples (at least five different concentrations) should also be used to estimate intra- and interrun accuracy/precision and stability [136, 172, 173].

3. Cytokines Network in RA

Over the years, increasing numbers of cytokines have been involved in RA pathology, further to those used as target of cytokine-blocking therapies which emerged from the hypothesis that the most abundant cytokines present in the joint were more likely to be pathogenic. A large number of cytokines are detected at the disease site (through both mRNA and protein quantification) in both synovial tissue and fluid, where they have a role in perpetuating inflammation, cartilage destruction, and bone remodelling associated with RA. Several methods of detection (ELISA, immunohistochemistry) identified TNF-alpha and IL-1 as major players in the network of cytokines, notably directly expressed at the disease site in joint tissue or fluid. IL-6 and IFN-gamma are also present as well as GM-CSF and LIF. More recently, other cytokines were added to this list (IL-7, IL-15, IL-17, IL-18, IL-21, and MIP-1 notably) together with cytokines with activities targeted towards fibroblasts (TGF-betas notably) and finally several growth factors (PDGF, EGF, and VEGF) [174] and chemokines (IL-8, SDF-1, RANTES, and MCP-1). Cytokines favouring survival of infiltrating cells have also been detected (such as the pairs between IL-7 and T cell or BAFF and B cells). However, if proinflammatory cytokines (TNF-alpha, IL-1, and IL-6) are abundant in all patients, cytokines classically defined as anti-inflammatory and regulatory (IL-4, IL-10, IL-13, and TGF) [175, 176] as well as antagonist receptors (IL-1RA, or soluble IL-2R, or TNF-R)

are also present. Most of these cytokines have dual roles with anti- and proinflammatory aspects depending on the context and the network they form; hence, studying their roles and actual effects is particularly complex. The redundancy and synergy between the effects of all cytokines in such an intricate network may further explain the inadequate response to single blockade therapy notably in established disease [175].

The interplay between cytokines, where excess of one may result in suppressed production of another, further complicated by interactions with soluble receptors for some of these cytokines, renders data interpretation challenging (notably for TNF-alpha and IL-1) [88, 89]. The relationship between blood and tissue is often complex and translating findings often proves difficult if not conflicting. Data on cytokine levels in humans in relation to disease activity is still limited. Increased levels of cytokines such as IL-l, IL-6, and TNF have been interpreted as an indicator of the inflammatory state. It is unlikely that these cytokines could serve as "biomarkers" in inflammatory disease, as they are linked to the disease biological processes, hence not specifically associated with a particular disease. Additionally, lack of correlation is often observed between cytokine levels (in serum/plasma) and clinical endpoints.

On the other hand, the absence of a cytokine in disease is particularly difficult to interpret. As indicated above, there may be multiple reasons for the inability to detect a cytokine when actually it is expected to be found. Even in the absence of specific or nonspecific inhibitors, excessive consumption of a cytokine versus lack of its synthesis is hard to dissociate. As an example, IL-7 levels were reported to be low in RA serum [177–179]; however, they are high in synovial fluid and tissue. The presence of high levels of sIL-7R in serum [180] may explain this discrepancy and the associated loss of biological activity [177, 181].

Despite these limitations, there are some cytokine biomarkers, which appear to be relevant in RA. IL-6, despite not being disease specific [78, 92, 182], was shown to be more sensitive than CRP (despite being directly correlated with it) for the prediction of therapeutic response of RA patients to rituximab [183]. Similarly, IL-7 was shown to have some value as diagnostic biomarker associated with potential for more erosive disease [179].

3.1. Differential Cytokine Expression between Diseases. Over the years, many studies provided evidence of differential expression of cytokines between healthy control (HC) and diseases such as RA, osteoarthritis (OA), ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), systemic lupus erythematosus (SLE), or gout. These initially used functional assays measuring the production of cytokines in variable cell subsets using intracellular expression of cytokines (in CD4+ or CD8+, T cells or B cells, or monocytes), ELISA, ELISOPT, or mRNA quantification. Several important observations were derived from these experiments and the tables below summarise all this data as well as tissue sources and technology/experiment.

