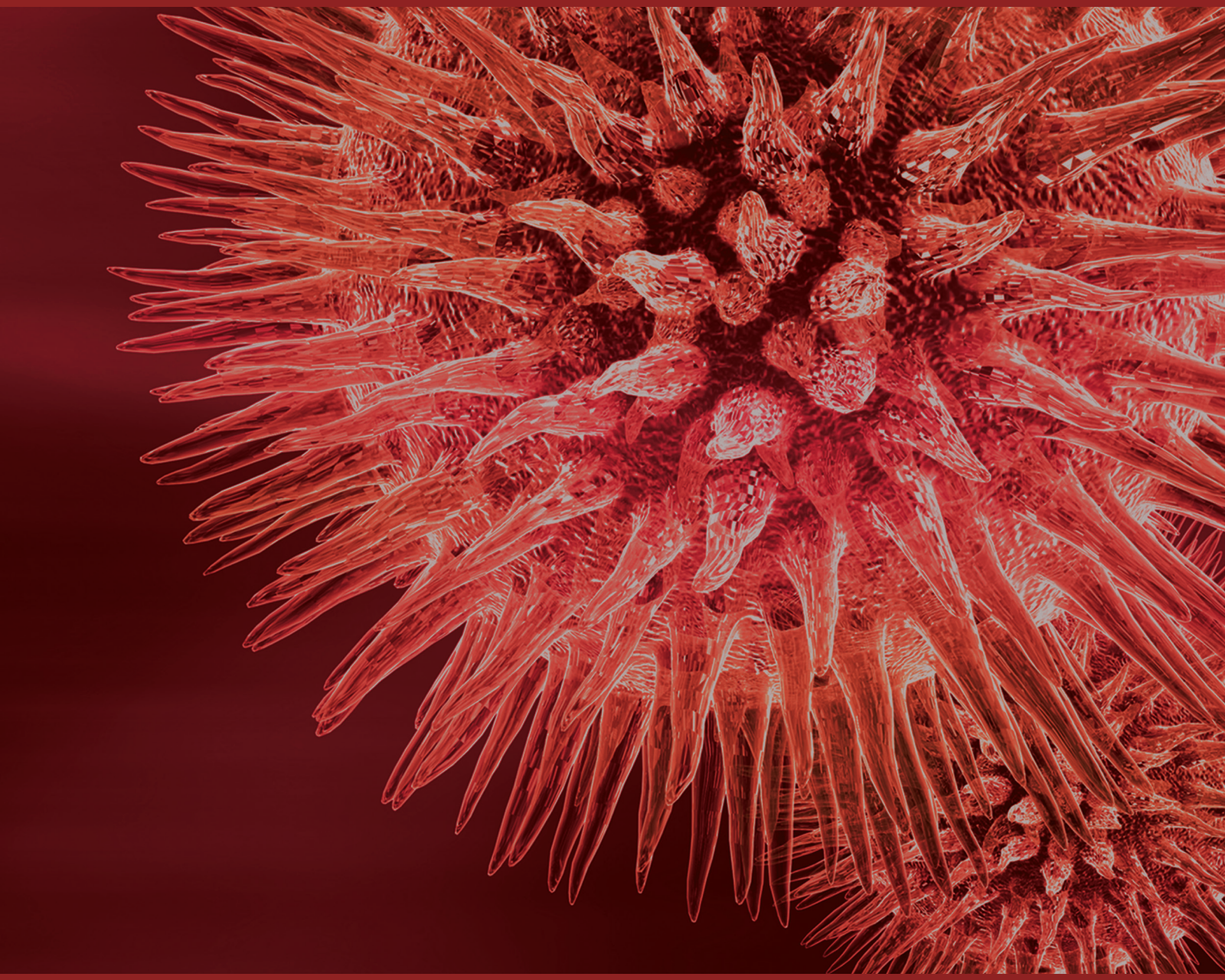
BioMed Research International

Molecular Mechanism of Rheumatic Diseases and Efficacy of Current Therapies

Guest Editors: Sadiq Umar, Abdul M. Tyagi, Anil K. Singh, and Abdul Haseeb



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Editorial

Molecular Mechanism of Rheumatic Diseases and Efficacy of Current Therapies

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Arthritis represents one of the most prevalent chronic health problems and is a leading cause of disability; it was 52.5 million Americans in 2010–12 with an estimate of 78 million by 2040 where two-thirds will be women, suffering from this disease. Arthritis includes more than 100 rheumatic diseases' condition which affects joint and tissue which surround the joint and other connective tissue, the most common being osteoarthritis which affects around 30 million US adults while others include juvenile arthritis, fibromyalgia, gout, rheumatoid arthritis, and systemic lupus erythematosus (SLE). It occurs often in people with chronic conditions, such as heart disease and diabetes, as well as those who are obese.

Antigen-activated $CD4^+$ T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce the cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). These cytokines act as potent inducer of inflammatory responses through upregulation of many genes, including cytokines, chemokines, and adhesion molecules. The focus of treatment for arthritis is to control pain, minimize joint damage, and improve or maintain function and quality of life. Current treatment modalities for rheumatic diseases either produce symptomatic relief (NSAIDs) or modify the disease process (DMARDs) and biological agents, mainly TNF blockers. Other potential experimental promising therapies are IL-17 blockers and IL-23 blockers. Though effective, their use is also limited by cost and their side effects. As a result, the interest in alternative, well tolerated anti-inflammatory remedies has reemerged. Targeting the pathogenic pathway of chronic inflammation represents an unmet challenge for controlling disease activity, preventing functional disability and maintaining an adequate quality of life in patients with rheumatic diseases.

In this special issue, we invited the researchers to contribute their work in understanding rheumatic disease to better take care of the people affected by these disease. P. Klinger et al. investigate the role of pigment epithelium-derived factor PEDF on the genome-wide gene expression, a pluripotent protein expressed in multiple tissues and involved in multiple signaling pathways, including the IP3-AKT, MEK-ERK, or PLA2-PPAR pathway, and showed it as marker and future therapy to stabilize the chondrocyte phenotype of articular cartilage and to prevent its degradation. I. P. Perpétuo et al. studied the role of TNF inhibitors (TNFi) in the differentiation and activity of OC in rheumatoid arthritis (RA) patients. They proposed that TNFi arrests bone loss and erosions, either by direct reduction of osteoclast precursor numbers or by inhibiting of intracellular signaling pathways acting through TRAF6. C. Lin et al. present their findings in the paper "Gray Matter Atrophy within the Default Mode Network of Fibromyalgia: A Meta-Analysis of Voxel-Based Morphometry Studies." E. V. Zakharova et al. published their finding in the paper "Immunosuppressive Treatment for Lupus Nephritis: Long-Term Results" in 178 patients. A. J. Ruiz-Padilla et al. published "The -174G/C Interleukin-6 Gene Promoter Polymorphism as a Genetic Marker of Differences in Therapeutic Response to Methotrexate and Leflunomide in Rheumatoid Arthritis."

Y.-F. Liu et al. showed that the effect of berberine alleviates monosodium urate crystals-induced inflammation by downregulating NLRP3 and IL-1β expressions and connects

it with inactivation of NLRP3 inflammasome. W. Hu et al. showed their analysis "Association between Toll-Like Receptor 4 Polymorphisms and Systemic Lupus Erythematosus Susceptibility: A Meta-Analysis." S. Kotake et al. published "Elevated Ratio of Th17 Cell-Derived Th1 Cells ($CD161^+$Th1 Cells) to $CD161^+$Th17 Cells in Peripheral Blood of Early-Onset Rheumatoid Arthritis Patients."

We hope this special issue covered many important aspects in current updates and therapeutics strategies for rheumatic diseases, which will surely provide us with a better understanding about the pathogenesis, diagnosis, and treatment of these rheumatic diseases.

