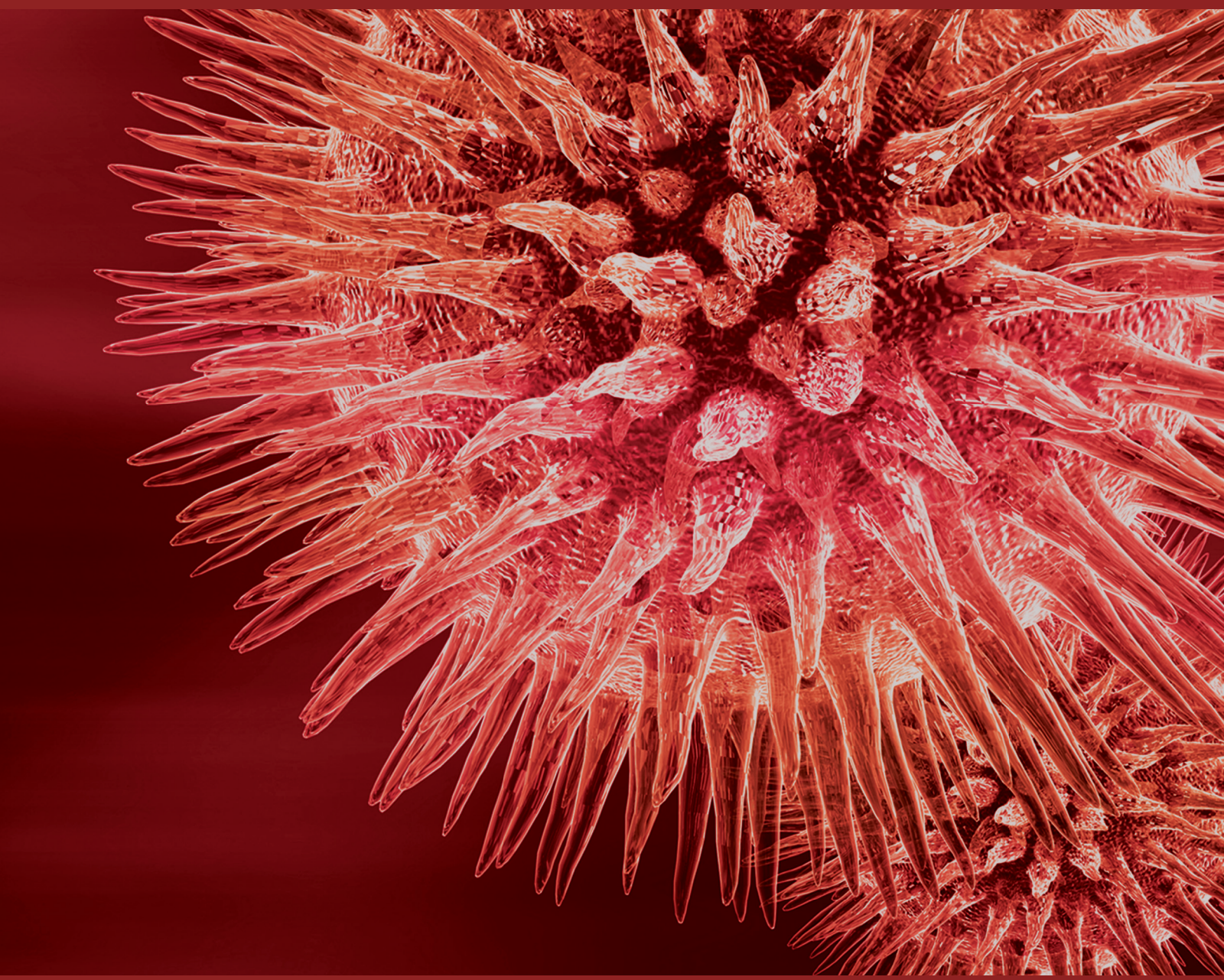
BioMed Research International

Novel Approach to the Treatment in Rheumatic Diseases: From Molecule to Value for Patient

Lead Guest Editor: Ewa Mojs
Guest Editors: Marek Brzosko and Włodzimierz Samborski



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Editorial

Novel Approach to the Treatment in Rheumatic Diseases: From Molecule to Value for Patient

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The treatment of rheumatic diseases changed with the appearance of biological drugs modifying the disease. This happened 20 years ago. The presence of the first anti-TNF agents, including infliximab (chimeric), adalimumab (humanized), and etanercept (soluble receptor), changes the treatment in the efficacy as well as in the aims of treatment. First, biologic treatment gave many options in choice and decisions regarding the drug and, second, the aim of treatment was the quality of life and cooperation with patients as a partner in the process. Then prognosis in these diseases is not so poor as some years ago.

Parallel to the appearance of those therapies, rheumatologists improve the management of traditional drugs, such as methotrexate, and added other nonpharmacological methods of work and therapy of patients, which gave the optimal approach toward patient and its disease.

This special issue presents the newest data in area of genetics and role of DNA and miRNA dysregulations in pathogenesis of rheumatic diseases.

The pathogenesis of lupus is contributed by genetic factors and its epigenetic modifications. miRNAs play important function in the posttranscriptional regulation of most gene-regulatory pathways and regulate both the innate and the adaptive immune responses. Dysregulation of miRNAs paths in lupus appearance is presented in the special issue. The authors show the newest data in that area.

Investigators present also work in area of genetics and answer the question whether osteopontin (OPN) variants are associated with susceptibility to ankylosing spondylitis (AS) in a Chinese population. There are interesting data polymorphisms at the 9175th position in exon 7 of OPN.

Another part of the special issue is a paper focused on orally administered small molecules, kinase inhibitors, which target blocking the pathogenic signalling process and open new way in the management of RA. A major milestone presented in the review study was the use of kinase inhibitors, tofacitinib. Author discusses the rationale for the use of kinase inhibitors in RA and shows new way of treatment with use of JAK family.

The special issue presents an article that presented socioeconomic factors which may reduce access to the biologic treatment in Romania. The data are actual not only in Romania but also in some other countries in Europe, for example, in Poland.

An interesting point in the special issue is that there are data on mud therapy as additional way of treatment in knee osteoarthritis—data presented from therapy centre in Italy.

We hope that readers will be satisfied with the modern, multidisciplinary data presented in the special issue.

Ewa Mojs
Marek Brzosko
Wlodzimierz Samborski

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Research Article

Investigation of MicroRNA in Mitochondrial Apoptotic Pathway in Systemic Lupus Erythematosus

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Background. Accumulating evidence indicates that microRNAs play a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE). This study tested the hypothesis that microRNA is associated with the mitochondrial apoptotic pathway in patients with SLE. *Methods*. Thirteen patients were in the clinical comparison study and microRNA study and overall 19 patients in the study of intracellular protein. Levels of microRNAs were determined by miRNeasy kit in 13 patients with SLE and 29 volunteer normal controls. Intracellular levels of caspase-9, caspase-10, MAVS, MDA5, and pIRF7 in mononuclear cells from 19 patiens and the SLE disease activity index (SLEDAI) were determined in all SLE patients. Correlation analyses were performed among microRNAs, intracellular adaptor proteins, and caspase levels and mean SLEDAI. *Results*. The ΔCT, defined by test reading difference between the target and the internal control microRNA (miR-451a), of miR-21-5p, miR-150-5p, and miR221-3p were significantly higher in plasma from SLE patients than in normal controls. miR-150-5pΔCT was positively correlated with both CRP and SLEDAI value. miR-150-5pΔCT was negatively associated with MAVS 70 kD. Caspase-10 protein levels were negatively associated with plasma miR-22-3pΔCT and miR-21-5pΔCT levels. *Conclusions*. Our study confirmed the hypothesis that these microRNAs were associated with the mitochondrial apoptotic pathway in SLE. miR-150-5pΔCT was positively associated with SLE disease activity and it was negatively correlated with MAVS 70 kD, which may facilitate viral survival and further enhance inflammation. On the other hand, miR-22-3pΔCT and miR-21-5pΔCT, were negatively correlated with caspase-10 levels, which may repress extrinsic apoptosis and increase cell survival.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic disease affecting mostly women of child-bearing age. It is the prototype of autoimmune diseases because of the variety of its proposed pathogenesis mechanisms. Chronic or acute viral infection or reactivation is one of several important mechanisms involved in the pathogenesis of this

condition [1–6]. Few markers reflect antiviral immunity clinically, with the exception of the antiviral immunoglobulins (e.g., IgG, IgA, or IgM). The peripheral blood mononuclear cells, PBMCs, include both lymphocytes and monocytes by definition. In SLE patients, these two leukocyte lineages are key players in disease pathogenesis and are key cells that fight viral infection. The major functions of these two leukocyte lines are antigen presentation and the execution of adaptive immunity and interferon production against infection [7, 8].

Aside from mononuclear cells of leukocytes, viruses play a role in inducing lupus and lupus flare-ups [4, 9–11]. In addition to the incorporation of the interferon pathway, we focused on antiviral molecules such as mitochondrial antiviral signaling protein (MAVS), melanoma differentiation-associated protein 5 (MDA5), and interferon regulatory factor 7 (IRF7) in this study. The postviral immune response should activate IRF genes [12]. Changes in IRF7 phosphorylation levels could be explained by aberrant activation of the NLRP3 pathway [13], STAT1 pathways [14], IRF3 [15], or downstream MAVS signaling due to inflammation. On the other hand, it might be caused by autoimmunity or cytokine milieu in SLE [16–18].

Levels of plasma microRNAs are deliberately controlled, requiring multiple layers of regulation involving the participation of various protein regulators and posttranscriptional modifications [19–23]. This study explored the associations between circulating microRNA and intracellular proteins involved in the mitochondrial apoptotic pathway including caspase, pIRF7, MAVS, and MDA5. Because of the possible benefits of choosing the appropriate immunosuppressant regimen, there is a need to improve our understanding of the clinical significance of antiviral immunity in SLE.

2. Patients and Methods

2.1. Study Patients. The patients with definitive diagnosis of SLE who were followed up at the Rheumatology Outpatient Clinic for more than six months were prospectively evaluated and compared to 29 healthy subjects. The diagnostic of SLE was based on the 1997 revision of the 1982 American College of Rheumatology classification criteria for SLE [24], and the assessment of SLE disease activity was based on the SLE disease activity index (SLEDAI) [25].

There were 19 SLE patients enrolled, and all patients did not undergo changes in steroid dose or immune-modifying medication during the study period. For comparison, 29 age- and sex-matched healthy subjects were enrolled as healthy controls. The individual plasma microRNA was retrieved in 13 SLE subjects, but the experiment from the rest of six SLE patients was suboptimal. In total, there were 13 patients accomplished in the plasma microRNA and clinical comparison study and 19 patients in the study of intracellular protein study.

The Institutional Review Committee on Human Research reviewed and approved the study protocol and all participants provided informed consent. Patients were excluded if they had autoimmune diseases other than SLE.

2.2. Clinical Assessments. All 19 subjects had complete medical examinations upon enrollment. Clinical data including complement levels and anti-double strand DNA levels were performed regularly and collected upon enrollment. Biomarkers, including demography data, complement levels, anti-ribosomal p autoantibody (a-rib p), anti-double strand DNA autoantibody (a-dsDNA) levels, and disease activity index were also collected.

2.3. Assessment of Protein Expression and MicroRNA Levels

2.3.1. Western Blot Analysis. Levels of intracellular proteins, including MAVS (57 kD and 70 kD), pIRF7 (65 kD), caspase-9 (37 kD), caspase-10 (59 kD), and MDA5 (135 kD), were determined by western blotting. The MAVS were defined as the larger one (70 kD) and the smaller one, mini-MAVS (57 kD) [26–29]. Blood samples were collected by venipuncture of forearm veins of the 19 SLE patients. Peripheral blood mononuclear cell (PBMC) intracellular protein levels of phosphorylated interferon regulator factor 7 (pIRF7), mitochondrial antiviral signaling protein (MAVS), and melanoma differentiation-associated protein 5 (MDA5) were detected by western blotting. Detailed procedures were described in the previous study [30]. The reagents and antibodies were rabbit polyclonal antibodies recognizing caspase-9 (Cell Signaling, #9501), phospho-IRF-7 (Cell signaling, #5184), rat polyclonal antibodies recognizing caspase-10 (Biolegend, #645202), and anti-mitochondrial antiviral signaling antibody (MAVS) (Abcam #ab25084).

Caspase-9 activation was demonstrated by observing cleaved caspase-9 (active caspase-9, caspase-9c, 37 kD) from original caspase-9 (caspase-9, 47 kD) [18]. Caspase-10 activation was demonstrated by observing cleaved caspase-10 (active caspase-10, caspase-10c, 43 kD) from original caspase-10 (caspase-10, 59 kD) [19]. The MAVS were shown to have two types with similar activities: 70 kD (full-length MAVS) and 57 kD (mini-MAVS) [27, 29, 31, 32].

2.3.2. MicroRNA Measurement. Blood samples were collected by venipuncture of forearm veins of the 19 SLE patients and 29 normal subjects. Samples were centrifuged at 1000×g for 10 min to pellet cellular debris. The supernatant was used for RNA extraction. Total RNA was extracted from 300 μL of fluid using the miRNeasy kit (Qiagen) as described by Weber et al. [33]. Amplification was performed according to the manufacturer's instructions (Qiagen). We assessed the extracted RNA for quality and quantity using an Agilent 2100 Bioanalyzer and NanoDrop 1000 spectrophotometer (Thermo Scientific). For the bioanalyzer, the RNA 6000 Pico chip was used for quantification and an initial quality measurement, followed by the use of a Small RNA chip to gain a more detailed view of RNAs in the 6- to 150-nucleotide size range. We performed quantitative real-time PCR (qPCR) according to manufacturer's instructions (Qiagen) to profile the miRNA distribution in body fluid samples. In brief, 5 μL total RNA was collected and pooled from the samples of the same fluid type, and the cDNA was produced using the miScript Reverse Transcription kit (Qiagen). We used the Matrix Hydra eDrop (Thermo Scientific) to mix the cDNA

sample and the qPCR master reagent [Human miScript Assay 384 set v10.1 (Qiagen)] to reduce pipetting error. Any wells with multiple melting temperature values were excluded from further analysis. We also used individual Human miScript Assays to validate the 384 miRNA qPCR set. Data was analyzed using SDS Enterprise Database 2.3 (Applied Biosystems) and normalized to a global mean instead of specific miRNA or noncoding RNA signals.

2.4. Statistical Analysis. Data were expressed in the form of mean ± SD or median (interquartile range). Categorical variables were compared by Chi-square test or Fisher's exact test. Continuous variables were arcsine-transformed to improve normality, and then comparisons between two groups were performed using Student's t-test. Correlation analysis was used to explore the relationship between the SLEDAI score and variables such as microRNA and intracellular protein levels. Spearman's rho for nonlinear distributed variables and Pearson correlation were used for linear distributed variables. The statistical significance threshold was set at $p < 0.05$. All statistical calculations were performed by using the SAS software package, version 9.1 (2002, SAS Statistical Institute, North Carolina).

3. Results

3.1. Baseline Characteristics of the Study Patients. The baseline characteristics, laboratory data, and microRNA of the SLE patients and healthy controls are listed in Table 1. The age and gender distributions were similar between SLE and normal controls ($p = 0.07$ and 0.37, respectively). The disease activity (SLEDAI-2k) of the 13 lupus patients was 6.08 ± 4.87, with the highest at 17 and lowest at 2. The clinical symptoms of the 13 SLE patients included neurologic involvement in three patients, musculoskeletal involvement in ten patients, hematologic involvement in three patients, renal involvement in one patient, cardiac involvement in one patient, respiratory involvement in two patients, and mucocutaneous involvement in two patients. Six SLE patients had involvement of more than one organ. Overall, these SLE patients were under medication control in a relatively stable disease condition who were regularly followed up with at outpatient clinics. The leukocyte, hemoglobin, c-reactive protein (CRP), liver enzymes, and creatinine levels were similar between the two groups (all $p > 0.05$), which demonstrated the stable and steady state of the SLE patients. The only difference between the two groups was the total cholesterol and the triglyceride levels, which were significantly higher in SLE patients than in normal controls (both $p < 0.05$), but this conferred no clinical significance (comparable statin usage between the two groups, $p = 0.64$) (Table 1).

3.2. MicroRNAs Expression in Patients with SLE. The levels of plasma microRNAs were significantly lower in three out of four microRNAs selected in this study (Table 1). Among them, the ΔCT of miR-21-5p, miR-150-5p, and miR221-3p were significantly higher in plasma from SLE patients than in normal controls (all $p < 0.05$, higher ΔCT indicates lower plasma level), except miR-22-3p. The levels of miR-22-3p were similar between SLE patients and normal controls ($p > 0.05$).

3.3. Correlations Analysis between MicroRNA and Leukocyte Viral Infection/Activation Markers. The association between microRNA and the intracellular protein levels including caspase-9, caspase-10, MDA5, full-length MAVS (70 kD), and mini-MAVS (57 kD), is listed in Table 2, and the western blot data is shown in Figure 1. The ΔCT of miR-150-5p was positively correlated with SLEDAI ($r = 0.63$, $p = 0.01$). The ΔCT of miR-150-5p was also negatively associated with full-length MAVS level ($r = -0.49$, $p = 0.04$, Table 2). Caspase-10 protein levels were negatively associated with plasma miR-22-3p ($r = -0.47$, $p < 0.05$) and miR-21-5p ($r = -0.62$, $p = 0.01$). Further, the ΔCT of miR-150-5p was positively correlated with CRP ($r = 0.56$, $p < 0.01$).

