Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda
Polskiej Akademii Nauk

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Analiza wybranych cytokin i profilu miRNA u pacjentów z reumatoidalnym zapaleniem stawów i zesztywniającym zapaleniem stawów kręgosłupa

Rozprawa doktorska

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Laboratorium Immunogenetyki Klinicznej i Farmakogenetyki

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Hirszfeld Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences

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Analysis of selected cytokines and miRNA profile in patients with rheumatoid arthritis and ankylosing spondylitis

Doctoral Thesis

Supervisor:

Prof. dr hab. Katarzyna Bogunia-Kubik

Laboratory of Clinical Immunogenetics and Pharmacogenetics

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Praca doktorska została wykonana w ramach projektu OPUS 11 pt. „W poszukiwaniu biomarkerów chorób reumatycznych - kompleksowa analiza profilu metabolomicznego, polimorfizmów i ekspresji genów szlaku IL-23/Th17 i cząsteczek miRNA” (2016/21/B/NZ5/01901) sfinansowanego ze środków Narodowego Centrum Nauki; kierownik: prof. Katarzyna Bogunia-Kubik.

The doctoral dissertation was carried out as part of the OPUS 11 project entitled “Looking for biomarkers of rheumatic diseases - comprehensive study of metabolomic profile, polymorphisms and expression of IL-23/Th17 pathway genes and miRNA molecules” (grant number: 2016/21/B/NZ5/01901) funded by the National Science Centre (Poland); principal investigator: prof. Katarzyna Bogunia-Kubik.

„Człowiek nigdy nie ogląda się na to, co zrobione, ale na to patrzy, co ma przed sobą do zrobienia.”

“One never notices what has been done; one can only see what remains to be done.”

Maria Skłodowska-Curie

Składam serdeczne podziękowania

Prof. dr hab. Katarzynie Boguni-Kubik,
za przyjęcie mnie do swojego zespołu badawczego,
opiekę merytoryczną, cenne uwagi i motywację.

Współpracownikom,
za wprowadzenie do świata reumatologii klinicznej
oraz dzielenie się swoim doświadczeniem i wiedzą.

Najbliższym,
za troskę, wsparcie i wiarę w moje możliwości.

Wszystkim,
którzy towarzyszyli mi na tym etapie mojej naukowej podróży.

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Abstract (in Polish)

Choroby reumatyczne są przewlekłymi schorzeniami zapalnymi o podłożu autoimmunologicznym, do których zalicza się reumatoidalne zapalenie stawów (RZS) i zesztywniające zapalenie stawów kręgosłupa (ZZSK). Charakterystycznym objawem RZS jest symetryczny stan zapalny stawów prowadzący do destrukcji tkanki chrzęstnej i kostnej. ZZSK dotyczy stawów krzyżowo-biodrowych i kręgosłupa, może również obejmować stawy obwodowe. W RZS stwierdza się obecność czynnika reumatoidalnego, natomiast ZZSK zaliczane jest do chorób seronegatywnych. Częstość występowania obu schorzeń na świecie jest różna. W Polsce RZS dotyka około 0,9%, natomiast ZZSK - 0,01% populacji. Zachorowania na RZS są nieproporcjonalnie częstsze u kobiet niż u mężczyzn, odwrotnie niż w przypadku ZZSK. Wszyscy pacjenci cierpią z powodu bólu, sztywności, ograniczenia ruchomości i złej jakości życia. Mimo że podatność na zachorowanie zarówno na RZS, jak i ZZSK jest stosunkowo dobrze poznana i związana ze środowiskowymi oraz genetycznymi czynnikami ryzyka, patogeneza nie została w pełni wyjaśniona. Wskazuje się, że kluczową rolę w tych chorobach odgrywa różnicowanie komórek T pomocniczych, a zwłaszcza zaburzenie równowagi między komórkami Th17 i regulatorowymi limfocytami T. Strategie leczenia pierwszego rzutu obejmują niesteroidowe leki przeciwzapalne, glikokortykoidy oraz leki przeciwreumatyczne modyfikujące przebieg choroby. Wprowadzenie terapii biologicznej znacznie poprawiło jakość życia pacjentów. Niestety, co najmniej 30% pacjentów nie odpowiada na leczenie inhibitorami TNF-α, co stanowi istotny, nierozwiązany problem opieki zdrowotnej. Innymi dominującymi cytokinami prozapalnymi zaangażowanymi w RZS i ZZSK są, odpowiednio, interleukina (IL)-6 i IL-17. Cytokiny te są również mediatorami bólu, a zatem blokowanie ich sygnalizacji nie tylko hamuje progresję choroby, ale również łagodzi przewlekły ból.

Rozprawa doktorska została przygotowana w formie cyklu publikacji. Celem pracy była analiza biomarkerów związanych z podatnością na wybrane schorzenia reumatyczne, progresją choroby i wynikami leczenia anty-TNF. Dane kliniczne i doświadczalne uzyskano od polskich pacjentów cierpiących na RZS i ZZSK przed, a także po trzech i sześciu miesiącach leczenia inhibitorami TNF i porównano je ze zdrowymi osobami z grupy kontrolnej.

W pierwszej publikacji, w grupie 130 chorych na RZS i 112 zdrowych osób z grupy kontrolnej, przeprowadzono genotypowanie polimorfizmu pojedynczego nukleotydu (ang. *single nucleotide polymorphism*, SNP) *IL6* rs1800795 zlokalizowanego w regionie promotora.

Przeanalizowano również poziomy IL-6 w surowicy. Zaobserwowano, że pacjenci przed rozpoczęciem terapii anty-TNF mieli istotnie wyższe stężenie IL-6 niż osoby z grupy kontrolnej. Ponadto, chorzy z homozygotycznym genotypem *CC* charakteryzowali się najwyższym poziomem tej cytokiny przed leczeniem biologicznym i wysoką aktywnością choroby w porównaniu z nosicielami allelu *G*. Wyniki te sugerują, iż związany ze zwiększoną produkcją IL-6 genotyp rs1800795 *CC* odgrywa niekorzystną rolę u chorych z RZS.

W drugiej pracy analizie poddano zmienność genów dla IL-17A, IL-17F i ich receptorów oraz zbadano potencjalne asocjacje między genotypami *IL17A* rs2275913, *IL17F* rs763780, *IL17RA* rs4819554 i *IL17RC* rs708567 z parametrami klinicznymi i odpowiedzią na leczenie anty-TNF. Badana grupa składała się z 138 pacjentów z ZZSK i 190 zdrowych osób z grupy kontrolnej. Wykazano zależność genotypu *IL17F* rs763780 *AG* z wyższą aktywnością choroby oraz brakiem odpowiedzi na leczenie po sześciu miesiącach terapii. Ponadto, zidentyfikowano allel *IL17RA* rs48419554 *G* jako potencjalny marker nasilenia ZZSK.

W trzecim artykule określono repertuar miRNA w egzosomach pochodzących z surowicy chorych na RZS i ZZSK, przed i po trzech miesiącach terapii anty-TNF. Analizę przeprowadzono z wykorzystaniem technologii NanoString umożliwiającej analizę profilu ekspresji 800 cząsteczek miRNA. Wykryto dwanaście miRNA różniących się ekspresją pomiędzy chorymi na RZS i ZZSK przed leczeniem. Porównanie profilu ekspresji u pacjentów przed i po trzech miesiącach stosowania inhibitorów TNF-α wykazała różnice w ekspresji, odpowiednio, czterech i czternastu miRNA u pacjentów z RZS i ZZSK. Dodatkowo przeprowadzono analizę *in silico* potencjalnych szlaków i genów docelowych dla miRNA.

Cykl publikacji zamyka praca poglądowa na temat wykorzystania miRNA jako potencjalnych markerów odpowiedzi na leczenie w chorobach reumatycznych.

Przeprowadzone badania wykazały istotne znaczenie polimorfizmów genów kodujących prozapalne cytokiny oraz profilowania miRNA w chorobach reumatycznych. Wykazano, że badane SNP mogą modulować odpowiedź terapeutyczną na inhibitory TNF-α oraz wpływać na parametry kliniczne, takie jak markery stanu zapalnego, czy aktywność choroby. Powyższe wyniki sugerują, że profilowanie miRNA w surowicy może mieć potencjał diagnostyczny i prognostyczny.

Abstract (in English)

Rheumatic diseases are autoimmune chronic inflammatory disorders including rheumatoid arthritis (RA) and ankylosing spondylitis (AS). Symmetric inflammation of joints leading to the destruction of cartilage and bone tissue is a characteristic manifestation of RA. AS affects sacroiliac joints and the spine, and may also involve peripheral joints. RA is described with the presence of rheumatoid factor, while AS is classified as a seronegative disease. The prevalence of both disorders differs worldwide. In Poland, RA affects approximately 0.9%, whereas AS affects 0.01% of the population. RA incidence is disproportionately more common in women than men, and the opposite is true in AS. All patients suffer from pain, stiffness, reduction of mobility and poor quality of life. Even though susceptibility for both RA and AS is relatively well-known to be associated with environmental and genetic risk factors, the pathogenesis has not been fully elucidated. However, it has been indicated that T helper (Th) cell differentiation plays a crucial role in these diseases, especially the imbalance between Th17 and regulatory T lymphocytes. First-line treatment strategies comprise non-steroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying antirheumatic drugs. The introduction of biological therapy remarkably improved patients' quality of life. Unfortunately, at least 30% of patients do not respond to the treatment with TNF-α, inhibitors, which constitutes a major, unsolved healthcare problem. Other dominant proinflammatory cytokines involved in RA and AS are interleukin (IL)-6 and IL-17 respectively. These cytokines are also mediators of pain, thus blocking their signalling not only impairs disease progression but also alleviates the chronic pain.

This doctoral thesis is presented as a series of publications. The aim was to identify biomarkers associated with susceptibility to selected rheumatic disorders, disease progression, and anti-TNF treatment outcomes. Clinical samples were collected from Polish RA and AS patients at baseline, as well as after three and six months of treatment with TNF-α inhibitors. Additionally, experimental data were compared to healthy controls.

In the first publication, a group of 130 patients with RA and 112 healthy controls were genotyped for the *IL6* rs1800795 single nucleotide polymorphism (SNP) located in the promoter region. Serum IL-6 levels were also analysed. It was observed that IL-6 concentrations were significantly higher in RA patients before initiation of anti-TNF therapy compared to healthy controls. In addition, RA patients with the homozygous *CC* genotype were characterized with the highest level of this cytokine before biological treatment and high disease activity compared to

G allele carriers. These results suggest that the rs1800795 *CC* genotype, associated with increased IL-6 production, plays an adverse role in RA patients.

In the second paper, genetic variants of IL-17A, IL-17F, and their receptors were analysed. Potential associations between the *IL17A* rs2275913, *IL17F* rs763780, *IL17RA* rs4819554 and *IL17RC* rs708567 alleles with clinical parameters and anti-TNF treatment responses were investigated. The study cohort consisted of 138 AS patients and 190 healthy controls. The *IL17F* rs763780 *AG* genotype was found to be associated with higher disease activity, as well as a lack of response to therapy after six months. Moreover, the *IL17RA* rs48419554 *G* allele was identified as a potential marker of AS severity.

In the third paper, repertoires of miRNAs in exosomes from serum of RA and AS patients before and after three months of anti-TNF therapy were determined. The analysis was performed using NanoString technology, which enabled the analysis of the expression profile of 800 miRNA molecules. Twelve miRNAs differentially expressed between RA and AS patients at baseline were detected. A comparison of the expression profiles in patients before and after three months of TNF-α inhibitor intake showed differences in four and fourteen miRNAs in RA and AS patients respectively. Additionally, we carried out an *in silico* analysis of potential miRNA targets and associated pathways.

The publication series closes with a review paper describing the usage of miRNAs as potential markers of treatment response in rheumatic diseases.

The studies described herein established an important role of polymorphisms within genes encoding proinflammatory cytokines and miRNAs profiles in predicting rheumatic disease outcome. The investigated SNPs were shown to modulate therapeutic responses to TNF-α inhibitors and influence clinical parameters such as inflammatory markers or disease activity. These results demonstrate that serum miRNA profiling may have a diagnostic and prognostic potential in rheumatic disease.

List of publications

1. Joanna Wielińska, Marta Dratwa, Jerzy Świerkot, Lucyna Korman, Milena Iwaszko, Barbara Wysoczańska, Katarzyna Bogunia-Kubik. Interleukin 6 gene polymorphism is associated with protein serum level and disease activity in Polish patients with rheumatoid arthritis. *HLA*. 2018, 92(Suppl. 2):38-41.

2. Joanna Wielińska, Jerzy Świerkot, Katarzyna Kolossa, Bartosz Bugaj, Monika Chaszczewska-Markowska, Sławomir Jeka, Katarzyna Bogunia-Kubik. Polymorphisms within Genes Coding for IL-17A and F and Their Receptor as Clinical Hallmarks in Ankylosing Spondylitis. *Mediators Inflamm.* 2021, 2021:3125922.

3. Joanna Wielińska, Rachel E Crossland, Piotr Łacina, Jerzy Świerkot, Bartosz Bugaj, Anne M Dickinson, Katarzyna Bogunia-Kubik. Exploring the Extracellular Vesicle MicroRNA Expression Repertoire in Patients with Rheumatoid Arthritis and Ankylosing Spondylitis Treated with TNF Inhibitors. *Dis Markers*. 2021, 2021:2924935.

4. Joanna Wielinska, Katarzyna Bogunia-Kubik. miRNAs as potential biomarkers of treatment outcome in rheumatoid arthritis and ankylosing spondylitis. *Pharmacogenomics*. 2021, 22(5):291-301.

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Contributor	Contribution [%]	Description of main tasks
Joanna Wielińska	40%	Investigation – data collection, genotyping of patients; formal analysis – data analysis, statistical analysis; visualization – preparation of tables and figures; writing – original draft preparation
Marta Dratwa	20%	Investigation – determination of serum cytokine concentration; raw data analysis
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Barbara Wysoczańska	5%	Supervision of serum studies
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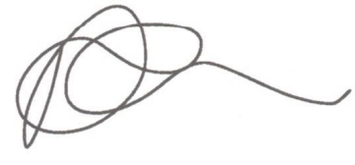

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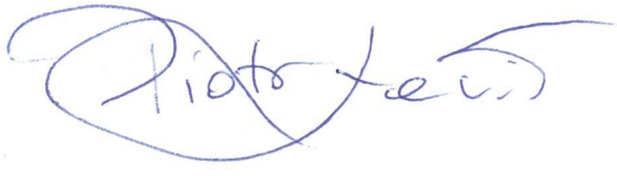

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WILEY HLA Immune Response Genetics

BRIEF COMMUNICATION

Interleukin 6 gene polymorphism is associated with protein serum level and disease activity in Polish patients with rheumatoid arthritis

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Interleukin 6 (IL-6) is a pro-inflammatory cytokine involved in the development of rheumatoid arthritis (RA). The present study aimed to determine the possible association of the *IL6* (rs1800795, G > C) polymorphism with RA susceptibility, disease progression and protein serum levels. Distribution of *IL6* alleles and genotypes was similar in RA patients and controls. As expected, patients before induction of anti-tumour necrosis factor agents had significantly higher IL-6 levels as compared with controls ($P = 0.002$). The *CC* homozygous patients were characterised with the highest average concentrations of this pro-inflammatory cytokine before treatment ($P = 0.028$), and they also more frequently presented with more active disease ($P = 0.048$). These results imply that the *IL6* rs1800795 *CC* homozygosity may play a rather unfavourable role in RA.

KEYWORDS
gene polymorphism, IL-6, rheumatoid arthritis, rs1800795

Rheumatoid arthritis (RA) is an autoimmune disorder distinguished by progressive inflammation of joints and cartilage that more frequently affects women. It is a multifactorial disease influenced by genetics and environmental features.[1] The use of anti-tumour necrosis factor (TNF) agents has revolutionised treatment of this disease and significantly improved RA therapy outcome, but unfortunately, almost 30% of patients do not respond to treatment with TNF inhibitors. Cause of this therapy resistance is still unknown,[2] although its inefficiency could be partially attributed to genetic differences between patients. Our previous studies have shown that polymorphisms within genes coding for TNF-α and its receptor,[3] as well as IL-17A,[4] may be considered factors affecting therapy outcome. In this present study, we concentrated on another pro-inflammatory cytokine involved in RA aetiology, interleukin 6 (IL-6).

IL-6 is a cytokine with pleiotropic activity. This glycoprotein stimulates signalling pathway via classical and trans-signalling pathways. In the classical mechanism, IL-6 binds to its transmembrane receptor (IL-6R) leading to signal activation in target cells, while in trans-signalling, secreted cytokine forms a complex with the soluble IL-6 receptor (sIL-6R).[5] The first pathway is thought to be anti-inflammatory, whereas trans-signalling promotes inflammation.[6] IL-6 plays numerous roles within the immune system, including T cell differentiation and activation, B cell proliferation, production of antibodies and regulation of hepatic acute-phase response.[7] Prevalence of Th17 over Treg in the CD4+ T cell subsets, which is possibly influenced by immune disease development, is promoted by IL-6. Moreover, this cytokine mediates joint destruction caused by local inflammation and increased C-reactive protein (CRP) level, characteristic for RA development. Consequently, IL-6 has an essential role in chronic inflammatory diseases such as RA.[8]

Genetic factors are crucial, but not necessarily sufficient for RA progression. However, some cytokine gene polymorphisms were identified as being associated with different

TABLE 1 Characteristics of RA patients

RA patients (*N*)	130
Females/males (% of females)	101/29 (77.7%)
Age (years) (mean ± SD)	49.8 (±14.1)
Disease onset (years) (mean ± SD)	38.2 (±12.8)
Disease duration (years) (mean ± SD)	12.1 (±9.1)
DAS28 baseline (mean ± SD)	6.5 (±0.8)
CRP baseline (mean ± SD)	17.3 (±20.2)
RF-positive (%)	92 (70.8%)
Anti-CCP positive (%)	64 (49.2%)

Abbreviations: anti-CCP, anti-cyclic citrullinated peptide autoantibodies; CRP, C-reactive protein; DAS28, disease activity score; RA, rheumatoid arthritis; RF, rheumatoid factor.

clinical pictures.[9] Fishman et al[10] in 1998 described for the first time a single-nucleotide polymorphism (SNP), a G to C substitution at position −174 in *IL6* gene promoter, as being possibly functional in RA pathogenesis. Recent meta-analyses showed that this SNP constitutes a genetic risk factor, especially in Asians, but not in Caucasians.[11] These findings prompted us to analyse the role of the *IL6* polymorphism in disease activity and susceptibility in an independent cohort of Polish RA patients.