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Research Article

Effect of Tumor Necrosis Factor Inhibitor Therapy on Osteoclasts Precursors in Rheumatoid Arthritis

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Objective. Tumor necrosis factor (TNF) increases circulating osteoclast (OC) precursors numbers by promoting their proliferation and differentiation. The aim of this study was to assess the effect of TNF inhibitors (TNFi) on the differentiation and activity of OC in rheumatoid arthritis (RA) patients. *Methods*. Seventeen RA patients treated with TNFi were analyzed at baseline and after a minimum follow-up period of 6 months. Blood samples were collected to assess receptor activator of nuclear factor kappa-B ligand (RANKL) surface expression on circulating leukocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers, in vitro OC differentiation assays, and qRT-PCR for OC specific genes was performed. *Results*. After TNFi therapy, patients had reduced RANKL surface expression in B-lymphocytes and the frequency of circulating classical $CD14^{bright}CD16^{-}$ monocytes was decreased. Serum levels of sRANKL, sRANKL/OPG ratio, and CTX-I were reduced in RA patients after TNFi treatment. Moreover, after exposure to TNFi, osteoclast differentiation and activity were decreased, as well as the expression of TRAF6 and cathepsin K. *Conclusion*. We propose that TNFi arrests bone loss and erosion, through two pathways: direct reduction of osteoclast precursor numbers and inhibition of intracellular signaling pathways acting through TRAF6.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by systemic inflammation, bone erosion, and secondary osteoporosis [1].

The immune and skeletal systems have several regulatory factors in common and immune system cells have a profound influence on bone metabolism, particularly in the context of chronic inflammatory diseases. Receptor activator of nuclear factor-κB ligand is present on osteoblasts' surface but is also expressed by activated immune cells, both in its membrane form and as a soluble molecule [2]. Tumor necrosis factor (TNF) increases the trafficking of immune system cells that efflux from bone marrow and peripheral blood into secondary lymphatic organs and sites of inflammation and is abundantly found in rheumatoid joints [3]. TNF, together with other cytokines, acts synergistically with the RANK-RANKL system [3, 4], further enhancing osteoclast (OC) differentiation from its circulatory precursors (monocytes) and contributing to bone resorption [2, 5]. It also increases the number of circulating OC precursors and the proinflammatory cytokine levels in RA patients. These effects are achieved with low levels of circulating TNF and thus TNF quantification is frequently unreliable in RA patients [6–8]. Of interest, TNF inhibitors (TNFi) have a beneficial effect in delaying radiographic damage in RA patients, even in the absence of clinical improvement, suggesting a specific effect of TNF inhibition, independent of inflammation control [9].

Whether this specific effect of TNFi in preventing bone damage in fact occurs independently of the overall inflammatory burden and whether it occurs because of reduced OC number and/or function are still unclear.

Our hypothesis was that, in RA patients, TNFi decrease the OC circulating precursors' differentiation potential and activity. Thus, the aim of this study was to assess the effect of TNFi in the differentiation and activity of OC precursors in a cohort of RA patients, evaluating also the correlation between clinical manifestations of inflammation and OC related parameters.

2. Patients and Methods

2.1. Patients. Patients with RA fulfilling the 2010 American College of Rheumatology/European League Against Rheumatism criteria [10] were recruited from the Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. All RA patients included were TNFi naïve and were followed up during a minimum of 6 months after starting TNFi therapy. Information regarding patients' demographics, duration of symptoms, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), tender and swollen joints counts, presence of erosion, presence of rheumatoid factor (RF), and presence of anticitrullinated protein antibodies (ACPA) was collected. Disease activity score (DAS28-CRP) was evaluated, as well as the Health Assessment Questionnaire (HAQ) [11].

Heparinized blood and serum samples were analyzed in baseline and follow-up samples after TNFi treatment approximately 6 months later. Whole blood samples were taken for flow cytometry and for isolation of peripheral blood mononuclear cells (PBMCs). Samples were stored at the Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. Patients were managed with the standard practice and all participants gave their informed consent. The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013).

2.2. Flow Cytometry. Identification of B- and T-cells and granulocytes in peripheral blood, RANKL surface expression, and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of anti-human murine mAbs as previously described [12]. Heparinized whole blood was used for flow cytometry and absolute cell counts were calculated from differential leukocyte count determined for all participants. Mononuclear cells were isolated from freshly drawn peripheral blood using density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich). Subpopulations of monocytes were identified based on the surface expression of CD14 and CD16 [13]. Median fluorescence intensity (MFI) was calculated based only on positive cells as determined by isotype control gating. FlowJo software (Tree Star, Stanford University) was used for analyzing flow cytometry data.

2.3. Bone Turnover Markers and Bone Metabolism Proteins Detection in the Serum. Carboxyterminal type I collagen cross links (CTX-I) for bone degradation products, human type I procollagen amino terminal propeptide (P1NP, Sunred Biological Technology) for bone formation, sclerostin (SOST), osteoprotegerin (OPG), Dickkopf-related protein-1 (DKK1), and soluble RANKL (ampli-sRANKL, Biomedica Gruppe) were analyzed with enzyme-linked immunosorbent assay [14] in serum samples according to the manufacturer's instructions.

2.4. PBMC Isolation and Cell Culture. PBMCs were isolated by density gradient centrifugation and plated in 96-well culture plates at a density of 7.0×10^5 cells/well as described previously [12]. PBMCs were left overnight for OC precursors to adhere on bone slices and were further cultured for 21 days with macrophage-colony stimulating factor (M-CSF, 25 ng/mL, Peprotech), sRANKL (50 ng/mL, Peprotech), dexamethasone (10 nM, Sigma-Aldrich), and transforming growth factor-β (TGF-β, 2.5 ng/mL, R&D Systems), as described by our group [12]. Adherent cells at day 1 and cells cultured on bone slices for 7, 14, and 21 days [15] were used for functional assays and gene expression.

2.5. Functional Assays. OCs were stained for tartrate-resistant acid phosphatase (TRAP) at days 7, 14, and 21 using the Acid Phosphate Leukocyte Kit (Sigma-Aldrich) according to the manufacturer's instructions. Multinuclear cells containing three or more nuclei [16, 17] were counted as TRAP positive OCs. After visualization, cells were removed from bone slices using sodium hypochlorite and stained with 0.1% toluidine blue for the measurement of resorbed area at days 7, 14, and 21 of culture [18]. Bone slices were photographed in an area of 1.25 mm^2 with a bright field microscope (Leica DM2500, Leica). The number of TRAP stained OCs was counted at each time point and resorption pits were traced using ImageJ software (NIH, Bethesda, MD). The resorbed area was expressed in % of total area.

2.6. Gene Expression. RNA was extracted from cells cultured over bone slices at days 1, 7, 14, and 21 of culture using NZYol (NZYTech) and complementary (c)DNA was synthesized as described previously [12]. Genes that encode osteoclast proteins such as RANK, TNF-receptor associated factor-6 (TRAF6), Fos-related antigen-2 (FRA-2), a subunit of H^+-dependent ATPase (ATP6V0D2), TRAP, and cathepsin K (CTSK) were studied by real-time quantitative PCR (RT-qPCR) using the DyNAmo™ Flash SYBR Green qPCR Kit (Thermo Scientific). Primers (Suppl. Table 1 in Supplementary Material available online at https://doi.org/10.1155/2017/2690402) were designed using the primer-BLAST software [19]. The results were normalized with the housekeeping gene ribosomal RNA 18s and the standard curve method was used to determine the efficiency of qPCR as described previously [20, 21].

2.7. Statistical Analysis. Statistical analysis was performed with SPSS Statistics 17.0 (IBM) and GraphPad Prism 5 (GraphPad Software Inc.). Categorical variables were expressed as frequencies and comparisons were tested using

Table 1: Baseline and follow-up characteristics of patients.