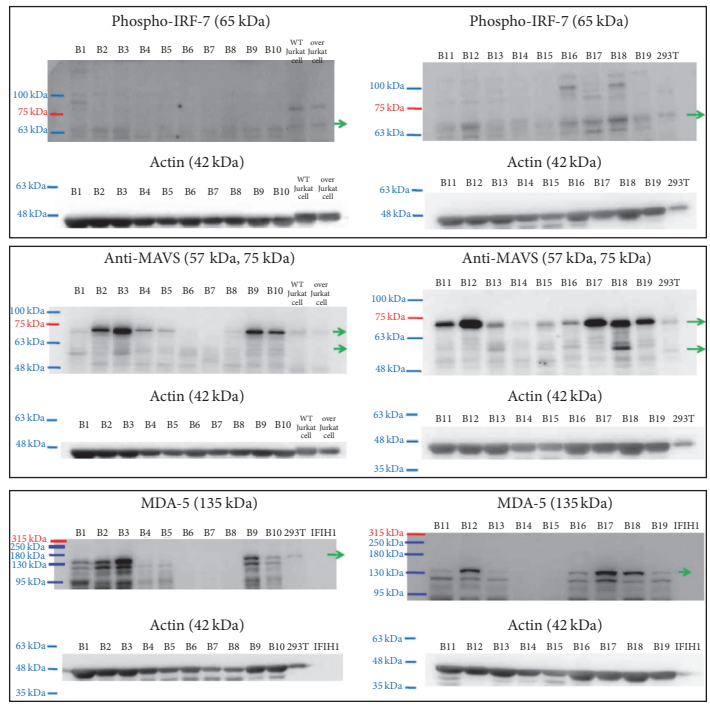
4. Discussion

The present study examined the role of microRNA in mitochondrial apoptotic pathway in SLE, with several major findings. First, the ΔCT of miR-21-5p, miR-150-5p, and miR221-3p were significantly higher in plasma from SLE patients than in normal controls (all $p < 0.05$) (Table 1). Second, the ΔCT of miR-150-5p was positively correlated with both the SLEDAI and CRP. Third, the ΔCT of miR-150-5p is negatively associated with active MAVS and ΔCT of miR-22-3p ($r = -0.47$, $p < 0.05$) and ΔCT of miR-21-5p ($r = -0.62$, $p = 0.01$) were negatively associated with caspase-10 protein levels.

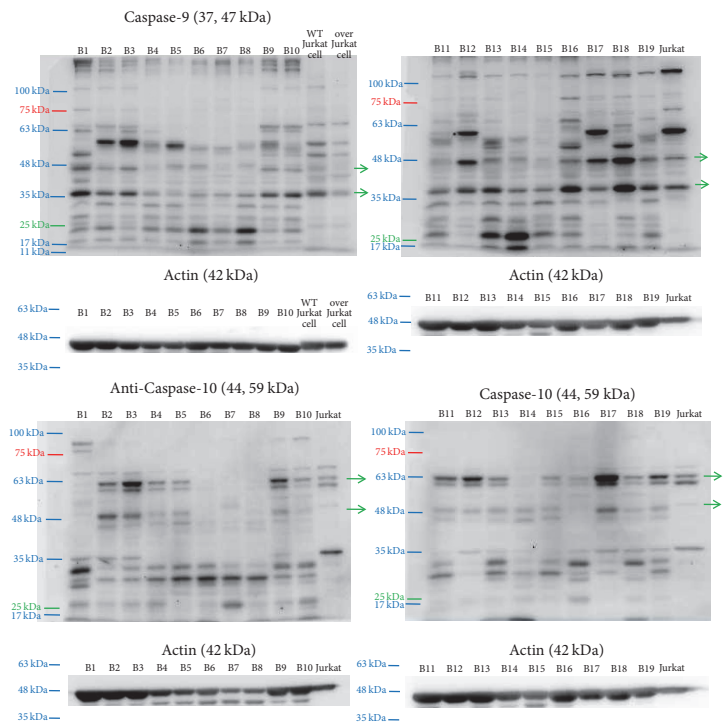
There is accumulating evidence about the role of microRNA in the pathogenesis of SLE and autoimmune diseases. For example, mir-150 regulates various immune cells, including B cells, T cells, and NK cells [34], and it is a biomarker in lupus nephritis [35], but its expression does not differ in T cells from lupus patients and normal controls [21]. On the other hand, this miRNA is downregulated in skin with psoriasis [20] but increased in plasma during osteogenesis [36] and scleroderma [34] or after a 10-km race [37]. These results suggest that microRNA expression and effects could be tissue-specific and that plasma microRNA reflects a general condition of a patient.

This study linked mir-150-5p with intracellular MAVS protein expression. The MAVS protein levels were significantly higher in patients with lupus than in normal controls and were negatively correlated with lupus activity in our previous study [30]. The dynamic changes of mir-150-5p relating to lupus activity are worthy of further investigation.

Cell catabolism is upregulated in SLE, and several recent articles have mentioned apoptosis and microRNAs in SLE [19, 20, 23, 38]. Wang et al. showed that miR221/222 downregulates caspase-10 *in vitro* [23]. As seen in Table 2, we noted that caspase-10 is negatively associated with ΔCT of several plasma microRNAs, including miR-22-3p and miR-21-5p. This observation suggests the activation of the extrinsic apoptotic caspase-10-related pathway in SLE [22, 39] and that caspase-10 is influenced by microRNA, which could be reflected by plasma microRNA levels in this study. Furthermore, we previously demonstrated that caspase-10 positively correlates with pIRF7, and caspase-9 and caspase-10 both



(a)



(b)

Figure 1: Protein expression in peripheral blood mononuclear cells from systemic lupus erythematosus patients. (a) Intracellular proteins (pIRF7, MAVS, and MDA5) in western blots. (b) Intracellular proteins (caspase-9 and caspase-10) in western blots. pIRF7, phosphorylated interferon regulator factor 7; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; B1~B19, peripheral mononuclear cell lysate from systemic lupus erythematosus patients.

Table 1: Demographic clinical data of SLE patients and healthy controls.

	Normal controls ($n = 29$)	SLE ($n = 13$)	p value
Age (year)	57.76 ± 5.47	51.31 ± 11.10	0.07
Leukocytes (×1000/ml)	5.53 ± 1.40	6.68 ± 2.24	0.11
Hemoglobin (mg/dL)	13.37 ± 1.66	12.44 ± 1.74	0.12
Hematocrit (%)	40.57 ± 3.75	37.40 ± 4.71	0.05
c-reactive protein (mg/dL)	1.75 ± 2.29	5.98 ± 7.54	0.23
Aspartate aminotransferase (U/dL)	23.88 ± 7.63	40.88 ± 56.02	0.42
Alanine aminotransferase (U/dL)	24.65 ± 17.12	19.13 ± 8.36	0.39
Total cholesterol (mg/dL)	196.9 ± 26.89	221.57 ± 34.07	<0.05*
high-density lipoprotein (mg/dL)	59.86 ± 14.85	67.17 ± 19.41	0.31
low-density lipoprotein (mg/dL)	118.62 ± 23.05	117.83 ± 26.23	0.94
Triglyceride (mg/dL)	102.83 ± 52.50	191.71 ± 86.69	0.04*
Creatinine (mg/dL)	0.71 ± 0.15	0.86 ± 0.49	0.37
Gender (female: male)	21:08	11:02	0.47
Use of statins (yes: no)	03:26	01:12	0.64
plasma microRNA: miR-541a as control (CT)			
miR-22-3p ΔCT	6.24 ± 1.51	6.36 ± 0.84	0.79
miR-150-5p ΔCT	7.07 ± 1.60	8.71 ± 1.46	<0.01*
miR-221-3p ΔCT	5.88 ± 2.19	7.65 ± 1.56	0.01*
miR-21-5p ΔCT	2.55 ± 1.43	4.10 ± 0.92	<0.01*

SLE, systemic lupus erythematosus; §, data presented with mean ± SD (standard deviation); continuous variables between two groups were compared using Student's T-test, between α, healthy group, and β, SLE; Gender and use of statins were compared using Fisher's exact test; ∗ indicates p value < 0.05.

Table 2: Correlation analysis between microRNA and leukocyte viral infection/activation markers.

$n = 13$	SLEDAI	casp-9 (37 kD)	casp-10 (59 kD)	MDA5	MAVS (70 kD)	MAVS (57 kD)	CRP
miR-22-3p ΔCT							
r	−.12	−.08	−.47*	.08	.10	−.38	−.09
p	.63	.75	<.05*	.77	.69	.13	.65
miR-150-5p ΔCT							
r	.70*	−.03	−.13	−.36	−.49*	−.06	.56*
p	.00*	.91	.60	.15	.04*	.81	<.01
miR-221-3p ΔCT							
r	−.12	−.12	−.02	.13	.21	−.39	.09
p	.65	.64	.93	.61	.40	.11	.65
miR-21-5p ΔCT							
r	.02	−.23	−.62*	.01	−.07	−.21	.23
p	.92	.35	.01*	.98	.78	.40	.21

Method: Spearman's rho for nonlinear distributed variables or Pearson correlation for linear distributed variables; r, correlation coefficient; p, p value; n, number; casp, caspase; SLEDAI-2K, systemic lupus erythematosus disease activity index 2000; ΔCT, compared with miR-541a as control (CT); pIRF7, phosphorylated interferon regulator factor 7; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; CRP, c-reactive protein (mg/dL); ∗, correlation is significant at the 0.05 level.

positively correlate with each other, pIRF7, and MAVS 70 kD [30], indicating that interferons are links to cellular apoptosis and anti-virus immunity in SLE [16, 40–42]. The pathways of MAVS and caspase-10 were examined by detecting plasma microRNA in SLE (Figure 2).

A new direction of SLE studies in microRNA shed light on SLE pathogenesis [43]. Our preliminary study adds several microRNAs that were significantly different from normal controls to the list of previously identified microRNAs. It is worth mentioning that miR-150-5p levels were positively

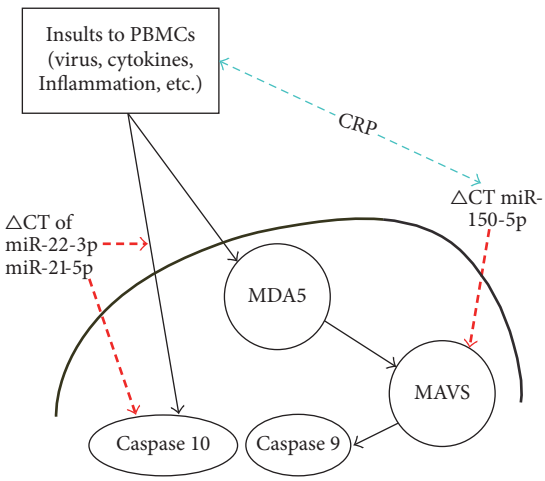


Figure 2: The simplified link between ΔCT of each microRNA and apoptotic molecules in peripheral blood mononuclear cells (PBMCs) in our study. Bidirection (blue) arrow-head thin dash-line: positive correlation between ΔCT microRNA and serum protein; arrow-head (red) bold dash-line: negative correlation between ΔCT microRNA and intracellular protein levels. CRP, C-reactive protein; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; PBMCs, peripheral blood mononuclear cells.

correlated with clinical inflammatory indicator CRP level and the SLEDAI but were negatively associated with MAVS. The idea that interplay between disease activity and infectious disease could be linked by microRNA has been mentioned in other studies [34, 44].

This study had several limitations. First, this was a cross-sectional observational study. More detailed studies are required to determine the real function of plasma microRNA levels in lupus. The concentration of plasma microRNA is low and it needs delicate handling during experiment procedure, and six of our patients' plasma microRNAs were either undetectable or with poor quality which prevent further analysis. A longitudinal study is also required to detect the trend of plasma microRNA in lupus and could reduce variance and improve our ability to predict the prognoses. Second, the case number in this study was small. The difference data number between the plasma microRNA and the intracellular protein levels was due to experiment difficulty of the retrieving plasma microRNA. Large-scale prospective and longitudinal studies are needed to evaluate the prognostic contribution of microRNAs on clinical outcome.

Our study confirmed the hypothesis that these microRNAs were associated with the mitochondrial apoptotic pathway in SLE. MiR-150-5p ΔCT was positively associated with SLE disease activity and was negatively correlated with MAVS 70 kD. The level of microRNA concentration is reversed to the ΔCT, so it is suggesting that this miR-150-5p is positively correlated with MAVS 70 kD and might facilitate anti-viral activity during viral infection and this might be reflected by elevation of CRP levels clinically. The miR-150-5p could be one useful marker demonstrating virus related lupus disease flare-up clinically (Figure 2). On the other hand, miR-22-3p ΔCT and miR-21-5p ΔCT were negatively correlated with caspase-10 levels, where these microRNAs may associate increased extrinsic apoptosis and decreased cell survival, which could reflect monocyte activation-induced cell death.

In conclusion, the plasma microRNA could be a maker demonstrating complex immune milieu in lupus. Some specific microRNA markers could be useful makers for differentiating intracellular immune pathways, such as miR-150-5p in MAVS pathway and miR-22-3p and miR-21-5p in extrinsic apoptosis pathway.

All the underlying research materials related to our article can be accessed on demand by email notification.

Ethical Approval

The study was approved by Chang Gung Memorial Hospital's Institutional Review Committee on Human Research.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yu-Jih Su participated in the design of the study and drafted the manuscript. Chia-Te Kung, Hung-Chen Wang, Wei-Che Lin, Chih-Cheng Huang, Ya-Ting Chang, Chih-Min Su, Yi-Fang Chiang, Ben-Chung Cheng, and Yu-Jun Lin participated in the sequence alignment and clinical evaluation of patients. Nai-Wen Tsai performed the statistical analysis. Cheng-Hsien Lu conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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Review Article

Efficacy of Spa Therapy, Mud-Pack Therapy, Balneotherapy, and Mud-Bath Therapy in the Management of Knee Osteoarthritis. A Systematic Review

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Background. Osteoarthritis (OA) is the most common musculoskeletal disease in the world. OA is the result of an inflammatory and degenerative process affecting the entire joint. Osteoarthritis, especially involving the knee, has a relevant socioeconomic impact in terms of drugs, hospital admissions, work absences, and temporary or permanent invalidity. Therapy of knee osteoarthritis is based on pharmacological and nonpharmacological measures. *Methods*. We conducted a systematic review of the studies published between 2002 and 2017 on spa therapy, mud-pack therapy, balneotherapy, and mud-bath therapy in the treatment of knee osteoarthritis in order to investigate the evidence of the efficacy of such treatment on pain, functional limitation, drug use, and quality of life. Overall, 35 studies were examined among which 12 were selected and included in the review if they are trial comparative. We have been able to illustrate the main results obtained in the individual studies and to elaborate these results in order to allow as much a unitary presentation as possible and hence an overall judgment. *Results*. Because the studies we reviewed differed markedly from one another in terms of the methods used, we were unable to conduct a quantitative analysis (meta-analysis) of pooled data from the 12 studies. For the purposes of the present review, we reevaluated the results of the different studies using the same statistical method, Student's *t*-test, which is used to compare the means of two frequency distributions. Among all the studies, the most relevant indexes used to measure effectiveness of spa therapy were improved including VAS, Lequesne, and WOMAC Score. *Conclusions*. The mud-pack therapy, balneotherapy, mud-bath therapy, and spa therapy have proved to be effective in the treatment and in the secondary prevention of knee osteoarthritis, by reducing pain, nonsteroidal anti-inflammatory drug consumption, and functional limitation and improving quality of life of affected patients.

1. Introduction

Osteoarthritis (OA) is the most common musculoskeletal disease in the world, especially in the elderly; nonetheless it should not be considered exclusively an aging disease. It affects approximately 10% of people over 60 years old with a significant impact on the quality of life of patients who are limited in carrying out normal daily activities and on the healthcare systems [1–3].

In Italy, rheumatic and musculoskeletal diseases (RMDs) are the second most common chronic condition in the population, and among them OA is the most frequent representing the main cause of people's disability [4]. The prevalence of OA varies according to the definition of OA, the specific joint(s) under study, and the characteristics of the study population [3].

According to ISTAT data, osteoarthritis affects 15.9% of the population; the prevalence is age-increasing and

sex-dependent, being higher in women [5]. Symptomatic OA is generally defined by the presence of pain, aching, or stiffness in a joint with radiographic. On the basis of a survey conducted in northwest Italy, 27% of the general population (31,2% women and 22,1% men) were affected by joint pain, defined by any pain lasting more than four weeks [6]. OA was defined as an articular disease resulting from loss of cartilage integrity in association with modifications of the adjoining bone tissue, due to an imbalance between catabolic phenomena and chondrocytic repair phenomena [7]. Osteoarthritis is low-grade inflammatory disease of synovial joints and is now defined as "a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness." Inflammatory flares may be associated with swelling, redness, and pain [8, 9]. Indeed, synovitis, which is secondary to metabolic, biochemical, and mechanical alterations, is of importance in its pathogenesis. For the purposes of classification, it should be specified whether the knee osteoarthritis is of unknown origin (idiopathic, primary) or is related to a known medical condition or event (secondary). Clinical criteria for the classification of idiopathic OA of the knee were developed through a multicenter study group [10]. Regarding the prevalence of symptomatic knee OA in Italy, it ranges from 5.39% to 29.8% with an age-increasing incidence from 27% of subjects under the age of 70 to 44% of patients over 80 years of age. Moreover, women are more affected than men (11% versus 7%) [6, 11, 12]. OA, particularly knee osteoarthritis, has a high socioeconomic impact in terms of drug spending, hospital admissions, work productivity, and temporary or permanent incapacity [13, 14]. Treatment of OA is based on pharmacological and nonpharmacological measures [15]. The latter include the use of spa therapy (in a broad sense, balneotherapy and/or mud-pack therapy) integrated or alternated with other therapeutic prescriptions. Also, spa therapy (multiple interventions in spa resorts and in particular of mud-bath therapy) can be considered a cost-saving measure in the management of knee OA [16, 17]. Mud is a heated slurry, which is the result of the combination of solid material (mainly clay) and mineral water, used for external application after an adequate maturation period, at a temperature between 45°C and 50°C for the duration of 20-30 minutes. The baths consist of diving a patient in a bathtub with thermal (with a temperature of 36–38°C) and mineral (with high mineral content) water for 20 min. The spa therapy comprises a broad spectrum of therapeutic modalities including mud-pack therapy, balneotherapy, mud-bath therapy, hot showers, massage, and supervised water exercises in spa resorts, adding other benefits as a pleasant climate, relaxing natural scenery, and clean air [18]. The objective of this review is to summarize the currently available information on clinical effects and briefly discuss the possible mechanisms of action of spa therapy in knee OA.