We recruited 130 patients diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria. Patients' characteristics are given in Table 1. The IL-6 concentration was measured in patients' sera at three time points: before, at 12th and 24th week after initiation of anti-TNF therapy, and in 73 controls using ProcartaPlex Multiplex Immunoassay (eBioscience, Vienna, Austria), following the protocol provided by the manufacturers. All 130 patients and 112 healthy individuals were genotyped for *IL6* (rs1800795, G > C) promoter region by real-time polymerase chain reaction (PCR) amplification and using TaqMan assay (ThermoFisher, Massachusetts). The LightCycler 480 Real-Time PCR system (Roche Diagnostics, Rotkreuz, Switzerland) was used in this analysis.

Potential associations between the rs1800795 polymorphism and clinical parameters of RA patients were analysed applying the non-parametric Mann-Whitney U test or the Fisher's exact test. *P* values less than 0.05 were considered statistically significant. All statistical calculations were performed in the GraphPad7 Prism software. Power of the study was calculated using G*Power.[12]

As shown in Table 2, no significant differences in genotype and allele distribution were observed between patients and the control group. In agreement with our findings, in another study involving Polish RA patients, Pawlik et al[13] reported that 26.5%, 54.1%, and 19.4% of patients were the *GG* homozygotes, *GC* heterozygotes or were homozygous for the *C* allele, respectively. Thus, in both analysed groups (ours and that of Pawlik et al), the *GC* heterozygosity appeared as the most frequent genotype, followed by the *GG* and the *CC* homozygosity. Most of the studies in Caucasians did not show the correlation between *IL6* polymorphism and

TABLE 2 Distribution of *IL6* alleles and genotypes in RA patients and in the control group

	RA patients *N* (%)	Controls *N* (%)
IL6 genotype		
GG	40 (30.8%)	37 (33.0%)
GC	65 (50%)	53 (47.3%)
CC	25 (19.2%)	22 (19.6%)
IL6 allele		
G	147 (56.0%)	127 (57.0%)
C	115 (44.0%)	97 (43.0%)

Abbreviation: RA, rheumatoid arthritis.

risk of acquiring RA.[14–16] However, in Egyptian population, the *C* allele was associated with RA susceptibility.[17] The same polymorphic variant was correlated with a significantly increased risk of RA in Chinese patients.[18,19] Some potential associations were also suggested for Europeans.[20]

As shown in Figure 1, patients before induction of anti-TNF agents presented with significantly higher IL-6 levels

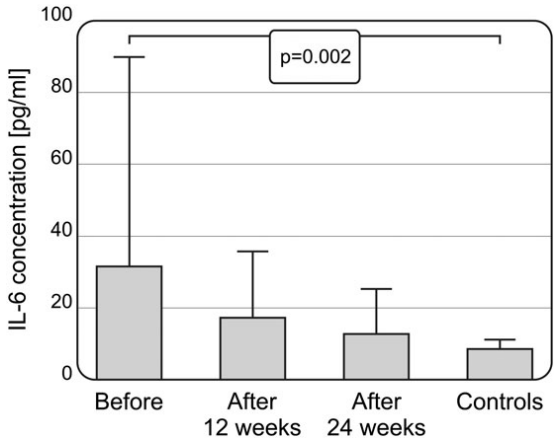

FIGURE 1 Interleukin 6 (IL-6) serum levels in rheumatoid arthritis patients before, at 12th and 24th week after initiation of anti-tumour necrosis factor therapy and in healthy controls. Significant difference was observed between IL-6 concentrations in patients before treatment vs controls ($P = 0.002$)

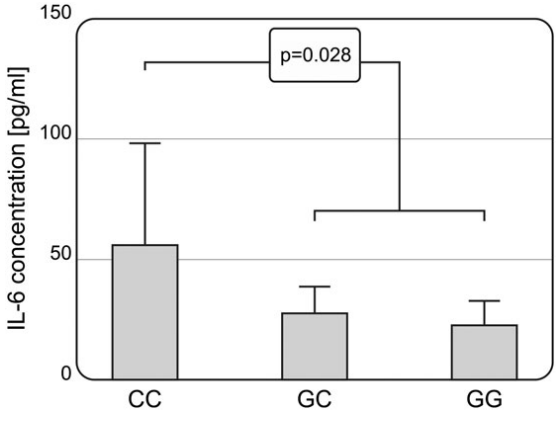

FIGURE 2 Average baseline *IL6* concentrations in patients with various *IL6* genotypes. The *CC* homozygotes presented with higher serum levels than the *G* allele carriers (CC vs GC + GG, $P = 0.028$)

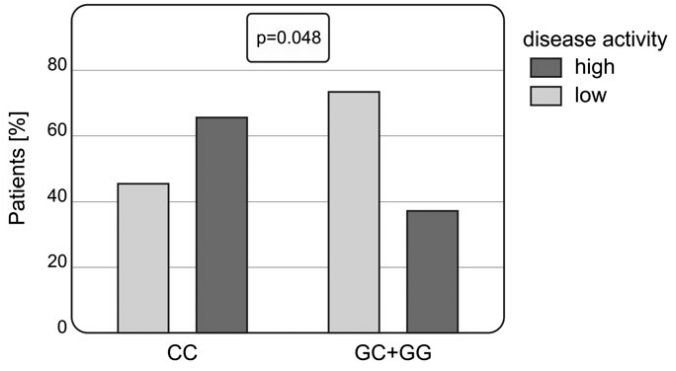

FIGURE 3 Disease activity in patients carrying various *IL6* genotypes. The *CC* homozygotes presented with higher DAS28 as compared with those carrying the *G* allele (CC vs GC + GG, $P = 0.048$)

as compared with controls (31.51 ± 58.47 vs 8.488 ± 2.501, $P = 0.002$) with 95.7% power.

Moreover, the *CC* homozygous cases were characterised with higher average concentrations of IL-6 before anti-TNF treatment as compared with patients carrying the *G* allele (55.84 ± 102.8 vs 25.55 ± 39.66, $P = 0.028$, Figure 2). Power of this test was calculated as 85.8%. Similar although not significant relationships were also seen after 12 weeks and 24 weeks of the treatment.

Our results are in line with recent reports showing the higher IL-6 concentration in Egyptian (mainly *CC* homozygous) patients with RA than in healthy subjects,[21,22] and increased IL-6 serum levels among European patients with the *C* allele.[23]

In this study, we also investigated a possible relationship between the *IL6* gene polymorphism and selected clinical parameters. Nevertheless, the rs1800795 polymorphism was not found to be significantly associated with parameters such as the rheumatoid factor, CRP level, the presence of the anti-cyclic citrullinated peptide autoantibodies, response to therapy with TNF inhibitors or age at disease onset. However, other authors reported higher age at disease onset in the *GG* homozygotes than in the *CC* carriers and the lowest in *GC* heterozygous patients.[21] Consequently, the *IL6* rs1800795 *C* allele may favour earlier disease onset.[24] Different studies also suggested that the *IL6* polymorphism might be a genetic marker of biologic therapy outcome, *G* allele carrying patients being better responders.[25–27] A favourable effect of the *G* variant was also observed in our study. It appeared that the presence of the *G* allele was more frequently detected among patients with less active disease (disease activity score [DAS28]) as compared with those being homozygous for the *C* allele ($P = 0.048$, Figure 3). On the other hand, Pawlik et al[13] observed a significant relationship between the presence of the *GG* homozygosity and more active disease.

A positive correlation between IL-6 concentration in patients and disease activity was also previously reported by van Leeuven et al,[28] while Gaber et al[22] observed a negative correlation of this cytokine level with DAS28 in heterozygous patients. However, we did not observe any direct association between IL-6 serum levels and DAS28 in our cohort of RA patients.

We evaluated a possible role of the *IL6* rs1800795 gene polymoprhism in the Polish cohort of patients with RA showing that the *IL6* rs1800795 *CC* homozygosity seems to play a rather unfavourable role in RA. A relatively small size of patients cohort analysed in the present study constitutes the most important limitation of our work. Some disparities between our findings and those reported in other studies may be attributed to the differences in patients' origins and numbers of investigated cases or therapeutic agents used. Therefore, more extended studies involving higher number of cases are needed to confirm our current observations.

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Conflict of interest

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTION

J.W. performed the experiments, analysed the data and drafted the manuscript; M.D. and M.I. performed the experiments and analysed the data; J.Ś. contributed with patients recruitment, diagnosis and provided clinical data; L.K. collected patients samples; B.W. supervised serum studies; K.B.-K. designed and supervised the study, provided reagents, participated in data analyses, preparation of the manuscript and revised its final version.

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Research Article

Polymorphisms within Genes Coding for IL-17A and F and Their Receptor as Clinical Hallmarks in Ankylosing Spondylitis

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IL-17A and IL-17F together with their coreceptor (IL-17RA/RC) were reported to play a significant role in the pathogenesis of spondyloarthritis. The group of axial spondyloarthritis comprises ankylosing spondylitis (AS), a rheumatic disease characterized by chronic inflammation of the joints in the spine. This study is aimed at investigating *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms as potential biomarkers of disease susceptibility, clinical parameters, and anti-TNF treatment outcome in a cohort of Polish ankylosing spondylitis patients. In total, 328 subjects, including 138 AS patients and 190 healthy volunteers, participated in the study. Genotyping of *IL-17A* rs2275913 (G/A), *IL-17F* rs763780 (A/G), *IL-17RA* rs4819554 (A/G), and *IL-17RC* rs708567 (G/A) was performed on real-time PCR instrument using LightSNiP assays. No significant differences were revealed in genotype and allele distribution between patients and controls despite the association of the *IL-17RC* rs708567 *AA* homozygosity with the earlier onset of the disease. Moreover, some relationships between *IL-17F* rs763780 and *IL-17RA* rs4819554 polymorphisms with clinical parameters related to the disease activity and anti-TNF treatment outcome were observed. *IL-17F* rs763780 G allele was found to be associated with high disease activity and BASDAI after 6 months and poor response to the treatment while higher VAS values were more common among *IL-17RA* rs4819554 G variant carriers. In conclusion, the *IL-17F* rs763780 polymorphism should be considered as a promising biomarker of disease activity and anti-TNF treatment outcome. The *IL-17RA* rs48419554 G allele may serve as a potential marker of disease severity in Polish AS patients.

1. Introduction

Ankylosing spondylitis (AS) is characterized by visible radiographic changes within the spine or sacroiliac joints. The axial spondyloarthritis (axSpA) group comprises AS, radiographic axial spondyloarthritis, and a nonradiographic (nr-axSpA) form of the disease [1]. AS patients suffer from inflammatory back pain and morning stiffness. Symptoms can also involve enthesitis and peripheral arthritis manifestations. The disease affects mostly men (ratio men to women is 2 to 1), those under thirty years of age, and with a strong genetic association linked to HLA-B27 [2]. Prevalence differs

between geographical regions and ethnicity, reaching 0.23% in the general European population [3] and roughly 0.083% in Polish people [4].

The standard pharmacological treatment against AS involves tumour necrosis factor-alpha (TNF-α) inhibitor (anti-TNF) dosage after the primary failure of nonsteroidal anti-inflammatory (NSAIDs) administration. The long-term anti-TNF approach has positive effects on patient's functional outcome, lessens disease activity, and reduces radiographic progression [5].

The IL-17 family consists of six cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. Those proteins transmit signals through defined heterodimeric transmembrane receptors (IL-17R). IL-17A, IL-17F, and IL-17A/F heterodimer act via the IL-17RA/RC receptor complex. IL-17E triggers responses through IL-17RA/RB, and IL-17C induces the IL-17RA/RE heterodimer. For other proteins, the heterotrimeric receptor compound has not been fully elucidated. IL-17A and IL-17F have a high degree of homology, and both are secreted by Th17 cells, $\gamma\delta$ T cells, innate lymphoid cells, cytotoxic T cells, and natural killer T (NKT) cells [6].

IL-17 was reported to have a crucial role in the immunopathogenesis of spondyloarthritis [7]. Elevated levels of IL-17 in serum have been observed in ankylosing spondylitis patients [8]. Besides, associations between IL-17 level and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [9, 10] have been described.

However, our knowledge of *IL-17* gene polymorphisms in AS is still limited. Thus, based on previous research focused on rheumatoid arthritis [11] and osteoarthritis [12], we hypothesized that *IL-17A* rs2275913 and *IL-17* rs763780 might influence AS susceptibility. The targeted single nucleotide polymorphisms were selected based on the available literature, especially on Caucasians, as well as our preliminary experiment on rheumatoid arthritis patients [13]. We also decided to investigate polymorphisms of IL-17 receptors *IL-17RA* and *IL-17RC*. *IL-17RA* rs4819554 was previously linked with response to etanercept in psoriatic arthritis [14], while *IL-17RC rs708567* was associated with lupus arthritis [15] and was described in Tunisians with rheumatoid arthritis [16]. Moreover, the newly performed study considered the *IL-17RA* polymorphism as an AS risk factor [17]. To the best of our knowledge, no investigations have been conducted to assess the association between *IL-17RA* and *IL-17RC* polymorphisms and rheumatic diseases in the Polish population.

This study examines the *IL-17A* rs2275913, *IL-17F* rs763780, *IL-17RA* rs4819554, and *IL-17RC* rs708567 genetic variants as potential biomarkers of disease susceptibility, clinical parameters, and anti-TNF treatment outcome in a cohort of Polish AS patients.

2. Materials and Methods

2.1. Patients and Controls. One hundred thirty-eight AS patients and one hundred ninety controls were involved in the study. AS patients were recruited from the Department of Rheumatology and Internal Medicine, Wroclaw Medical University, Poland, and from the Department of Rheumatology and Connective Tissue Diseases, Jan Biziel University Hospital No. 2 in Bydgoszcz, Poland. All the participants diagnosed with AS were Caucasians over 18 years of age, and 74% (102/138) were male. Included criteria comprise a resistance to treatment with at least two nonsteroidal anti-rheumatic drugs (NSAIDs), high disease activity before starting biological treatment, initialization of anti-TNF therapy at the time of the research, and complete medical history. Subjects with the coexistence of acute or chronic disorders besides AS, other autoimmune diseases, malignancies, or current infections, during pregnancy and breastfeeding, as well as with insufficient clinical records, and an unwillingness or inability to cooperate were excluded from the study.

AS patients were diagnosed according to the 1984 modified New York Criteria [18].

Data such as gender, age, disease onset, disease duration, body mass index (BMI), presence of HLA-B27, C-reactive protein (CRP) level, pain visual analogue scale (VAS, range: 0-100 mm), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI, range: 0-10) were collected from patients.

90.4% of patients were HLA-B27 positive, and most of them (76.7%) had the axial form of AS. Drug administration comprised MTX in 27.5% of cases, corticosteroids (20.3%) of subjects and NSAIDs have been taken by 71.3% of patients.

Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) was used to calculate disease activity, which was considered to be high (BASDAI ≥ 4), moderate ($3 \leq$ BASDAI < 4), or low (BASDAI < 3). The clinical outcome was assessed after 3 and 6 months of anti-TNF treatment. Significant improvement after therapy was defined as a reduction of BASDAI (ΔBASDAI ≥ 2.0), good outcome as ΔBASDAI ≥ 2.0 and BASDAI < 3.0 at the endpoint, moderate response as ΔBASDAI ≥ 2.0 and BASDAI ≥ 3.0 at the endpoint, and no improvement as ΔBASDAI < 2.0 [19].

The patient's demographic and clinical characteristics are described in Table 1. The data are presented as median with range (minimum to maximum).

The control group was enrolled from the healthy volunteers, 63 females (33%) and 127 (67%) males, from the Regional Centre of Transfusion Medicine and Blood Bank in Wroclaw without a personal history of rheumatic diseases.

Informed consent was obtained from all participants involved in the study. The research was approved by the Wroclaw Medical University Ethics Committee (identification code KB-625/2016).

2.2. SNP Selection and Genotyping. Tested genetic variants were selected based on analysis of previous publications and search results from NCBI Database of Short Genetic Variations (dbSNP) and SNPinfo Web Server [20]. Minor allele frequency (MAF) in EUR was above 5% (1000 Genomes Project) [21].

In total, four single nucleotide polymorphisms (SNPs) were chosen for analysis: *IL-17A* rs2275913 (G/A) and *IL-17F* rs763780 (A/G) located on chromosome 6, *IL-17RA* rs4819554 (A/G) located on chromosome 22, and *IL-17RC* rs708567 (G/A) located on chromosome 3. Two of them,

Table 1: Clinical characteristics of the study cohort.

Characteristic	*N*	Median (range)
Age (years)	138	43.5 (22-75)
Disease duration (years)	135	10 (0-48)
Disease onset (years)	135	33 (6-56)
BMI	113	25.32 (18.61-40.31)
CRP before treatment (mg/l)	108	16.83 (0.3-561)
CRP at 3 months (mg/l)	79	5.75 (0.2-175)*
CRP at 6 months (mg/l)	72	5.495 (0.2-204.3)*
VAS before treatment (mm)	132	80 (45-100)
VAS at 3 months (mm)	138	30 (0-80)*
VAS at 6 months (mm)	131	20 (0-100)*
BASDAI before treatment	138	8 (4.05-10)
BASDAI at 3 months	138	3.2 (0.7-6.7)*
BASDAI at 6 months	132	2.25 (0.2-9.75)*
Treatment (anti-TNF drug)	*N* = 138	*n* (%)
Adalimumab		63 (45.6%)
Etanercept		44 (31.9%)
Certolizumab		17 (12.3%)
Golimumab		12 (8.69%)
Infliximab		2 (1.45%)

N: number of patients with clinical information; BMI: body mass index; CRP: C-reactive protein; MTX: methotrexate; NSAIDs: nonsteroidal anti-inflammatory drugs; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; VAS: visual analogue scale; HLA-B27: human leukocyte antigen B27. *$p < 0.001$; p value comparing the clinical variables between baseline and after 3 or 6 months of treatment.