	RA patients ($n = 17$)		p-value
	Baseline	Follow-up	
Age (years)	50 [38–63]		—
% Females	71%		—
Symptoms duration (years)	6 [3.5–9.5]		—
Rheumatoid factor (% positive)	71		—
ACPA (% positive)	53		—
Erosive (% y)	59		—
Treatment with NSAIDs (% y)	47		—
Treatment with DMARD (% y)	100		—
DMARD duration (months)	15 [3–51]		—
ESR (mm/h)	28 [18–48]	21 [13–26]	0.0257
CRP (mg/dl)	1.4 [0.7–2.0]	0.3 [0.04–0.8]	0.0018
Tender joint count	9 [4–14]	0 [0–2]	0.0005
Swollen joint count	7 [4–9]	0 [0-0]	0.0005
DAS28-CRP	5.6 [5.2–6.3]	2.9 [2.2–3.5]	<0.0001
HAQ	1.7 [0.8–2.0]	0.1 [0.0–1.0]	0.0059
TNFi duration (months)	—	6 [6–12]	—

Data is represented as median [Interquartile range] unless stated otherwise; p-value < 0.05 is considered significant; ACPA - anti-citrullinated protein antibodies; CRP – C-reactive protein; DAS – disease activity score; DMARDs – disease modifying antirheumatic drugs; ESR – erythrocyte sedimentation rate; HAQ - Health assessment questionnaire; NSAIDs - non-steroidal anti-inflammatory drugs; RA – rheumatoid arthritis; TNFi – tumor necrosis factor inhibitors; y – yes.

Table 2: Monocyte subpopulation frequency and osteoclastogenic marker expression.

	Baseline	Follow-up	p value
Classic (%)[a]	88 [82–89]	78 [70–83]	0.0065**
Classic CD51/CD61 MFI	130 [119–148]	125 [111–137]	0.4258
Classic RANK MFI	133 [116–160]	122 [100–135]	0.1849
Intermediate (%)[a]	4.4 [2.4–5.6]	4.0 [2.1–7.1]	0.6013
Intermediate CD51/CD61 MFI	222 [139–400]	193 [146–240]	0.8203
Intermediate RANK MFI	197 [117–361]	188 [120–272]	0.9102
Nonclassic (%)[a]	5.7 [4.1–11]	14 [11.5–18.1]	0.0005***,†
Nonclassic CD51/CD61 MFI	192 [80–290]	142 [127–167]	0.5703
Nonclassic RANK MFI	139 [122–157]	138 [126–146]	1.0000

Flow cytometry results are shown as median and interquartile range; [a]gated on the monocyte subpopulation from peripheral blood mononuclear cells. RANK: receptor activator of nuclear factor-κB; MFI: median fluorescence intensity (arbitrary units); ** $p < 0.01$, *** $p < 0.001$. †Remained significant after correction for multiple comparisons.

chi-square test. Continuous variables were expressed by median and interquartile range. Spearman's correlations were performed between the analyzed parameters and clinical variables (ESR, CRP, tender and swollen joint count, and DAS28). Baseline and follow-up values of each sample were compared using Wilcoxon's matched-pairs signed-rank test or paired t-test according to normal distribution. p value less than 0.05 was considered significant.

3. Results

3.1. Patient Background. Seventeen RA patients, evaluated before and after starting TNFi therapy, were included in this study. All patients were receiving methotrexate (10–20 mg weekly), 15 of whom were also under low dose prednisolone and 2 were additionally under bisphosphonates. These therapies had been introduced more than 6 months before TNFi was started and were stable over the study period. Patients were treated with one of four TNFi: one of the monoclonal antibodies (adalimumab, golimumab, or infliximab; 41%) or etanercept (59%). A blood sample was obtained before the start of TNFi and after at least 6 months of treatment. Thirteen patients (76%) were good responders to TNFi and 4 (24%) were moderate responders according to the EULAR response criteria [22]. Joint counts, ESR, CRP, DAS28, and HAQ were significantly decreased after TNFi therapy. The clinical and demographic characteristics of patients both at baseline and at follow-up are described in Table 1.

3.2. TNFi Treatment in RA Patients Decreases the Frequency of Circulating Osteoclast Precursors. After TNFi treatment, the frequency of the classical monocyte subpopulation ($CD14^{bright}CD16^{-}$) was decreased ($p = 0.0065$; Table 2) and

Table 3: Whole blood cell distribution and RANKL expression.

	Baseline	Follow-up	p value
Neutrophils (%)[a]	82 [71–91]	90 [84–091]	0.2662
Neutrophils ($\times 10^8$ cells/L)	12.7 [8.0–15.6]	9.6 [8.4–12.9]	0.2642
RANKL$^+$ neutrophils (%)[b]	22 [3–41]	53 [21–77]	0.0856
RANKL$^+$ neutrophils ($\times 10^8$ cells/L)	1.5 [0.3–4.3]	5.9 [1.8–7.1]	0.1475
Neutrophil RANKL MFI	33.2 [25.5–44.9]	24.1 [21.7–28]	0.0830
T-cells (%)[c]	62 [58–74]	68 [52–72]	0.5265
T-cells ($\times 10^8$ cells/L)	4.2 [2.4–5.2]	3.4 [2.4–11.7]	0.4131
RANKL$^+$ T-cells (%)[b]	6.2 [0.8–24]	6.7 [4.6–15.7]	0.8984
RANKL$^+$ T-cells ($\times 10^8$ cells/L)	0.30 [0.03–1.03]	0.20 [0.16–0.69]	0.7646
T-cell RANKL MFI	49 [41–55]	32 [25–53]	0.2061
B-cells (%)[c]	7.3 [4.8–14]	9.2 [4.9–15.0]	0.7364
B-cells ($\times 10^8$ cells/L)	0.40 [0.18–0.94]	0.44 [0.23–1.51]	0.9658
RANKL$^+$ B-cells (%)	4.7 [2.0–6.7]	14 [3–28]	0.0088**
RANKL$^+$ B-cells ($\times 10^8$ cells/L)[b]	0.02 [0.01–0.06]	0.06 [0.02–1.22]	0.0029**,†
B-cell RANKL MFI	48 [38–80]	30 [25–63]	0.0401*

Flow cytometry results are shown as median and interquartile range; [a]gated on granulocytes from whole blood; [b]gated on the correspondent parent gate (neutrophil, T- or B-cell); [c]gated on the nongranulocyte cells from whole blood (also called the "monolymph" gate). RANKL: receptor activator of NF-$\kappa\beta$ ligand; MFI: median fluorescence intensity (arbitrary units); $^{*}p < 0.05$, $^{**}p < 0.01$. †Remained significant after correction for multiple comparisons.

Table 4: Serum levels of bone turnover markers and bone metabolism proteins.

	Baseline	Follow-up	p value
sRANKL (pmol/L)	0.32 [0.21–0.67]	0.18 [0.11–0.35]	0.0085**
OPG (pmol/L)	4.34 [2.60–5.82]	4.22 [3.05–5.08]	0.7990
sRANKL/OPG	0.08 [0.04–0.17]	0.05 [0.03–0.07]	0.0031**,†
DKK1 (pmol/L)	25.5 [18.1–43.3]	26.4 [21.9–31.7]	1.000
Sclerostin (pmol/L)	25.2 [16.94–33.8]	25.2 [19.2–29.3]	0.8577
CTX-I (ng/mL)	194.6 [176.6–430.7]	163.6 [152.1–173.9]	0.0005***,†
P1NP (ng/mL)	55.7 [46.3–61.3]	45.8 [39.6–48.9]	0.0252*
CTX/P1NP	3.36 [3.09–3.82]	3.71 [3.34–4.30]	0.5590

Enzyme-linked immunosorbent assay results are shown as median and interquartile range. sRANKL: soluble receptor activator of NF-$\kappa\beta$ ligand; OPG: osteoprotegerin; DKK1: Dickkopf-related protein-1: CTX: carboxyterminal telopeptide of type I collagen; P1NP: total procollagen type 1 N-terminal propeptide; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. †Remained significant after correction for multiple comparisons.

that of the nonclassical subpopulation (CD14^{dim}CD16$^+$) was increased ($p = 0.0005$) [13]. No differences were identified in either CD51/CD61 ($\alpha_v\beta_3$ integrin) or RANK surface expression. After statistical correction for multiple comparisons, only the increase in the nonclassical subpopulation remained significant.