2. Methods

We conducted a systematic review of the literature on spa therapy in the treatment of knee osteoarthritis in order to investigate the evidence of the efficacy of this treatment. We searched MEDLINE via PubMed for articles published between 2002 and 2017 using the terms "osteoarthritis", "knee osteoarthritis", "SPA therapy", "mud-bath therapy", and "randomized controlled clinical trials". Studies were included in this review if in accordance with the eligibility criteria: clinical trials with patients conforming to the American College of Rheumatology (ACR) criteria relating to knee OA [10]; randomized controlled clinical trials (RCTs); clinical trials whose the main objective was the effectiveness of spa therapy [10]. The studies that were excluded from the review were those that analyzed the effects of spa therapy in different joints other than the knee, reviews, and those that are not in English and not on clinical effect of spa therapy, bath therapy, mud-pack therapy, and mud-bath therapy; moreover the studies in which data are expressed as median, with percentage but without absolute values or only with a graphic representation, were excluded. Overall, 35 studies were examined among which 12 were selected and included in the review if they were trial comparative. The Study Flow Diagram according to the Prisma Statement is reported in Figure 1 [19].

Each report was reviewed to identify the criteria used for study enrolment and for assignment to experimental versus control groups, sample size, type and characteristics of treatment, features of mineral water, control intervention, assessment point, endpoints, outcome measures, and tests used for statistical analysis of the results. Instead, data from each study were critically analyzed and the main findings compared with those of the other studies. For the quality assessment of the studies that were included in the review after the preliminary selection we considered the scientific value of the international journals that published these researches, the number of patients included in the studies, the methods used to study the patients, and the possibility of exclusion of more frequent studies bias. Moreover, we cannot exclude the existence of publication bias that can occur for any scientific research especially if related to topics concerning therapeutic methods.

Of 12 studies included in the review, 5 were carried out in Italy: 2 in *Chianciano SPA (Siena)* [20, 21], 2 in *Rapolano SPA (Siena)* [22, 23], and 1 in *Levico SPA (Trento)* [24]. The remaining 7 studies were carried out in other European or non-European countries, namely, 1 in France [25], 3 in Hungary [26–28], 2 in Turkey [29, 30], and 1 in Israel [31]. Overall studied subjects were 1044 of which 582 in the experimental groups and 462 in the control groups (by excluding patients treated only by physical therapy and those not completing the study, the total number of subjects evaluated in this review is 553), although no control group was included in two researches [23, 28]. The total number of patients according to the American College of Rheumatology (ACR) [10] criteria included in each study varies considerably from 382 to 20. Number of patients included in the experimental and control groups and treatment characteristics is shown in Tables 1, 2,

Table 1: Mud-bath therapy: number of patients included in the experimental and control groups.

Study	Treated Patients	Treatment	Control Group	Ongoing treatment	Total Patients
Fraioli A. et al. 2011	17	Mud-bath therapy	44	yes	61
Fioravanti A. et al. 2010	40	Mud-bath therapy	40	yes	80
Fioravanti A.et al. 2011	30	Mud-bath therapy	--	---	30
Fioravanti A. et al. 2015	49	Mud-bath therapy	46	yes	95
Cantarini et al. 2007	30+24 (∘)	Mud-bath therapy (∗)	20	yes (∘∘)	74
Total Patients	190		150		340

(∘) 2 experimental groups included, the first one treated by mud-bath therapy, the second one with physical therapy.
(∗) Ongoing treatment.
(∘) Not allowed any physical or pharmacological treatment during the study.
(∘∘) Not allowed glucocorticoids and intra-articular treatment with hyaluronic acid.

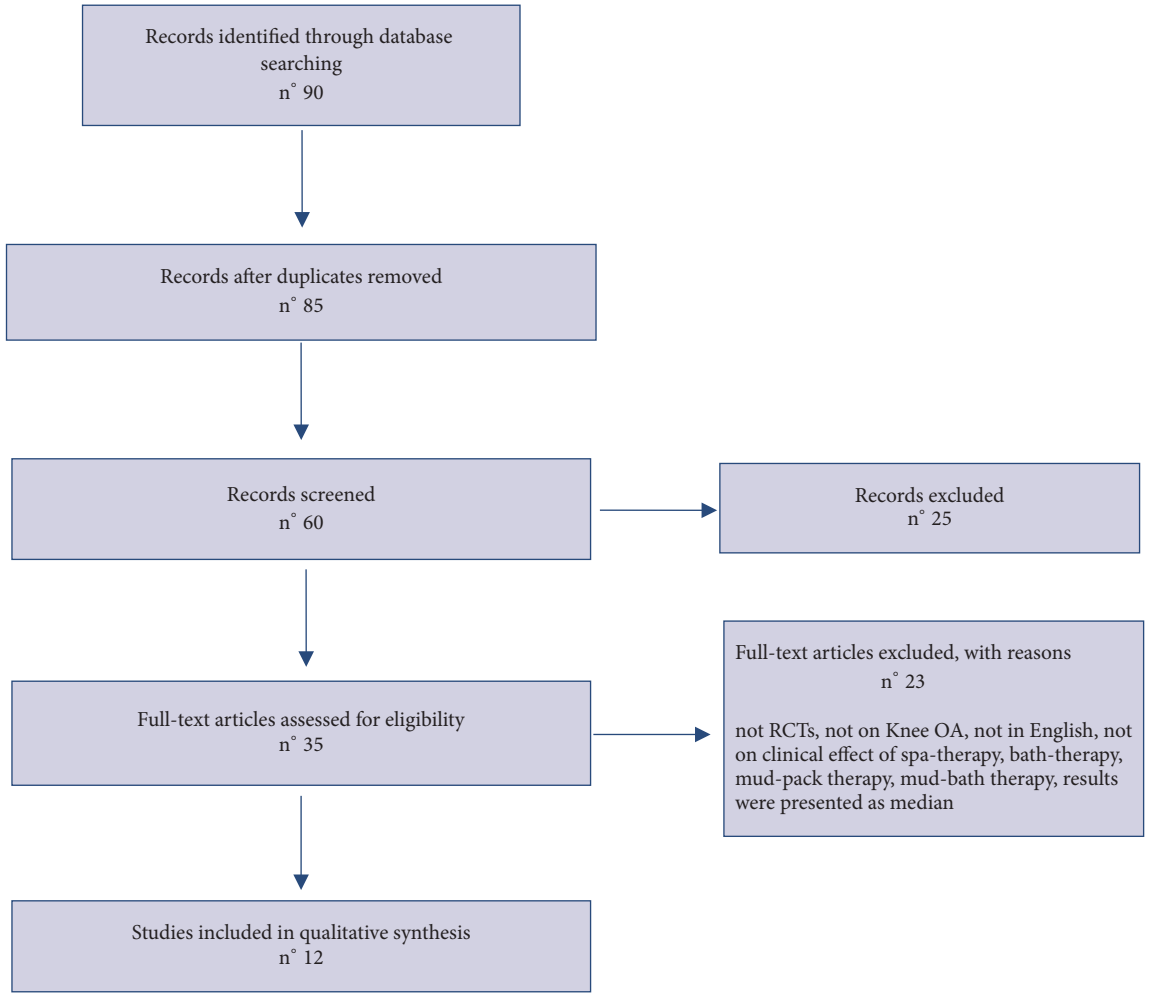

Figure 1: Study Flow Diagram.

3, and 4. Four types of spa therapy were employed for patients placed in the experimental groups: mud-bath therapy in 5 studies (Table 1) [20–24], bath therapy in 5 studies (Table 2) [26–29, 31], mud-pack therapy in 1 study (Table 3) [30], and spa therapy (massages, showers, mud, and pool sessions) alone in 1 study (Table 4) [25] while patients in the control groups were allowed to continue their ongoing therapy. Two studies were double blinded using tap water in the control groups [26, 27].

Even though the methods used to evaluate the results of the therapy of the experimental groups may vary across studies, they are generally based on quantitative evaluations. In all studies the end points were evaluated at the beginning and at the end of the therapy but in some studies additional evaluation schedules were observed. Statistical analysis was performed using common tests (Student's t-test, Pearson's χ^2 test, Wilcoxon's test, Z value, Mann–Whitney U test, and Friedman's test). Instead, data from each study were critically

Table 2: Bath therapy: number of patients included in the experimental and control groups.

Study	Treated Patients	Treatment	Control Group	Ongoing treatment	Total Patients
Gaal J et al. 2008	38	Bath therapy (#)	---	---	38
Kovàcs I. et al. 2002	31	Bath therapy (∘)	27	yes	58
Tishler M., et al. 2004	48 (§)	Bath therapy (#)	24	yes	72
Karagülle M. et al. 2007	10 (^)	Bath therapy (#)	10	yes	20
Kulisch A et al. 2014	38	Bath therapy (##)	39	yes	77
Total Patients	165		100		265

(§) 4 patients did not complete the study.
(^) 1 patient has not completed the study.
(∘) 2 experimental groups included, the first one treated by balneotherapy, the second one with physical therapy.
(∗) Ongoing treatment.
(#) Allowed NSAIDs treatment.
(∘) Not allowed any physical or pharmacological treatment during the study.
(##) Not allowed physical treatment and changes in ongoing NSAIDs therapy.

Table 3: Mud-pack therapy: number of patients included in the experimental and control group.

Study	Treated Patients	Treatment	Control Group	Ongoing treatment	Total Patients
Odabaşi E et al. 2009	32	Mud-pack therapy (∗∗)	25	yes	57

(∗∗) Allowed treatment with analgesic drugs in case of worsening pain, by informing the study authors.

Table 4: Spa therapy: number of patients included in the experimental and control group.

Study	Treated Patients	Treatment	Control Group	Ongoing Treatment	Total Patients
Forestier R.et al. 2010	195	Multiple interventions in spa resorts	187	yes	382

analyzed and the main findings compared with those of the other studies. For the purposes of the present review, we reevaluated the results of the different studies using the same statistical method, Student's *t*-test, which is used to compare the means of two frequency distributions. Assessment of pain was performed in all studies through Visual Analogue Scale (VAS) [32]. Assessment of OA was performed by WOMAC (Western Ontario and McMaster Universities Osteoarthritis Index) [33] in 7 studies and by the Lequesne's Index [34] in 6 studies.

The research carried out in *Chianciano SPA (Siena)* tested the effects of three mud-bath therapy cycles once a day for 12 days with sulphate-bicarbonate-calcium-magnesium mineral water over 1 year's time in patients with knee OA treated with analgesics and nonsteroidal anti-inflammatory drug (NSAID's). The control group was only required to continue the ongoing therapy. The patients who were already being treated with anti-inflammatory agents and analgesics were divided into two groups: the first group also underwent spa therapy, and the second did not [20]. At the end of the trial period, all patients were evaluated with VAS, Lequesne's Index, and the physical examination of each knee to assess the persistence of pain at palpation and during flexion-extension movements [32, 34].

In the second study performed in *Rapolano SPA (Siena)*, a 14-day mud-bath therapy cycle with bicarbonate-sulfate mineral water was planned in the experimental group, while ongoing therapy was allowed in experimental and control groups. Patients were assessed at baseline time, after 2 weeks and after 3, 6, and 9 months after the beginning of the treatment [22]. VAS, WOMAC, and Lequesne's Index were used and also the Arthritis Impact Measurement Scales (AIMS) [32–35].

Another study at *Rapolano SPA (Siena)* evaluated patients with knee OA treated with a cycle of a mud-bath therapy with sulphurous-calcium-bicarbonate mineral water. Ongoing therapy was allowed. No control group was considered in the study [23]. VAS, Lequesne's Index, and leptin and adiponectin plasma levels were assessed at baseline and after 2 weeks, upon completion of the mud-bath therapy [32, 34].

Another study based in *Chianciano SPA (Siena)* evaluated 12 mud-bath therapy with sulphate-bicarbonate-calcium-magnesium mineral water applications provided within two weeks. Ongoing therapy was allowed in experimental and control group [21]. Patients were assessed at basal time and at the end of spa treatment period with VAS, WOMAC and serum levels of adiponectin, resistin, and visfatin [32, 33].

The study at *Levico SPA (Trento)* evaluated patients with knee OA and considered two experimental groups, one of which was treated with cycle mud-bath therapy with arsenical-ferruginous mineral water (3 weeks) and the other with short wave therapy for the same period. Both the experimental group and the control group patients were allowed to continue the ongoing therapy [24]. The results were evaluated at baseline, at the end of the treatment period, and 12 weeks later by VAS, Lequesne's Index, and AIMS [32, 34, 35].

The study performed in *Cserkeszölö Spa (Hungary)* provided for the patients of the experimental group a 15-day bath therapy cycle with sodium bicarbonate mineral water and for

the control group a cycle of similar duration of baths with tap water (placebo treatment) [26]. Results were evaluated at baseline, at the end of the spa therapy, and 3 months later with VAS and symptom scores [32].

The study in *Chamei Yoav SPA (Israel)* evaluated the effects of only bath therapy with salso-sulphate-bicarbonate-calcium mineral water applied once a week for 6 consecutive weeks. Ongoing therapy was allowed in experimental and control groups. Evaluation was done at baseline, at weeks 4 and 6 and 4 weeks following completion of treatment (week 10) [31]. VAS, WOMAC, and Lequesne's Index were used in this study [32–34].

The research conducted in *Alaçati Baths (Turkey)* assessed if patients undergoing bath therapy consisting of two baths with sodium-chloride-sulphate-calcium-magnesium mineral water daily (morning and afternoon) for 10 consecutive days were able to discontinue ongoing therapy. Control group subjects continued ongoing pharmacological treatment [29]. The results were evaluated at baseline, at the end of balneotherapy, at 2 weeks, and during follow-up period at 12 and 24 weeks later by VAS and Lequesne's Index [32, 34].

In the study from SPA Hévíz *(Hungary)*, baths with sulphurous-carbonated-calcium-magnesium and very light radon of Lake Hévíz water were provided for 30 minutes, 5 times a week for 3 weeks in the experimental group, while the control group patients performed the same number with the same duration of baths with tap water (placebo treatment). Both the experimental group and the control group patients were allowed to continue the ongoing therapy [27]. The research evaluated the outcome of bath therapy at baseline, at the end of the treatment and after 15 weeks by VAS, Womac, angle of knee flexion, joint circumference, stair-climb time, and questionnaire of general health-related quality of life [32, 33, 36].

The study at *Bank SPA (Hungary)* evaluated patients with osteoarthritis of the knee underwent a 15-day course of balneotherapy with mineral water contains sodium bicarbonate, fluoride, and metaboric acid. The patients were allowed to take NSAID's or analgesic drug of their choice. No control group was considered in the study [28]. The results were evaluated before the start of balneotherapy and at least 2 weeks and between 10 and 14 weeks after the end of spa treatment period by VAS, WOMAC, and the SF-36 Health Survey [32, 33, 37].

The study in Denizli SPA (Turkey) evaluated knee OA patients treated with mud-pack therapy rich in organic substances (lignin and humin) for 30 minutes/day for 15 days in 3 weeks. Patients were required to suspend any analgesic therapy during trial except in case of intense pain. The control group patients continued ongoing pharmacological treatment [30]. The results were evaluated at the baseline, after the end of spa therapy, and 30 days after the end of the treatment by VAS, WOMAC, and the patient's and physician's global assessments of disease status and response to therapy scores [32, 33].

The study performed in three *French SPAs (Aix-les-Bains, Balaruc, and Dax)* on 382 patients tested the effects of 18 spa therapy (massages, showers, mud, and pool sessions) cycles over three weeks (features of mineral water not indicated). Ongoing therapy was allowed in experimental and control groups. Follow-up was at 1, 3, and 6 months after the beginning of the treatment [25]. The patient's and physician's opinion and the Minimal Clinic Important Improvement (MCII) were adopted for the measurement of results [38].

3. Results

The main results reported by the authors of each study are summarized below. In the study by Fraioli et al., the parameters used to measure the severity of the symptoms of knee OA (VAS, Lequesne's Index, flexion/extension, and tenderness) were significantly reduced after 3 mud-bath therapy cycles during 1 year; in the control group no significant differences were observed. Even in the comparison between the patients of the two groups that were following the therapy with drug, the results were that, in experimental group, the percentage of patients with no symptoms or mild symptoms was higher than that in control group [20]. In the studies, Fioravanti et al. observed a significant improvement of all evaluated parameters VAS, Lequesne's Index, W-TPS (WOMAC Total Pain Score), W-TSS (WOMAC Total Stiffness Score), and W-TFS (WOMAC Total Function Score) at the end of the cycle of mud-bath therapy, whereas in the control group no significant differences were noted [21–23]. Fioravanti et al. found that adiponectin and resistin were reduced after mud-bath therapy in patients with knee OA, while no decrease was detected in the control group [21, 23].

Cantarini et al. evaluated results by VAS and Lequesne's Index and observed a marked improvement in the first experimental group (mud-bath therapy) at both 3 and 12 weeks of treatment, an improvement at 3 weeks in the second experimental group (short wave diathermy on both knees) and a deterioration at both steps in the control group. Also AIMS was improved in others steps. All the groups continued ongoing therapy. The drugs dosage in the groups varied according to pain intensity: in the first experimental group a significant reduction in drug intake was observed at both steps versus baseline (before starting treatment), in the second experimental group the reduction was observed at 3 weeks versus baseline time, and in the control group an increase in drugs intake was observed at both 3 and 12 weeks in comparison with baseline [24].

Kovacs et al. reported a significant amelioration of initial pain, tenderness on palpation, walking ability, time to climb and descend 20 steps, and patient's and physician's opinion in patients treated with bath therapy and three months later this improvement persisted only in the actively treated group. The values reduction were significant for experimental group and not significant for control group [26].