IL-17F rs763780 and *IL-17RC* rs708567 are missense variants in exon 3 (His161Arg) and exon 4 (Ser111Leu), respectively. *IL-17A* rs2275913 and *IL-17RA* rs4819554 are substitutions within gene promoter regions with a predicted transcription factor binding site (TFBS).

Whole blood samples were collected in EDTA tubes (BD Vacutainer® Blood Collection Tubes). Genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Midi/Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The genotyping of selected SNPs: *IL-17A* rs2275913 (G/A), *IL-17F* rs763780 (A/G), *IL-17RA* rs4819554 (A/G), and *IL-17RC* rs708567 (G/A) was performed using LightSNiP assays (TIB MOLBIOL, Berlin, Germany) on the LightCycler 480 Real-Time PCR Instrument (Roche Diagnostics, Basel, Switzerland).

2.3. Statistical Analysis. The genotype frequencies were tested for the Hardy-Weinberg equilibrium (HWE). Potential differences in allele and genotype distributions between the patient and control groups were calculated using Fisher's exact test. Continuous variables were tested for normal distribution by the Shapiro–Wilk test. Quantitative variables that were normally distributed were presented as mean ± SEM, while medians with interquartile ranges (IQRs) were calculated for nonnormally distributed variables. The unpaired two-sample Wilcoxon test (for nonparametric data) or unpaired two-sample *t*-test (for normally distributed data) were performed to identify associations within genetic variants and clinical parameters. Fisher's exact test was also applied to detect relationships between genotypes and categorical variables such as disease activity or treatment outcome. A p value lower than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analysis was performed using R Software (http://www.r-project.org) and GraphPad Prism 7 for Windows.

Table 2: The distribution of *IL-17* genotypes and alleles in AS patients and the control group.

	Patients	Controls
IL-17A rs2275913	*N* = 138	*N* = 190
G	174 (63.0%)	234 (61.6%)
A	102 (37.0%)	146 (38.4%)
GG	50 (36.2%)	69 (36.3%)
GA	74 (53.6%)	96 (50.5%)
AA	14 (10.1%)	25 (13.2%)
IL-17F rs763780	*N* = 138	*N* = 189
A	265 (96.0%)	359 (95.0%)
G	11 (4.0%)	19 (5.0%)
AA	127 (92.0%)	170 (89.9%)
AG	11 (7.97%)	19 (10.1%)
GG	0 (0%)	0 (0%)
IL-17RA rs4819554	*N* = 138	*N* = 190
A	215 (77.9%)	311 (81.8%)
G	61 (22.1%)	69 (18.2%)
AA	83 (60.1%)	126 (66.3%)
AG	49 (35.5%)	59 (31.1%)
GG	6 (4.35%)	5 (2.63%)
IL-17RC rs708567	*N* = 138	*N* = 189
A	150 (54.3%)	205 (54.2%)
G	126 (45.7%)	173 (45.8%)
AA	41 (29.7%)	47 (24.9%)
AG	68 (49.3%)	111 (58.7%)
GG	29 (21.0%)	31 (16.4%)

3. Results

3.1. Distribution of IL-17A, IL-17F, IL-17RA, and IL-17RC Alleles and Genotypes in Patients and Controls. The distribution of genotypes and alleles of *IL-17A* rs2275913, *IL-17F* rs763780, *IL-17RA* rs4819554, and *IL-17RC* rs708567 did not differ between AS patients and healthy individuals (Table 2). Also, no significant gender-dependent differences were detected between patients and healthy subjects (not shown). Please note that none of the patients or controls were homozygous for the *IL-17F* rs763780 G allele. Thus, in the further analyses, *AA* homozygotes were being compared with *AG* genotype reflecting also the *G* allele carriers.

On the other hand, the significant association between disease onset and genotype frequency was observed for *IL-17RC* rs708567 SNP. Patients with *AA* genotype had a lower age of disease onset (29.39 ± 1.405) than those with *G* allele (33.43 ± 1.001) (*AA* vs. *AG*+*GG*, $p = 0.022$; *AA* vs. *AG*, $p =$

0.015). However, disease duration was not found to be affected by any of the analysed SNPs.

3.2. Associations between IL-17A, IL-17F, IL-17RA, and IL-17RC Genotypes and Clinical Parameters. The potential associations between *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* genotypes and CRP level, VAS, and BASDAI values were analysed.

During anti-TNF therapy, these major clinical parameters were decreased. CRP level was significantly lower after 3 and 6 months compared to baseline ($p < 0.0001$). Also, VAS and BASDAI were reduced after 3 and 6 months related to baseline, as well as after 6 months in comparison to 3 months of therapy ($p < 0.0001$) (Table 1). A significant improvement in clinical parameters was achieved after administration of anti-TNF agents.

Higher VAS values at the baseline were found in *IL-17A* rs2275913 *GG* (*GG* vs. *GA*+*AA*, $p = 0.005$; *GG* vs. *GA*, $p = 0.006$) and *IL-17F* rs763780 *AG* (*AA* vs. *AG*, $p = 0.027$) genotype carriers (Table 3(a)).

The *IL-17RA* rs4819554 G allele was found to be more common among patients who presented with higher VAS and BASDAI values after anti-TNF treatment induction.

Patients possessing the *IL-17RA* rs4819554 G allele had higher VAS values after 3 months of anti-TNF therapy (*AA* vs. *AG*+*GG*, $p = 0.002$). This result was also observed after 6 months of treatment (*AA* vs. *AG*+*GG*, $p = 0.002$).

Besides, *IL-17RA* rs4819554 G patients demonstrated greater BASDAI values at 6 months of therapy than *AA* homozygotes (*AA* vs. *AG*+*GG*, $p = 0.046$) (Table 3(b)). The *IL-17RA* results concerning VAS at 3 and 6 months and BASDAI at 6 months were confirmed by the overdominant model (*AA*+*GG* vs. *AG*, $p = 0.008$, $p = 0.006$, and $p = 0.045$, respectively). Additionally, a significant relationship with an absolute BASDAI change (ΔBASDAI 0-3 m.) (*AA*+*GG* vs. *AG*, $p = 0.027$) was noted. Tendencies were observed regarding BASDAI score at 3 months (*AA* vs. *AG*+*GG*, $p = 0.066$; AA+GG vs. AG, $p = 0.064$) and absolute BASDAI change (ΔBASDAI 0-6 m.) (*AA*+*GG* vs. *AG*, $p = 0.057$).

The BASDAI parameter at 6 months was also higher in the group of AS individuals bearing *IL-17F* rs763780 *AG* genotype (*G* allele) (*AA* vs. *AG*, $p = 0.035$) in comparison to *AA* carriers (Table 3(a)).

As for CRP levels, *IL-17RC* rs708567 and *IL-17A* rs2275913 polymorphisms were identified as significantly associated with CRP level after 3 months of TNF inhibitor administration. At that time point, *IL-17A* rs2275913 *GG* was more frequently observed among patients with an elevated level of CRP (>10 mg/l) (*GG* vs. *GA*+*AA*, $p = 0.022$, OR = 3, and 95%CI = 1.237-7.046), and *IL-17RC* rs708567 G patients showed a significantly higher CRP level as compared to the *AA* patients (*AA* vs. *AG*+*GG*, $p = 0.043$; *AA* vs. *AG*, $p = 0.018$) (Table 3(b)).

No other significant differences between clinical parameters of AS patients and their *IL-17* genotype distribution were detected.

3.3. Effect of IL-17F Polymorphisms on the Disease Activity and Anti-TNF Treatment Outcome. Before the anti-TNF administration, all patients were characterized with high disease activity (BASDAI > 4). After 3 months of therapy, 25.4% (35/138), 44.9% (62/138), and 29.7% (41/138) of subjects had a high, moderate, and low disease activity, respectively. After 6 months, only 3.03% (4/132) and 1.52% (2/132) were described with high and moderate disease activity, respectively. The remaining 95.5% (126/132) of patients presented low disease activity. After 3 months of anti-TNF treatment, 97.8% (135/138) achieved a good or moderate outcome, and 2.2% (3/128) were nonresponders. Similarly, after 6 months, 3% (4/132) of patients did not respond positively to treatment.

Out of *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms studied, a significant association concerning disease activity was detected for the *IL-17F* rs763780 variant. AS patients homozygous for the *A* allele more likely presented low or moderate disease activity (BASDAI < 4) after 6 months of treatment than heterozygotes (*AA* vs. *AG*, $p = 0.035$, OR = 13.22, and 95%CI = 1.82-87.84). The same genotype was significantly more common among subjects with a good or moderate response to TNF inhibitor therapy (*AA* vs. *AG*, $p = 0.035$, OR = 13.22, and 95%CI = 1.82-87.84).

The other studied *IL-17A* rs2275913, *IL-17RA* rs4819554, and *IL-17RC* rs708567 genetic variants were not found to significantly differ among AS patients in respect to disease activity and biological agent treatment outcome.

4. Discussion

In the present study, patients with ankylosing spondylitis and controls were genotyped for the *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-17RC* polymorphisms to assess whether their genetic variants may be associated with susceptibility to the disease, clinical parameters, and anti-TNF treatment outcome in our Polish population.

Comparison made between our patients and controls did not show any significant association with predisposition to the disease as in both groups, similar distributions of alleles and genotypes of all SNPs studied were observed. However, disease onset was found to be affected by the *IL-17RC* rs708567 SNP and the presence of *AA* homozygosity prevailed in patients that had a lower age of disease onset than those with *G* allele.

Among currently analysed genetic variants, *IL-17A* rs2275913 and *IL-17F* rs763780 have been previously extensively studied for associations with various rheumatic disorders.

In our current study, neither *IL-17A* rs2275913 nor *IL-17F* rs763780 was found to be associated with AS risk. Nevertheless, association with AS susceptibility and *IL-17A* rs2275913 in Chinese [22] and *IL-17F* rs763780 in Turkish [23] populations has been reported. Erkol et al. did not find the relationship between *IL-17A* rs2275913 and AS susceptibility in Turkish patients [23]. More recently, Rocha Loures et al. reported rs2275913 *A* variant and rs763780 G allele as risk factors for AS, spondyloarthritis (SpA), and psoriatic arthritis (PsA) in Brazilian patients [24].

As for the associations of *IL-17* polymorphisms with other diseases, many previous studies focused on

Table 3: The distribution of (a) *IL-17A* and *IL-17F* and (b) *IL-17RA* and *IL-17R* genotypes with respect to clinical parameters of ankylosing spondylitis patients.

(a)

	IL-17A rs2275913						*IL-17F* rs763780			
	GG		*GA*		*AA*		*AA*		*AG*	
	N	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)
CRP 0 m.	43	13.99 (7.06-47.09)	54	18 (6.983-38.86)	11	15.3 (7.85-33)	97	18.33 (7.825-39.27)	11	9.38 (5-16.73)
CRP 3 m.	30	10.11 (1.39-21.12)	40	5.1 (1.325-10.08)	9	2.6 (0.95-7.9)	71	6.2 (1.52-13.55)	8	1.3 (0.375-11.84)
CRP 6 m.	25	7.06 (0.8-12.67)	39	5.41 (0.9-15.6)	8	3.35 (0.9-9.758)	64	5.71 (1.55-13.22)	8	1.1 (0.35-19.76)
VAS 0 m.	48	87 (80-90)[a]	70	80 (70-90)	14	80 (70-90)	122	80 (72.5-90)[b]	10	88.5 (84.5-92.5)
VAS 3 m.	50	35 (24.5-40)	74	30 (29.5-40)	14	30 (25-30)	127	30 (27-40)	11	30 (22-35)
VAS 6 m.	47	20 (10-29)	71	20 (15-29)	13	20 (16-23.5)	120	20 (14.25-25)	11	20 (11-30)
BASDAI 0 m.	50	7.95 (7-9)	74	7.95 (6.775-8.6)	14	8.3 (6.375-9)	127	8 (6.8-8.8)	11	8.6 (7-9)
BASDAI 3 m.	50	3.275 (2.744-4.1)	74	3.2 (2.65-3.94)	14	3 (2.8-3.063)	127	3.2 (2.75-4)	11	3 (2.725-3.3)
BASDAI 6 m.	48	2.35 (1.925-2.8)	71	2.25 (2-2.7)	13	2 (2-2.825)	121	2.15 (2-2.7)[c]	11	2.5 (2.3-2.9)

(b)

	IL-17RA rs4819554						*IL-17RC* rs708567					
	AA		*AG*		*GG*		*AA*		*AG*		*GG*	
	N	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)	*N*	Median (IQR)
CRP 0 m.	63	16.93 (6.9-39.11)	41	18.46 (7.825-42.09)	4	9.62 (9.248-25.34)	32	14.8 (6.928-28.73)	54	18.4 (8.29-45.73)	22	14.12 (7.17-33.51)
CRP 3 m.	46	5.745 (1.3-13.78)	31	6.54 (1-12.27)	2	5.51 (1.52-9.5)	26	2.15 (0.675-10.53)[g]	40	9.46 (2.55-14.79)	13	3 (0.9-9.555)
CRP 6 m	44	6.49 (0.75-18.33)	26	4.655 (2.05-12.07)	2	5 (3.8-6.2)	23	3.6 (0.7-12.88)	34	6.06 (1.35-13.37)	15	4.2 (2-15.94)
VAS 0 m.	80	80.5 (80-90)	46	80 (70-90)	6	84.5 (68.25-91.25)	39	80 (70-90)	65	81 (79-90)	28	84 (76-90)
VAS 3 m.	83	30 (23-40)[d]	49	35 (30-40)	6	38 (25-46.25)	41	30 (25-38.5)	68	30 (26.5.5-40)	29	35 (30-40)
VAS 6 m.	81	20 (10-23.5)[e]	45	20 (20-30)	5	21 (15-35)	40	20 (11.25-26.5)	62	20 (15-25)	29	20 (10-30)
BASDAI 0 m.	83	8 (7-8.8)	49	7.9 (6.6-8.95)	6	8.213 (6.675-9.25)	41	7.8 (6.1-8.6)	68	7.95 (7-8.8)	29	8.2 (7.4-9)
BASDAI 3 m.	83	3.1 (2.55-3.7)	49	3.325 (3-4)	6	3.3 (1.975-4.2)	41	3 (2.175-3.95)	68	3.2 (3-4)	29	3.2 (2.5-3.9)
BASDAI 6 m.	81	2.1 (1.925-2.6)[f]	46	2.375 (2-2.8)	5	2.3 (1.45-2.9)	40	2.2 (2-2.5)	63	2.25 (2-2.8)	29	2.3 (1.95-2.8)

[a]*GG* vs. *GA+AA*, $p = 0.005$; *GG* vs. *GA*, $p = 0.006$; *GA* vs. *GG+AA*, $p = 0.016$; [b]*AA* vs. *AG*, $p = 0.027$; [c]*AA* vs. *AG*, $p = 0.035$; [d]*AA* vs. *AG+GG*, $p = 0.002$; *AA* vs. *AG*, $p = 0.004$; *AA+GG* vs. *AG*, $p = 0.008$; [e]*AA* vs. *AG+GG*, $p = 0.002$; *AA* vs. *AG*, $p = 0.004$; *AA+GG* vs. *AG*, $p = 0.006$; (f) *AA* vs. *AG+GG*, $p = 0.046$; *AA* vs. *AG*, $p = 0.040$; *AA+GG* vs. *AG*, $p = 0.045$; [g]*AA* vs. *AG+GG*, $p = 0.043$; *AA* vs. *AG*, $p = 0.018$; *AA+GG* vs. *AG*, $p = 0.011$. *N*: number of patients in groups; IQR: interquartile range; CRP: C-reactive protein; VAS: visual analogue scale; DAS28: disease activity score 28; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; 0 m.: at the baseline; 3 m.: after 3 months of therapy; 6 m.: after 6 months of treatment.

osteoarthritis (OA) and rheumatoid arthritis (RA). Results of analysis performed on an Asian OA group suggested that the *IL-17A* rs2275913 *A* allele [25–27] and *IL-17F* rs763780 G variant [26] increased susceptibility to knee OA. In Caucasians, no association between *IL-17A* rs2275913 polymorphism and risk of hip or knee OA was found [28], but the *IL-17F* rs763780 G allele had a significant impact on the risk of the hip [28] and knee OA [29]. *IL-17A* rs2275913 *GA* [29], *IL-17F* rs763780 *AA* [28], and *IL-17A-F* G-A haplotype [30] seem to play a rather protective role in the knee, hip, or hip and knee OA, respectively. Further meta-analysis performed by Lu et al. highlighted higher susceptibility to OA in patients with *IL-17A* rs2275913 *A* allele and *IL-17F* rs763780 G allele among Asians, as well as with *IL-17F* rs763780 *G* genetic variant in a Caucasian cohort [12].

Interestingly, papers concerning the role of *IL-17A* rs2275913 and *IL-17F* rs763780 in RA are inconsistent. *IL-17A* rs2275913 *GG* genotype [31, 32] and *G* allele [33] have been found to increase susceptibility to RA, whereas Shen et al. described *AA* genotype as being linked to lower RA risk [34]. Other studies showed no significant correlations between *IL-17A* rs2275913 variant and prevalence to develop RA in Polish [13, 35], Turkish [36], Brazilian [37], Tunisian [16, 38], Algerian [39], Mexican [40], and Egyptian [41] patients. Growing evidence suggests that *IL-17F* rs763780 G is associated with susceptibility to the disease [13, 33, 38]. However, many studies did not confirm this polymorphism as an RA risk factor [35–37, 39, 41, 42]. Recent meta-analysis findings led to the identification of *IL-17A GG* and *IL-17F AG* genotypes as more frequently distributed among RA patients [11].