RANKL surface staining was performed in CD66b$^+$ neutrophils, CD3$^+$ T-cells, and CD19$^+$ B-cells (Table 3). No difference was found in the total number of circulating neutrophils and T- or B-cells after therapy. Although the frequency of RANKL$^+$ neutrophils or T-cells was not significantly different after treatment, both frequency and absolute number of RANKL$^+$ B-cells were higher after treatment ($p = 0.0088$ and 0.0029, resp.). However, B-cell RANKL surface expression was significantly decreased after treatment ($p = 0.0401$). When statistically corrected for multiple comparisons, the increase in RANKL$^+$ B-cells remained significant.

3.3. The sRANKL/OPG Ratio and CTX-I Circulating Levels Are Reduced in RA Patients after TNFi Treatment. Circulating levels of sRANKL were significantly decreased after TNFi ($p = 0.0085$; Table 4), leading to decreased sRANKL/OPG ratio ($p = 0.0031$). We found no differences in the circulating levels of DKK1 or SOST. CTX-I and P1NP levels were lower in patients at 6 months of follow-up, when compared to patients at baseline ($p = 0.0005$ and 0.0252, resp.), and no difference was found in the CTX-I/P1NP ratio. After correcting for multiple comparisons, the differences in sRANKL/OPG and CTX-I after treatment remained significant.

3.4. Osteoclast Differentiation and Activity in RA Patients Are Decreased after TNFi Treatment due to Decreased TNF Intracellular Signaling and Cathepsin K Expression. Under stimulating conditions, adhering precursors from patients treated with TNFi formed fewer OCs than adhering precursors from patients at baseline ($p = 0.0094$ at culture day 14, $p = 0.0203$ at culture day 21; Figure 1).

Although the number of resorption pits was not significantly different before and after treatment, the area resorbed per pit was significantly reduced in cultures from patients

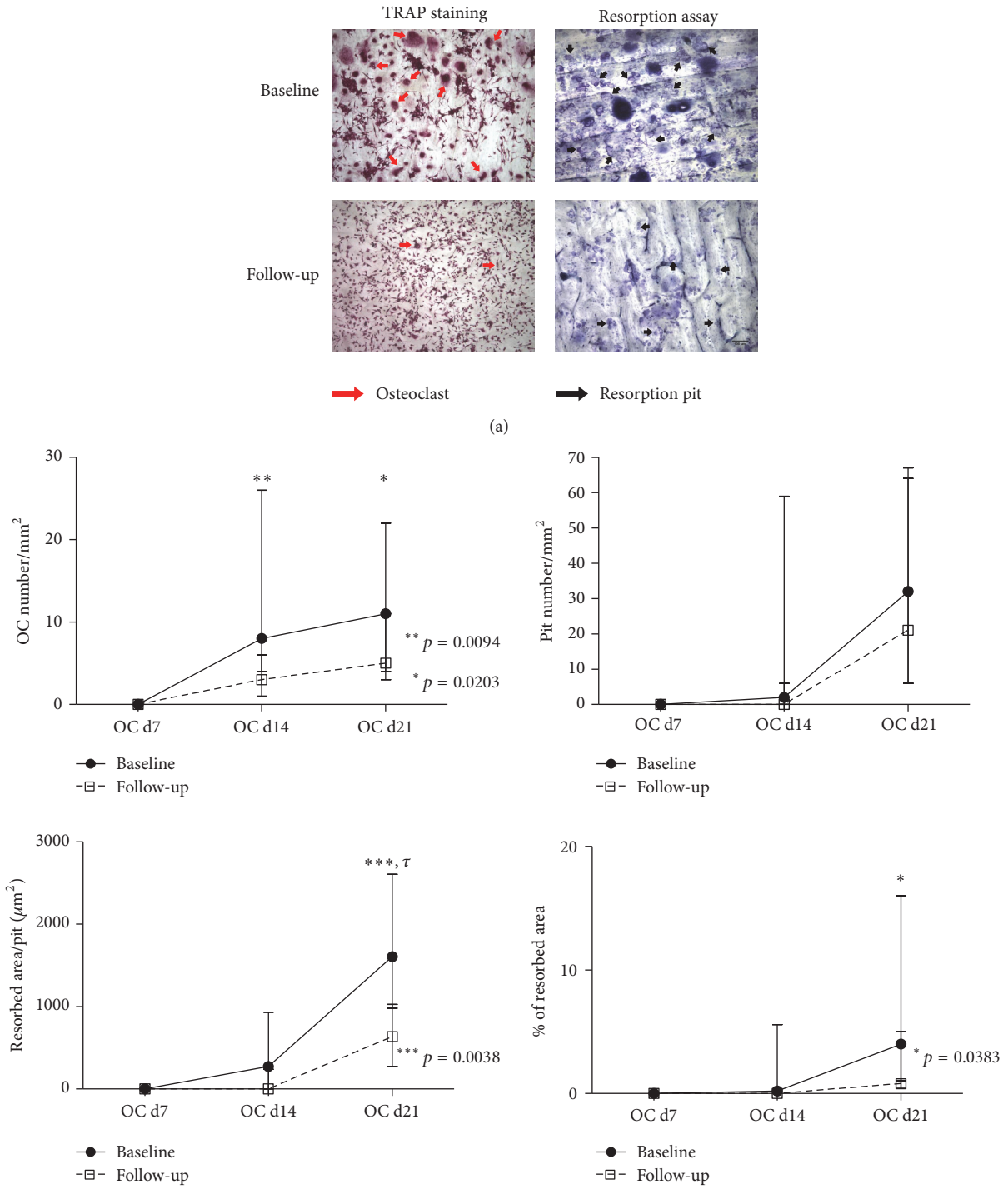

FIGURE 1: Functional assays of in vitro differentiated OC. (a) Representative images, at culture day 21, of adhering precursors stimulated with M-CSF, RANKL, dexamethasone, and TGF-β stained for TRAP, where the pit assay was performed. (b) OC number increased throughout time and, at culture days 14 and 21, patients at follow-up had significantly fewer osteoclasts than at baseline (p = 0.0094 and 0.0203, resp.). No differences were found in the number of resorption pits/mm^2; patients at follow-up had significantly smaller pits at culture day 21 (resorbed area/pit, p = 0.0038) and significantly less resorbed area at culture day 21, when compared to their baseline (p = 0.0383). Dots represent median counts for each group at each time point and bars represent interquartile range. d: day; OC: osteoclast. Scale bars: 100 μm; red arrows: osteoclasts; black arrows: resorption pits. τ: remained significant after adjusting for multiple comparisons.