Moreover, Tishler et al. found that balneotherapy was effective in improving all measuring indexes (VAS, WOMAC, and Lequesne's Index), except for WOMAC-Stiffness Index, as well as a reduction in analgesic and NSAID consumption, which was also noted in experimental group. The values reduction were significant for experimental group and not significant for control group, and the improvement remained significant after 10 weeks in the experimental group [31].

Karagülle et al. by using Lequesne Algo Functional Index (LAFI) and VAS, as well as the time taken to climb and descend 10 steps, to make a 15-foot walk and to crouch and raise three times observed a significant reduction in LAFI and VAS which was found at week 2, week 12, and week 24 in the bath therapy group compared to baseline. Comparing the two groups (experimental and control) differences, bath therapy was superior to drug therapy in pain reduction and in physician's global assessment at all time points [29].

Kulish et al. evaluated results by VAS, WOMAC, right and left knee bend angle measured with goniometer, circumference of the right and left knee, time to climb 22 stairs of the Stage Climb Time (SCT), and patient questionnaire on quality of life in relation to health (Quality of life) observed before, immediately after treatment, and after 15 weeks. The obtained results were more favourable in the experimental group than in the control group with regard to the various parameters considered, even after 15 weeks, except for those referring to the knee circumferences and time used for climb the 22 steps [27].

In the study, Gaal et al. compared baselines and all monitored parameters (VAS, WOMAC, and SF-36) were significantly improved by balneotherapy after 2 and 14 weeks of therapy. Moreover, patients taking NSAIDs dropped from 60.5% to 10.5% and 0% for 2 and 14 weeks of SPA treatment [28].

Odabaşi et al. used VAS and WOMAC scales for the evaluation of results as well as a global health status expressed by the patient's and the physician's assessment of disease status. A significant decrease was observed in both groups (superior in the study group as compared to the controls) in terms of disease severity index scores. Compared to the baseline an improvement was observed in the experimental group in all the considered parameters 3 weeks after the beginning of the study, and a greater improvement was observed 7 weeks after the beginning of the study. The number of patients who had MCII was significantly higher in the study group at week 3 and remained high till the end of the follow-up period [30].

Finally Forestier et al. reported that a three-week period of spa treatment combined with a pharmacologic and home exercise program was superior to conventional treatments and exercise alone at the end of sixth month, and it was better tolerated. The author found that the percentage of patients who reached the MCII was significantly higher in the experimental group than in the control group, 50,8% versus 36,4%, and a significant reduction of VAS and WOMAC was observed after 6 months from the end of treatment. The overall opinions of the patient (SF36 scores) and the examining physician at 6 months showed an improvement in both groups (54.4%) [25].

4. Discussion

Therapy of knee osteoarthritis is based on pharmacological and nonpharmacological measures. These include the use of SPA therapy (comprises a broad spectrum of therapeutic modalities including hydrotherapy, balneotherapy, mud-pack therapy, mud-bath therapy, massage, and exercise) to supplement or alternate pharmacological and/or physiotherapeutic therapy, with favourable results on pain, drug use, and improving the patient's general well-being. The biological effects of mud-bath therapy in osteoarthritis are mainly secondary to thermal and chemical stimuli. The thermal effects are characterized by an increase in the temperature of the skin, subcutaneous tissue, and muscles, with decreasing muscular tone. Hyperaemia at periarticular sites (capsules, ligaments, and tendon insertions) caused by heat stimulation of the thermal mud can contribute to the removal of inflammatory cytokines and chemokines thus reducing pain [19, 39]. The heat component plays a fundamental role together with the organic and inorganic properties of the thermal medium (mineral content) [40, 41]. Numerous studies have highlighted the effects of mud-pack therapy, bath therapy, or mud-bath therapy on prostaglandin E2 (PGE2), leukotriene B4 (LTB4), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), metalloproteinases (MMP-3), prolactin and growth hormone (GH), insulin-like growth factor I (IGF1), transforming growth factor beta (TGF-β), reactive oxygen species (ROS), catalase, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-peroxidase), nitric oxide (NO) and myeloperoxidase (MPO), adiponectin, cartilage oligomeric matrix protein (sCOMP), and systemic modification of the chondrocyte markers, suggesting a protective action on articular cartilage [42–48]. The general effect of mud-bath therapy with thermal waters is achieved through influences on the hypothalamus-pituitary-adrenal axis, with increased secretion of Adreno Cortico Tropic Hormone (ACTH) and cortisol and greater production of endogenous opioids (β-endorphin) [49, 50].

Some systematic reviews on spa therapy for knee OA have recently been published [51–55].

Because the studies we reviewed differed markedly from one another in terms of the methods used, we were unable to conduct a quantitative analysis (meta-analysis) of pooled data from the 12 studies. We finally summarized the results obtained from the literature on the basis of the main outcomes of the different studies included in this review. The results of SPA therapy according to measures employed in the studies evaluated are shown in Table 5.

Concerning the VAS, 297 patients were homogeneously evaluated among 12 studies. The mean VAS values before and after SPA therapy were 54.71 and 29.36, respectively, showing a significant reduction corresponding to 46.34% if compared with the initial value (Table 5-line 1).

With regard to Lequesne's Index, it was used in 6 of the studies included in this review over a total of 140 patients in the SPA treated groups [20, 22–24, 29, 31]. Mean Lequesne's Index significantly improved with an overall improvement of 33.78% compared to the initial value (Table 5-Line 2).

The WOMAC was evaluated in 7 studies. WOMAC includes three different measurement scales: WOMAC Total Pain Score (TPS), WOMAC Total Stiffness Score (TSS), and WOMAC Total Physical Function Score (TPFS). Four studies [21, 22, 27, 31] evaluated results from all the three WOMAC scales; in other two researches [25, 28] only the third version

Table 5: Results of SPA therapy according to measures employed in the studies evaluated.

Measure scales applied	Number of patients (1)	Mean values		b1- b2	Reduction % (2)
		b1 before treatment	b2 after treatment		
VAS	297	54.71	29.36	- 25.35	- 46.34
Lequesne's Index	140	56.61	37.49	- 19.12	- 33.78
WOMAC – TPS (3)	170	21.49	13.74	- 7.75	- 36.06
WOMAC – TSS (4)	126	27.78	16.98	- 10.80	- 38.87
WOMAC – TPFS (5)	387	40.26	26.71	- 13.55	- 33.65

(1) Number of patients included in the experimental groups participating in the studies using a specific measure scale, excluding the studies in which data are expressed differently from practice (for instance, with percentage but without absolute values, only with a graphic representation, etc.).
(2) Calculated according to the following procedure: values at the treatment end if the starting values are = 100 (referring to VAS) 29,36:54,71 = x:100; x = 53,66 = value observed at the end of treatment if starting value is 100; 100-53,66 = 46,34 = percentage reduction of VAS value at the treatment end.
(3) TPS =Total Pain Score.
(4) TSS =Total Stiffness Score.
(5) TPFS =Total Physical Function Score.

(W-TPFS) was measured; finally, in the last study [30] a global index was derived from the sum of scores of the three WOMAC scales. WOMAC Total Pain Score was examined in 4 studies, for a total of 170 patients [21, 22, 27, 31]. WOMAC improved before and after SPA therapy from 21.49 to 13.74, respectively, suggesting a decrease in pain of 36.06% (considering the initial value = 100) (Table 5-line 3).

Similarly, the results evaluated with the WOMAC Total Stiffness Score (total patients =126), were equally favourable. In fact, the mean values of the index calculated on the total of patients before and after the treatment were 27.78 and 16.98, respectively. Hence, WOMAC was reduced by 38.87% (Table 5-line 4). Finally, the third WOMAC scale (WOMAC Total Physical Function Score) was investigated in 6 studies [21, 22, 25, 27, 28, 31]. The total number of patients for the evaluation was 387; the mean scores were 40.26 and 26.71 before and after therapy, respectively. Thus, reduction was 33.65% from the initial WOMAC value (Table 5-line 5).

AIMS was evaluated in 2 studies [22, 24] and SF36 in other 2 studies [25, 28]. AIMS was improved in all studies suggesting that psychological aspects, such as anxiety and depression, can also be positively influenced by SPA therapy. SF36 also improved in all considered studies. Two studies evaluated adipokines, specifically adiponectin, resistin, and visfatin, showing that adiponectin and resistin can be reduced by mud-bath therapy [21, 23].

5. Conclusions

To conclude, SPA therapy on the ground of the researches objective of this review is effective in the management of knee OA and significantly improves the pain and functional status of patients with knee OA. SPA therapy is a noninvasive, complication-free, and cost-effective alternative modality for the conservative treatment of knee osteoarthritis.

The mud-pack therapy, bath therapy, mud-bath therapy, and SPA therapy have proved to be effective in the treatment and in preventing progression of the disease and the onset of disability (secondary prevention) of knee osteoarthritis, by reducing pain and functional limitation and improving quality of life of affected patients. Turkish League against Rheumatism states that balneotherapy may be recommended for at least two weeks of treatment because of its thermal and nonthermal effects. In addition to this treatment, peloidotherapy may be advised [56]. In the OARSI guidelines for the nonsurgical management of knee osteoarthritis, balneotherapy was considered appropriate only for the subphenotype with multiple-joint OA (symptomatic OA of the knee) in addition to other joints (e.g., hip, hand, and spine) and comorbidities (diabetes, hypertension, cardiovascular disease, renal failure, gastrointestinal bleeding, depression, physical impairment limiting activity, and obesity) due to paucity of treatment alternatives for that group [57]. Indications based on the clinical guidelines of the French National Authority suggest that patients with knee osteoarthritis might gain the benefit of a persistent improvement (at least twelve weeks) of pain, analgesic and nonsteroidal anti-inflammatory drug consumption, functional capacity, and/or quality of life [58].

RCTs findings suggest that spa therapy is part of an integrated and synergistic multidisciplinary approach with other treatments (pharmacotherapy, physiotherapy), allowing also the reduction of conventional treatment dosage, possibly resulting in lesser costs and drug-related adverse events, and further improving the quality of life of affected patients. Moreover, patients benefit from staying in an environment with a favorable and relaxing climate with a positive impact on their perception of wellbeing [59].

Conflicts of Interest

There are no potential conflicts of interest of any financial or personal relationship with other people or organizations that could inappropriately bias conduct and findings of this study.

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Review Article

Are Janus Kinase Inhibitors Superior over Classic Biologic Agents in RA Patients?

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The Janus Kinases (JAKs) are a family of intracellular tyrosine kinases that provide transmission signals from cytokine, interferons, and many hormones receptors to the nucleus resulting in synthesis of many biologically active compounds and changing cell metabolism and function. That was theoretical background to synthetize the JAK inhibitors (Jakinibs). In recent years a substantial battery of evidence has been collected indicating the potential role of Jakinibs to interact with the specific elements of the immune system, therefore changing the inflammatory response. JAK kinase blockade offers a unique opportunity to block most of the key cytokines enabling the deep interaction into immune system functioning. Following discovery first Jakinibs were intensively studied in various forms of autoimmune diseases, including rheumatoid arthritis, and finally two Jakinibs tofacitinib and Baricitinib have been approved for the treatment of rheumatoid arthritis. Some clinical data indicated that under special circumstances Jakinibs may be even superior to biologics in the treatment of RA; however this suggestion should be verified in large clinical and observational studies.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, devastating polyarthropathy with symmetrical involvement of peripheral joints [1]. Synovial inflammation in joints directly leads to cartilage damage with formation of bone erosions followed by joint space narrowing. The disease leads to disability particularly if poorly controlled and is also a leading cause of premature death. Having a prevalence of 1% RA is recognized as the most common form of inflammatory polyarthropathy. The disease affects three times more females than men. The etiology of the disease although not fully understood comprises a variety of factors including environmental, genetic, and lifestyle related factors [2]. Recent advances in genetic studies using single nucleotide polymorphisms enabled the characterization of more than a hundred loci associated with rheumatoid arthritis risk. Most of them are directly involved in proper immune system functioning; some of them already played a role in pathogenesis of the other immune driven disorders [3]. At the current level of knowledge the HLA system (particularly HLA-DRB1) is believed to be one of the most important players, strongly supporting hypothesis of antigens or (and self-antigens) recognition in RA pathogenesis. This region encodes many important molecules and transmitters which are directly involved in areas such as immune processes as costimulation, T cell recognition of antigens, cytokine receptors expressions, posttranslational citrullination, and synthesis of intracellular regulatory molecules directly responsible for immune signals transmitting [4].

The inflammatory states start with breaking the tolerance of T and B cells against self-antigen (antigens). This ultimately leads to uncontrolled immune response [5]. Recent advances in understanding the pathogenesis highlighted the role of the cytokine network in the initiation and progression of the disease [6–8]. This led to development of a novel class of drugs for rheumatoid arthritis directly targeting cytokines and costimulatory molecules or causing depletion of whole lines of immune cells [9]. This new class of drugs called biologics or biological DMARDs (bDMARDs) revolutionized treatment of RA [10–12]. This kind of treatment has, however, some limitations. The most important one is primary or secondary lack of efficacy. It is estimated that up to 30% of patients still do not respond adequately to the treatment, which requires switching the treatment to the second-line

agents [13]. The other important issue is biologics-related toxicity, increased risk for severe infection, and infusion-related adverse effects [14]. With the exception of abatacept and rituximab, all agents available so far interact with cytokine network (anti-TNF, anti-IL-6) [15]. All of those agents are high molecular weight proteins with complicated molecular structure and they have to be administered parenterally. The other important consequence that should be kept in mind is the fact that biologics may generate immune system response that leads to the formation of neutralizing antibodies, causing secondary lack of efficacy [16, 17]. Given the efficacy of biologics against different targets, the open question remains whether patients who do not respond to first-line biologic (usually anti-TNF) may differentially respond to another drug from the same group (another TNFi) and why some patients respond to anti-TNF although they do not respond to anti-IL-6 and vice versa? This clinical observation gives some insight into pathogenesis of RA indicating diversity of causative factors, cytokines, and transmission molecules creating a unique immunological environmental in a given patient.

This limitation may be overcome by the targeted synthetic DMARDs (tsDMARDs) or biologics that should be considered when treatment target is not achieved with conventional synthetic DMARD and poor prognostic factors are present. Current recommendations, however, indicate to start treatment with bDMARDs [18]

Due to their crucial roles as signal transducers downstream of cytokine receptor activation, the Janus Kinase (JAK) family of tyrosine kinases have attracted much attention since their discovery more than 20 years ago [19–21]. Cytokine receptors are specific type of receptors since they lack intrinsic protein kinase domains and entirely rely on the enzymatic activities of Janus Kinase attached to cytoplasmatic part of cytokine receptors [22]. This makes receptor-JAK interaction the most important step in signal transmission. In line with it JAK inhibition blocks action of all dependent cytokines *("many birds with one shot").*

2. The Janus Kinases Structure and Function

The Janus Kinases (JAKs) are a family of intracellular tyrosine kinases that provide transmission signals from cytokine, interferons, and many hormones receptors to the nucleus resulting in synthesis of many biologically active compounds and changing cell metabolism and function [23]. With this ability to transmit cytokine-related signals JAKs play a key role in proper function of innate and adaptive immune systems as well as an important role in such pathophysiological processes as hematopoiesis, immune cells development, and many others [24]. In mammalians the JAK family consists of four members (JAK1, JAK2, JAK3, and TYK2) that are specifically associated with different types of cytokine receptors [25]. Cytokine (ligand) binding activates JAKs which in turn facilitate binding of the other transmission molecules, namely, STAT (signal transducer and activator of transcription). STATs are DNA-binding proteins which underwent phosphorylation, which allows dimerizing them, translocating to the nucleus, and regulating gene expression. Ligand-JAK activation is not restricted to one cytokine, but one specific JAK could be activated by several cytokines. JAK1, JAK2, and TYK2 are expressed by many cells while JAK3 expression is restricted to hematopoietic, myeloid, and lymphoid cells [26]. In line with this JAKs play a crucial role in normal hematopoiesis and kinases malfunction results in hematopoiesis dysfunction and immunodeficiency [27].

JAKs are an essential part of receptor activity. The majority of known cytokine receptors utilize JAKs as a catalytic center to transmit signals from cytokines and hormone and growth factors to the nucleus to promote transcription of ligand-related genes. Based on structural homologies in receptor sequences and similarities in structure of cytokines, cytokine receptors are typically divided into two subgroups, namely, class I and class II [28, 29]. Class I and class II receptors are protein complexes expressed on the surface of cells. They consist of one to four receptor chains. The typical structure of receptor consists of an extracellular cytokine R homology domain (CHD) and a sequence responsible for cytokine binding. Based on structural differences within CHD cytokines receptors may belong to a class I or class II family [30]. Class I receptor chains have two disulfide bridges linking cysteines in two chains of receptors. But the most important mark of class I is the presence of a highly conserved WSXWS motif [28]. Contrary to class I, class II receptors may form only one disulfide bridge as receptor chains have only one cysteine pair in their CHD [31]. Based on the presence of signal transducing chains that build the active receptors and are entirely responsible for ligand recognition the class I receptor family may interact with four cytokine subfamilies and one hormone-like cytokine receptors. The common gamma family (γc) transmits signals from IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [32]. The common beta family (βc) is involved in activating GM-CSF, IL-3, and IL-5 followed by family receptors that utilize gp 130 protein and act as a transducer for IL-6, IL-11, IL-31 (gp130 homolog), IL-35, and IL-27. The last family in this group consists of Il-12 and Il-23 interleukins receptors for heterodimeric cytokines that share the common subunit p40 [33, 34]. Type I receptors are also involved in recognition of signals from some hormone-like cytokines as erythropoietin, thrombopoietin growth hormone, and leptin (Figure 1) [35].