With regard to *IL-17RA* and *IL-17RC* polymorphisms, the present analysis did not show differences in genotype and allele distribution between patients and controls. This observation confirms previous results for *IL-17RC* among RA patients [16] but stays in contrast with *IL-17RA* developments in AS Spanish cohorts [17]. *IL-17RA* rs48419554 was also identified as a risk factor for psoriasis [43, 44]. However, other *IL-17RA* SNPs were not found to be associated with PsA [45].

Our current results also show that *IL7RC* rs708567 G variant has an effect on disease onset and is more frequently detected among patients that developed the diseases approximately 4 years later than *AA* homozygotes. To the best of our knowledge, no one has studied *IL-17RC* rs708567 in AS so far. This genetic variant and its homozygosity were also described to affect arthritis among systemic lupus erythematosus Bulgarian patients [46]. On the other hand, in a study conducted by Dhaouadi et al., *IL7RC A* allele of this polymorphism tended to show higher DAS28 in RA subjects [16]. However, the functional consequence of *IL-17RC* rs708567 polymorphism remains unknown.

In the present study, some interesting results were described regarding the *IL-17F* rs763780 SNP and our cohort of patients with AS and unfavourable effect of the *IL-17F* rs763780 G allele.

Likewise, the *IL-17F* rs763780 *G* allele was observed by Paradowska-Gorycka et al. to be positively correlated with the number of tender joints, as well as to tend to reach insignificantly higher values of DAS28-CRP and health assessment questionnaire (HAQ) score [42].

According to earlier findings established in Turkey, the *IL-17F* rs763780 *GG* genotype was prone to greater BASFI scores and *AG* variant to higher CRP level [23]. As suggested, the evidence we found points to an association between this polymorphism and disease activity in AS patients. Our results show that the *AG* genotype is significantly correlated with higher, both VAS values before treatment and BASDAI score after 6 months. Also, we link *AA* genotype with moderate/low disease activity and good/moderate response to treatment after 6 months. This concurs well with results obtained by Prieto-Peréz et al., who observed that rs763780 can predict response to adalimumab at 6 months, in psoriasis [47].

Of note, our previous analysis showed the association of the *IL-17F* rs763780 G allele with higher IL-17F secretion [48]. Also, Braga et al. observed this association between the *IL-17F* rs763780 *G* allele and increased IL-17F serum levels in Brazilian AS patients and controls [49]. These results suggest that alleles or genotypes associated with higher IL-17F production may play an unfavourable role.

Recently, the novel insight into functional consequences of the *IL-17F* polymorphism was described by Nisar et al. The change at position 161 (His to Arg) is located in the C-terminal end of IL-17, which interacts with IL-17RA. This substitution resulted in more favourable conformation, enhanced stability of the trimeric IL-17A/F/IL-17RA complex. The stronger binding may induce the proinflammatory effect and influence the severity of RA [50].

One of our previous studies performed on RA patients found that *IL-17A* rs2275913 *GG* homozygous females were characterized with the most active disease after 3 months and poor response to anti-TNF therapy [13]. On the other hand, de la Peña et al. reported that *A* allele carriers were predicted to present more severe RA and needed more than three DMARDs to control the disease [51].

It has been reported that the *IL-17A* rs2275913 polymorphism located in the promoter region can regulate gene transcription and stimulate IL-17 cytokine secretion (-197A allele) [52].

Current analysis demonstrates a significant correlation between the *IL-17A GG* genotype and higher VAS values before starting therapy in AS patients. The same genotype more frequently characterized patients with elevated CRP after 3 months. We did not find any significant correlation between *IL-17A* variants and response to the therapy. However, it was reported in the literature that rs2275913 was associated with response to anti-TNF therapy among patients with inflammatory bowel disease [53].

Our results are also in line with the findings of Vidal-Castiñeira et al. concerning significantly higher BASDAI scores in AS patients carrying the *IL-17RA* rs4819554 G allele. Moreover, our study reveals associations between the *IL-17A G* allele and greater VAS after 3 and 6 months of anti-TNF treatment. These correlations are worth mentioning because they indicate an impact of *G* variant, located in the gene promoter, on AS severity. This region was also noted to affect the response to anti-TNF therapy outcome in psoriasis [54].

The *IL-17RA* rs4819554 variant is encoded within the promotor region and may have a functional effect by modulating the gene transcription. This SNP was found to be in linkage disequilibrium with rs4819553 and rs4819958. Those polymorphisms are predicted to be related to transcription factor binding sites (TFBSs) belonging to the Ikaros (IK) family. These are involved in Th17 cell differentiation [54].

In *G* allele carriers, the increase of Th17 cytokines could promote the pathogenic mechanism via IL-23/Th17 pathway [54]. It could explain the higher VAS values and BASDAI scores that we observed.

These findings shed some light on common genetic variants in *IL-17A*, *IL-17F*, and *IL-17RA* genes. The investigated polymorphisms can affect biological activity of the protein and thus influence immunological features like a response to etanercept [14].

There is still considerable controversy surrounding *IL-17* SNP relationships and AS development. Therefore, our results need to be interpreted with caution. In fact, population diversity and treatment approach may explain the differences between studies. Although the advantage of our methodology is homogeneity of the Polish population, we are aware that the main limitation of our study is the relatively limited number of cases included in the analysis. Therefore, further data collection from AS patients is required to confirm these observations.

Other interesting genetic variants within *IL-17F* include rs11465553 [35] and rs2397084 [33, 35, 36, 38, 39, 42], which were investigated in RA. In a Polish cohort, the rs2397084 polymorphism was correlated with longer disease duration [42], whereas in Tunisians, it was associated with disease severity [38]. Additionally, rs2397084 [29] and rs1889570 [28, 30] were studied in osteoarthritis patients. Regarding the *IL-17A* gene, rs3804513 was associated with radiographic progression in early RA [55]. Other *IL-17A* polymorphisms were studied in a Chinese population. *IL-17A* rs4711998 and rs8193037 were not associated with RA, whereas rs3819024, rs3819025, and rs8193036 were correlated with the risk of RA [34]. These polymorphisms may be of interest for further study on ankylosing spondylitis.

5. Conclusions

The analysis shows that *IL-17* polymorphisms are associated with clinical parameters in Polish patients with ankylosing spondylitis and have influence on AS severity and potential course of the disease and may be biomarkers of response to anti-TNF drugs in Polish patients. The *IL-17F* rs763780 polymorphism should be considered as a candidate biomarker of disease activity and anti-TNF treatment outcome. The *IL-17RA* rs48419554 G allele may serve as a potential marker of disease severity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Conceptualization was contributed by K.B.-K. and J.W.; methodology was done by J.W. and K.B.-K.; formal analysis was carried out by J.W. and K.B.-K.; investigation was done by J.W. and K.B.-K.; resources were contributed by J.S., K.K., B.B., M.Ch.-M., S.J., and K.B.-K.; data curation was performed by J.W., J.S., K.K., B.B., M.Ch.-M., and S.J.; writing—original draft preparation was contributed by J.W. and K.B.-K.; writing—review and editing was contributed by J.S., K.K., B.B., M.Ch.-M., S.J., and K.B.-K.; supervision was contributed by J.S., S.J., and K.B.-K.; project administration was done by K.B.-K.; funding acquisition was contributed by K.B.-K. All authors have read and agreed to the published version of the manuscript.

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Research Article

Exploring the Extracellular Vesicle MicroRNA Expression Repertoire in Patients with Rheumatoid Arthritis and Ankylosing Spondylitis Treated with TNF Inhibitors

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Rheumatoid arthritis (RA) and ankylosing spondylitis (AS) belong to the most common inflammatory rheumatic diseases. MicroRNAs (miRNAs) are small 18–22 RNA molecules that function as posttranscriptional regulators. They are abundantly present within extracellular vesicles (EVs), small intercellular communication vesicles that can be found in bodily fluids and that have key functions in pathological and physiological pathways. Recently, EVs have gained much interest because of their diagnostic and therapeutic potential. Using NanoString profiling technology, the miRNA repertoire of serum EVs was determined and compared in RA and AS patients before and after anti-TNF therapy to assess its potential use as a diagnostic and prognostic biomarker. Furthermore, possible functional effects of those miRNAs that were characterized by the most significant expression changes were evaluated using in silico prediction algorithms. The analysis revealed a unique profile of differentially expressed miRNAs in RA and AS patient serum EVs. We identified 12 miRNAs whose expression profiles enabled differentiation between RA and AS patients before induction of anti-TNF treatment, as well as 4 and 14 miRNAs whose repertoires were significantly changed during the treatment in RA and AS patients, respectively. In conclusion, our findings suggest that extracellular vesicle miRNAs could be used as potential biomarkers associated with RA and AS response to biological treatment.

1. Introduction

MicroRNAs (miRNAs, miRs) are a family of single-stranded, noncoding endogenous regulatory RNAs derived from double-stranded precursors, typically composed of 21-23 nucleotides. They are involved in the regulation of gene expression at the posttranscriptional level [1, 2]. They function by the way of complementary binding to the 3′UTR regions of target mRNA via the RNA-induced silencing complex (RISC), resulting in inhibition of the translation process [3]. One miRNA molecule can regulate the expression of several genes (transcripts), and a transcript may have a 3′-UTR region that is recognized by many miRNAs [4, 5]. It is estimated that encoding miRNA genes constitute 1-5% of all the genes in humans and animals [6]. Regulatory miRNAs and exosomes are becoming increasingly important in identification of molecular markers related to pathogenesis and prognosis of disease [7].

Extracellular vesicles (EVs) are lipid membrane-enclosed vesicles, released by cells into the extracellular space. EVs are a heterogeneous collection of exosomes, microvesicles, and apoptotic bodies, ranging in size from 40 nm to 4000 nm.

Classification is based on their size, origin, and biological function. EVs are present in blood, saliva, urine, milk, and amniotic fluid and are secreted by all mammalian cell types [8]. Their main function is the transport of lipids, proteins, miRNAs, and mRNAs. Extracellular vesicles are also mediators in intercellular communication and immune-regulatory processes such as bone remodelling, which implicates them in pathogenesis of rheumatic diseases [9]. Although the unique mechanism of immune complex formation remains unclear, it has been observed that synovial exosomes contain citrullinated peptides, which are well-known autoantigens in RA [10]. It has been reported that EVs widely participate in RA development, including antigen presentation and immune complex formation, inflammation, delivery of miRNA, and destruction of extracellular matrix [11]. Moreover, EVs are considered to be promising biomarkers in joint diseases such as RA [12]. Recent studies have indicated a significantly higher level of plasma EVs in RA patients compared to healthy individuals [13]. Other studies established a role of EVs as biomarkers in arthritis by showing an association of exosomal amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 with disease activity in RA [14] and identified higher expression of long noncoding RNA, HOX Transcript Antisense RNA (HOTAIR), in serum exosomes of RA cases [15].

Pathogenesis of rheumatoid arthritis (RA) and ankylosing spondylitis (AS) is considered to be multifactorial, with disease susceptibility being associated with genetic, environmental, and stochastic factors. RA and AS are the most common inflammatory rheumatic diseases [16, 17]. They are characterized by different clinical, laboratory, and imaging hallmarks [18, 19]. RA is an autoimmune disease characterized by symmetric and erosive arthritis typically affecting small- and medium-sized joints. It is marked by inflammation of joint synovial tissue, resulting in progression of cartilage and bone tissue damage, ultimately leading to disability [20]. AS is a systemic inflammatory disorder that affects the sacroiliac joints and the spine and can also affect peripheral joints, causing characteristic inflammatory back pain, which can lead to structural and functional impairments [21].

A number of studies have reported that alteration of miRNA profiles may play an important role in the pathogenesis of rheumatic diseases [22, 23], and thus, these profiles may constitute potential biomarkers [24]. Increased expression of miRNAs has been detected in various cell types of RA patients, and miR-146a was shown to mediate negative feedback in the immune response of RA [25]. This specific miRNA was found to be overexpressed in synovial fibroblasts, synovial tissue, synovial fluid monocytes, peripheral blood mononuclear cells, and serum plasma of RA patients [26]. miR-146a regulates gene expression of TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) in inflammation and participates in a negative feedback loop [27]. Moreover, single-nucleotide polymorphisms (SNPs) in miR-146a may alter its expression. The rs2910164 SNP was found to reduce the expression level of miR-146a, which led to less efficient inhibition of target genes, including two molecules important for RA development, TRAF6 and IRAK1, suggesting that miR-146a rs2910164 could contribute to RA development [28]. Moreover, our previous study in a group of Polish patients with RA showed an association between rs2910164 C variant and higher expression of miRNA-146a in serum after three months of therapy with TNF inhibitors [29].

Despite the fact that anti-TNF treatment constitutes a breakthrough in management of RA and other rheumatic diseases, approximately 30% of patients do not achieve any improvement. Because patients with RA or AS have a variable response to treatment, identification of biomarkers capable of predicting therapeutic response is imperative.

Both RA and AS would benefit from discovery of biomarkers that could be detected when disease is present, distinguish between the two disorders, that are associated with disease progression and outcome, and help to predict the response to treatment.

This study is aimed at investigating whether analysis and comparison of miRNA profiles in patients with RA and AS could be used (i) for detection of diagnostic miRNA markers to distinguish RA from AS and (ii) before and after TNF-α inhibitor treatment to predict the outcome and effectiveness of this biological therapy on modulation of proinflammatory response.

2. Materials and Methods

2.1. Patients. AS patients were classified according to the 1984 modified New York criteria [30]. Adult Caucasians (age $\geq$ 18 years) included in the study were characterized with high-disease activity (defined as BASDAI $\geq$ 4 and back pain $\geq$ 4) before initiation of anti-TNF therapy and failed to respond to at least two nonsteroidal anti-inflammatory drugs (NSAIDs) for at least four weeks at maximum doses (if there were no contraindications). Effectiveness of current drug therapy was assessed using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), which is based on a 0-10 scale measuring discomfort, pain, and fatigue.

RA patients enrolled in this project were classified according to the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria, as well as the presence of active disease represented by the disease activity score in 28 joints (DAS28 $\geq$ 5.1). Patients qualified for the study were also before biologic agents therapy initiation and failed to respond to at least two disease-modifying antirheumatic drugs (DMARDs). They were $\geq$18 years, of Caucasian origin, and with complete medical records.

Exclusion criteria for both RA and AS patients comprised clinically significant impairment of hepatic and renal function, coexistence of connective tissue diseases, infections with hepatotropic viruses, infections resistant to therapy, an ongoing history of cancer or uncontrolled diabetes, alcohol abuse, pregnancy or breastfeeding, and insufficient clinical records.

The ASAS/EULAR criteria were used to assess the clinical outcome of anti-TNF treatment.

All RA patients responded to therapy. Good response was a reduction of the DAS28 score ΔDAS28 > 1.2 to a

posttreatment value of DAS28 ≤ 3.2, and moderate response was interpreted as ΔDAS28 > 1.2 and a posttreatment DAS 28 > 3.2 or 0.6 < ΔDAS28 ≤ 1.2 and DAS28 ≤ 5.1 [31].

For AS, a good response was determined as a reduction of ΔBASDAI ≥ 2.0 from baseline and BASDAI < 3.0 at the endpoint. A moderate response was defined as a reduction of ΔBASDAI ≥ 2.0 from baseline and BASDAI ≥ 3.0 at the endpoint [32].

The study was approved by the Wrocław Medical University Ethics Committee (identification code KB-625/2016), and written informed consent was obtained from all participants.

Clinical characteristics of the patients are presented in Table 1 as mean and standard deviation (± SD).

2.2. Sample Preparation. Serum samples were collected from three patients (two men and one woman, aged: 23-46, mean age: 35) with RA and three patients (two men and one woman, aged: 36-70, mean age: 48) with AS at two-time points before and after three months of anti-TNF treatment initialization. All RA patients were treated with Etanercept. In the AS group, two patients were treated with Etanercept and one with Adalimumab. Further characteristics of patients enrolled in this study are detailed in Table 1. Sera were prepared from whole blood samples, which had been collected in 8.5 ml BD Vacutainer® SST™ II advance blood tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with clot activator and acrylic gel separation. Samples were centrifuged at 1500 g for 10 min and stored at −70°C.

2.3. EV and RNA Isolation. Extracellular vesicles (EVs) were isolated from 3 ml of serum using Total Exosome Isolation Reagent (from Serum) (ThermoFisher Scientific), following supplier's guidelines. EV pellets were stored at -80°C.

The EV pellets were resuspended in particle-free PBS, and RNA was isolated using Total Exosome RNA and Protein Isolation Kit (ThermoFisher Scientific), following supplier's protocol. RNA was concentrated incorporating Amicon Ultra-0.5 centrifugal filter unit with Ultracel-3 membrane (Merck Millipore), according to NanoString recommendations.

RNA concentration was determined using the 2100 Bioanalyzer and the RNA 6000 Pico Kit (Agilent Technologies). While accurate RIN numbers could not be generated, due to the lack of ribosomal RNA in EVs, RNA concentration and peak area were assessed. All RNA was stored at −80°C.

2.4. EV Morphology and Size Measurement. To find out whether EVs were properly isolated, EV morphology and size distribution were assessed. For EV morphology, transmission electron microscopy (TEM) was used. 300-mesh grids were filmed with Pioloform® resin (SPI Supplies), carbon coated, and plasma etched before use. EVs were directly applied on the grid, stained with 10 μl of 2% aqueous uranyl, and air-dried. Examination was conducted on a Hitachi HT7800 transmission electron microscope equipped with an Emsis Xarosa camera with Radius software, in cooperation with the Electron Microscopy Research Services, Newcastle University.