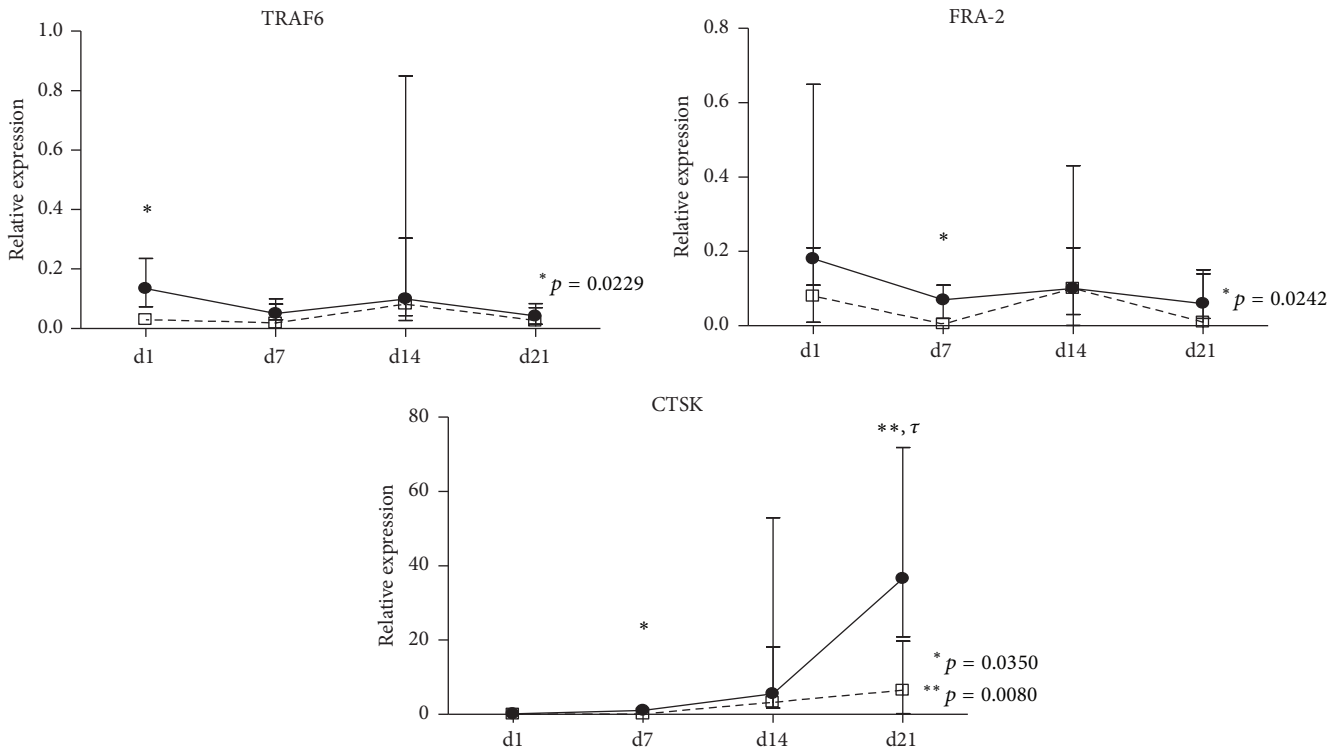

Figure 2: Gene expression profile of stimulated adhering precursors in culture for 21 days. At day 1, TRAF6 expression in patients at follow-up was significantly reduced ($p = 0.0229$). At day 7, both FRA-2 and CTSK expressions were significantly decreased ($p = 0.0242$ and 0.035, resp.). At day 21, patients at follow-up had significantly reduced expression when compared to patients at baseline ($p = 0.008$). Gene expression shown as a ratio to housekeeping expression ($2(-\Delta CT)/2(-\Delta CT)$). Dots in graphs represent median gene expression for each group at each time point and lines represent interquartile range [25–75]. d: day; TRAF6: gene encoding tumor necrosis factor receptor-associated factor-6; FRA-2: gene encoding Fos-related antigen-2; CTSK: gene encoding cathepsin K. τ: remained significant after adjusting for multiple comparisons.

at follow-up at culture day 21 ($p = 0.0038$), which resulted in significantly decreased total resorbed area ($p = 0.0383$). After statistical correction for multiple comparisons, only the differences in OC number at day 14 and the resorbed area per pit at day 21 remained significant.

Gene expression by RT-qPCR was performed for OC genes that are known to be important during the adhering precursors' differentiation and OC activity. At culture day 1, TRAF6 expression in patients at follow-up was significantly lower than in patients at baseline ($p = 0.0229$; Figure 2). At culture day 7, expression of both FRA-2 and CTSK was significantly decreased after TNFi treatment ($p = 0.0242$ and 0.0350, resp.). No differences were found in any of the studied genes at culture day 14, but at culture day 21 there was a significant decrease in CTSK expression in the differentiated OC from patients after treatment. This difference in CTSK expression remained significant after multiple comparisons adjustment.

No differences were found in any of the studied parameters when comparing monoclonal antibodies (adalimumab, infliximab, or golimumab) with the fusion protein etanercept (data not shown). No correlation was found between clinical or laboratorial inflammatory parameters for any of the studied variables.

4. Discussion

With this study, we aimed to test the effect of TNFi in the differentiation and activity of OC precursors in RA patients.

We have shown that RA patients treated with TNFi have reduced frequency of classic monocytes. We also found a decrease in the circulating levels of soluble RANKL and consequently a reduction in the sRANKL/OPG ratio after TNFi treatment. Although no differences in circulating levels of SOST or DKK1 were detected, serum CTX-I and P1NP levels were decreased after TNFi treatment, reflecting decreased bone turnover in these patients. Accordingly, we found that the ex vivo differentiation and resorptive activity of OC precursors from TNFi-treated patients were reduced, mainly due to early downregulation of TNF signaling proteins, such as TRAF6 or FRA-2, and to a later reduction of CTSK expression. Moreover, when comparing all studied parameters, we found no differences between the use of monoclonal antibodies (adalimumab, golimumab, and infliximab) and the fusion protein [23], suggesting that they have similar effects on OC precursors. Previous studies have compared the effects of different TNFi in disease activity, sRANKL/OPG ratio, and circulating leukocytes without finding significant differences [24, 25]. It has been previously reported that granulocyte numbers were reduced in circulation after 2 and

14 weeks of infliximab treatment [26]; however, this study identified granulocytes as $CD16^+$ cells instead of $CD66b^+$ cells. We found no significant differences in the frequency of neutrophils and T-lymphocytes or in RANKL surface expression in these cells, but we observed a significant increase in $RANKL^+$ B-lymphocytes accompanied by a decrease in RANKL surface expression. There have been a number of studies addressing the effect of TNFi in RA patients' peripheral lymphocytes; however, there is no consensus among different reports, mainly due to sampling differences. In 2005, Toubi and colleagues have shown that infliximab decreased apoptosis in Tregs of RA patients [27]. Other studies showed that short in vitro exposure of PBMCs to infliximab or etanercept had no effect in peripheral lymphocyte apoptosis [28] or in synovial membrane biopsies [29]. It has previously been shown that RA patients under TNFi have increased number of T-regulatory cells and a reduced number of T-effector cells [30]. Other studies showed that in TNFi-treated RA patients there were no changes in T-regulatory cells frequency [24] or in the frequency of total T-cells, monocytes, or granulocytes and only a transient unspecified effect on B-cells [31]. To our knowledge, a comparative study of RANKL expression in RA patients before and after therapy with TNFi has never been published.

Three monocytes subpopulations, based on their expression of CD14 and CD16 surface markers, have been described in humans [13]. In RA patients, it has been shown that the intermediate subpopulation is increased when compared to healthy donors [13] and apoptosis of local and peripheral monocytes/macrophages was also increased after etanercept or infliximab treatment [29, 32]. Another study has shown no differences in $CD14^{dim}$ or $CD14^{bright}$ subpopulations after 4 months of infliximab therapy [26]. In our cohort, 6 months after TNFi therapy, patients showed decreased classic ($CD14^{bright}CD16^-$) and increased nonclassical ($CD14^{dim}CD16^+$) subpopulations. These changes in frequency were accompanied by a nonsignificant decrease in CD51/CD61 ($\alpha_v\beta_3$ integrin) and RANK surface expression in all subsets. In accordance with our results, a recent study showed a reduction in classical monocyte subpopulation and an increase in the nonclassical subpopulation following infliximab therapy [33]. Moreover, Sprangers et al. observed that although nonclassical monocytes can also differentiate into OC, these cells have lower resorptive ability [34], which might explain why we did not observe bone resorption increase.