In vitro assays removed the microenvironment context; however, they reflect good the capabilities acquired through exposure to the priming effect that such microenvironment may exert (i.e., Th1/Th2 polarization, transition from naïve to memory). Altogether, they demonstrate the dysregulated expression of certain cytokines in T cells subsets notably and increased expression by monocytes in RA patients. Importantly, all cytokines tested were shown to be increased, with the exception of IL-2 and IL-4. Interestingly, RA patients' T cells showed hyporesponsiveness to stimulation of the T cell receptor (TCR) pathways and hardly produced any cytokines despite evidence of previous activation (memory phenotype) [184]. This deficit was attributed to chronic exposure to TNF-alpha [185] and/or abnormal RAP1 signalling [186–188]. The classic model of T cell naïve/memory differentiation is perturbed in RA. T cells despite being naïve with respect to antigen stimulation [189] express chemokine receptors which facilitate trafficking to sites of inflammation [7, 177]. This phenomenon was hypothesized to result from cytokine activation notably of naïve T cells (by IL-6 and TNF-alpha) bypassing the need for an antigen to achieve activation [190, 191]. Similar cells were found in RA joint (but not OA) [192] where they enable TNF-alpha production by monocytes in an antigen-independent manner. These properties of cytokine activated T cells were further extended to chemokine production and were confirmed *in vivo* using a cytokine cocktail containing IL-2, IL-6, and TNF-alpha [193]. Such increased ability to produce all types of cytokines reflects the chronic stage of the disease but nevertheless gives insight into potential candidates for further biomarker program.

3.2. Differential Cytokine Levels in RA Sera or SF. There are several studies comparing circulating levels of cytokine, they often show discrepancy in their results, and most do not use the appropriate biomarker development strategy. IL-1beta and TNF-alpha are increased in RA [194] and such profile is accentuated in active diseases compared to clinical remission [195]. In contrast, low levels of IL-2 and IL-7 were reported [177, 179, 194, 196]; however, those may be due to high levels of soluble sIL-2R and sIL-7R which are also present. IL-6 could not be detected in HC serum, while serum IL-6 levels are substantially increased in RA with significant circadian variations corresponding to the circadian rhythm of symptoms in RA [79]. High IL-7 [197] and IL-16 [198] were detected in sera and SF of RA patients compared to OA and are also confirmed in synovial tissues by mRNA levels. Certain cytokine levels were related to disease parameters such as IL-1RA and the number of tender and swollen joints [199], IL-18 (both sera and SF) and disease activity [200, 201], and IL-7 in the tissue (both mRNA and protein) with local levels of inflammation measured during arthroscopy [196]. IL-21 is highly produced in the synovial fluid of RA patients compared to paired serum specimens as well as healthy control sera. The increased levels of IL-21 correlate with those of IL-17 [202] and an association between levels of IL-21 and Th17 cells responses in the RA synovium was shown [202].

Similar increased serum levels of many cytokines were indeed found in other rheumatic diseases: notably PSA [203–205], SLE [206, 207], AS [208–210], and scleroderma [211, 212] (IL-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-16, IL-17, IL-18, and IFN-gamma, TGF-beta, or TNF-alpha, as well as IL-1RA

and sIL-2R or leptin) suggesting that such rises may reflect inflammation rather than being disease specific. Therefore, the biomarker value of either one of the cytokines, or a combination of them, will likely depend on whether their disease specificity can be verified.

3.3. Cytokines as Diagnostic Biomarkers for RA. The early diagnosis of RA is critical, as it has been demonstrated that a therapeutic window of opportunity is available very early in the development of RA, when disease can be stopped efficiently, preventing structural and functional damage and leading to remission if treated. In face of such a need, clinical diagnosis remains difficult. At the (very) early stage, inflammatory arthritis often has an atypical presentation with progression towards RA that can vary in speed. Autoantibodies (RF and ACPA) are useful in RA diagnosis as recently recognised by their inclusion in the new diagnostic EULAR 2010 criteria. However, they both lack sensitivity in early disease (<50%) [213] even if ACPA specificity is quite high (over 95%) [214].

The ideal RA diagnostic biomarker should therefore be characterised by high specificity and sensitivity, both close to 100%. An ideal biomarker should also detect the presence of RA at early stages. Few, if any, biomarker testing systems achieve these levels of sensitivity and specificity although this can be approached by improvement of the assays. In advanced disease (i.e., fully developed RA), biological differences between healthy and disease states are easily detected. In contrast, in early disease, the biological distinctions between healthy and disease states or alternative diagnosis are often more subtle, and clear differentiation even for a gold standard becomes more challenging. Therefore, the evaluation of a candidate diagnostic biomarker requires an infallible diagnosis to be established which in RA remains difficult [215].