For EV size distribution analysis, nanoparticle tracking analysis (NTA) was performed employing a NanoSight LM10 microscope supplied with NTA software version 3.0 (NanoSight Ltd., UK). Background extraction with blur settings and maximum jump distance was applied automatically, and 5 × 60 second recordings were taken for each sample.

Table 1: Characteristics of RA and AS patients included in the study.

	RA ($N = 3$)	AS ($N = 3$)
Age [years]	34.67 ± 11.50	5
Sex (female/male)	1/2	1/2
Disease duration [years]	9.333 ± 9.074	15.33 ± 15.63
BMI	27.02 ± 2.728	26.47 ± 8.474
VAS baseline [mm]	82.67 ± 6.429	73.00 ± 18.38
VAS after therapy [mm]	30.00 ± 20.00	19.67 ± 11.68
DAS28 baseline	5.61 ± 0.71	—
DAS28 after therapy	1.85 ± 1.20	—
BASDAI baseline	—	7.90 ± 0.36
BASDAI after therapy	—	2.00 ± 0.87

BMI: body mass index; VAS: visual analogue scale; DAS28: disease activity score 28, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; RA: rheumatoid arthritis; AS: ankylosing spondylitis.

2.5. miRNA Profiling. Serum EV microRNA expression profiling was performed using the nCounter® Human v3 miRNA Expression Assay kit (NanoString Technologies) as previously described [33]. This code set comprises 98% of microRNA sequences found in miRbase v22 and includes 798 mature microRNAs, six positive and eight negative controls, six ligation controls, and five reference controls. The procedure was performed according to manufacturer's guidelines. Data normalization was performed using nSolver Analysis Software v4.0 (NanoString Technologies), with positive control normalization using the geometric mean and normalization flagging outside the normalization factor range 0.3-3.0. Codeset content normalization was performed using the top 100 microRNAs for normalization, based on geometric mean and flagging outside the normalization factor range 0.1-10.0.

2.6. Statistical Analysis. Data normalization and fold change (FC) expression differences between groups were conducted using nSolver v4.0 software (NanoString Technologies). Further analyses were performed using the R software (version 3.6.1) with RStudio 1.2.5001 (RStudio, Inc., USA) applying an analysis pipeline designed by Newcastle University, Haematological Sciences Department. "ggplot2" (v2.1.0) package function was used to construct Volcano plots. Heatmaps with unsupervised clustering were generated using "gplots" (v2.17.0) and "RColorBrewer" (v1.1-2). p values between the two groups were calculated using a two-tailed t-test. Significance was set at $p < 0.05$.

2.7. Target Prediction and Pathway Analysis. To perform gene prediction and pathway analyses for miRNAs obtained

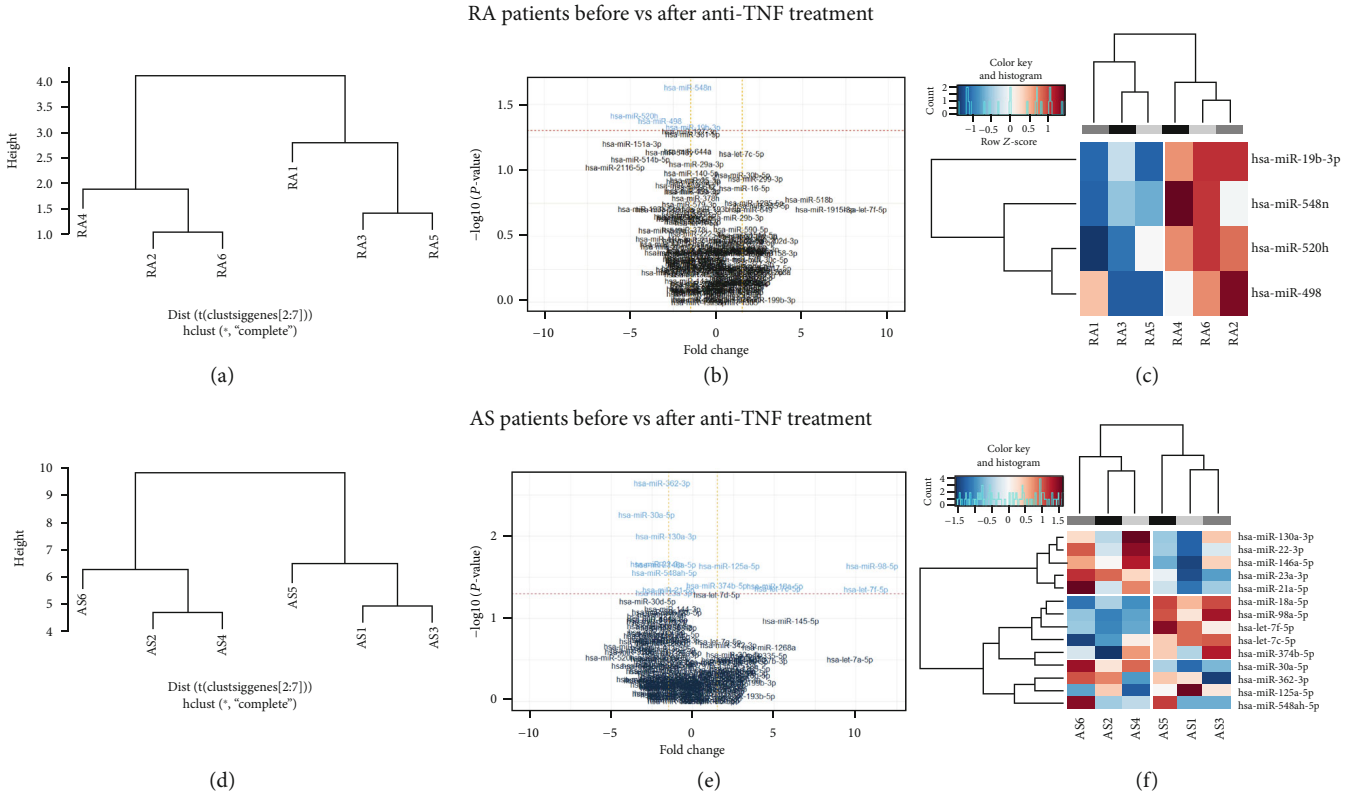

Figure 1: Serum EV microRNA expression in RA and AS patients before vs. after anti-TNF treatment. (a, d) Unsupervised hierarchical clustering analysis. Samples before (1, 3, and 5) vs. after (2, 4, and 6) anti-TNF therapy. (b, e) Volcano plots showing the relationship between fold change and significance for RA and AS patients before and after anti-TNF therapy. The horizontal dashed line indicates cutoff for significance $p < 0.05$ (−log10 p value > 1.3) and the vertical lines for fold change ≥ 1.5/≤−1.5. Significantly different miRNAs are highlighted in blue. (c) Heatmaps showing unsupervised hierarchical clustering of differentially expressed miRNAs in serum EVs of patients before ($n = 3$) vs. after Etanercept treatment. The colour scale indicates relative fold change (red: high; blue: low).

from NanoString, we incorporated an approach described by Lou et al. [34]. Potential microRNA gene targets were identified by the miRNet database (http://www.mirnet.ca/) [35]. The STRING database (http://string-db.org) was used to construct networks of protein-protein interactions based on target genes obtained from miRNet [36]. Hub genes, or genes from the protein-protein interaction network that have the highest degree of connectivity, were determined using the Cytoscape software (version 3.7.2) [37]. They were subsequently used as input in the KEGG pathway enrichment analysis performed in the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifc http://rf.gov/) [38].

3. Results

3.1. NanoString Experimental Setup and MicroRNA Expression Counts. In this study, serum samples from three RA patients and three AS patients before and after administration of biological treatment were analysed. Samples were labelled according to diagnosis (RA or AS), time points of sample collection: before anti-TNF treatment induction (indicated by odd numbers, for example: RA1 and RA3) and after three month of drug administration (indicated by respective even numbers, RA2 and RA4). This labelling method resulted in the following groups: six RA samples, including three before treatment (RA1, RA3, and RA5) and three after therapy (RA2, RA4, and RA6), and similarly six AS samples, three before treatment (AS1, AS3, and AS5) and additional three samples after three months after treatment initialization (AS2, AS4, and AS6).

All NanoString samples passed quality control parameters according to their microRNA expression profiles. A total of 159 microRNAs were positively expressed in >2 RA and AS samples and were included in the final analysis.

3.2. MicroRNA Expression Analysis in RA EV Samples before vs. after Therapy. Unsupervised hierarchical clustering analysis separated RA serum EV samples before and after three months of anti-TNF treatment as shown in Figure 1(a). EVs from serum collected after three months of anti-TNF therapy were characterized by a unique miRNA signature consisting of four miRNA molecules that were overexpressed ($p < 0.05$), compared to samples collected before treatment: miR-520 h (4.77-fold, $p = 0.038$), miR-498 (3.28-fold, $p = 0.042$), miR-548n (1.66-fold, $p = 0.023$), and miR-19b-3p (1.35-fold, $p = 0.047$) (Figures 1(b) and 1(c), Table 2).

3.3. MicroRNA Expression Analysis in AS EV Samples before vs. after Therapy. AS samples were similarly evaluated and unsupervised clustering analysis separated serum EV samples taken before and after three months of anti-TNF

Table 2: Comparison of differentially expressed miRNAs in RA and AS patients before and after three months of the anti-TNF treatment. Statistical characteristics of depicted miRNAs. FC: fold change.

RA samples before vs. after therapy			AS samples before vs. after therapy			RA vs. AS patients before anti-TNF treatment			RA vs. AS patients after anti-TNF treatment		
miRNA	p	FC	miRNA	p	FC	miRNA	p	FC	miRNA	p	FC
hsa-miR-19b-3p	0.047	-1.35	hsa-let-7c-5p	0.042	5.21	hsa-miR-125a-5p	0.028	4.31	hsa-let-7e-5p	0.005	6.13
hsa-miR-548n	0.023	-1.66	hsa-let-7f-5p	0.044	10.61	hsa-miR-130b-3p	0.031	4.91	hsa-let-7i-5p	0.004	-1.77
hsa-miR-520 h	0.038	-4.77	hsa-miR-125a-5p	0.023	2.23	hsa-miR-151a-5p	0.012	11.58	hsa-miR-125a-5p	0.020	6.00
hsa-miR-498	0.042	-3.28	hsa-miR-130a-3p	0.010	-1.65	hsa-miR-301a-3p	0.029	4.61	hsa-miR-145-5p	0.012	12.76
			hsa-miR-146a-5p	0.022	-1.67	hsa-miR-324-5p	0.031	5.45	hsa-miR-151a-3p	0.027	3.42
			hsa-miR-18a-5p	0.040	5.05	hsa-miR-376c-3p	0.018	-1.08	hsa-miR-151a-5p	0.027	2.96
			hsa-miR-21-5p	0.045	-1.49	hsa-miR-378 h	0.048	-2.47	hsa-miR-1915-3p	0.033	-2.62
			hsa-miR-22-3p	0.022	-2.22	hsa-miR-411-5p	0.037	-1.77	hsa-miR-221-3p	0.021	1.62
			hsa-miR-23a-3p	0.048	-1.74	hsa-miR-548a-5p	0.008	-3.50	hsa-miR-28-3p	0.044	3.04
			hsa-miR-30a-5p	0.005	-2.82	hsa-miR-548n	0.001	-2.11	hsa-miR-30a-5p	0.050	-2.27
			hsa-miR-362-3p	0.002	-1.86	hsa-miR-548q	0.013	-1.95	hsa-miR-3158-3p	0.033	-2.62
			hsa-miR-374b-5p	0.040	1.48	hsa-miR-579-3p	0.029	-1.60	hsa-miR-324-5p	0.019	4.56
			hsa-miR-548a-5p	0.028	-1.72				hsa-miR-379-5p	0.039	-2.84
			hsa-miR-98-5p	0.023	10.99				hsa-miR-496	0.030	-2.83
									hsa-miR-503-5p	0.012	2.90
									hsa-miR-612	0.024	-3.08
									hsa-miR-649	0.033	-2.62
									hsa-miR-98-5p	0.006	7.32

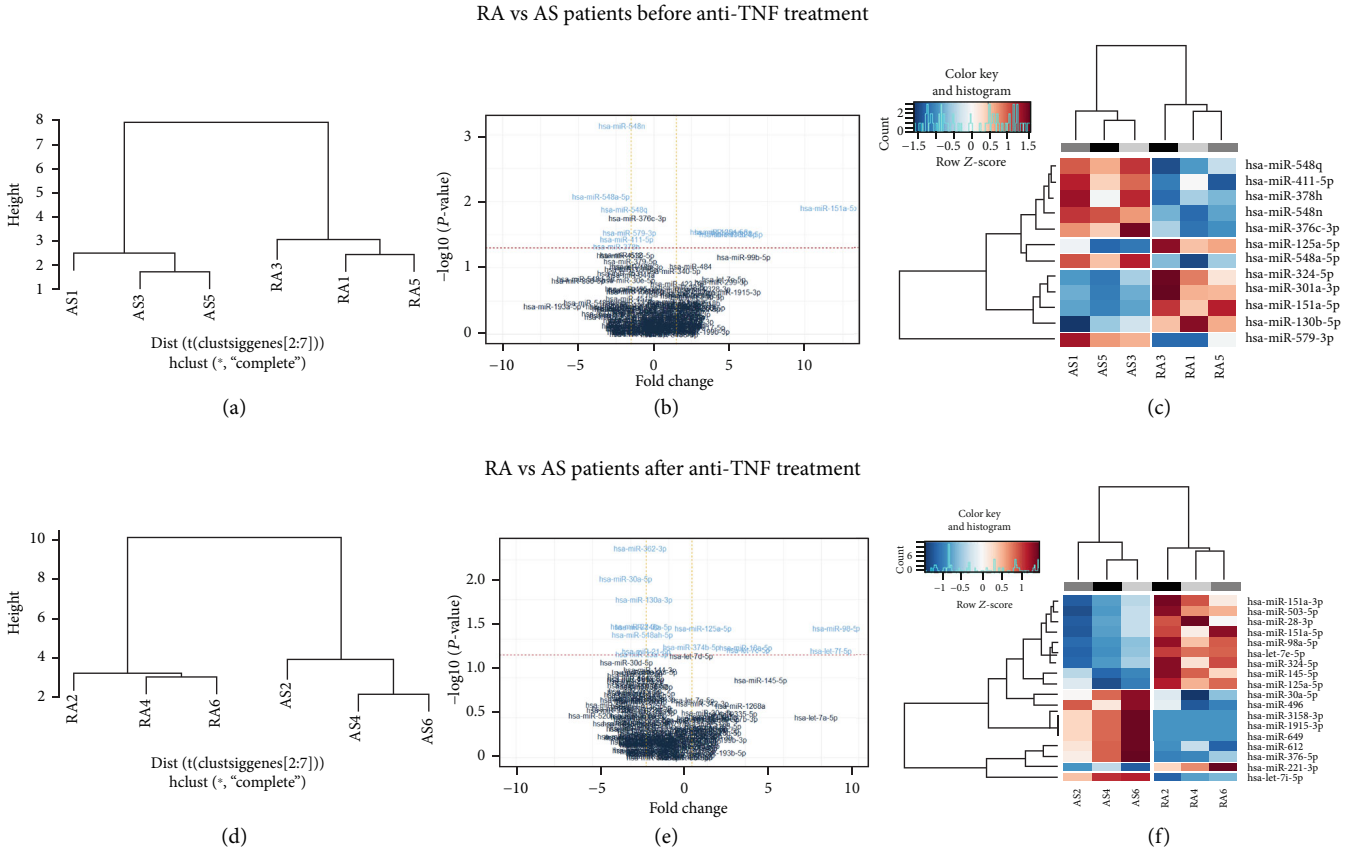

Figure 2: Serum EV microRNA expression in RA vs. AS patients before and three months after anti-TNF treatment. (a, d) Unsupervised hierarchical clustering analysis. RA vs. AS patients before biological treatment initialization and after agent administration. (b, e) Volcano plots showing the relationship between fold change and significance for RA vs. AS patients before and after anti-TNF therapy. The horizontal dashed line indicates cutoff for significance $p < 0.05$ (−log10 p value > 1.3) and the vertical lines for fold change ≥ 1.5/≤−1.5. Significantly different miRNAs are highlighted in blue. (c) Heatmaps showing unsupervised hierarchical clustering of differentially expressed miRNAs in serum EVs of RA vs. AS patients before ($n = 3$) and after treatment. The colour scale indicates relative fold change (red: high; blue: low).

treatment (Figure 1(d)). Expression profiling analysis identified 14 miRNAs significantly ($p < 0.05$) differentially expressed in AS after therapy, of which eight were overexpressed: miR-130a-3p, miR-146a-5p, miR-21-5p, miR-22-3p, miR-23a-3p, miR-30a-5p, miR-362-3p, miR-548ah-5p (with FC range 1.49-2.82, p value range: 0.002-0.048), while 6 were downregulated: let-7c-5p, let-7f-5p, miR-125a-5p, miR-18a-5p, miR-374b-5p, and miR-98-5p (FC range: 1.48-10.99, p value range: 0.023-0.044). The greatest fold change (FC = 10.99) was detected for hsa-miR-98-5p (Figures 1(e) and 1(f), Table 2).

3.4. Comparison of RA vs. AS EV miRNA Expression before Anti-TNF Therapy Implementation. To assess whether RA and AS patients can be distinguished based on their serum EV expression profiles, we compared miRNA repertoires at the baseline, before induction of biological treatment (Figure 2(a)). The comparison of RA and AS serum EVs before anti-TNF treatment implementation identified 12 miRNAs with significantly different expression (FC range: 1.08-11.58, p value range: 0.001-0.048) (Figures 2(b) and 2(c)). Among those miRNA molecules, higher expression of miR-125a-5p, miR-130b-3p, miR-151a-5p, miR-301a-3p, and miR-324-5p characterized patients with RA (FC range: 4.31-11.58, p value range: 0.012-0.031), while miR-376c-3p, miR-378h, miR-411-5p, miR-548a-5p, miR-548n, miR-548q, and miR-579-3p were overexpressed in AS patients, but with a smaller FC range (1.08-3.50, p value range: 0.001-0.048) (Figures 2(b) and 2(c), Table 2).