Patients under TNFi had reduced levels of sRANKL, sRANKL/OPG, CTX-I, and P1NP, suggesting a decrease in OC activity and a return to balanced coupling of bone resorption and bone formation. No differences were found in the circulating levels of DKK1 and SOST after TNFi treatment. Previous studies have shown discrepancies in the determination of these bone remodeling-associated proteins. Studies have found no differences in sRANKL or OPG serum levels after infliximab or etanercept [35, 36]. However, contradictory results have emerged regarding both OPG and sRANKL circulating levels after TNFi therapy [37, 38]. DKK1 and sclerostin have a direct effect on bone formation through interaction with the Wnt signaling pathway [39] but they have not been extensively studied in RA patients under TNFi. Previous reports have shown that etanercept has no effect on circulating levels of DKK1 but it increases sclerostin in circulation after treatment [35]. However, infliximab has been shown to decrease DKK1 levels in patients responding to therapy [40]. It has been previously shown that TNFi have a beneficial effect, reducing radiographic damage in RA patients, even in the absence of clinical improvement [9, 41]. Reports have described a decrease in CTX-I or urinary markers of bone resorption after TNFi therapy [35, 42]. However, some discrepancies have been found when studying bone formation markers. Studies with etanercept and infliximab showed no alteration in circulating P1NP levels after treatment [35, 42], while another study with etanercept showed reduced levels of urinary bone formation markers [43].

Although the classical monocyte subpopulation has been considered the OC precursor subset, all three subpopulations can differentiate in vitro into OC [44]. To understand the effect of TNFi in OC differentiation and function, we isolated PBMCs from RA patients before and after TNFi treatment and cultured them in vitro over bone slices. After TNFi treatment, we found a decrease in OC number and both in the total resorbed area and in the average resorbed area per pit. No differences in pit number and in the number of nuclei/OCs, aspects associated with OC activity, were identified [45]. These observations suggest that TNFi reduces the number and mobility of OCs.

Complex in vivo studies with animal models also showed that infliximab and etanercept reduced the bone resorbed area [46, 47] and etanercept decreased $\alpha_v\beta_3$ integrin expression [48]. In a study similar to ours, Gengenbacher and colleagues studied RA patients under infliximab therapy for 6 months and observed decreased pit number after in vitro cell culture in OC differentiating conditions [36]. There have been reports that infliximab inhibits directly (in vitro) murine and human OC formation [49, 50]. Other authors show that although TNFi reduce the number of murine pre-OCs in vitro, there is no effect in the total number of formed OCs [51]. Another study has shown that infliximab directly inhibits OC formation in high density healthy PBMC cultures without any further stimuli [52]. Etanercept was also shown to inhibit in vitro OC formation induced by M-CSF and IL-23 from healthy subjects [53]. Controversially, Takita and colleagues cultured PBMCs from RA patients, exposing them to M-CSF, RANKL, and infliximab in vitro, and observed that infliximab increased bone resorption when compared to M-CSF and RANKL alone [54].

There is evidence that TNF contributes to expression of specific OC proteins and that it directly activates OC differentiation through cross activation of the NF-κB pathway or c-Jun N-terminal kinase (JNK) signaling cascade [55]. We were interested in understanding the underlying mechanisms of reduced OC formation and bone resorption after TNFi, so we conducted gene expression assays and observed that OC precursors from RA patients after TNFi exposure had decreased expression of TRAF6 at culture day 1, followed by a reduction of FRA-2 and CTSK at day 7, and finally decreased expression of CTSK at culture day 21, when

compared to patients before TNFi exposure. RANK/RANKL signaling cross-talks with TNF signaling, as RANK is a TNF-superfamily member [56]. Upon activation, both RANK and TNF activate cytoplasmic kinases and adaptor proteins, including TRAF6, which further activate FRA-2 [57]. FRA-2 is a protein that when associated with Fos and AP-1 promotes the transcription of OC differentiating genes, including CTSK [58]. In TNFi-treated patients, we have observed not only a decrease in serum CTX-I (cleaved by CTSK), but also a reduction in CTSK expression after adhering precursors differentiation in vitro, as well as a decline in the resorbed area/OC. This has previously been observed in a RA patient with concomitant pycnodysostosis, an autosomal recessive mutation in the cathepsin K gene characterized by absence of this enzyme. Osteoclasts from these patients form very small resorbing pits and do not release CTX-I into the culture media [59].

The main limitations of this work were the lack of healthy controls and the reduced number of patients and the diversity of TNF blockers studied, which we tried to overcome by studying the same patient before and after therapy.

Taken together with the results found in the literature, these findings suggest that TNFi decrease bone resorption, independently of the control of disease activity. We propose that this is due to the direct reduction of OC classical precursors and downregulation of intracellular signaling pathways involving TRAF6 resulting in a reduction of CTSK expression and consequent lack of OC motility. Further investigation of the signaling pathways involving TRAF6, such as the ASK1-TRAF6 interaction, is of clear interest in this context.

Additional Points

Key Messages. (i) TNFi decrease bone resorption through the direct reduction of OC precursor numbers. (ii) TNFi downregulate the intracellular signaling pathways involving TRAF6 resulting in a reduction of CTSK expression and consequent lack of OC motility.

Disclosure

The opinions expressed in this paper are those of the authors and do not necessarily represent those of Merck Sharp & Dohme Corp. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

The authors declare no competing interests regarding the publication of this paper.

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References

[1] G. S. Firestein, "Evolving concepts of rheumatoid arthritis," *Nature*, vol. 423, no. 6937, pp. 356–361, 2003.

[2] G. Schett, S. Hayer, J. Zwerina, K. Redlich, and J. S. Smolen, "Mechanisms of disease: the link between RANKL and arthritic bone disease," *Nature Clinical Practice Rheumatology*, vol. 1, no. 1, pp. 47–54, 2005.

[3] G. Schett, "Review: immune cells and mediators of inflammatory arthritis," *Autoimmunity*, vol. 41, no. 3, pp. 224–229, 2008.

[4] R. Cascão, R. A. Moura, I. Perpétuo et al., "Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis," *Arthritis Research & Therapy*, vol. 12, no. 5, article R196, 2010.

[5] H. Appel, C. Loddenkemper, and P. Miossec, "Rheumatoid arthritis and ankylosing spondylitis—pathology of acute inflammation," *Clinical and Experimental Rheumatology*, vol. 27, no. 4, supplement 55, pp. S15–S19, 2009.

[6] G. P. Eng, P. Bouchelouche, E. M. Bartels et al., "Anti-drug antibodies, drug levels, interleukin-6 and soluble TNF receptors in rheumatoid arthritis patients during the first 6 months of treatment with adalimumab or infliximab: a descriptive Cohort Study," *PLoS ONE*, vol. 11, no. 9, Article ID e0162316, 2016.

[7] M. Takeshita, K. Suzuki, J. Kikuchi et al., "Infliximab and etanercept have distinct actions but similar effects on cytokine profiles in rheumatoid arthritis," *Cytokine*, vol. 75, no. 2, pp. 222–227, 2015.

[8] S. M. Zivojinovic, N. N. Pejnovic, M. N. Sefik-Bukilica, L. V. Kovacevic, I. I. Soldatovic, and N. S. Damjanov, "Tumor necrosis factor blockade differentially affects innate inflammatory and Th17 cytokines in rheumatoid arthritis," *Journal of Rheumatology*, vol. 39, no. 1, pp. 18–21, 2012.

[9] M. Hoff, T. K. Kvien, J. Kälvesten, A. Elden, A. Kavanaugh, and G. Haugeberg, "Adalimumab reduces hand bone loss in rheumatoid arthritis independent of clinical response: subanalysis of the PREMIER study," *BMC Musculoskeletal Disorders*, vol. 12, article 54, 2011.