The type II cytokines consist of more than 300 signaling molecules including mainly but not exclusively interferons types I, II, and III but also cytokines of IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) [29]. Although many cytokines, hormones, and growth factors interact with receptors coupled with JAKs, each cytokine receptor family interacts with the specific JAK/JAK composition [36]. Hormone-like cytokines interact with receptors that transmit signals through homodimers of JAK2. The γc family utilized heterodimers of JAK1 and JAK3, and the βc family uses JAK2. Type II cytokine receptors are linked to JAK1, JAK3, and TYK2 [37, 38]

JAKs share seven homology domains termed the JAK homology (JH). JH1 and JH2 are located at the C terminal end of the enzyme encoding a kinase and a pseudokinase, respectively [39]. Contrary to JH1, JH2 domain is characterized by dual kinase specificity and acts as a regulator of

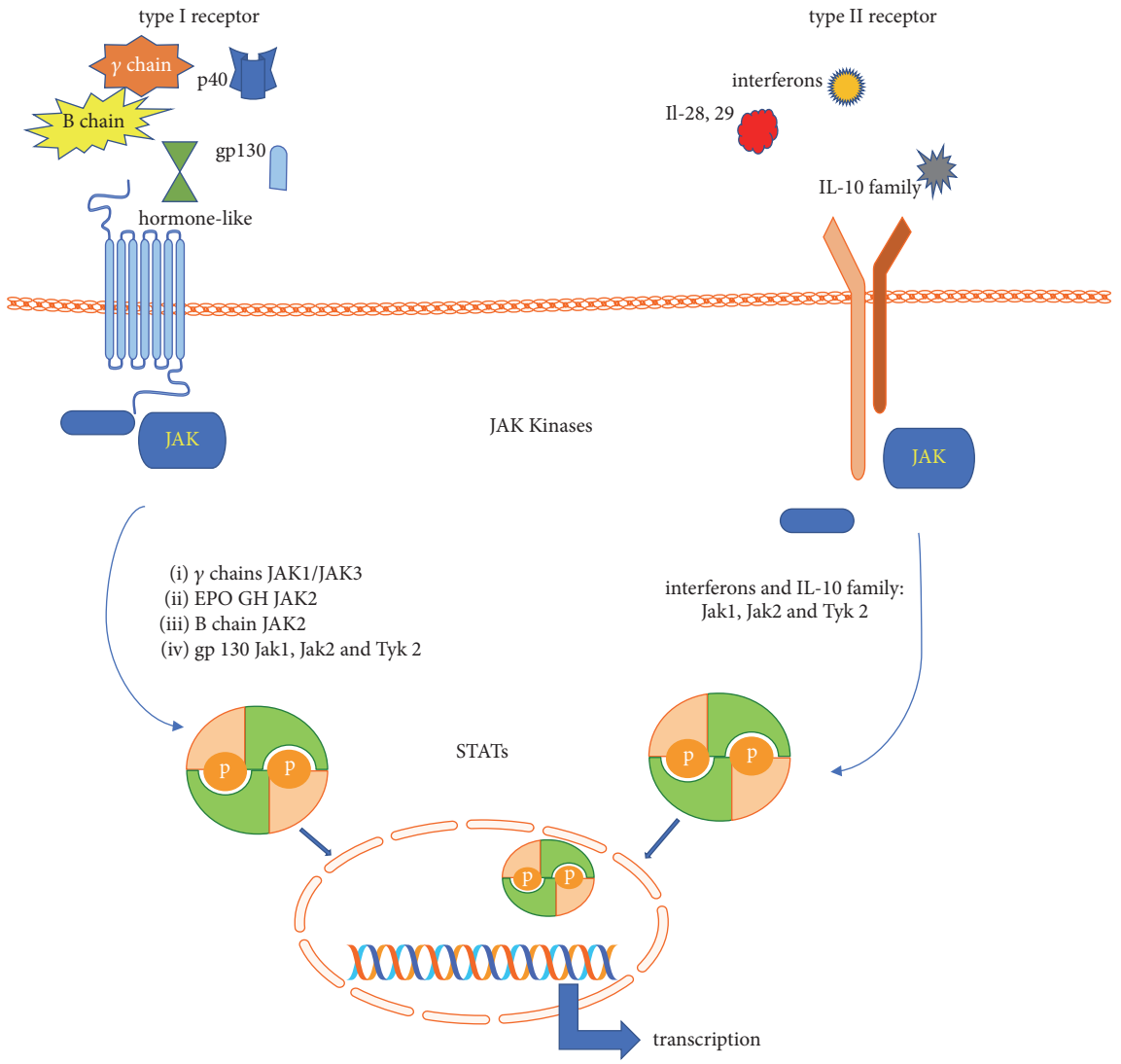

Figure 1: JAK/STAT transmission pathways from type I and type II cytokines receptors. Type I: several cytokines utilize type I cytokine receptor: (i) Il-2, Il-4, Il-7, IL-9, Il-15, and Il-21 that use gamma chain of cytokine receptor; (ii) GM-CSF Il-3 and Il-5 utilize beta chain of cytokine receptor; (iii) IL-6, Il-11, and Il-27 are cytokines that interact with receptors containing gp 130 subunit; (iv) Il-12 and Il-23 with common p40 subunit; (v) erythropoietin, thrombospondin G-CSF GH, and leptin also use type I receptor homolog. TYPE II: (i) type II receptors are main receptors for interferons α, β, and γ; (ii) IL-28 and IL-29 (IFN lambda) also interact with type II; (iii) Il-10 family cytokines are ligands for type II.

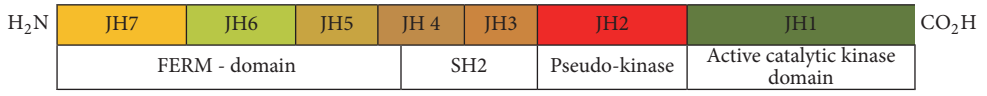

Figure 2: Structure of JAK kinase. Seven JAK homology regions (JHs) built the structure of four structural domains. JAK active catalytic domain is regulated by pseudokinase, which exerts regulatory role over catalytic centre of enzyme. The remaining domains (FERM and SH2, resp.) are responsible for maintaining the structure of kinase and for interaction with cytokine receptor.

JH1 kinase domain activity [40]. The other domains do not encode enzymatic domains but are involved in binding the kinases to the cytoplasmic tails of receptors which contain box 1 and box 2 motifs; both of them are required for proper JAK engagement. Structure of box 1 and box 2 varies substantially between receptors; however proper function and structure are essential for receptor-JAK interaction [41]. This binding part of the enzyme consists of four domains, specifically JAK-FERM (JH5-7) and SH2- (Src homology-2-) like domains (JH3-4) (Figure 2) [37]. The structure of JAK-FERM resembles the canonical FERM and consists of three subdomains, ubiquitin-like F1, acyl-CoA-binding protein like

F2, and pleckstrin homology domain-like F3. There are however many differences: JAK FERMs are characterized by a longer L1 linker usually placed between 29 and 42 amino residues and the SH2 domain is packed against an F1 α1 helix F3-SH2 linker and L3 linker. Some deviations may be observed in regard to F2 domains with additional residues F2α1 and F2α2. It is speculated that this elongation facilitates binding JAK FERMs to the cytokine receptor [42].

3. CytoR-JAK-STAT Signal Transmission Pathway

Receptors associated with JAK kinases are dimers, assembling with two receptor chains. After activation two chains dimerize to mount the active receptor. After ligand ligation by receptor, receptor subunits are oligomerized leading to reorientation of receptor-associated JAK enabling them to take a position that facilitates their transphosphorylation and activation [43]. Phosphorylated JAK then phosphorylates tyrosine residues within the cytoplasmic tail of the receptor leading to conformational changes enabling creation of docking sites for signal transducers and activators of transcription (STAT) proteins acting as downstream regulatory factors [44]. Interaction between STATs and receptor chains is realized by interaction between phosphotyrosine recognition domains on receptor chain and SH2 domains expressed by STAT proteins. Depending on the type of receptor and structure and position of phosphotyrosine domains in the receptor tail one of seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, or STAT6) may be recruited by the receptor. Close proximity of JAK to the STAT enables it to activate STAT followed by STAT translocation to the nucleus, where it acts as transcriptor factor [45]. This process is regulated by the Suppressor of Cytokine Signaling (SOCS) family of negative regulators, which modulate signaling by inactivation of the Janus Kinases (JAKs), preventing access of STATs to receptor binding sites, blocking signaling proteins access to the proteasome [46, 47]. This shows the potential of SOCS family members to block multiple cytokine-induced signaling pathways. Obviously the physiological effect depends on which cytokine action is abolished [48].

4. Primary Immunodeficiencies and Autoimmunity

Primary immunodeficiencies may serve as a model to understand a key role of JAKs in normal immune response as well as to understand pathophysiological consequences of JAK blockade. Mutations in JAKs lead to development of myeloproliferative diseases but also lead to autoimmunity and immunodeficiency states [49]. As an example we can consider a loss of function mutation in JAK3 [50–52]. Working together with JAK1, JAK3 is responsible for transmission of signals provided by γ chain receptors thereby enabling physiological activity of cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21)—cytokines that utilize γ chain receptors to transmit their signals [53]. JAK3 blockade resembles (and in fact is clinically indistinguishable from) a pure switch off mutation in γ chain resulting in severe combined immune deficiency (X-linked SCID) [54]. This disease is characterized by a combination of impaired T cell development and reduced immunoglobulin synthesis leading to the development of a clinical picture with severe diarrhea, recurrent severe infection, and atopic dermatitis being the most important ones [55].

Lack of immunoglobulin synthesis is the result of impaired cooperation between T and B cells, although the structure of B cells is not impaired [56]. This illustrates the basic role of γ chain transmission in the proper function of immune system. Specifically, reduced IL-15 signaling results in impaired development of T and NK cells ($T^-NK^-B^+$ SCID). In contrast, blockade of signaling from the interleukin-7 receptor specifically impairs T cell development, leading to $T^-B^+NK^+$ SCID, since interleukin-15 signaling, which is required for NK-cell development, is maintained. Finally impaired IL-21 signaling substantially explains the nonfunctional B cells in this disease [57–59]. Contrary to this, elevated levels of the other cytokines, which transmit their signals through JAKs, may be recognized as pathogenic factors or at least serve as a marker of autoimmune diseases. For example in early rheumatoid arthritis synovial fluid is characterized by high amount of IL-4. Recent data suggest the role of IL-9 in such autoimmune disorders as systemic lupus erythematosus, multiple sclerosis, psoriasis, rheumatoid arthritis, atopy, and inflammatory bowel disease [60]. The other striking example of the substantial role of JAK-STAT function is the autosomal dominant hyper IgE (HIES or Job's syndrome) syndrome that is characterized by eczema, recurrent pneumonia, chronic mucocutaneous candidiasis, and very high level of IgE [61, 62]. The syndrome is caused by mutation in the DNA-binding Src homology 2 domain of STAT3 [63]. STAT3 activity is essential for cytokines that orchestrated development of IL-17 dependent immune cells (although signaling by IL-17 is not mediated via JAK-STAT system) [64].

5. Neoplasms

The crucial role of the JAK-STAT system in host defense, immune response, and autoimmunity suggested the role that JAK may play in cancerogenesis. It was established that several gains of function mutations (activation mutation) in JAK1, JAK2, and JAK3 are entirely responsible for hematopoietic disorders such as T and B cell acute lymphocytic leukemias, acute myeloid leukemia, polycythemia vera, essential thrombocytopenia, or Hodgkin Lymphoma [65].

This is especially true for JAK2 activation mutation, where the most frequent mutation V617F is seen in over 95% of cases of polycythemia vera and up to 57% in patients with primary myelofibrosis or essential thrombocythemia [66]. Augmented cytokine signaling is also a hallmark of some solid tumors [67]. With the key role of STAT3 that is now commonly accepted to support tumorigenesis by various mechanism of immunocompetent cells cross talk. This indicates the role that the JAK-STAT axis plays in neoplasm development but also indicates possible medical interventions.

6. Rationale for JAKs Blockade in the Treatment of Autoimmune Diseases

In recent years a substantial battery of evidence has been collected indicating the potential role of JAK kinase inhibitors (Jakinib) in interacting with the specific elements of the immune system, therefore changing the inflammatory response. JAK kinase blockade offers a unique opportunity to block most of the key cytokines enabling the deep interaction into immune system functioning. There are however many limitations of such a treatment. Firstly the first generation of Jakinibs (pan-inhibitors) target many of the known JAKs. Taking into account the fact that JAKs are a group of signal transmitters, panblockade may not only result in reduction of inflammatory response but also contribute significantly to development of serious adverse events, toxicity, increased risk of infection, bone marrow suppression, and higher rate of cardiovascular events [68]. Secondly JAKs blockade may potentially reduce anti-inflammatory response provided by anti-inflammatory cytokines such as IL-10 (JAK1/TYK2) and IL-4 (JAK1/JAK3). Moreover JAK cannot transmit signals provided by TNF, IL-1, IL-8 TGFβ, MCSF, and IL-17, which maintain normal immune response to infectious agents on one side but reduce efficacy of drugs in treatment of some autoimmune diseases on the other. This limitation should be borne in mind when starting treatment with Jakinibs.

Tofacitinib was the first Jakinib approved for treatment of autoimmune diseases in humans. The background for its introduction to clinical practice was the role of JAK3 in transmission of many inflammatory stimuli provided by type I and II cytokines. Initially it was believed that tofacitinib is a selective Jakinib blocking only JAK3. Therefore it may exert high therapeutic potential parallel with an acceptable adverse effect profile. Subsequently it was clear that tofacitinib also blocks JAK1 and to a lesser degree JAK2 [69]. This paradoxicality may be an advantage of tofacitinib as mild inhibition of JAK1 and JAK2 does not change the safety profile of the drug but provides enhanced efficacy of the compound in the treatment of autoimmune diseases. In line with this finding FDA approved tofacitinib for patients with RA refractory or intolerant to methotrexate. This was based on a synthesis of data accumulated from phase II and III trials where tofacitinib was extensively tested against methotrexate and placebo in patients with rheumatoid arthritis [70]. Studies in phase II where tofacitinib 5 and 10 mg daily was compared to placebo showed significantly higher ACR 20 response rate at week 12. The improvement in tofacitinib group was seen as early as weeks 1 and 2 and the therapeutic effect was sustained to the end of the treatment. Patients treated with tofacitinib showed higher ACR 50 and ACR 70 response rate versus placebo arm and the effect was seen in both tofacitinib doses [71–73]. Moreover in trials with RA patients tofacitinib was noninferior to a TNF inhibitor—adalimumab [72]. This is an important finding as JAK3 kinase does not directly transmit signals provided by TNFα and anti-TNFα biologics actually increase level of this cytokine [74]. This also gives an interesting insight into pathogenesis of RA, suggesting that disease is not driven by a single cytokine but it is the result of interaction of several proinflammatory cytokines building together a proinflammatory milieu. Phase III trials confirmed observations driven from phase II [75]. Significant improvement in almost all measured outcomes has been recorded in tofacitinib group regardless of the previous treatment (biologic DMARDs naïve, biologic DMRDs resistant) [76]. Subsequent studies in phases III and IIIb/IV confirmed that tofacitinib treatment is noninferior to standard care with TNFi (adalimumab) [77, 78].

Inhibition of inflammation is not obviously a target but rather the way of the treatment. The real target of treatment is to halt structural damage of joint and prevent disability. In this field tofacitinib also showed high therapeutic potential halting the progression of joint damage [79].

Deep interference in the immune system has to bring many safety issues, as a blockade of JAK-STAT transmission inhibits the action of several cytokines involved in normal immune response thus reducing organism self-defense. Therefore this issue has also been extensively studied. The adverse events (AE) incidence ratio did not differ significantly between tofacitinib and placebo arms. The most common AEs in the initial phase of study (months 0–3) were diarrhea, nasopharyngitis, headache and urinary tract infection within later phase (months 3–6), upper respiratory tract infections, nasopharyngitis, and bronchitis [80]. Of note is a smaller increment in hemoglobin concentration observed in tofacitinib group (10 mg bid) in comparison with smaller dose tofacitinib group (5 mg bid). That may be due to direct blockade of erythropoietin signals in patients on higher doses of drug that superimposes beneficial effect of inflammatory cytokines with blockade on the hematopoietic system.

Blocking of signal transmission by JAK inhibition may be therefore potentially dangerous. The special issue is JAK inhibitors selectivity as JAK1 and JAK2 blockade are lethal in the mouse [81, 82]. Fortunately, contrary to the permanent inhibition of JAKs that would lead to severe immunodeficiency, many accumulated data suggest that temporary and reversible JAKs inhibition may provide safe and efficacious treatment for many autoimmune diseases.

7. BARICITINIB: A JAK1/JAK2 Inhibitor

The therapeutic potential of reversible blockade of JAK1 and JAK2 in autoimmune diseases has been intensively studied. JAK1 is associated with β chain of IL-2 receptor as well as the other cytokines as interferons, γ-chain cytokines, interleukins of IL-10, IL-12 family, and those that utilize gp130 receptor subunit. JAK2 is coupled with receptors expressed on variety of hematopoietic cells and is involved in transmission of signals provided by erythropoietin, thrombopoietin, GM-CSF, IL-3, and IL-5. Therefore JAK2 function is essential for hematopoiesis. Selective blockade of JAK1 and JAK2 may cover many of signaling transmission pathways, most of them involved into pathogenesis of RA. This was a background to develop a second generation of Jakinibs. Baricitinib, an oral selective inhibitor of JAK1 and JAK2, has proven its safety and efficacy in RA patients naïve to csDMARD therapy with no prior bDMARD [83] with inadequate response to methotrexate [84, 85] and conventional DMARD [86] and patients with an inadequate response to or side effects associated with the

treatment with one or more tumor necrosis factor inhibitors and/or the other biologic DMARDs [87]. Moreover, as it was shown in phase 3, double-blind, placebo- and active-controlled trial with 1307 patients with active rheumatoid arthritis and treatment with Baricitinib showed superiority over adalimumab as Baricitinib for the ACR20 response and mean change in DAS28-CRP at week 12 [88]. This indicates that Baricitinib may be more potent drug for rheumatoid arthritis than tofacitinib which is characterized by similar potency in ACR responses as compared to adalimumab [78]. The results, however, should be interpreted with caution as the studies differ with regard to their design. This finding is not surprising in light of the potency of Baricitinib to block JAK1, a key transmitter of signals provided by IL-6. As it was shown in Adalimumab-Tocilizumab study, Il-6 blockade may provide more powerful therapeutic effect than inhibition of TNFα [89]. Moreover treatment with Baricitinib was associated with halting the progression of bone erosions to a similar extent to that observed during the treatment of TNFi-adalimumab [88]. What is of special interest is that the therapeutic response is maintained through the treatment [90]. Quite recently Fleischmann et al. performed a post hoc analysis of two phase III studies of Baricitinib 4 mg in classic synthetic DMARD-resistant patients with RA, which showed similar response to treatment in young and elderly patients [91].