3.5. Comparison of RA vs. AS EV miRNA Expression after Therapy. A similar comparison of miRNA repertoires between patients with RA vs. AS was performed on the RA and AS samples collected three months after initialization of anti-TNF treatment. A total of 18 microRNAs were significantly differentially expressed after therapy in RA compared to AS patients (FC range: 1.77-12.76, p value range: 0.004-0.050), of which eight were upregulated in AS (miR-7i-5p, miR-1915-3p, miR-30a-5p, miR-3158-3p, miR-379-5p, miR-496, miR-612, and miR-649; FC range: 1.77-3.08, p value range: 0.004-0.050) (Figures 2(e) and 2(f), Table 2).

Furthermore, miR-30a was upregulated in AS after therapy in comparison to its expression before treatment and its

Table 3: MicroRNAs differently expressed in RA and AS patients compared to healthy controls.

miRNA	Disease	Country	Ethnicity	Source	No. of cases/ controls	Investigation method	Changes of miR expression	Target gene	References
miR-146a	RA	Egypt	Arab	Whole blood	25/25	qPCR	↑	—	[104]
	RA	Japan	Asian	PBMC	6/5	qPCR*	↑	—	[62]
	RA	China	Asian	PBMC	69/69	qPCR	↑	—	[105]
	RA	Egypt	Arab	PBMC	52/56	qPCR	↑	—	[55]
	RA	Egypt	Arab	PBMC	70/60	qPCR	↑	—	[106]
	RA	Canada	—	PBMC	11/10	qPCR	↑	—	[107]
	RA	USA	—	PBMC	16/9	qPCR	↑	*TRAF6*, *IRAK1*	[26]
	RA	Switzerland	European	Serum	34/16	qPCR	↓	—	[60]
	RA	Poland	European	Serum	13/16	qPCR	↓	—	[29]
	RA	USA	European	Plasma	168/91	qPCR	↑	—	[108]
	RA	China	Asian	Plasma	25/20	qPCR*	↓	—	[109]
	RA	Japan	Asian	Plasma, synovial fluid	30/30	qPCR	↑	—	[61]
	RA	China	Asian	Synovial tissue	17/3	qPCR*	↑	—	[102]
	RA	China	Asian	FLS	12/10	qPCR	↓	*TLR4*	[57]
	RA	Japan	Asian	CD4+ T	33/12	qPCR	↑	*FAF1*	[54]
	RA	Germany	European	Treg	61/49	qPCR	↓	*STAT1*	[110]
	AS	China	Asian	Serum	70/68	qPCR†	↑	—	[66]
	AS	Spain	European	Plasma	53/57	qPCR‡	↑	—	[74]
	AS	Switzerland	European	Plasma	24/29	qPCR*	↓	—	[69]
	AS	China	Asian	Hip capsule	30/30	qPCR	↑	*DKK-1*	[67]
miR-155	RA	Canada	Native American	Whole blood	18/12	qPCR	↑	—	[111]
	RA	Japan	Asian	PBMC	6/5	qPCR*	↑	—	[62]
	RA	China	Asian	PBMC	45/25	qPCR	↑	*SOCS1*, *TNFa*, *IL-1B*	[112]
	RA	Canada	—	PBMC	11/10	qPCR	↑	—	[107]
	RA	USA	—	PBMC	16/9	qPCR	↑	—	[26]
	RA	China	Asian	PBMC, FLS	26/23	qPCR*	↑	*IKBKE*	[113]
	RA	Egypt	Arab	Serum	100/100	qPCR	↑	—	[114]
	RA	Japan	Asian	Plasma, synovial fluid	30/30	qPCR	↑	—	[61]
	RA	China	Asian	Plasma	25/20	qPCR*	↓	—	[109]
	RA	USA	European	Plasma	168/91	qPCR	↑	—	[108]
	RA	China	Asian	FLS	89/49	qPCR	↑	*FOXO3a*	[115]
	RA	UK	European	CD14+ T	9/8	qPCR*	↑	—	[116]
	RA	Germany	European	Treg	61/49	qPCR	↓	—	[110]
	RA	UK	European	Monocyte	24/22	qPCR	↑	*CCR2*, *CCR7*	[117]
miR-21	AS	China	Asian	Serum	70/68	qPCR	↑	—	[66]
	RA	China	Asian	Plasma	25/20	qPCR*	↑	—	[109]
	RA	Italy	European	Plasma	28/20	qPCR	↓	—	[94]
	RA	China	Asian	CD4+ T, PBMC	25/20	qPCR	↓	*STAT3*, *STAT5*	[93]
	AS	China	Asian	Whole blood	122/122	qPCR	↑	*PDCD4*	[91]

Table 3: Continued.

miRNA	Disease	Country	Ethnicity	Source	No. of cases/ controls	Investigation method	Changes of miR expression	Target gene	References
miR-22	RA	China	Asian	RASF	40/40	qPCR	↓	*SIRT1*	[118]
	RA	UK	European	Serum	12/12	Microarray	↑	—	[119]
	RA	China	Asian	FLS	48/30 (OA)	qPCR	↓	*Cyr61*	[120]
miR-30a	AS	Spain	European	Plasma	53/57	qPCR‡	↑	—	[74]
	RA	China	Asian	Synovial tissue	7/12 (OA)	qPCR	↓	*Beclin-1*	[121]
	AS	USA	—	Plasma	15/5	Microarray	↓	—	[98]
miR-125a	RA	China	Asian	Plasma	25/20	qPCR*	↓	—	[109]
	RA	Japan	Asian	Plasma	102/104	qPCR*	↑	—	[122]
	RA	USA	European	Plasma	168/91	qPCR	↑	—	[108]
	AS	Spain	European	Plasma	53/57	qPCR‡	↑	—	[74]
miR-221	RA	Egypt	Arab	PBMC	30/20	qPCR	↑	—	[123]
	RA	China	Asian	Serum, FLS	22/18	qPCR	↑	—	[124]
	AS	Switzerland	European	Plasma	24/29	qPCR*	↓	—	[69]
miR-29a	RA	China	Asian	Serum, synovial tissue, FLS	20/10	qPCR	↓	*STAT3*	[87]
	AS	Switzerland	European	Plasma	24/29	qPCR*	↓	—	[69]

↑/↓: up/downregulation; RA: rheumatoid arthritis; AS: ankylosing spondylitis; PBMC: peripheral blood mononuclear cell; FLS: fibroblast-like synoviocytes; RASF: RA synovial fibroblasts; *microarray screening; †sequencing screening; ‡NanoString screening.

expression in patients with RA after anti-TNF drug administration. On the other hand, miR-98 was downregulated after initialization of anti-TNF therapy in both cases. Interestingly, miR-151a and miR-125a were characterized with lower expression levels in AS patients compared to those with RA after, as well as before biological therapy.

3.6. Identification of Target Genes and Potential Pathways. The potential target genes were analysed separately for upregulated and downregulated microRNAs. 10 hub genes were identified for each of the analysed groups. In RA patients, all upregulated microRNAs after therapy were linked to *TP53*, *EP300*, *PTEN*, *MAPK1*, *STAT3*, *ESR1*, *EZH2*, *CCNB1*, *BRCA1*, and *CASP3*. In AS patients, both upregulated and downregulated microRNAs after treatment were associated with *TP53*, *AKT1*, *MYC*, *UBC*, *EGFR*, and *IL6*. Additionally, downregulated microRNAs after therapy were linked to *UBA52*, *CCND1*, *PTEN*, and *STAT3*, whereas the upregulated ones were associated with *RPS27A*, *MAPK1*, *UBB*, and *VEGFA*. In comparison between RA and AS patients before treatment, downregulated microRNAs were connected to *TP53*, *AKT1*, *MYC*, *UBC*, *EGFR*, *RPS27A*, *MAPK1*, *UBB*, *HSPA8*, and *PTEN*, while upregulated microRNAs were linked to *HSP90AA1*, *CCNB1*, *STAT3*, *CDC5L*, *MDM2*, *CASP3*, *ATM*, *SKP1*, *ACTB*, and *IGF1R*. The hub genes detected in comparison between RA and AS patients after therapy linked to downregulated, as well as upregulated microRNAs were *TP53*, *AKT1*, *MYC*, *EGFR*, and *CTNNB1*, although downregulated microRNAs were also related to *UBC*, *PTEN*, *CCND1*, *HSPA8*, and *VEGFA* and upregulated to *MAPK1*, *JUN*, *HDAC1*, *NOTCH1*, and *MAPK8*.

The KEGG pathway enrichment analysis showed that targets of miRNAs identified as upregulated/downregulated in the treatment-related analyses (RA before vs. after, AS before vs. after) were enriched for pathways associated with response to drugs and cellular response to drugs. Additionally, targets of miRNAs upregulated in AS vs. RA (after therapy) and in AS patients after treatment were enriched in pathways associated with the TNF signalling pathway.

4. Discussion

Anti-TNF treatment constitutes a breakthrough in management of rheumatic diseases, although many patients do not achieve significant or any improvement. This makes it essential to find new biomarkers capable of predicting therapeutic response. In the present study, we examined whether serum EV miRNA profiles could be used to distinguish rheumatoid arthritis from ankylosing spondylitis, as well as to predict outcome of TNF inhibitor treatment in both of these rheumatic diseases. We found that in RA patients, four miRNAs (miR-520h, miR-498, miR-548n, and miR-19b-3p) were differentially expressed after anti-TNF treatment, while fourteen were differentially expressed in AS patients (miR-130a-3p, miR-146a-5p, miR-21-5p, miR-22-3p, miR-23a-3p, miR-30a-5p, miR-362-3p, miR-548ah-5p, let-7c-5p, let-7f-5p, miR-125a-5p, miR-18a-5p, miR-374b-5p, and miR-98-5p). Additionally, we identified twelve miRNAs that distinguished the two diseases (miR-125a-5p, miR-130b-3p, miR-151a-5p, miR-301a-3p, miR-324-5p, miR-376c-3p, miR-378h, miR-411-5p, miR-548a-5p, miR-548n, miR-548q, and miR-579-3p).

Recently, there have been many studies investigating EV miRNAs as markers for evaluating RA progression. To date, the association between miRNAs and parameters of disease activity were reported in East Asian populations, specifically

in Korean [39] and Chinese [40] RA patients. Wang et al. showed downregulation of exosome-delivered miR-548a-3p in serum of RA patients in contrast with healthy controls, as well as a lower level of miR-548a-3p correlating with higher levels of CRP, RF, and ESR [40]. Similarly, serum exosomal miR-6089 was significantly decreased in RA Chinese patients compared to controls [41]. Regarding plasma exosomes, secretion of miR-17, miR-19b, and miR-121 was significantly higher in RA patients compared to healthy individuals. Moreover, it was found that transport of miR-17 into T cells represses Treg induction and differentiation [42].

MiRNAs delivered by exosomes were also described in the context of their therapeutic potential in RA. miR-150-5p reduced joint destruction by inhibiting angiogenesis mediated by downregulation of matrix metalloproteinase 14 (MMP14) and vascular endothelial growth factor (VEGF) [43]. However, upregulation of exosomal miR-92 boosted bone destruction by blocking apoptosis of fibroblast-like synoviocytes (FLSs) and inflammatory cytokine release [44]. Another microRNA, miR-let-7b, and its ligation to TLR-7 were able to induce joint inflammation through M1 macrophage differentiation [45]. Furthermore, overexpression of miR-221-3p in exosomes isolated from inflamed FLSs might suppress bone formation at erosion sites [46]. These findings provide evidence that exosomal miRNAs can modulate inflammatory responses during RA pathogenesis.

To date, researchers have focused mostly on microRNAs extracted from whole blood, serum/plasma, peripheral blood mononuclear cells (PBMC), and rheumatoid arthritis synovial fibroblasts (RASF) to investigate their role in RA progression [23, 47, 48]. Herein, we present isolated serum exosomes as a novel source of microRNAs. Various techniques can be used to study the miRNA profile. The most common practice is to employ microarray profiling to analyse a wide range of microRNAs on a qRT-PCR instrument [49]. However, a novel technique, NanoString, described previously in a study on serum miRNAs in patients with graft-versus-host disease (GvHD) [33, 50], allows analysis of around 800 microRNAs using digital barcoding technology. This removes the need for reverse transcription or preamplification of RNA, as the technology is able to directly count isolated microRNA molecules. During our study, NanoString was used to assess the repertoire of serum EV microRNAs in RA and AS cases subjected to biological treatment with anti-TNF agents. In the present study, we established for the first time that miR-498, miR-520h, and miR-548n are present in serum EVs of RA patients. Furthermore, expression of miR-7c-5p, miR-7f-5p, miR-362-3p, miR-3746-5p, and miR-548ah-5p has been characterized by us for the first time in AS EVs; in fact, these miRNAs have never been described in any rheumatoid disease at all.

As described in the literature (summary presented in Table 3), miR-155 [51] and miR-146a [52] are among the most widely studied microRNAs in RA and AS patients. Although our analysis did not identify miR-155 expression as significantly different between RA and AS patients, our results regarding miR-146a are consistent with those previously described. Higher expression of miR-146a in RA is well-characterized in multiple components such as synovial fibroblasts [53], CD4+ T cells [54], serum [55], and peripheral blood cells [26]. Moreover, upregulation of miR-146a in various samples from RA patients can distinguish them from osteoarthritis (OA) patients [56]. Interestingly, miR-146a is an NF-κB-dependent gene and controls inflammatory responses through inhibition of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 proteins [25] and downregulation of TLR4/NF-κB pathway [57]. By studying exosomes from rheumatoid arthritis synovial fibroblast cell line, Takamura et al. observed upregulation of miR-146a caused by TNF-α stimulation [58]. A positive correlation between the miR-146a level and IL-1β, IL-6, and TNF-α expression was also observed in AS [59].

There is some evidence to suggest a role for miR-146a in the course of the disease. Filková et al. characterized a decreased level of circulating miR-146a at an early stage of RA, compared to established disease [60]. Furthermore, the expression of miR-146a correlated positively with disease activity in RA [61, 62]. A baseline level of miR-146a was lower in methotrexate responders than in nonresponders [63]. Regarding anti-TNF therapy, previous studies reported increased levels of serum miR-146a in patients under treatment [29, 64]. Besides, Liu et al. demonstrated the potential predictive value of miR-146a measurement for biological agent therapy outcome in RA patients [65].

Recent studies also highlight a role of miR-146a in AS. Significantly higher expression levels have been found in patient serum samples compared to healthy controls [66]. miR-146a overexpression can also cause inhibition of fibroblast proliferation and osteogenic potential while its knockdown blocked disease progression by regulating Dickkopf Wnt Signalling Pathway Inhibitor 1 (DKK1) expression [67]. Other results suggested a positive correlation between miR-146a expression in peripheral blood mononuclear cells and duration of morning stiffness, ESR, CRP, and BASDAI [68]. Prajzlerová et al. also established the role of miR-146a in the pathogenesis of Axial Spondyloarthritis (AxSpA) [69]. Our current study does not show any significant differences in miR-146a expression between RA patients in two time points (before and after three months of the treatment) or RA group compared to AS. However, we detected upregulation of miR-146a-5p in AS cases after anti-TNF therapy. This observation is consistent with previous results obtained by our group for RA patients [29] where the expression level of miR-146a was lower in serum of patients before therapy compared to that after three months of treatment. Taken together, those conclusions implicate miR-146a as a potential noninvasive biomarker in RA and AS prognosis.

Another microRNA, miR-125b, can also serve as a predictor of anti-TNF therapy response [70]. We identified downregulation of miR-125a-5p in AS patient serum EVs after therapy. In contrast, Castro-Villegas et al. observed that patients responding to anti-TNF/DMARD combination therapy exhibited overexpression of miR125b-5p after treatment and lower RF, CRP, TNFα, IL-17, and IL-6 levels [64]. Previous studies confirmed that the upregulation of miR-125a and miR-125b directly affects NF-κB [71, 72]. Likewise,

decreased miR-125a-5p expression enhanced the protein level of TNFRSF1B and reduced osteoclast activity [73].

On the other hand, Perez-Sanchez et al. found that expression levels of miR-146a-5p, miR125a-5p, miR-151-3p, miR-22-3p, and miR-451a were higher in AS patients compared to psoriatic arthritis patients. Furthermore, miR-146a-5p, miR-125a-5p, and miR-22-3p can distinguish the active and nonactive stage of the disease. Expression of miR-125a-5p, miR-151a-3p, miR-150-5p, and miR-451a was also related to the presence of syndesmophytes in AS patients [74]. Although we demonstrated that miR-146a-5p and miR-22-3p were upregulated in AS patient EVs after therapy, miR-125a-5p was downregulated. Moreover, miR-125a-5p was characterized by lower expression in AS patient EVs compared to RA EVs before and after treatment. Similarly, miR-151-5p was decreased in AS EV samples before and after anti-TNF treatment compared to RA samples. These results implicate miR-125a-5p as a potential diagnostic biomarker in AS.

Little is known about miR-130a and miR-301a-3p. In AS patients after biological therapy, we observed an upregulation of miR-130a. A molecular mechanism involving histone deacetylase 3 (HDAC3) was published by Jiang and Wang [75]. A study on miR-130a in human chondrocytes identified its crucial role in regulating TNF-α expression [76]. miR-301a-3p was found to be overexpressed in the PBMCs and associated with Th17 cell frequency in RA patients [77]. We hypothesize that miR-130b-3p and miR301a-3p EV expression may distinguish RA patients from AS patients based on our observation that those microRNAs are higher in RA cases.