[10] D. Aletaha, T. Neogi, A. J. Silman et al., "2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against Rheumatism collaborative initiative," *Arthritis and Rheumatism*, vol. 62, no. 9, pp. 2569–2581, 2010.

[11] M. C. Hochberg, R. W. Chang, I. Dwosh, S. Lindsey, T. Pincus, and F. Wolfe, "The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 35, no. 5, pp. 498–502, 1992.

[12] I. P. Perpétuo, R. Raposeiro, J. Caetano-Lopes et al., "Effect of tumor necrosis factor inhibitor therapy on osteoclasts precursors in ankylosing spondylitis," *PLoS ONE*, vol. 10, no. 12, Article ID e0144655, 2015.

[13] K. L. Wong, W. H. Yeap, J. J. Y. Tai, S. M. Ong, T. M. Dang, and S. C. Wong, "The three human monocyte subsets: implications for health and disease," *Immunologic Research*, vol. 53, no. 1–3, pp. 41–57, 2012.

[14] G. Furneri, L. G. Mantovani, A. Belisari et al., "Systematic literature review on economic implications and pharmacoeconomic issues of rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 30, no. 4, supplement 73, pp. S72–S84, 2012.

[15] M. Husheem, J. K. E. Nyman, J. Vääräniemi, H. K. Vaananen, and T. A. Hentunen, "Characterization of circulating human osteoclast progenitors: development of in vitro resorption assay," *Calcified Tissue International*, vol. 76, no. 3, pp. 222–230, 2005.

[16] L. K. Osier, S. N. Popoff, and S. C. Marks Jr., "Osteopetrosis in the toothless rat: failure of osteoclast differentiation and function," *Bone and Mineral*, vol. 3, no. 1, pp. 35–45, 1987.

[17] N. Kurihara, T. Suda, Y. Miura et al., "Generation of osteoclasts from isolated hematopoietic progenitor cells," *Blood*, vol. 74, no. 4, pp. 1295–1302, 1989.

[18] T. R. Arnett and D. W. Dempster, "A comparative study of disaggregated chick and rat osteoclasts in vitro: effects of calcitonin and prostaglandins," *Endocrinology*, vol. 120, no. 2, pp. 602–608, 1987.

[19] J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden, "Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction," *BMC Bioinformatics*, vol. 13, article 134, 2012.

[20] M. L. Wong and J. F. Medrano, "Real-time PCR for mRNA quantitation," *BioTechniques*, vol. 39, no. 1, pp. 75–85, 2005.

[21] J. Caetano-Lopes, A. Rodrigues, A. Lopes et al., "Rheumatoid arthritis bone fragility is associated with upregulation of IL17 and DKK1 gene expression," *Clinical Reviews in Allergy and Immunology*, vol. 47, no. 1, pp. 38–45, 2014.

[22] J. Fransen and P. L. C. M. van Riel, "The Disease Activity Score and the EULAR response criteria," *Clinical and Experimental Rheumatology*, vol. 23, no. 5, supplement 39, pp. S93–S99, 2005.

[23] D. van der Heijde, G. Burmester, J. Melo-Gomes et al., "Inhibition of radiographic progression with combination etanercept and methotrexate in patients with moderately active rheumatoid arthritis previously treated with monotherapy," *Annals of the Rheumatic Diseases*, vol. 68, no. 7, pp. 1113–1118, 2009.

[24] C. Blache, T. Lequerré, A. Roucheux et al., "Number and phenotype of rheumatoid arthritis patients' $CD4^{+}CD25^{hi}$ regulatory T cells are not affected by adalimumab or etanercept," *Rheumatology*, vol. 50, no. 10, Article ID ker183, pp. 1814–1822, 2011.

[25] A. I. Catrina, E. Af Klint, S. Ernestam et al., "Anti-tumor necrosis factor therapy increases synovial osteoprotegerin expression in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 1, pp. 76–81, 2006.

[26] L. R. Coulthard, J. Geiler, R. J. Mathews et al., "Differential effects of infliximab on absolute circulating blood leucocyte counts of innate immune cells in early and late rheumatoid arthritis patients," *Clinical & Experimental Immunology*, vol. 170, no. 1, pp. 36–46, 2012.

[27] E. Toubi, A. Kessel, Z. Mahmudov, K. Hallas, M. Rozenbaum, and I. Rosner, "Increased spontaneous apoptosis of $CD4^{+}CD25^{+}$ T cells in patients with active rheumatoid arthritis is reduced by infliximab," *Annals of the New York Academy of Sciences*, vol. 1051, pp. 506–514, 2005.

[28] A. I. Catrina, C. Trollmo, E. Af Klint et al., "Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report," *Arthritis and Rheumatism*, vol. 52, no. 1, pp. 61–72, 2005.

[29] C. A. Wijbrandts, P. H. Remans, P. L. Klarenbeek et al., "Analysis of apoptosis in peripheral blood and synovial tissue very early after initiation of infliximab treatment in rheumatoid arthritis patients," *Arthritis and Rheumatism*, vol. 58, no. 11, pp. 3330–3339, 2008.

[30] Z. Huang, B. Yang, Y. Shi et al., "Anti-TNF-α therapy improves treg and suppresses teff in patients with rheumatoid arthritis," *Cellular Immunology*, vol. 279, no. 1, pp. 25–29, 2012.

[31] L. W. Moreland, R. P. Bucy, M. E. Weinblatt, K. M. Mohler, G. T. Spencer-Green, and W. W. Chatham, "Immune function in patients with rheumatoid arthritis treated with etanercept," *Clinical Immunology*, vol. 103, no. 1, pp. 13–21, 2002.

[32] T. J. M. Smeets, M. C. Kraan, M. E. van Loon, and P.-P. Tak, "Tumor necrosis factor α blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue," *Arthritis & Rheumatism*, vol. 48, no. 8, pp. 2155–2162, 2003.

[33] D. Aeberli, R. Kamgang, D. Balani, W. Hofstetter, P. M. Villiger, and M. Seitz, "Regulation of peripheral classical and non-classical monocytes on infliximab treatment in patients with rheumatoid arthritis and ankylosing spondylitis," *RMD Open*, vol. 2, no. 1, Article ID e000079, 2016.

[34] S. Sprangers, T. Schoenmaker, Y. Cao, V. Everts, and T. J. de Vries, "Different blood-borne human osteoclast precursors respond in distinct ways to IL-17A," *Journal of Cellular Physiology*, vol. 231, no. 6, pp. 1249–1260, 2016.

[35] M. J. Lim, S. R. Kwon, K. Joo, M. J. Son, S.-G. Park, and W. Park, "Early effects of tumor necrosis factor inhibition on bone homeostasis after soluble tumor necrosis factor receptor use," *Korean Journal of Internal Medicine*, vol. 29, no. 6, pp. 807–813, 2014.

[36] M. Gengenbacher, H.-J. Sebald, P. M. Villiger, W. Hofstetter, and M. Seitz, "Infliximab inhibits bone resorption by circulating osteoclast precursor cells in patients with rheumatoid arthritis and ankylosing spondylitis," *Annals of the Rheumatic Diseases*, vol. 67, no. 5, pp. 620–624, 2008.

[37] I. González-Alvaro, A. M. Ortiz, E. G. Tomero et al., "Baseline serum RANKL levels may serve to predict remission in rheumatoid arthritis patients treated with TNF antagonists," *Annals of the Rheumatic Diseases*, vol. 66, no. 12, pp. 1675–1678, 2007.

[38] M. Ziolkowska, M. Kurowska, A. Radzikowska et al., "High levels of osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in serum of rheumatoid arthritis patients and their normalization after anti–tumor necrosis factor α treatment," *Arthritis and Rheumatism*, vol. 46, no. 7, pp. 1744–1753, 2002.