The therapy with Baricitinib may potentially bring some safety issues. As far as safety of the treatment is concerned, rates of adverse events were more frequent with Baricitinib than with placebo but similar to that observed in the adalimumab group. But what may be of special interest in the light of deep immunosuppression is that rates of serious infection were similar in the placebo, Baricitinib, and adalimumab groups.

8. Conclusions

The Janus Kinase family inhibitors represent a novel group of small molecules successfully introduced to the treatment of RA and other autoimmune diseases. With unique potential to inhibit signal transmission provided by a wide branch of inflammatory cytokines these compounds may provide a stable and pronounced therapeutic effect. As the drugs are still under clinical judgement with only two JAK inhibitors (tofacitinib and Baricitinib) currently approved for clinical use, it is too early to speculate whether these compounds may substitute biologics that are currently being used. There are however some advantages over classical biologics that Jakinibs potentially may have. The first one is the blockade of a wide spectrum of cytokines that may cover many existing and potential inflammatory pathways. As it comes from lessons from anti-TNF, inhibition of single cytokine does not guarantee therapeutic effect in all patients with RA. So, blockade of multiple cytokines with one agent may be of special interest. Moreover the therapeutic potential of single biologics has a tendency to exhaust while continuing the treatment leading to secondary lack of efficacy. It is largely due to formation of anti-drug antibodies which are able to neutralize activity of biologics. It may be also speculated that inhibition of one cytokine pathway contributes to activation of alternative inflammatory pathways which do not use the cytokine currently blocked. Secondly Jakinibs are small nonprotein substances lacking potential to generate antidrug response and therapeutic effect may be more stable. It is also worth underlining that treatment with Jakinibs is not associated with allergic reaction, making this treatment safer compared with typical biologics. Similar to biologics (in some study higher) the anti-inflammatory potential of Jakinibs seems similar to biologics (in one study higher) is undoubtedly a great advantage, but again it is too early to draw the final conclusion on the base of the results from one study [78, 88]. Finally Jakinibs as small chemical compounds are easy to synthesize, which indicates that in the future the price for treatment may be substantially lower than biologics with advanced and complicated chemical structure.

At the moment we have been given a new therapeutic option for patients who do not respond to TNF inhibitors, with the hope that our potential to reach targets in RA would be easier.

Conflicts of Interest

The author declares that he has no conflicts of interest.

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Research Article

Area of Residence and Socioeconomic Factors Reduce Access to Biologics for Rheumatoid Arthritis Patients in Romania

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Introduction. The study aimed to evaluate the influence of socioeconomic factors on rheumatoid arthritis (RA) patients' access to biologics in Romania. *Method*. Cross-sectional data were collected in January 2014 from the Romanian Registry of Rheumatic Diseases (RRRD) comprising all RA patients on biologics from 42 Romanian counties. "Territorial" access to biologics was defined by patients receiving biologics in their home county. A county was "equitable" if <25% of RA patients received biologics outside it. *Results*. The RRRD included 4507 RA patients aged 56.7 ± 12.1 years, with a disease duration of 12.1 ± 8.3 years. Urban dwellers (67.8%) had a significantly higher prevalence of territorial biologic access than rural dwellers (83.1% compared to 74.1%; $p < 0.001$). Gross domestic product (GDP) in 1000 €/capita/county (odds ratio (OR) = 1.224) and number of physicians/1000 inhabitants/county (OR = 2.198) predict territorial access to biologics and also predict the number of territorially treated RA patients. Inequitable counties exhibited significantly lower socioeconomic indicators than equitable counties. *Conclusion*. In Romania, RA patients' access to biologics varies significantly between counties. Urban dwellers and patients living in counties/regions with high living standards are more likely to receive biologics locally than those living in more deprived areas.

1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disorder affecting approximately 1% of the general population [1]. Recent epidemiological data show an increase in disease incidence in women in the past ten years [2]. RA can lead to functional disability, low quality of life, increased morbidity, and mortality [3].

Since RA usually affects professionally active people, it has important economic consequences, adding costs to patients and their families and to society through extensive use of health resources and loss of work productivity [4, 5]. The main goal of RA treatment is to induce and maintain remission whenever possible, or at least low disease activity [6]. Biological disease modifying antirheumatic drugs (bDMARDs) have revolutionized the therapy of RA and their efficacy is largely documented in clinical trials worldwide [7–9]. However, the high cost of bDMARDs is limiting their use, particularly in developing countries (since 2000, the Romanian public healthcare system reimburses adalimumab, etanercept, infliximab original and biosimilar, and rituximab, and since 2015 it also reimburses abatacept, certolizumab, golimumab, and tocilizumab).

In order to be eligible for bDMARDs in Romania, RA patients must be nonresponders to two different conventional synthetic DMARDs (csDMARDs) just like in other European countries, but they must fulfill other severity criteria which are stricter [10]. While some European countries do not reimburse bDMARDs yet, others offer free prescription, regardless of disease duration and previous therapies [10]. In 2011, among Central and Eastern European countries, Romania had the fifth highest prevalence of bDMARDs treatment among RA patients (2.2%) [11]. This variation of bDMARD uptake in Europe is coupled with observational evidence that socioeconomic status, area of residence, and income influence disease activity, disease outcome, and treatment access [12–15], which shows that significant inequities exist among different European countries with respect to RA

management. Therefore, it is reasonable to hypothesize that these same limiting factors also create inequities at a national level especially because the regions of Romania differ in terms of socioeconomic and development characteristics. The Romanian territory is divided into 4 macroregions, 2 regions, and 42 counties: macroregion 1 consists of the Northwest (6 counties) and Center (6 counties) regions; macroregion 2 consists of Northeast (6 counties) and Southeast (6 counties) regions; macroregion 3 consists of South (6 counties) and Capital (2 counties) regions; macroregion 4 consists of Southwest (5 counties) and West (4 counties) regions. Historically and economically, macroregions 1 and 3 are the most developed areas of Romania, while macroregions 2 and 4 are significantly below the European Union's mean. This uneven distribution could hypothetically generate similar differences regarding access of RA patients to expensive medication (biologics). In this context, the study aims to evaluate the influence of socioeconomic factors on RA patients' access to biological therapy in Romania.

2. Materials and Methods

2.1. Romanian Eligibility Criteria for bDMARD Therapy in RA. In order to be considered for bDMARDs therapy, Romanian RA patients must fulfill four criteria: (a) the diagnosis of RA to be be made by a rheumatologist and it should fulfill the 2010 RA classification criteria [16]; (b) either high disease activity (HDA) irrespective of disease duration or early RA (under 2 years) with moderate disease activity (MDA) and with at least five poor prognosis factors, both with at least 5 swollen and/or tender joints and at least two of the following three criteria: morning stiffness above 60 minutes, erythrocyte sedimentation rate (ESR) above 28 mm/h, and C reactive protein (CRP) more than 3 times the upper limit of normal (ULN); (c) lack of response to at least two csDMARDs used for 12 weeks each; (d) no known contraindications for bDMARDs.

RA activity is assessed using the composite 28-joint count disease activity score (DAS28), which is based on the number of tender joints, number of swollen joints, ESR or CRP, and the visual analog scale for patient-reported general health [17]: patients with HDA have a DAS28 > 5.1, while patients with MDA have a DAS28 > 3.2.

The mentioned poor prognosis factors include age under 45 years; rheumatoid factor and/or anticitrullinated protein antibodies 10 times the ULN; ESR > 50 mm/h or CRP > 5 times the ULN; erosions on X-rays, ultrasound or magnetic resonance imaging; Health Assessment Questionnaire score above 1.5; extra-articular manifestations.

2.2. The Romanian Registry of Rheumatic Diseases (RRRD). As soon as a patient fulfills the above criteria for bDMARDs use, the attending rheumatologist uploads the data into the RRRD, which is a national electronic database comprising all RA patients treated with biologics in Romania. Prior to treatment and inclusion in the RRRD, all patients signed an informed consent form for both bDMARD therapy and scientific use of their data.

For the purpose of this study, the variables were collected in January 2014 using a cross-sectional study design: demographics (age, sex, and area of residence) and RA-specific variables (disease duration, bDMARDs). The geographical distribution of patients (Romania has 42 administrative divisions: 41 counties and the capital) was reconfigured from a treatment perspective: if the patient received the bDMARD in home county, the case was considered "territorial" access; if the patient received the bDMARD outside home county, the case was considered "extraterritorial" access. Similarly, if a county had less than 25% of its RA patients treated outside, the county was considered to have "equitable" access; if more than 25% of its RA patients were treated outside, the county was considered to have "inequitable" access. The local and RRRD ethics committees approved the study protocol.

2.3. Socioeconomic Indicators. Socioeconomic indicators were collected from the National Institute of Statistics Yearbook (EUROSTAT [18]) and included the population distribution by county, indicators of living standards: gross domestic product (GDP) per capita per county in local currency (Romanian Leu) and Euro, physicians' distribution nationwide and by county, and the rheumatologists' distribution per counties.

2.4. Statistical Analysis. The normal distribution of the data was assessed using descriptive statistics, normality and stem-and-leaf plots, and the Lilliefors corrected Kolmogorov-Smirnov test. The age of the patients and their disease duration were distributed normally and therefore they were reported as "mean ± standard deviation."

On a patient-based analysis (4507 patients), the association between residence (rural or urban) and bDMARD access (territorial and extraterritorial) was assessed using a χ^2 test with a Cramer's V statistic for effect size. The differences between continuous variables (age, GDP, number of physicians, and number of rheumatologists) between patients with territorial or extraterritorial biologic access were assessed using t-tests. Effect size for these t-tests was calculated by Cohen's d statistic, approximated by running the t-tests using the standardized values (Z scores) of the independent variables and observing the mean difference output of these t-tests. A binary logistic regression model was computed in order to predict the likelihood that RA patients will have territorial bDMARD access using the following predictors: age (years), area of residence (coded "0" for rural and "1" for urban), GDP (expressed in 1000 €/capita/county) and number of physicians/1000 inhabitants/county. The results were reported in terms of odds ratios (OR) with 95% confidence intervals (CI).

On a county-based analysis (42 counties), GDP/capita, number of physicians/1000 inhabitants, and number of rheumatologists/county exhibited a nonnormal distribution; therefore the differences of these scale variables according to type of county (equitable and inequitable) were studied using Mann–Whitney tests (effect size for these tests was evaluated by estimating Glass rank biserial correlations using bivariate Spearman's correlations between the nominal and scale variables). For the same reason, the correlations

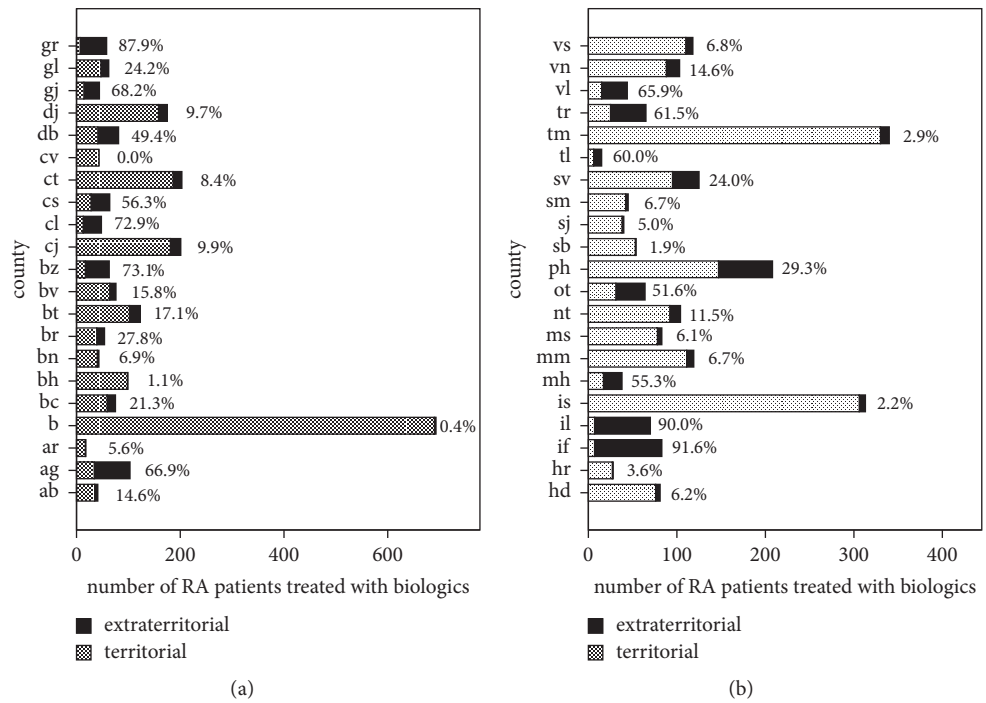

Figure 1: The distribution of RA patients treated with biologics according to county and treatment access (territorial and extraterritorial). The percentages represent the fraction of RA patients who benefited from extraterritorial access to biologics: for example, in the capital ("b", (a)), there were 693 RA patients on biologics, but only 3 (0.4%) were treated extraterritorially, while in other counties more than 90% were treated extraterritorially. Counties are designated by their Romanian abbreviation.

between these scale variables among themselves and with the number of territorially and extraterritorially treated RA patients per county were computed using Spearman's rho coefficients. Two hierarchical multiple linear regression models were constructed in order to predict the number of RA patients with territorial access to biologics. Both models used GDP/capita as predictor in the first step. For the second step, in the first model the number of physicians/1000 inhabitants was added as predictor, while in the second model the number of rheumatologists/county was added as predictor. These predictors were previously normalized by extracting square roots (number of rheumatologists/county) or by calculation of their natural logarithms (number of physicians/1000 inhabitants). Since the number of physicians and the number of rheumatologists are not independent variables (the number of physicians includes the number of rheumatologists), they were not included together in the same regression model.

The statistical tests were considered significant if $p < 0.05$. All the statistical analysis was done using IBM SPSS Statistics version 22.0 for Windows (Armonk, NY, IBM Corp.).

3. Results

3.1. Patient-Based Analysis. Until January 2014, the RRRD included 4507 patients with RA: 4267 (94.7%) treated with biologics and 240 (5.3%) with approved biological therapy, but treatment not yet started. The patients had a mean age of 56.7 ± 12.1 years and a mean RA duration of 12.1 ± 8.3 years. The majority of patients were women (3842 patients—85.2%) and the majority of patients lived in urban areas (3056 patients—67.8%). In the sample, 80.2% (3614) of RA patients benefited from territorial bDMARD access, while 19.8% (893) had extraterritorial bDMARD access (Figure 1, Table 1). Urban dwellers had a significantly higher prevalence of territorial bDMARD access than rural dwellers (Figure 2).

Compared to RA patients with territorial bDMARD access, those with extraterritorial bDMARD access had equivalent mean ages but came from counties with significantly lower socioeconomic indicators (Figure 3).

The logistic regression model ($\chi^2(5) = 1018.9$; $p < 0.001$) explained 32.1% of bDMARD accessibility (Nagelkerke $R^2 = 0.321$) and it correctly classified 81.7% of patients. Accounting for all other predictors included/present in the model, GDP expressed in 1000 €/capita/county (OR = 1.224; 95% CI: 1.186–1.263) and number of physicians/1000 inhabitants per county (OR = 2.198; 95% CI: 1.845–2.618) significantly increased the likelihood that patients will have (equitable) territorial bDMARD access.

3.2. County-Based Analysis. The majority of RA patients who are treated with bDMARDs outside their home environments chose the capital (Bucharest): 53.7% (805/1498) of patients treated in Bucharest come from a different county. Compared to equitable counties regarding bDMARD treatment, the inequitable counties exhibited significantly lower socioeconomic indicators (Figure 4).

Table 1: Characteristics of Romanian divisions: macroregions (4), regions (8), and counties (42).