To date, publications describing miR-18a referred to osteoarthritis [78, 79], primary Sjögren's syndrome [80], and rheumatoid arthritis [81]. Herein, we demonstrate for the first time downregulation of miR-18a-5p in AS patient EVs after anti-TNF therapy.

We also observed that EVs isolated from sera of RA patients showed overexpression of miR-19b-3p, miR-498, miR-520h, and miR-548n. miR-19b-3p was previously investigated in knee osteoarthritis patients and associated with disease severity [82]. Duan et al. revealed miR-19b-3p involvement in OA through the GRK6-NF-κB pathway [83]. Gantier et al. suggested that miR-19 regulates NF-κB signalling [84]; however, data collected from an experiment with RA FLSs underline the role of miR-19a/b in the stimulation of TLR2 expression [85].

To the best of our knowledge, we showed for the first time that miR-548n and miR-548ah-5p are overexpressed after therapy in RA and AS patients, respectively. Moreover, we identified miR-548n, miR-548ah-5p, and miR-548q as possible biomarkers of AS. Even though no results describing miR-548n, miR-548ah-5p, or miR-548q in rheumatic diseases have been published before, our results are consistent with those reporting about miR-548a-3p, which belongs to the hsa-miR-548 family. miR-548a-3p has previously been shown to be downregulated in serum EVs of RA compared to healthy individuals. Furthermore, expression was negatively correlated with ESR, CRP, and RF levels in patients [40]. In our study, expression of miR-548a-5p was also decreased in RA patients compared to AS patients, before initialization of anti-TNF therapy.

Several studies reported the differential expression of miR-29a and miR-21, making them potentially involved in AS pathogenesis [86–89]. Additionally, overexpression of miR-29a was described in PBMCs from AS patients after etanercept treatment [90]. However, we did not observe differential expression of miR-29a in either RA or AS samples. The analysis showed a significant increase in expression of miR-21 in AS patients after anti-TNF treatment. In contrast, Huang et al. reported greater miR-21 expression in whole blood of patients with AS compared to controls [91]. Recently, Zou et al. showed that upregulation of miR-21 was associated with radiographic severity of AS [92]. The studies regarding RA reported decreased miR-21 level in PBMCs, CD4+ T cells [93], and plasma [94]. Balzano et al. hypothesized that low expression of miR-21-5p was a result of corticosteroids that inhibit NF-κB [94]. Further analyses revealed that miR-21 may be implicated in several signalling pathways such as the IL-34/STAT3/miR-21 pathway, essential for synovial fibroblast survival in RA [95], as well as a mediator between inflammation and bone formation through the JAK2/STAT3 pathway in AS [96]. Additionally, miR-21 plays a role in mediation of RANKL-induced osteoclastogenesis and downregulation of programmed cell death 4 (PDCD4) protein levels [97].

Other microRNA considered to be involved in AS are miR-30a and let-7i. MiR-30a was downregulated in serum/-plasma of radiographic axial spondyloarthritis patients compared to healthy individuals [98]. In turn, let-7i was upregulated in T cells [99] and plasma [100] of AS patients compared to controls. In our analysis, both miR-30a-5p and let-7i-5p levels were higher in AS patient EVs after therapy compared to that before treatment and RA cases, respectively. In the study conducted by Lai et al., expression of let-7i in T cells was also positively correlated with the Bath Ankylosing Spondylitis Radiology Index (BASRI) of the lumbar spine, a scoring system which we did not include in our analysis. The authors concluded that overexpression of these microRNAs suppressed TLR-4 expression, which led to downregulation of TLR-4 [99].

The present work is in line with the previous finding identifying miR-23a as a potential predictor of etanercept response [101]. miR-23b can repress IL-17-associated autoimmune inflammation in human fibroblast-like synoviocytes [102]. This observation was confirmed for miR-23a in articular cartilage tissues from RA patients [103].

In summary, to the best of our knowledge, the present study identified for the first time 12 microRNAs differently expressed in serum EVs between RA and AS patients before biological agent administration. miR-125a-5p, miR-130b-3p, miR-151a-5p, miR-301a-3p, and miR-324-5p were upregulated in RA EVs, and miR-376c-3p, miR-378h, miR-411-5p, miR-548a-5p, miR-548n, miR-548q, and miR-579-3p in AS EVs. We believe that these microRNAs have the potential to distinguish RA pathogenesis from AS. The pathway prediction analysis performed using mirPATH v.3 DIANA tools (data not shown) found that all the microRNAs are involved in the Wnt signalling pathway.

5. Conclusions

Our analysis has revealed a unique profile of differentially expressed miRNAs in RA and AS patient serum EVs, both before and three months after anti-TNF treatment administration. These results suggest that EV miRNA profiling of RA and AS patients can be used for detection of diagnostic and predictive biomarkers. They confirm a potential role of these miRNAs in distinguishing the two diseases and in the prediction of response to treatment in RA and AS patients. Nevertheless, the results reported herein should be considered as a pilot study that was conducted on a limited number of patients; therefore, validation in larger verification cohorts is required.

Data Availability

All relevant data are within the manuscript. More detailed information cannot be shared publicly because it allows identification of the patients. It can be accessed from the Hirszfeld Institute of Immunology and Experimental Therapy (contact via katarzyna.bogunia-kubik@hirszfeld.pl) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare no conflict of interest.

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Review

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Pharmacogenomics

miRNAs as potential biomarkers of treatment outcome in rheumatoid arthritis and ankylosing spondylitis

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Common autoimmune, inflammatory rheumatic diseases including rheumatoid arthritis and ankylosing spondylitis can lead to structural and functional disability, an increase in mortality and a decrease in the quality of a patient's life. To date, the core of available therapy consists of nonsteroidal anti-inflammatory drugs, glucocorticoids and conventional synthetic disease-modifying antirheumatic drugs, like methotrexate. Nowadays, biological therapy including anti-TNF, IL-6 and IL-1 inhibitors, as well as antibodies targeting IL-17 and Janus kinase inhibitors have been found to be helpful in the management of rheumatic conditions. The review provides a summary of the current therapy strategies with a focus on miRNA, which is considered to be a potential biomarker and possible answer to the challenges in the prediction of treatment outcome in patients with rheumatoid arthritis and ankylosing spondylitis.

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The rheumatic diseases consist of autoimmune and autoinflammatory conditions, with the involvement of the adaptive and innate immune responses, respectively, having been established [1].

Rheumatoid arthritis (RA) is characterized by progressive synovitis, cartilage degradation, bone erosion and, as a consequence, joint damage, leading to patient disability [2]. In Poland, the average age of RA cases is 56 years with strong female predominance (78%) among patients. RA prevalence for women is 1.06% compared with 0.74% for men; overall for the Polish population the prevalence is -0.9% [3]. This is in agreement with a worldwide prevalence of RA calculated at 0.5–1.0% but which is higher than in Hungary (0.37%), Estonia (0.46%) or neighboring Czech Republic (0.61%). It has been shown that the prevalence of RA differs geographically [4].

Ankylosing spondylitis (AS) affects the axial skeleton and is clinically described with inflammatory back pain leading to functional and structural destruction. AS belongs to a group of rheumatic diseases called spondyloarthritides. Characteristic features of the disease comprise enthesitis and asymmetrical peripheral oligoarthritis, and the disease can affect other organs such as eyes, skin, lungs or heart [5]. Patients suffering from AS are typically under 30 years old and they constitute around 0.1% of the Polish population [6]. One study estimated the burden of AS at 23.8 per 10,000 in Europe. Reports from clinical diagnosis showed a lower prevalence than those estimations based on the modified New York criteria [7].

AS and RA involve chronic, inflammatory processes reducing the quality of people lives and increasing mortality. The pathogenesis of AS and RA is different but has not been fully elucidated to date. However, it is known that genetic factors play a pivotal role in both cases [8]. A comparison of the two diseases shows significant differences in many areas. The HLA-B27 antigen occurs in 95% of AS patients, while the presence of HLA-DR1 or -DR4 predisposes to RA in 60% of cases. The age of onset is lower in AS than in RA, as well as in the first disease, and the disease is predominantly seen in men, in contrast to RA. Also, arthritis in AS is described as oligoarthritis involving mainly knees, hips and shoulders, whereas in RA peripheral polyarthritis affecting hands and feet is seen [9].

Future Medicine

Specific diagnostic markers detected only in RA are rheumatoid factor (RF) and antibodies against cyclic citrullinated peptides (anti-CCP). The novel diagnostic strategies and established clinical recommendations allow AS to be distinguished more precisely from RA [8]. Coexistence of the two diseases in one patient is extraordinary. However, such a case has been diagnosed. The female patient was HLA-B27 and HLA-DR4 positive and demonstrated AS before RA [10].

In clinical practice, the modified New York criteria have been widely used for classification of AS since 1984. The accomplishment of those criteria requires identification of distinct sacroiliitis and existence of other clinical criteria such as lumbar spine movement limitation, low back pain longer than 3 months or reduction of chest expansion. To identify the disease at an early stage and to diagnose patients at the first signs of axial inflammation, the Assessment of Spondyloarthritis International Society (ASAS) Classification Criteria were developed. The ASAS criteria introduced a new term 'axial spondyloarthritis' (axSpA), which includes SpA patients having visible (radiographic axSpA) or not (nonradiographic axSpA [nr-axSpA]) radiographic sacroiliitis. The criteria combine sacroiliitis detectable on an x-ray or MRI, or HLA-B27 presence with features characteristic for SpA [11]. Disease activity is determined by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index [12].

The classification of RA patients is based on a scale scoring symptom duration, number of painful and swollen joints, presence of serologic factors (RF and/or anti-CCP) and a measure of acute-phase response (erythrocyte sedimentation rate [ESR] or C-reactive protein [CRP]) [13].

Treatment

Therapy of rheumatic diseases such as RA and AS is carried out based on conventional synthetic, biological and targeted synthetic disease-modifying antirheumatic drugs (DMARDs). Conventional synthetic DMARDs (csDMARDs) applied for RA treatment include methotrexate (MTX), sulfasalazine, hydroxychloroquine and leflunomide. Currently, biological DMARDs (bDMARDs) comprise anti-TNF drugs (adalimumab [ADA], etanercept [ETA], certolizumab pegol, golimumab [GOL], infliximab [IFX]) used in RA and AS management, as well as, IL-6 receptors antagonists (tocilizumab [TCZ], sarilumab) and recombinant IL-1R blocker (anakinra), used only in RA treatment. An alternative to TNF inhibitors for AS patients is secukinumab, a monoclonal antibody (mAb) against IL-17A. A newly developed treatment for RA is costimulation T-cell blockers acting via anti-CD80/86 inhibition (abatacept) and B-cell depletion drug, which is a chimeric anti-CD20 mAb (rituximab). Moreover, approved treatment of RA also includes inhibitors of Janus kinase (JAK) that belong to the targeted synthetic DMARDs (tsDMARDs) (tofacitinib, baricitinib) [14–16].

MTX can block proinflammatory cytokine secretion, increase reactive oxygen species production and inhibit the expression of adhesion-molecule. The mechanism of MTX function in RA treatment involves folate antagonism and most likely the adenosine pathway [17].

Cytokines delivered by synovial macrophages, such as TNF, IL-1, IL-6, play a key role in the pathogenesis of RA (Figure 1) [18]. TNF promotes inflammatory processes through interaction with two receptors (TNFR1 and TNFR2) and activates the NF-κB pathway. Inhibitors were originally designed for RA treatment and later their usage was extended to AS. They act by binding TNF, and preventing interaction with membrane receptors and in consequence neutralize biological activity, but there is some difference between anti-TNF drugs in their mode of action. IFX was the first approved anti-TNF and is a chimeric mouse-human mAb. ADA and GOL are human IgG1 mAb. Certolizumab pegol is a humanized Fab fragment of mAb covalently linked to polyethylene glycol. In contrast to described above mAbs (IFX, ADA, GOL), ETA belongs to a different type of anti-TNF agents. ETA acts as a soluble receptor and is a fusion of Fc immunoglobulin and human TNFR2 [19].

The IL-1 receptor blocker called anakinra is a recombinant, nonglycosylated antagonist of natural, human proinflammatory cytokine. The modified protein has an extra methionine residue at the N-terminal [20].

Treatment of RA can be also carried out under TCZ administration. TCZ is a human mAb against IL-6 receptor subunit alpha (IL-6R). The mechanism of action involves inhibition of *cis*- and *trans*-mediated cascades and modulation of signaling via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [21].

Among JAK inhibitors, tofacitinib was the first to be accepted for the treatment of autoimmune disorders by blocking JAK1 and JAK3. Barticitinb is a second-generation JAK blocker targeted JAK1 and JAK2 [22].

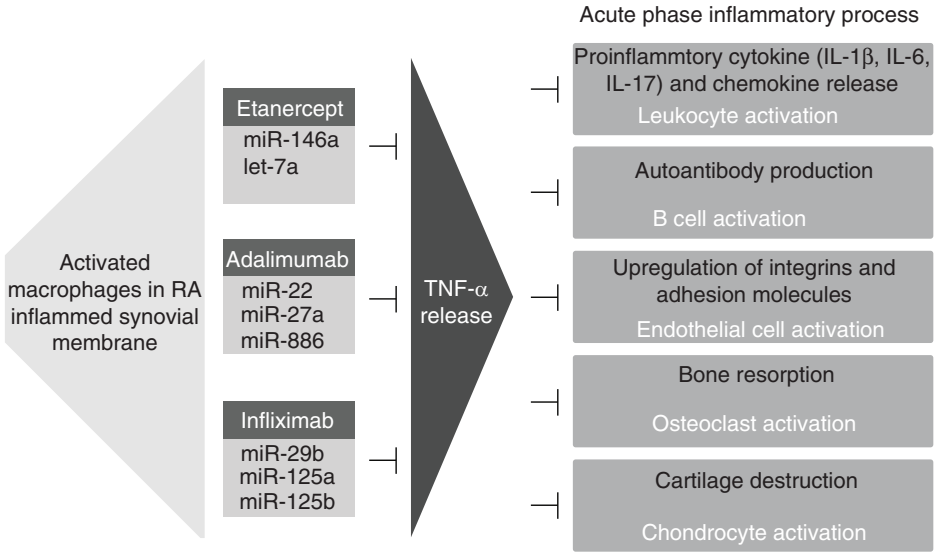

Figure 1. Schematic representation of TNF-α activity, a cytokine constituting the target for biological therapy in rheumatoid arthritis patients, clinically used anti-TNF therapeutic antibodies and miRNA molecules associated with their activity as disease-modifying drugs.

Table 1. miRNA molecules as treatment outcome predictors in patients with rheumatic diseases.

Disease	Drug		miRNA as a treatment outcome predictor	Ref.
Rheumatoid arthritis	csDMARDs		miR-125b, miR-125a, miR-132, miR-155, miR-146a, miR-10a, miR-16, miR-223	[36,48,57–59]
Rheumatoid arthritis	Anti-TNF	Infliximab	miR-29b, miR-125b, miR-125a	[49,66]
		Etanercept	miR-146a, let-7a	[41]
		Adalimumab	miR-27a, miR-886, miR-22	[63,64]
		Golimumab	–	
		Certolizumab	–	
Rheumatoid arthritis	Anti-TNF/DMARDs		miR-23, miR-223, miR-146a	[61,62]
Rheumatoid arthritis	Anti-IL-6	Tocilizumab	–	
Rheumatoid arthritis	Anti-CD20	Rituximab	miR-125b	[65]
Ankylosing spondylitis	Anti-TNF	Infliximab	–	
		Etanercept	miR-29a, miR-126-3p	[53]
		Adalimumab	–	
		Golimumab	–	
		Certolizumab	–	
Ankylosing spondylitis	Anti-IL-17A	Secukinumab	–	
Nonradiographic spondyloarthropathy	Anti-TNF	Certolizumab	–	

csDMARD: Conventional synthetic disease-modifying antirheumatic drug.

The mechanism of action of JAK inhibitors involves blocking the signal transmission between the proinflammatory cytokines receptor and cellular nucleus. Tofacitinib causes a blocking of phosphorylation and activation of JAKs, which in turn suppresses inflammatory responses (Table 1).

Therapy with tofacitinib was considered to be effective for RA patients who did not respond or showed intolerance against csDMARDs. This JAK inhibitor can be used as monotherapy [23].

RA therapy in Poland is performed according to EULAR guidelines described previously in this section. Patients include treatment program are characterized with high disease activity evaluated using disease activity score with an assessment of 28 joints (DAS28 $\geq$5.1) and are taking MTX or/and one of the other csDMARDs for 4 months. The rheumatologist can apply no more than five bDMARDs including two anti-TNF drugs [24].

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Polish RA patients are commonly treated with MTX. In 91% of patients who have taken MTX, treatment in 80% of cases was prolonged as monotherapy or simultaneously with other DMARDs. Dosage was started from 10 to 15 mg/week and could be increased up to 25 mg/week. Those modifications were necessary for 76% of patients and there were conducted because of MTX side effects, remission or due to therapy failure [25]. The biological therapy has been received by 2.94% of RA-affected patients [3].

Currently, management with AS in Poland is conducted using TNF inhibitors. Patients have to meet modified New York criteria and be described as having high disease activity (BASADAI $\geq$4) with an assessment on a visual analogue scale (VAS). Biological drugs can be prescribed after poor treatment outcome with a minimum of two, single administration of nonsteroidal anti-inflammatory drugs after 4 weeks. In the case of nr-axSpA, the biologics are limited to certolizumab in the polish healthcare programme [24]. However, studies showed that anti-TNF treatment response does not differ between nr-axSpA and AS patients [26].

miRNA as a biomarker

miRNAs are noncoding, endogenous, single-stranded and are an average of 22 nucleotides long. They play a pivotal role in gene regulation by repressing the degradation of a translation or promoting mRNA [27]. miRNAs have been found to be one of the most significant regulatory molecules because their genes make up approximately 1–2% of the complete genome and they may have influence on around 30% of total protein-coding genes. It has been estimated that greater than 60% of genes contain a minimum of one miRNA-binding region generally located on the 3′-untranslated region of destined mRNA [28].

miRNAs have been found to be involved in the pathogenesis, development and progression of rheumatic diseases. RA and AS pathogenesis have also been linked with dysregulation of miRNAs expression [29–31]. The role of miRNA in RA progression was initially established by Bhanji *et al.* in 2007 [32]. Since then, miRNAs have been considered as novel, noninvasive biomarkers of RA and seem to be a promising candidate for diagnostic purposes.