Cytokines and other soluble factors are prime candidates for diagnostic biomarkers. Several studies investigated their expression using variable methods (ELISA, multiplex assays, or gene expression) and material (tissue and body fluids). However, few studies actually compared very early inflammatory arthritis with differential outcome and still use healthy individuals or established disease patients as controls. Cytokines detected in joints were not different in 12- month disease duration compared to more advanced RA [216]; however, these findings remain to be established in very early disease. Even if right and left RA knee showed similar profiles (IL-6, IL-8, IL-10, and IFN-gamma, high expression of IL-1beta, TNF-alpha, and TGF-beta, low levels of IL-2 and GM-CSF, and no detectable IL-4 or IL-5) [217], the same pattern was observed in other diseases such as seronegative spondyloarthropathy or OA with different levels of expression.

Using Luminex technology with the blocking of heterophilic antibody, increased levels of TNF-alpha, IL-1beta, IL-6, IL-12P40, IL-13, and several chemokines (CXCL10, CCL11, CCL2, and IL-8) were observed in sera from RA patients with <6-month symptom duration compared to HC [23]. The profile was specific to RA and not reproduced in established AS or SpA but was not investigated in patients with early inflammatory symptoms who did not progress towards RA. The profile was also restricted to ACPA-positive patients suggesting increased inflammation associated with autoreactivity. In addition, ACPA was closely related to RF in this study (titres were directly correlated), questioning the efficiency of the RF-blocking methodology used as most cytokine levels were also related to ACPA levels.

In a similar study [158] comparing already diagnosed RA patients of less than 6-month symptom duration with established AS and PsA, a multiplex biomarker platform (combining cytokines, bone turnover markers, metalloproteinases, inflammatory markers, and several citrullinated epitopes) established a signature again including cytokines such as TNF-alpha, IL-1alpha and beta, IL-6, IL-12p40, IL-15, IL-17, GM-CSF, and eotaxin. However, most were also present in AS and PsA (TNF-alpha, IL-1beta, IL-6, IL-17, and eotaxin) and others were associated with autoantibody positive disease (IL-1alpha, IL-12p70, and IL-15).

Studies truly investigating early diseases and the value of cytokines as diagnostic biomarkers in a predictive manner are few. SF from early inflammatory arthritis patients before diagnosis established that patients with persistent symptoms on development of RA showed increase in Th2 cytokines (IL-4 and IL-13) but not Th1 (IFN-gamma) [218]. IL-17 was also increased however only in established RA [218]. In individuals who donated serum samples and later developed RA, a multiplex study showed significant increased levels of cytokines related to T cell activation (IL-2, IL-6), inflammation (IL-1beta, IL-1RA, and TNF-alpha), Th1 (IL-12 and IFN-gamma), Th2 (IL-4, IL-13, and eotaxin), and immune regulation (IL-10), while chemokines, stromal cell-derived cytokines, and angiogenic-related markers were elevated in patients after the development of RA rather than in individuals before the onset of RA [219]. Levels were particularly increased in ACPA-positive and RF-positive individuals. However, in all three studies, every cytokine and chemokine tested were increased (even if not significantly) and again particularly in ACPA/RF-positive patients, whereas other studies demonstrated reduction (i.e., IL-2 and IL-7). Therefore, technical issues related to heterophilic antibody interference may have to be considered when interpreting these data. A similar preclinical RA study [220] showed no detectable cytokine more than 5 years before RA onset, but during the 5-year interval before diagnosis, increased levels were associated with an increased likelihood of the risk of developing RA (IL-1 alpha, IL-1beta, IL-1RA, IL-4, IL-10, TNF-alpha, and soluble TNF-RI).

In established RA as well as in patients with less than 24-month symptom duration, reduced levels of circulating IL-7 have been reported [177, 196]. IL-7 is a pleiotropic cytokine regulating peripheral T cell homeostasis, notably in RA [177, 221, 222]. However, IL-7 is highly expressed in the joints of RA patients [196, 197, 223, 224], and such discrepancies between low systemic levels and high expression at disease site have also been reported in systemic sclerosis [225] and recently in ulcerative colitis and Crohn's disease [226, 227]. A cohort of 250 sera from patients with very early symptoms suggesting a possible evolution towards RA (less than 6-month duration and 5-year follow-up) designed to discover

diagnostic biomarkers demonstrated the potential of IL-7 as a biomarker [2].