Division	Inhabitants	GDP	Physicians	Rheumatologists	RA biologics
Macroregion 1	**5468525**	**7123.2**	**2.45**	**50**	**872**
Northwest	*2834186*	*7049.7*	*2.72*	*32*	*547*
Bihor	619102	5095.0	2.93	6	99
Bistriţa-Năsăud	329188	4503.3	1.33	2	43
Cluj	721955	6977.6	5.08	16	201
Maramureş	525765	3809.4	1.56	3	119
Satu Mare	390639	4014.1	1.52	2	45
Sălaj	247537	4304.7	1.51	3	40
Center	*2634339*	*7406.4*	*2.29*	*18*	*325*
Alba	380976	5320.4	1.77	1	41
Braşov	630807	6521.9	2.16	5	76
Covasna	228732	4288.3	1.70	1	43
Harghita	333674	4361.5	1.38	1	28
Mureş	595948	4428.3	3.50	8	83
Sibiu	464202	6358.1	2.54	2	54
Macroregion 2	**6794269**	**6235.8**	**1.68**	**64**	**1358**
Northeast	*3922407*	*4887.6*	*1.72*	*45*	*858*
Bacău	746566	3899.3	1.43	3	75
Botoşani	455973	2922.3	1.33	2	123
Iaşi	919049	4227.4	3.25	30	313
Neamţ	577359	3118.8	1.40	4	104
Suceava	743645	3375.7	1.34	4	125
Vaslui	479815	2535.4	1.29	2	118
South-East	*2871862*	*7161.8*	*1.66*	*19*	*500*
Brăila	356196	4519.9	1.47	2	54
Buzău	478811	3582.4	1.19	1	63
Constanţa	769768	6414.3	2.62	10	203
Galaţi	631669	3766.2	1.38	2	62
Tulcea	244249	3755.8	1.47	1	15
Vrancea	391169	3262.1	1.31	3	103
Macroregion 3	**5757871**	**10892.3**	**3.22**	**89**	**1469**
South	*3260976*	*6724.4*	*1.38*	*13*	*633*
Argeş	646333	6487.6	2.11	3	103
Călăraşi	317293	3223.0	1.00	0	48
Dâmboviţa	528426	4090.3	1.23	2	81
Giurgiu	276781	3291.2	1.06	0	58
Ialomiţa	293658	3747.3	1.01	0	70
Prahova	809052	5828.3	1.45	7	208
Capital	*2496895*	*18653.8*	*4.74*	*76*	*776*
Ilfov	390751	9798.3	1.51	1	83
Bucureşti	2106144	13574.6	6.02	75	693
Macroregion 4	**4221053**	**6706.5**	**2.82**	**32**	**868**
Southwest	*2206321*	*5683.5*	*2.10*	*9*	*365*
Dolj	700117	4486.8	3.02	7	175
Gorj	366261	5498.3	1.75	0	44
Mehedinţi	286678	3527.0	1.70	0	38
Olt	450094	3090.2	1.49	0	64
Vâlcea	403171	4292.8	1.75	2	44

Table 1: Continued.

Division	Inhabitants	GDP	Physicians	Rheumatologists	RA biologics
West	*2014732*	*8045.6*	*3.13*	*23*	*503*
Arad	473946	5599.7	2.44	3	18
Caraş-Severin	328047	4703.2	1.79	0	64
Hunedoara	469853	4742.9	2.36	5	81
Timiş	742886	7938.1	4.99	15	340

Notes. Data from 2013; county names are in Romanian; for each county: GDP €/capita, number of physicians/1000 inhabitants, absolute number of rheumatologists, and absolute number of RA patients treated with biologics. GDP: gross domestic product; RA: rheumatoid arthritis.

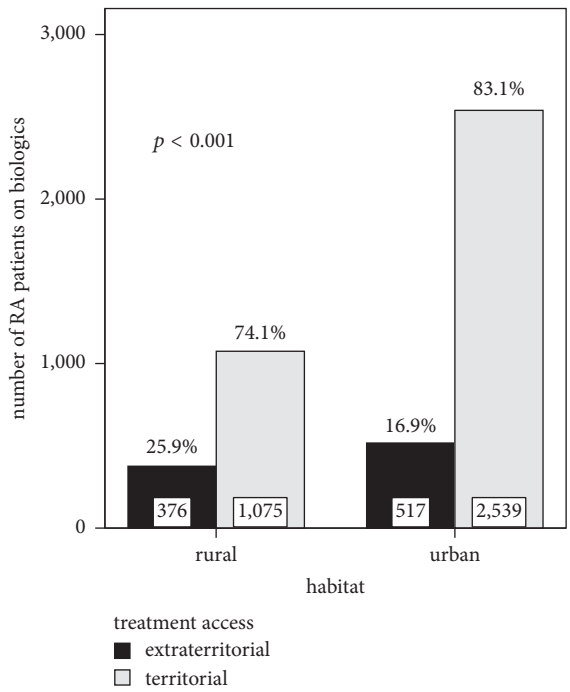

Figure 2: Access to biologics of RA patients according to habitat: 83.1% of urban dwellers had territorial access to biologics, compared to only 74.1% of rural dwellers ($p < 0.001$; χ^2 test; effect size Cramer's $V = 0.205$, $p < 0.001$).

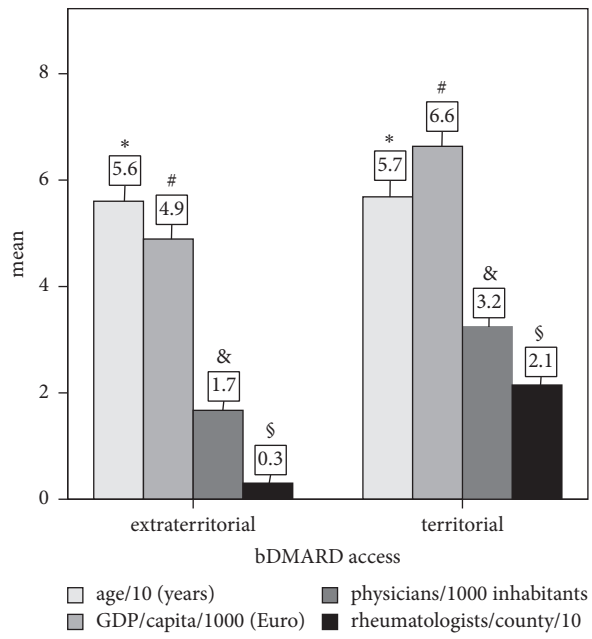

Figure 3: Differences between RA patients with territorial or extraterritorial access to biologics (bDMARD) regarding age and county socioeconomic indicators (gross domestic product – GDP/capita; number of physicians/1000 inhabitants and number of rheumatologists). The p values represent the significance of t-tests. Effect sizes are evaluated by Cohen's d statistics: −0.72 for rheumatologists/county, −0.89 for physicians/county; −0.51 for GDP/capita; −0.07 for age. The variables have been scaled to appropriate coillustration size by decimal division. $^{\#,\&,\S}p < 0.001$; $^{*}p = 0.067$.

The number of rheumatologists/county, the number of physicians/1000 inhabitants/county, and GDP/capita were significantly and positively correlated with the number of territorially treated RA patients/county (rho = 0.843, $p < 0.001$; rho = 0.448, $p = 0.003$; rho = 0.337, $p = 0.034$, resp.), and they were significantly and negatively correlated with the number of extraterritorially treated RA patients/county (rho = −0.340, $p = 0.027$; rho = −0.410, $p = 0.007$; rho = −0.337, $p = 0.034$ resp.).

The distribution of socioeconomic indicators among counties was uneven in terms of number of rheumatologists/county (mean = 5.6; median = 2.0; skewness = 4.84; kurtosis = 26.2; minimum = 0; maximum = 75), the number of physicians/1000 inhabitants/county (mean = 2.01; median = 1.52; skewness = 2.13; kurtosis = 4.48; minimum = 1; maximum = 6.02), and GDP/capita (mean = 4824 €; median = 4299 €; skewness = 2.48; kurtosis = 8.25; minimum = 2535 €; maximum = 13575 €).

Two hierarchical regression analysis models were computed to predict the number of territorially treated RA patients using GDP (1000 €/capita/county) in the first step and then adding either the number of physicians/1000 inhabitants/county or the number of rheumatologists/county as predictors (Table 2). GDP on its own significantly explained 43.4% of the variance of territorially treated RA patients. Adding either the number of physicians/1000 inhabitants/county or the number of rheumatologists/county produced significant models in which these variables predicted an additional 22.3% and 46.8%, respectively, in the variance of territorially treated RA patients. In both models GDP became an insignificant predictor when both independent variables

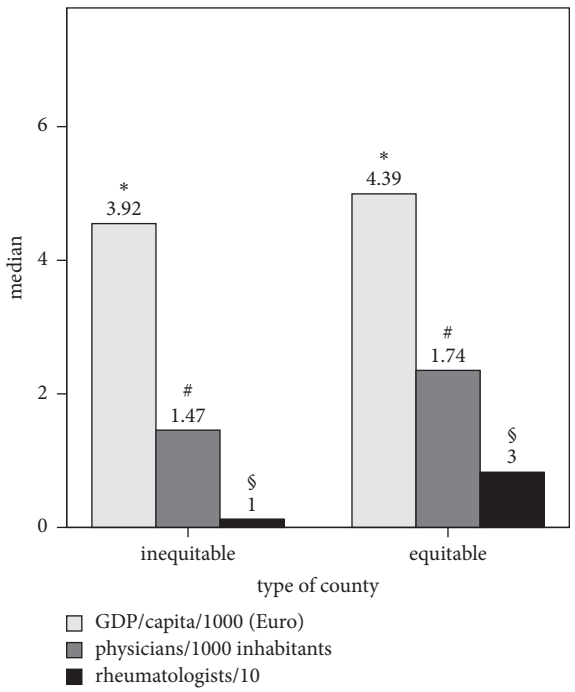

Figure 4: Differences between equitable and inequitable counties regarding access to biologics in terms of socioeconomic indicators (gross domestic product – GDP/capita; number of physicians/1000 inhabitants and number of rheumatologists). The p values represent the significance of Mann–Whitney tests. Effect size was evaluated using Glass rank biserial correlation: 0.34 for GDP/capita; 0.38 for physicians; 0.63 for rheumatologists. The variables have been scaled to appropriate coillustration size by decimal division. $^{*}p = 0.338$, $^{\#}p = 0.016$, and $^{\$}p < 0.001$.

Table 2: Hierarchical regression models predicting the number of territorially treated RA patients.

	GDP	GDP + physicians	GDP + rheumatologists
R^2	0.434	0.656	0.901
F	30.6	37.3	177.9
$p_R{}^2$	<0.001	<0.001	<0.001
R^2 change	-	0.223	0.468
$p_R{}^2$ change	-	<0.001	<0.001
B	93.1	72.1	−3.7
p_B	<0.001	0.203	0.718

Notes. GDP was expressed per 1000 €/capita/county; the number of physicians was expressed per 1000 inhabitants/county; the number of rheumatologists was expressed per county; there are 2 models: model 1 (GDP at the first step, GDP and physicians at the second step); model 2 (GDP at the first step, GDP and rheumatologists at the second step). GDP: gross domestic product; RA: rheumatoid arthritis.

(GDP and number of physicians or GDP and number of rheumatologists) were entered into the regression model, an observation explained by the degree of correlations of GDP with the number of physicians/1000 inhabitants/county (rho = 0.731; $p < 0.001$) and with the number of rheumatologists/county (rho = 0.438; $p = 0.004$).

4. Discussion

The aim of the study was to assess the impact of socioeconomic factors on Romanian RA patients' accessibility to bDMARDs. In this sense, we found that rural habitat and poor county socioeconomic indicators (GDP/capita, number of physicians/1000 inhabitants, and number of rheumatologists/county) are associated with lower access to bDMARDs. In order to discuss the relevance of these results, we must first review the characteristics of the sample. Compared to patients from other European RA registries [19], RA patients from the RRRD have an equivalent mean age, the same form of established disease according to disease duration, but a higher prevalence of female patients. In the absence of conclusive epidemiological studies of RA in Romania, this observation may be explained by a number of competing reasons: underdiagnosis of men; higher disease severity among women requiring biological therapy; a hypothesized genetic population trait. Detailed prevalence studies are needed to assess these possibly non-mutually exclusive hypotheses.

Observational studies have reported that financial factors such as macroeconomic conditions, income, and national health expenditure are major influencing factors of bDMARD accessibility in European countries [11, 15, 20–23]. Even though these studies made observations by comparing different countries, it is reasonable to expect that the same financial factors influence bDMARD accessibility of RA patients within different regions of the same country, given the existence of macroeconomic heterogeneity between these regions. As EUROSTAT data show, the 41 counties and the capital of Romania display an important amount of GDP/capita heterogeneity. Given these significant differences of GDP/capita among Romanian counties, we indeed showed that low GDP/capita predicts low bDMARD access. Furthermore, even though national guidelines attempt to create equal opportunities in terms of access to biologic therapy for all eligible RA patients, in practice many patients need to travel to another county in order to benefit from bDMARDs. Finally, following its highest GDP/capita among counties, we observed a strong centralizing effect of the capital on the number of RA patients treated with bDMARDs, a tendency noted by other authors in the literature [24]. Since every county has its own Health Insurance House which reimburses treatment cost, a revision of the distribution of funds would possibly increase accessibility to bDMARDs outside the capital.

The observed predictive power of the number of physicians/rheumatologists for bDMARD access of RA patients is intrinsically linked to the characteristics of the disease and the structure of the health system. In this sense, the number of physicians has been long used as an indicator of socioeconomic development. Nationwide, for a population of roughly twenty million inhabitants, there were 235 active rheumatologists. Out of the 42 administrative divisions of Romania, there were 7 counties without a rheumatologist, while in the capital there were 75 active rheumatologists. The distribution of rheumatologists among the 41 counties and the capital was uneven and it was correlated with territorial economic level, as measured by the GDP/county (rho = 0.731; $p < 0.001$).

While other more economically developed countries are also facing a shortage of rheumatologists [25, 26], the proportions are very different. The lack of specialized healthcare professionals in rheumatology leads to known barriers to bDMARD therapy in RA, such as long waiting time for medical visits and travel difficulties related to long distances to rheumatology clinical settings [27, 28]. A study investigating patient-reported barriers to access bDMARD treatment in Romanian RA patients would assess the full extent of the issue.

There are some limitations of this study which could influence interpretation of the results. There were no data regarding educational status (a known influencing factor of bDMARD access) [15] and the actual extent of disease activity above the protocol cutoff (DAS > 5.1). A geographical confounder, which we were unable to control, can be described, namely, the travel distances to clinics between different counties: these must have been patients who lived very close to county borders and therefore their extraterritorial bDMARD access could have been a matter of convenience. Additional variables (such as socioeconomic status of each patient and the number of patients who did not receive biologics because of travelling limitations) were not collected. All the analyzed variables came from different contributors to the RRRD (the attending rheumatologists) and their quality relies on the assumption of correct data input into the national electronic system.

5. Conclusions

In Romania, accessibility of RA patients to biological therapy varies significantly between different counties. Areas with low socioeconomic level do not offer equal and fair therapeutic opportunities for RA patients compared to other national areas: patients with RA living in urban areas and counties/regions with high living standards are more likely to receive biological agents locally than those living in more deprived areas. Studies investigating patient-reported barriers to biologic therapy are needed in the Romanian population.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Polymorphisms in the Osteopontin Are Associated with Susceptibility to Ankylosing Spondylitis in a Han Chinese Population

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The aim of this study was to investigate whether osteopontin *(OPN)* variants are associated with susceptibility to ankylosing spondylitis (AS) in a Chinese population. Polymorphisms at the 9175th position in exon 7 of *OPN* and rs17524488 were genotyped using direct sequencing in 186 unrelated AS patients and 188 ethnically matched healthy controls. Serum concentration of OPN was measured by enzyme-linked immunosorbent assay (ELISA) in all participants. AS patients displayed significantly higher OPN serum levels than the controls ($P < 001$). A heterozygous, novel 9175 T>A in exon 7 of the *OPN* gene was found in this study. In healthy controls, subjects carrying the rs17524488 G/G genotype of the *OPN* display significantly higher OPN serum levels than the GG/GG genotype ($P < 0.05$). Plasma OPN level is implicated as an early diagnostic marker of AS. The novel 9175th- (exon 7) position polymorphism of *OPN* and rs17524488 were related to susceptibility to AS in a Chinese population, the rs17524488 G/G genotype may be involved in the pathogenesis of AS, and the precise molecular mechanism underlying the influence of *OPN* polymorphisms on the development of AS remains to be determined in the further prospective studies.

1. Introduction

Ankylosing spondylitis (AS) is an autoimmune disease caused by chronic inflammation response and pathological mineralization and usually strikes the males [1, 2]. Incipient symptoms of AS appear before 40 years of and it is characterized by inflammatory back pain and the stiffness and ankylosis of spinal joints [3]. Approximately 0.2–1.4% of the general population suffers from AS, and its incidence is 0.2–0.54% in the Chinese [2, 4].

AS is highly heritable and many genetic polymorphisms have been reported to correlate the onset of AS [5]. *HLA B27* has been reported to be as a major genetic contributor to AS and 90% AS patients are accompanied with the positive HLA B27 [6]; in addition, there are other genetic polymorphisms, such as *JMY*, *PTGER4*, *JARID1A*, and *ANTXR2* [7, 8]. Although many studies focus on the pathogenesis of AS, the etiology and mechanisms behind AS remain unclear.

Osteopontin (OPN) is a secreted phosphoglycoprotein with several functions in different physiological and pathological processes, including bone remodeling process, inflammation, autoimmune responses, and tumorigenesis [9]. Studies have shown that OPN serum levels are higher in some kinds of autoimmune diseases than healthy controls and may influence development of these diseases through enhancing the proinflammatory T helper type 1 (TH1) and TH17 cell

responses and inhibiting the TH2 responses [10]. More than 10 SNPs have been identified in the *OPN* promoter. These polymorphisms may affect the transcriptional activity of *OPN* and some of them are thought to be genetic risk factors for disease susceptibility [11–13], of which the rs17524488 (-156 GG/G) polymorphism is most frequently studied, which has been found to associate with several diseases, including hip osteoarthritis [14], cancer [15], and diabetic nephropathy [16].

These observations led us to hypothesize that OPN polymorphisms may be involved in the pathogenesis of AS through inflammation and/or the bone remodeling process. However, there have been little studies investigating the association of the *OPN* polymorphisms and AS to date. Therefore, the aim of this study was to investigate if an association exists between *OPN* polymorphisms, OPN serum levels, and the risk of AS in a Chinese population.

2. Materials and Methods

2.1. Subjects. From May 2010 to October 2013, 188 healthy volunteers for physical examination and 186 unrelated AS patients were recruited from the Affiliated Hospital of Ningxia Medical University in a Han Chinese population. All AS patients are HLA-B27 positive. Patients diagnosed with AS according to the modified New York criteria developed in 1984 were included in the study. All of the subjects gave written informed consent. This study was approved by the Ethics Committee of Ningxia Medical University. Two mL fasting venous blood was collected from all subjects; one mL full blood sample was used to extract genomic DNA; however, the other one mL full blood sample was centrifuged (10 minutes at 1160g) to separate the clot material from the solution phase (serum) which was stored at −80°C for further analysis. Genomic DNA was extracted using TIANGEN reagent set (Beijing) following standard protocols; DNA samples were stored at −20°C. Exclusion criteria were patients with diabetes mellitus, cancer, severe liver and kidney failure, and being on therapy for any chronic inflammatory disease. Healthy volunteers as the control group were recruited from the department of physical examination at the same time, and those carrying HLA-B (rs13202464) were excluded.