Definition of a biomarker is as follows: a physical sign or molecular, genetic, biochemical or cellular change by which a normal or aberrant biologic process can be recognized or monitored, or both, and that may possess prognostic or diagnostic advantages. Measurement of the desired biomarker should be repetitive and characterized by high specificity and sensitivity, and biomarker itself should reveal an interesting pathogenic process [33].

miRNAs fulfill the above-mentioned criteria to be potential biomarkers. It has been established that miRNAs have the required sensitivity, specificity and stability, and in addition, they seem to be resistant to nuclease degradation. This could be due to the small size of the miRNA and due to the complexes they form with proteins, which could protect from degradation. miRNAs can be detected in multiple biological fluids like blood, serum, plasma, saliva, urine and also in tissue samples in a reproducible way [34].

Importantly, there are validated, trustworthy and available technologies for miRNA detection. Diagnostics is established on comparison of altered miRNA expression levels between patients and healthy individuals, and expression profile of miRNAs determined as significant in pathological condition. Widely used measurement methods include northern blot analysis, quantitative real-time PCR, microarrays and *in situ* hybridization [35].

Biomarkers may have several potential applications in prediction, early diagnosis, disease activity and treatment response in patients with rheumatic disorders thus, the issue will be discussed further in this review.

miRNA: biomarker of disease progression

A significant correlation between the expression of circulating miRNA and disease activity has been observed, for miR-223 and miR-16 at an early stage of RA [36]. However, it has been found that, in patients with early RA differential expression levels of plasma miR-143-3p, miR-145-5p and miR-99b-5p, may indicate a severe form of the disease [37]. In addition, high expression of miR-16 and miR-146a has been associated with active disease [38]. Plasma miR-16 was detected to be significantly associated with DAS28 and tender joint counts, and miR-146a, miR-155 and miR-223 levels were correlated with tender joint counts [39]. MiR-146a level also positively corresponded with ESR [40,41]. In addition, mir-146a expression pattern in Tregs was linked to clinical parameters of disease activity [42].

Moreover, DAS28 has been determined as being positively associated with let-7a-5p and inversely correlated with miR-335-5p expression levels [41]. Liu *et al.* established that plasma miR-23b expression is characterized by a positive correlation with ESR, CRP and DAS28 [43]. It also has been observed that DAS28 and ESR levels are elevated by high miR-155 expression level [44]. Thus, serum miR-155 and miR-210 have been considered as disease activity biomarkers [45]. Murata *et al.* found that another miRNA, miR-24, correlated with DAS28-CRP,

DAS28-ESR, VAS and CRP [46]. The high disease activity appeared to be proportional to the expression of miR-221 and miR-222 [47] and low levels of miR-125b in peripheral blood mononuclear cells (PBMC) [48]. In contrast, miR-125b PBMC level was established recently by Cheng *et al.* as being positively correlated with DAS28-ESR, CRP, ESR as well as with tender and swollen joint count [49]. Besides, a negative correlation has been observed between exosomal miR-548a-3p and RF, CRP, ESR detected in serum [50]. Described findings provide evidence that those miRNAs may be considered as biomarkers of disease activity and progression in RA patients.

In AS cases, BASDAI was associated with expression levels of miR-155 [51], miR-99b-5p, miR-133a-3p, miR-625-3p and miR-885-5p, while CRP corresponded to levels of miR-146a-5p, miR-151a-3p, miR-181a-5p and miR-221-3p [52]. Moreover, Prajzlerova *et al.* identified among AS patients with spinal involvement, miRNAs positively correlated with BASDAI (mi-29a-3p, miR-222-3p, miR-375, miR-625-3p, miR-885-5p) and with CRP (miR-29a-3p, miR-133a-3p, miR-151a-3p, miR-409-3p) [52]. On the contrary, Lv *et al.* reported that miR-29a did not correlate with ESR, CRP or BASDAI [53]. Wei *et al.* also identified a positive association between miR-146a and CRP, ESR and duration of morning stiffness in PBMC of AS patients [54]. Analysis of the expression profile of T cells showed that miR-221 and let-7i corresponding to the Bath Ankylosing Spondylitis Radiology Index of the lumbar spine [55]. So far, the study of PBMC from nr-AxSpA patients conducted by Li *et al.* revealed an inverse correlation between miR-27a and Ankylosing Spondylitis Disease Activity Score as well as CRP level [56].

miRNA: predictor of treatment response

miRNA has a potential significance in treatment results prognosis. Importance of selected miRNAs as predictors to MTX therapy in RA subjects has been described previously. miR-146a-5p, miR-132-3p, miR-155-5p expression levels in whole-blood were found to be higher in response to 4 months of MTX treatment [57]. Furthermore, miR-10a, which was upregulated in patients serum, could be a good predictor of MTX effectiveness [58].

Higher expression of miR-125b before treatment significantly improves therapy outcome after 3 months of conventional DMARDs usage [48]. Analysis of good and poor responders to RA treatment indicated a role of miR-125a-5p as a biomarker of treatment response [59]. Another study has shown increased levels of miR-223-3p and miR-16-5p as a response to conventional treatment in patients newly diagnosed with RA [36].

Cuppen *et al.* have hypothesized that expression of selected miRNA can predict treatment response to TNF inhibitors in RA cases including miR-99a and miR-143, which could be involved in the prediction of outcome to ADA, as well as miR-23a and miR-197, which may be helpful in the prognosis of ETA response [60]. Unfortunately, those results were not consistent. Some findings point out a lack of significant differences between RA patients receiving biologics and conventional drugs regarding the level of miR-146a [42], miR-210 and miR-155 [45].

Interestingly, miRNAs strongly associated with response to anti-TNF-α/DMARDs combination therapy have been identified. Serum expression of miR-146a-5p, miR-125b, miR-126-3p, miR-23-3p, miR-16-5p and miR-223-3p were upregulated in a cohort of RA patients who respond to applied treatment [61]. Also, Bogunia-Kubik *et al.* found lower baseline level of miR-146a-5p than in RA patients after 3 months of therapy with TNF inhibitors [62].

Therapy based on combination csDMARDs with novel biological drugs is an effective treatment strategy. Thus, researchers have been looking for a promising candidate to be a biomarker of treatment outcome. MiR-27a-3p appeared to correlate with remission at 12 months after the initiation of combination therapy, ADA with MTX. Before the therapy, levels of the miRNA detected in the serum of patients with early RA were higher, and then decreased during the first 3 months of treatment [63]. Krintel *et al.* studied a whole blood miRNA profile in early RA patients and identified the combination of increased expression of miR-886-3p and decreased of miR-22, highly predictive of ADA outcome in pretreatment samples [64].

Recently, the response to another TNF inhibitor was found to be related with miRNAs profile in PBMC. Differential expression of miR-146a-5p, let-7a-5p in combination with measurement of CRP, and biologic history may provide a valuable predictor of response to ETA in RA patients after 24 weeks of treatment. Increased mir-146a-5p and reduction of let-7a-5p expression level was observed in patients with better clinical outcome compared with those who did not achieve response [41].

Further analysis indicated that higher levels of miR-125b in RA serum before therapy suggested patients may achieve a better response to rituximab after 3 months [65]. Conversely, the level of miR-125b was lower in RA patients after the 24th week of IFX therapy, and those responders were characterized as having increased expression at the baseline compared with nonresponders. Those findings are consistent with previous observations that miR-125b may potentially improve clinical outcome to anti-TNF inhibitors [49].

Finally, miR-29b showed a decreased expression in RA patients with effective IFX therapy, but not in those administrated TCZ, which suggested that miR-29b levels may be informative with regards to immunotherapy choice [66]. Also, miR-29a level was higher after the 12th week of ETA administration in AS patients [53].

One study revealed miR-5196 as a biomarker of positive anti-TNF therapy outcome, both in RA and AS patients. As a result, miR-5196 was significantly upregulated in the serum of RA and AS cases before taking biologic drugs [67].

However, the predictive value of miRNAs in treatment outcome to JAK/STAT inhibitor, tofacitinib has not been found yet [68].

The differences in individual drug response could be explained by pharmacogenomics. Pharmacogenomics is one of the main routes in contemporary medicine, which can predict drug efficiency, safety, toxicity and which can allow for a personal therapy approach. The field combining pharmacogenomics with post-transcriptional gene regulation has been described as 'miRNA pharmacogenomics' [69]. Shomron in 2010 proposed an including miRNAs in pharmacogenomics analysis, due to their effect on drugs target and metabolism [70].

Significant for pharmacogenetic research in RA is the analysis of single-nucleotide polymorphisms and their associations with MTX or biological therapies, but data were limited, inconsistent and required further investigation [71,72]. In our study, we focused on differences in miRNAs expression.

The recent report of Latini *et al.* has suggested a potential utility of miRNAs as biomarkers for personalized medicine. Considering this report and data presented in our review, we believe that miRNAs, as important regulators of the expression of multiple genes (including genes incorporated in pharmacodynamics and pharmacokinetics of drugs) and modulators of biological processes, may be responsible for different drug responses. Aberrations in levels of circulating miRNAs can influence on variability in pharmacological response among individuals, thus may constitute biomarkers of therapy outcome [73].

miRNA: potential therapy target

Noncoding RNAs like miRNAs are now emerging as excellent drug targets. miRNA molecules can be used for therapeutic purposes employing miR-mimic-specific oligonucleotides that prevent the binding of other molecules to a specific location in the mRNA molecule leading to gene silencing [74]. This so-called miRNA mimics (miR-mimics) approach has already been successfully applied in the clinic [75,76], although not for the treatment of rheumatic disorders. miR-mimics contain non-natural or artificial ds-miRNA-like RNA fragments. These RNA fragments carry a sequence motif on their 5′-end that is partially complementary to the target sequence located within the 3′-untranslated region and thus are constructed to act in a gene-specific fashion. Due to these properties, their use for gene targeting, antisense and cell-reprogramming approaches have attracted the attention of many researchers, including those working on epigenetic therapies. Also, the antagomirs approach seems promising for inhibiting miRNAs as a therapeutic strategy. Antagomirs are synthetic antagonists of miRNAs. They are specific and complementary to their mature target miRNA and interfere with their function.

In fact, miR-mimics technology has been successfully used in animal models of many diseases, including rheumatic disorders, for example, the *in vivo* analysis of Zang *et al.* showed that miR-671 mimics alleviated the progression of osteoarthritis in mice [77]. Moreover, Ohnuma *et al.* [78] using osteoclastogenic culture and pit-formation assay found that a miR-124 mimic inhibited osteoclastogenesis in mouse bone marrow-derived macrophages stimulated by TNF-α, IL-6 and M-CSF in the presence of osteoprotegerin. They also showed that the expression levels of osteoclast-specific genes and NFATc1 protein were suppressed in the miR-124 mimic-transfected cells. The miR-mimic approach has been also employed in human *in vitro* studies investigating RA fibroblast-like synoviocyte cell line [79].

These promising results from *in vitro* and *in vivo* studies suggest that the targeting of miRNA sequences could soon become a valuable, clinically applicable tool for the treatment of patients with rheumatic diseases. These data imply that in addition, miRNA can serve not only as a diagnostic and prognostic biomarker but also as a target for epigenetic therapy.

Iwamoto and Kawakami, in their recent review, focused on miRNAs and the central role of these molecules in affecting fibroblast-like synovial cells (FLS) in RA pathogenesis. They showed that miRNAs act via various (NF-κB, TLR, Wnt, PI3K-AKT) signaling pathways. The authors concluded that RA-FLS are crucial effector cells in the pathogenic state of the disease and that why they may constitute promising therapeutic targets. In their opinion, elucidation of the mechanisms of the unfavorable effect of miRNAs on the modulation of FLS behaviors may be helpful in the development of another generation of RA drugs [80]. The observed effect may not only be

attributed to miRNA expression itself but also epigenetic modifications (such as methylation) leading to elevated miRNA levels. Also, Stanczyk *et al.* observed miR-203 upregulation in RA-FLS. The regulation of miR-203 level was dependent on methylation. Thus, elevated expression of miR-203 may be due to the effect of changes in an epigenetic modification in the gene promoter [81].

It is well known that epigenetic modifications and miRNA have influence on the regulation of gene expression and act as key players in this mechanism. Therefore, miRNAs are becoming novel and required pharmacogenomic biomarkers in rheumatology. They are a potentially perfect antirheumatic drug target because of their ability to regulate multiple immune processes and to be intercellular mediators [82]. The miRNA molecules can target cytokines, chemokines or matrix metalloproteases and this regulation can be either 'direct' on the target, or 'indirect' via other target genes that influence their production. They are also able to modulate apoptosis and cell invasion. Therefore, finding a unique miRNAs expression profile under RA conditions may help to establish prognostic factors in clinical applications [83].

Conclusion

These results highlighted the crucial role of miRNAs as safe, precise diagnostic and prognostic biomarkers in patients with rheumatic diseases. They also imply that miRNA molecules may be used for therapeutic purposes as potential targets for epigenetic therapy.

Future perspective

In the future, the interest in the role of miRNAs in different diseases is likely to rise or stay consistent at today's level. MiRNAs are major molecules involved in the genetic regulation of various pathological conditions. Current knowledge comprises a link between these molecules and pathogenesis or disease activity in RA and concerned less AS. Overall, a deeper knowledge of the functions of already established and newly identified miRNAs is desired. Hence, researchers may focus on other rheumatic diseases and compare them not only with healthy subjects but also with other patients to identify distinctive miRNAs. The studies should be also conducted on larger sample groups with different clinical characteristics and ethnicity. Further examinations should be a focus on the identification and selection of key miRNAs as useful diagnostic biomarkers. Next, those miRNAs should be exactly characterized together with delineating their functional network and impact on human genes. This approach would be able to identify signaling pathways and molecular processes underlying rheumatic diseases.

Introducing a novel biomarker to common clinical practice follows many validation steps, clinical testing would be a difficult and time-consuming process. However, biomarkers able to predict treatment responses to particular drugs are urgently required. Such biomarkers would optimize the benefit to risk ratio of therapy strategy in individual cases. It may also be possible to predict remission or relapse and change or modify treatment options in advance, due to monitoring the expression level of miRNAs.

Prognosis of disease development before the manifestation of first symptoms or at an early stage of the disease is clinically necessary to prevent progression and irreversible consequences for patient's mobility and quality of life. This progress allows administering the correct drug at the right time and dosage to the sufferer.

The idea that miRNAs may be novel candidates of immunomodulatory drugs is a matter of great interest and should be developed over coming years. miRNAs not only could be the desired drug itself but also a target for designed drugs.

Nevertheless, although the first progression miRNA-based therapeutic strategies in RA have been made and seem to be promising, multiple limitations have to be overcome before clinical trials. However, future therapy based on miRNA can be expected.

The objective of future studies should be to explore mechanisms and signaling pathways involving in disease progression and regulated through miRNAs, as well as explain different responses to treatment in each patient.

In future, personalized therapy should be based on an analysis of a number of biomarkers, and the clinical phenotypes of RA and AS, in order to provide the most effective and optimized treatment for each patient. But it will not be an easy task because of the complexity and heterogeneous nature of rheumatic conditions. Thus, only a pattern of expression levels of different miRNAs would be a sufficient approach. Ultimately, miRNA markers may represent a more realistic approach toward personalized medicine in rheumatic diseases than targeted drugs.

Executive summary

- Rheumatoid arthritis (RA) and ankylosing spondylitis (AS) are rheumatic, inflammatory disorders that can lead to structural and functional disability, increase mortality and decrease the quality of a patient's life.
- The current treatment strategy consists of nonsteroidal anti-inflammatory drugs, glucocorticoids and disease-modifying antirheumatic drugs (DMARDs).
- The most popular is combined therapy, which is the use of conventional synthetic DMARDs, usually methotrexate, and biologic drugs, and eventually targeted synthetic DMARDs, mostly JAK inhibitors.
- Biological drugs are designed against proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-17).
- Nowadays, treatment decisions are based on specific clinical or individual factors, side effects and patient or physician preference.
- Biomarkers may have several potential applications in prediction, early diagnosis, disease activity and treatment response in patients with rheumatic disorders.
- miRNAs are promising candidates as safe, precise diagnostic biomarkers.
- miRNA expression levels differ in RA or AS patients compared with healthy controls.
- There is a lack of reliable biomarkers that may predict treatment outcome in patients with RA or AS.
- Several miRNA molecules have been identified that can potentially act as treatment outcome predictors.
- miRNA sequences may be considered as future targets for epigenetic therapy.

Author contributions

J Wielinska and K Bogunia-Kubik designed the content of the review, wrote the manuscript and approved its final version.

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Conclusions

- IL-6 contributes to the pathogenesis of rheumatoid arthritis (RA), and the single nucleotide polymorphism within its gene promoter is associated with the level of this cytokine in patients' serum and disease activity.
- *IL17F* polymorphism can influence anti-TNF therapy outcome, and together with the *IL17RA* receptor genetic variants is associated with disease activity in ankylosing spondylitis (AS) patients after anti-TNF treatment.
- Polymorphisms within the *IL17A* and *IL17RC* genes are related to inflammatory parameters in AS patients.
- Genotyping of selected proinflammatory cytokines and their receptors may have a predictive value for the patients with rheumatic diseases in our Polish population.
- Profile of microRNA expression can distinguish rheumatoid arthritis from ankylosing spondylitis.
- MicroRNA repertoire was significantly changed during treatment with TNF-α inhibitors.
- MicroRNAs can be considered as promising diagnostic and prognostic markers for RA and AS and patients' responses to biological treatment.