3.4. Cytokines as Markers for Treatment Selection and Response to Therapy. Biological therapies (cytokine blockade or receptor antagonism) nowadays appear very effective in chronic inflammatory conditions such as RA, however, in a limited number of patients, with up to 40% nonresponse. Considering the cost of such therapies, biomarker prediction response and allowing for selection of the most appropriate biological treatment would have considerable impact. Most authorities recommend starting therapy with biologics after the failure to respond to at least one disease-modifying agent in RA. However, due to the limited number of studies, there is little guidance about which biological agent to select although anti-TNF remains the most commonly used.

RA patients not responding to anti-TNF showed higher synovial fluid IL-6 at baseline amongst elevated levels of IL-1beta, IL-1RA, IL-2, IL-4, IL-8, IL-10, IL-17, IFN-gamma, G-CSF, GM-CSF, and TNF-alpha. In contrast, responders had elevated IL-2 and G-CSF. In plasma, however, levels were not significantly predicting response, and IL-6 levels decreased posttreatment. In this study, SF cytokine clustering revealed 6 groups of patients with possibly underlying different cellular pathologies, and IL-6, IL-2, and G-CSF in SF may be useful in predicting response to anti-TNF [228]. Recently, we also showed that serum IL-6 was significantly higher at baseline in rituximab nonresponders and that a significant reduction followed treatment in responders only despite adequate B cell depletion in nonresponders [229]. Multivariate logistic regression analysis of synovial cytokine expression showed that TNF at baseline could only explain ~10–15% of the variance in response to TNF blockade [230], suggesting that TNF expression itself would have a limited role in relation to personalised health care. Synovial tissue analysis associated absence of sign of improvement with increased TNF and MMP-3 expression [231, 232]. In contrast, another study showed response to be associated with higher TNF bioactivity in the blood [233], which is more convenient for personalised medicine.

To date, several studies using blood have used gene expression rather than ELISA. CCL4, IL-8, and IL-1beta discriminated between responders and nonresponders to anti-TNF [234]. Several gene signatures have been published so far (some including IL-8, IL-2R) [235–238] with a sensitivity of 90% and a specificity of 70% [237] and 94.4% sensitivity and 85.7% specificity for the response to anti-TNF treatment [238]. Response to anti-TNF (etanercept) was associated with reduced levels of IL-6 and increased IL-23 and IL-32 posttreatment while there was no change in nonresponders; however, no baseline level had predictive value [239].

Recently, several interferon signalling related signatures have emerged as potential biomarkers of response to biological therapies [240–242] as well as for the progression of "at risk" individuals to symptomatic arthritis [243]. Such signatures are interesting as they most likely reflect an immunological status that is favourable to responding or not to therapy, although they are not really linked to the presence/absence of interferon. Indeed, these signatures combined different sets of intracellular signalling factors and transcriptional regulators (between 8 and 15 markers) and are measured through gene expression (using mostly qPCR).

4. Conclusion

Assays measuring known diagnostic biomarkers are commonly used in clinical practice. In fact, it has been reported that about 70% of the decisions made by physicians are based on the results provided by those tests [244]. However, the implementation of novel biomarkers into clinical practice proves to be a long and challenging process, which includes convincing physicians. The assessment of the impact of using the biomarker on general health is an essential step to guarantee the uptake of the biomarker into clinical practice and to further optimise its use. This area of research is likely to become increasingly important as more biomarkers enter clinical practice [245]. Given the complexity and heterogeneous nature of RA, it is unlikely that a single cytokine may provide sufficient discrimination. Many reliable cytokine assays are nowadays available with multiplex formats taking the lead (although this may not be an appropriate solution in RA due to RF interferences). These have established clinical utility for other diseases and purposes and should be easily (technically) transferable to rheumatology, although the exact performance characterization and quality assurance for the specific cytokines of interest in RA may need to be established. At present, limitation in RA lies more in the disease related complexity of networks, the elucidation of the respective role, and the redundant effect that one cytokine may have with another.

Finally, multiple biomarker signatures potentially using genetic as well as proteomic markers may represent a more realistic approach for the future of personalised medicine in RA. Such multifactorial analysis may potentially reveal patterns rather than individual biomarkers. As such, it is interesting that IL-7 alone was able to predict diagnostic at very early disease stage, whereas more complex combination of markers may be needed to predict response to therapy and define subsets of patients with more advanced and heterogonous disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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