2.2. Information Collection. The following information was collected: sex, age, duration of disease, body mass index (BMI), smoking status, family history of AS, and HLA-B27. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Global (BAS-G) score were applied to evaluate the disease activity, physical function, and global wellbeing, respectively. The modified Chinese versions of BASDAI, BASFI, and BAS-G have good intraclass correlation and Cronbach's alpha [17].

2.3. Analysis of Polymorphisms in the OPN Regulatory Region. The 9175th position in exon 7 of *OPN* and rs17524488 variants were genotyped by direct sequencing of the sense and antisense strands following polymerase chain reaction (PCR) amplification. A primer pair of the 9175th position was 5′-TACCCTGATGCTACAGACGAGG-3 (forward) and 5′-CTGACTATCAATCACATCGGAATG-3′ (reverse). A primer pair of rs17524488 was 5′-TGTCACTAGTGCCATTTGT-3′ (forward) and 5′-TGTACCTTGGTCGGCGTTTG-3′ (reverse). PCR was performed using 50 ng DNA as a template under the following conditions: 95°C for 3 min, then 35 cycles of 94°C for 30 s, an annealing temperature at 60°C for 45 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were direct sequencing using an automated ABI 3100 DNA sequencer by GeneCore BioTechnologies (Shanghai, China).

Table 1: Characteristics of AS patients and healthy control (mean ± SEM).

Variable	Case (%)	Control (%)	P
Gender			
Male	96 (51.61)	100 (53.19)	0.760
Female	90 (48.39)	88 (46.81)	
Age (years)	27.24 ± 0.68	28.90 ± 0.68	0.084
BMI (kg/m^2)	24.68 ± 0.29	25.17 ± 0.21	0.166
Disease duration (years)	5.67 ± 0.28	—	—
BASDAI (0–10)	4.23 ± 0.06	—	—
BASFI (0–10)	2.79 ± 0.06	—	—
BAS-G (0–10)	4.38 ± 0.09	—	—
Family history			
Yes	25 (13.44)	12 (6.38)	*0.022*
No	161 (86.56)	176 (93.62)	
Smoking status			
Yes	85 (45.70)	92 (48.94)	0.531
No	101 (54.30)	96 (51.06)	

Significant values are written in italics.

2.4. OPN ELISA Assay. Serum concentration of OPN was measured by ELISA according to the protocol provided by the manufacturer (Raybiotech, Norcross, USA) in all participants. Serum was diluted as 1 : 10 into sample diluents. The optical density was measured at 450 nm (BioRad, USA).

2.5. Statistical Analysis. Statistical analysis was performed using SPSS 15.0 software. One way ANOVA and t-test were used to compare mean differences for continuous variables. Allele frequency was determined via direct counting. Binary logistic regression analysis was used to assess independent predictors of AS. Receiver operating characteristic curve (ROC) analysis was used to find the cut-off point of OPN for predicting AS. Differences in the distribution of genotypes between AS patients and control subjects were examined using the χ^2 test, and a statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Subject Characteristics. As was shown in Table 1, a total of 374 subjects were enrolled in this study, containing 186 cases and 188 controls. The clinical characteristics such as age, sex, body mass index (BMI), and smoking status had no significant difference between the case and control groups

Table 2: Difference in the scores of BASDAI, BASFI, and BAS-G among AS patients stratified by different AS genotype.

SNP	Genotype	Number (%)	BASDAI	BASFI	BAS-G
9175 (exon 7)	TT	142 (76.34)	4.21 ± 0.07	2.79 ± 0.07	4.34 ± 0.10
	TA	44 (23.66)	4.30 ± 0.12	2.80 ± 0.12	4.50 ± 0.18
	Unadjusted *P* value		0.561	0.965	0.422
	Adjusted *P* value		0.614	0.795	0.334
rs17524488	GG/GG	102 (54.84)	4.20 ± 0.08	2.79 ± 0.08	4.37 ± 0.12
	G/G	84 (45.16)	4.27 ± 0.09	2.79 ± 0.11	4.38 ± 0.13
	Unadjusted *P* value		0.529	0.948	0.961
	Adjusted *P* value		0.545	0.988	0.821

Data represent means ± SEM, adjusted for the effects of age, sex, BMI, family history, smoking status, and disease duration.

Table 3: Risk factors for AS by binary logistic regression analysis.

	OR	95% CL for OR	*P*	OR*	95% CL for OR*	*P**
Sex	1.065	0.710–1.599	0.760	/	/	/
Age	0.981	0.959–1.003	0.085	/	/	/
BMI	0.959	0.904–1.018	0.167	/	/	/
Family history	2.277	1.108–4.682	*0.025*	/	/	/
Smoking status	0.878	0.585–1.318	0.531	/	/	/
OPN	1.011	1.007–1.014	*0.000*	1.011	1.007–1.014	*0.000*

CI, confidence interval. Logistic regression models were used to calculate OR. *Adjusted for gender, age, BMI, family history, and smoking status. Significant values are written in italics.

(all $P > 0.05$). Family history has significant difference between the case and control groups ($P < 0.05$). In AS subjects, all patients were HLA-B27 positive and their mean disease duration, mean BASDAI, mean BASFI, and mean BAS-G scores were 5.67 ± 0.28, 4.23 ± 0.06, 2.79 ± 0.06, and 4.38 ± 0.09, respectively.

3.2. Association of OPN Genetic Polymorphisms with the Disease Activity Index in AS Patients. We analyzed the relationship between disease activity index (BASDAI, BASFI, and BAS-G) and the two polymorphisms of *OPN* in AS patients. No significant association between *OPN* polymorphisms and BASDAI, BASFI, or BAS-G (all $P > 0.05$) was found. However, we failed to improve the significance even after adjustment for the effects of age, sex, BMI, smoking status, and family history (Table 2).

3.3. Increased Levels of OPN in AS Patients and Diagnostic Value of OPN for AS. Serum concentration of OPN was measured in 186 AS patients and 188 ethnically matched healthy controls by ELISA, even after adjusting for confounding risk factors (age, sex, BMI, smoking status, and family history), which shows that AS patients displayed significantly higher OPN serum levels (median, 217.76; range, 102.02–468.65 ng/mL) than the controls (median, 156.99; range, 51.56–321.54 ng/mL) in Figure 1 and Table 3 ($P < 001$).

Taking AS as the dependent variable, the risk factors (gender, age, BMI, family history, and smoking status) were entered into binary logistic regression analysis. After adjusting for the risk factors, plasma levels of OPN remained with a significant association with an increased odds ratio (OR) for AS ($P < 0.001$). ROC curve analysis was performed to verify the diagnostic accuracy of OPN for AS. The area under curve (AUC) of OPN was 0.71 (95% confidence intervals (CI) 0.662–0.764, $P < 0.001$) and the optimal cut-off point for siglec-5 was 131.7 ng/mL. At this level, the Youden index = 0.313, sensitivity was 42.2% (95% CI 0.349–0.494); specificity was 89.25% (95% CI 0.839–0.933). The AUC of OPN + confounding risk factors was 0.73 (95% CI 0.676–0.776, $P < 0.001$), which was higher than that of OPN, but this did not reach the level of statistical significance ($P > 0.05$) (Figure 2).

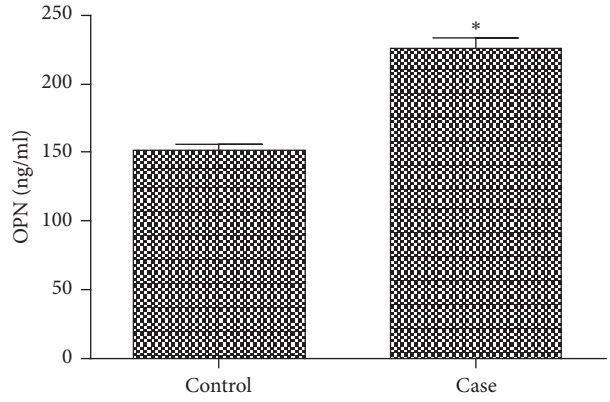

Figure 1: *Serum concentration of OPN in AS patients and healthy controls*. Compared with healthy controls, the serum levels of OPN in AS patients were significantly elevated (*$P < 0.01$).

3.4. OPN Polymorphisms Are Associated with AS Patients. DNA fragments from 9041 to 9292 in exon 7 of the *OPN* gene and one known polymorphism (rs17524488) were analyzed

Table 4: Frequencies of genotypes and alleles of *OPN* in AS patients and healthy control.

Genotype/allele	Case (n = 186), n (%)	Control (n = 188), n (%)	OR (95% CL)	P
9175 (exon 7)				
Genotype				
TT	142 (76.34)	164 (87.23)	1.853 (1.177–2.918)	*0.006*
TA	44 (23.66)	24 (12.77)		
Allele				
T	328 (88.17)	352 (93.62)	1.853 (1.151–2.983)	*0.01*
A	44 (11.83)	24 (6.38)		
rs17524488				
Genotype				
GG/GG	102 (54.84)	124 (65.96)	1.327 (1.029–1.711)	*0.028*
G/G	84 (45.16)	64 (34.04)		
Allele				
GG	204 (54.84)	248 (65.96)	1.327 (1.108–1.588)	*0.002*
G	168 (45.16)	128 (34.04)		

Significant values are written in italics.

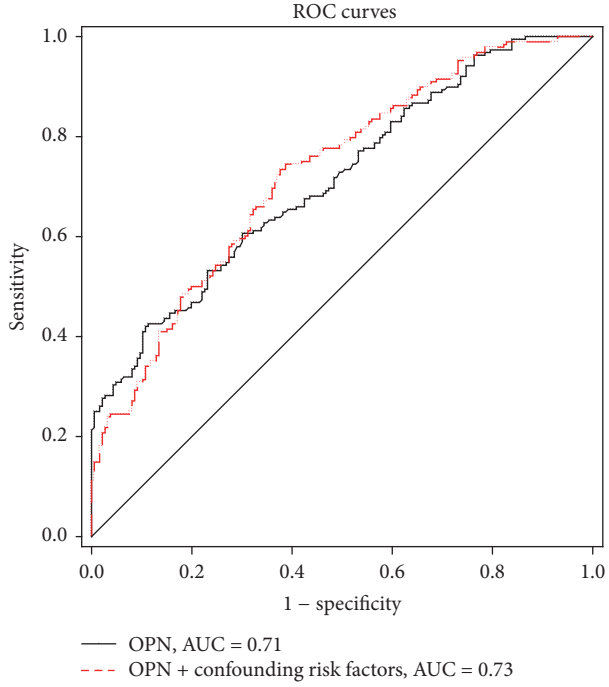

Figure 2: *Analysis of ROC curve to detect OPN in AS patients*. In ROC analysis, the AUC of Siglec-5 was 0.71; the AUC of confounding risk factors was 0.73.

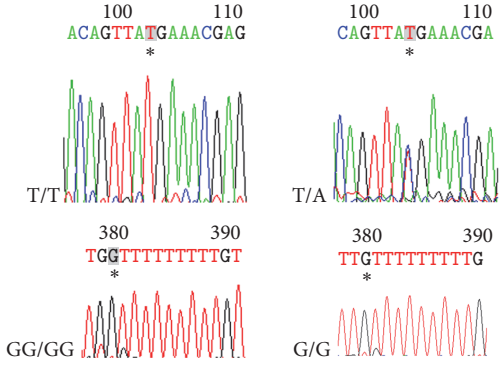

Figure 3: The results of DNA sequencing.

using direct sequencing in 186 AS patients and 188 ethnically matched healthy controls. We found a heterozygous, novel 9175 T>A in exon 7 of the *OPN* gene, and there is a small insertion at nt-156 (rs17524488), which has only two alleles: G/G and GG/GG. Overall distributions of genotypes of *OPN* gene 9175 and rs17524488 were significantly different in AS patients controls (all $P < 0.005$). Frequencies of allele A and allele G were all higher in AS patients than in the controls (all $P < 0.005$), indicating that subjects who carried allele A or allele G have a significantly higher risk of developing AS (Table 4). The results of DNA sequencing were shown in Figure 3.

3.5. OPN Polymorphisms Are Associated with the OPN Levels. As shown in Figure 4, subjects carrying the rs17524488 G/G genotype of the *OPN* display significantly higher OPN serum levels than the GG/GG genotype ($^*P < 0.05$), but there were no significant associations between TT or TA genotypes with OPN serum levels in healthy controls. However, there were no significant associations between *OPN* genotypes and OPN serum levels in AS patients.

4. Discussion

In this study, a heterozygous, novel 9175 T>A in exon 7 of the *OPN* gene and rs17524488 were significantly associated with a genetic predisposition for AS. Patients with AS had higher serum levels of OPN compared with controls, even after adjusting for confounding risk factors. However, the rs17524488 G/G genotype of the *OPN* was significantly correlated with an increased OPN serum level compared to

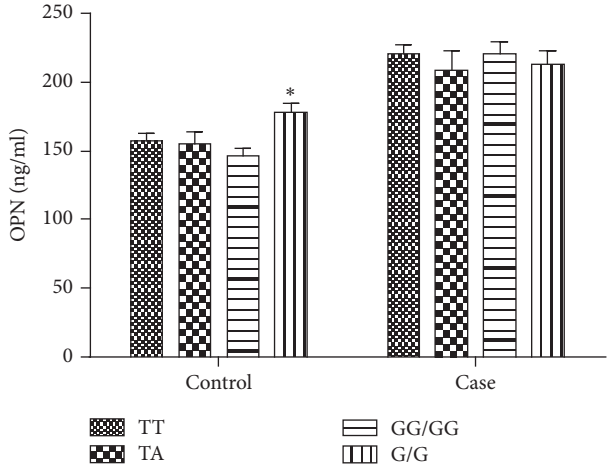

Figure 4: *OPN polymorphisms are associated with the OPN levels.* In healthy controls, subjects carrying the rs17524488 G/G genotype of the *OPN* display significantly higher OPN serum levels than the GG/GG genotype ($^{*}P < 0.05$).

the GG/GG genotype in healthy controls, which suggests that rs17524488 G/G genotype may be involved in the pathogenesis of AS. This is the first study to demonstrate a strong relationship between the 9175th- (exon 7) position polymorphism of *OPN* or rs17524488 and AS patients.

We first considered the possibility that *OPN* polymorphisms are related to the inflammatory process in AS. It has been reported that patients with AS had significantly higher plasma OPN, TNF-alpha, and IL-6 levels and more mRNA expression than healthy controls. The plasma OPN level was correlated with serum ALP, OCN, and CTX-I levels, but not with disease activity in AS. OPN might be involved in bone remodeling rather than in inflammation in AS [18]. In this study, no significant association between *OPN* polymorphisms and disease activity index (all $P > 0.05$) was found, and we also found that patients with AS had significantly higher plasma OPN than healthy controls which is consistent with them [18]. We found a heterozygous, novel 9175 T>A in exon 7 of the *OPN* gene, and distributions of genotypes of *OPN* gene 9175 and rs17524488 were significantly different in AS patients controls. Frequencies of allele A and allele G were all higher in AS patients than in the controls, indicating that subjects who carried allele A or allele G have a significantly higher risk of developing AS (OR = 1.853 or OR = 1.327). In addition, subjects carrying the rs17524488 G/G genotype of the *OPN* display significantly higher OPN serum levels than the GG/GG genotype in healthy controls, strongly indicating that the rs17524488 G/G genotype of the *OPN* may be involved in the pathogenesis of AS. Thus, the mechanism by which this polymorphism contributes to AS susceptibility is less clear. There is evidence suggesting that OPN acts as a proinflammatory cytokine and plays an important role in regulating inflammation [18]; subjects carrying polymorphism loci of the *OPN* will be more sensitive to the immune response; in addition, it might be relevant in the regulation of OPN production in response to the initial immunostimulating trigger.

So far, genetic variants in the *OPN* gene have shown being involved in susceptibility to other immune-mediated diseases such as systemic lupus erythematosus [19], Crohn's disease [20], rheumatoid arthritis [21], and lupus nephritis [22]. In addition, overexpression of OPN has been described in several basic inflammatory processes, such as arthritis [23], myocardial remodeling after infarction [24], kidney interstitial fibrosis after obstructive uropathy [25], wound healing [26], and several types of cancer [27], where it is demonstrated that *OPN* gene polymorphism and OPN levels play important roles in immune- and inflammatory-mediated diseases, including AS.

There are some limitations of the present study that should be considered. Large sample study needs to be explored further. Although this case-control study revealed a strong relationship between the 9175th- (exon 7) position polymorphism of *OPN* or rs17524488 and AS patients, the precise molecular mechanism underlying the influence of *OPN* polymorphisms on the development of AS remains to be determined in further prospective studies.

5. Conclusions

In conclusion, plasma OPN level is implicated as an early diagnostic marker of AS; the novel 9175th- (exon 7) position polymorphism of *OPN* or rs17524488 was related to susceptibility to AS in a Chinese population; the rs17524488 G/G genotype may be involved in the pathogenesis of AS, and the precise molecular mechanism underlying the influence of *OPN* polymorphisms on the development of AS remains to be determined in further prospective studies.

Conflicts of Interest

No conflicts of interest are associated with this work.

Authors' Contributions

Aiping Deng and Guoliang Yang conceived and designed the experiments. Juyi Li, Yi Cai, and Zhongjing Wang performed the experiments. Juyi Li, Zhongjing Wang, and Guoliang Yang analyzed the data. Juyi Li and Aiping Deng wrote the paper. Juyi Li and Yi Cai contributed equally to this paper.

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