[39] C.-G. Miao, Y.-Y. Yang, X. He et al., "Wnt signaling pathway in rheumatoid arthritis, with special emphasis on the different roles in synovial inflammation and bone remodeling," *Cellular Signalling*, vol. 25, no. 10, pp. 2069–2078, 2013.

[40] S.-Y. Wang, Y.-Y. Liu, H. Ye et al., "Circulating dickkopf-1 is correlated with bone erosion and inflammation in rheumatoid arthritis," *Journal of Rheumatology*, vol. 38, no. 5, pp. 821–827, 2011.

[41] B. Seriolo, S. Paolino, A. Sulli, and M. Cutolo, "Are there any positive effects of TNF-α blockers on bone metabolism?" *Reumatismo*, vol. 58, no. 3, pp. 199–205, 2006.

[42] F. Chopin, P. Garnero, A. Le Henanff et al., "Long-term effects of infliximab on bone and cartilage turnover markers in patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 67, no. 3, pp. 353–357, 2008.

[43] K. Yasunori, T. Masaaki, N. Tetsuyuki, K. Hayato, and N. Akira, "Reduction of urinary levels of pyridinoline and deoxypyridinoline and serum levels of soluble receptor activator of NF-kappaB ligand by etanercept in patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 27, no. 9, pp. 1093–1101, 2008.

[44] J. Costa-Rodrigues, A. Fernandes, and M. H. Fernandes, "Spontaneous and induced osteoclastogenic behaviour of human peripheral blood mononuclear cells and their $CD14^+$ and $CD14^-$ cell fractions," *Cell Proliferation*, vol. 44, no. 5, pp. 410–419, 2011.

[45] K. Piper, A. Boyde, and S. J. Jones, "The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro," *Anatomy and Embryology*, vol. 186, no. 4, pp. 291–299, 1992.

[46] H. Matsuno, K. Yoshida, A. Ochiai, and M. Okamoto, "Requirement of methotrexate in combination with anti-tumor necrosis factor-alpha therapy for adequate suppression of osteoclastogenesis in rheumatoid arthritis," *Journal of Rheumatology*, vol. 34, no. 12, pp. 2326–2333, 2007.

[47] L. M. Childs, J. J. Goater, R. J. O'Keefe, and E. M. Schwarz, "Efficacy of etanercept for wear debris-induced osteolysis," *Journal of Bone and Mineral Research*, vol. 16, no. 2, pp. 338–347, 2001.

[48] S. Y. A. Terry, M. I. Koenders, G. M. Franssen et al., "Monitoring therapy response of experimental arthritis with radiolabeled tracers targeting fibroblasts, macrophages, or integrin $\alpha_v\beta_3$," *Journal of Nuclear Medicine*, vol. 57, no. 3, pp. 467–472, 2016.

[49] T. Yago, Y. Nanke, N. Ichikawa et al., "IL-17 induces osteoclastogenesis from human monocytes alone in the absence of osteoblasts, which is potently inhibited by anti-TNF-α antibody: a novel mechanism of osteoclastogenesis by IL-17," *Journal of Cellular Biochemistry*, vol. 108, no. 4, pp. 947–955, 2009.

[50] C.-K. Lee, E. Y. Lee, S. M. Chung, S. H. Mun, B. Yoo, and H.-B. Moon, "Effects of disease-modifying antirheumatic drugs and antiinflammatory cytokines on human osteoclastogenesis through interaction with receptor activator of nuclear factor κB, osteoprotegerin, and receptor activator of nuclear factor κB ligand," *Arthritis & Rheumatism*, vol. 50, no. 12, pp. 3831–3843, 2004.

[51] N. B. Binder, A. Puchner, B. Niederreiter et al., "Tumor necrosis factor-inhibiting therapy preferentially targets bone destruction but not synovial inflammation in a tumor necrosis factor-driven model of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 65, no. 3, pp. 608–617, 2013.

[52] T. J. de Vries, J. Yousovich, T. Schoenmaker, N. Scheres, and V. Everts, "Tumor necrosis factor-α antagonist infliximab inhibits osteoclast formation of peripheral blood mononuclear cells but does not affect periodontal ligament fibroblast-mediated osteoclast formation," *Journal of Periodontal Research*, vol. 51, no. 2, pp. 186–195, 2016.

[53] T. Yago, Y. Nanke, M. Kawamoto et al., "IL-23 induces human osteoclastogenesis via IL-17 in vitro, and anti-IL-23 antibody attenuates collagen-induced arthritis in rats," *Arthritis Research and Therapy*, vol. 9, no. 5, article R96, 2007.

[54] C. Takita, Y. Fujikawa, I. Itonaga, H. Taira, M. Kawashima, and T. Torisu, "Infliximab acts directly on human osteoclast precursors and enhances osteoclast formation induced by receptor activator of nuclear factor κB ligand in vitro," *Modern Rheumatology*, vol. 15, no. 2, pp. 97–103, 2005.

[55] K. Kobayashi, N. Takahashi, E. Jimi et al., "Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction," *Journal of Experimental Medicine*, vol. 191, no. 2, pp. 275–286, 2000.

[56] B. Osta, G. Benedetti, and P. Miossec, "Classical and paradoxical effects of TNF-α on bone homeostasis," *Frontiers in Immunology*, vol. 5, article 48, 2014.

[57] S. L. Teitelbaum and F. P. Ross, "Genetic regulation of osteoclast development and function," *Nature Reviews Genetics*, vol. 4, no. 8, pp. 638–649, 2003.

[58] A. Bozec, L. Bakiri, A. Hoebertz et al., "Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia," *Nature*, vol. 454, no. 7201, pp. 221–225, 2008.

[59] M. Ainola, H. Valleala, P. Nykänen, J. Risteli, R. Hanemaaijer, and Y. T. Konttinen, "Erosive arthritis in a patient with pycnodysostosis: an experiment of nature," *Arthritis and Rheumatism*, vol. 58, no. 11, pp. 3394–3401, 2008.

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Research Article

PEDF Is Associated with the Termination of Chondrocyte Phenotype and Catabolism of Cartilage Tissue

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Objective. To investigate the expression and target genes of pigment epithelium-derived factor (PEDF) in cartilage and chondrocytes, respectively. *Methods.* We analyzed the expression pattern of PEDF in different human cartilaginous tissues including articular cartilage, osteophytic cartilage, and fetal epiphyseal and growth plate cartilage, by immunohistochemistry and quantitative real-time (qRT) PCR. Transcriptome analysis after stimulation of human articular chondrocytes with rhPEDF was performed by RNA sequencing (RNA-Seq) and confirmed by qRT-PCR. *Results.* Immunohistochemically, PEDF could be detected in transient cartilaginous tissue that is prone to undergo endochondral ossification, including epiphyseal cartilage, growth plate cartilage, and osteophytic cartilage. In contrast, PEDF was hardly detected in healthy articular cartilage and in the superficial zone of epiphyses, regions that are characterized by a permanent stable chondrocyte phenotype. RNA-Seq analysis and qRT-PCR demonstrated that rhPEDF significantly induced the expression of a number of matrix-degrading factors including SAA1, MMP1, MMP3, and MMP13. Simultaneously, a number of cartilage-specific genes including COL2A1, COL9A2, COMP, and LECT were among the most significantly downregulated genes. *Conclusions.* PEDF represents a marker for transient cartilage during all neonatal and postnatal developmental stages and promotes the termination of cartilage tissue by upregulation of matrix-degrading factors and downregulation of cartilage-specific genes. These data provide the basis for novel strategies to stabilize the phenotype of articular cartilage and prevent its degradation.

1. Introduction

Pigment epithelium-derived factor (PEDF), also known as Serpin F1 (SERPINF1), was originally isolated from human fetal retinal pigment epithelial cells and was defined as a potent antiangiogenic factor [1, 2]. PEDF is also expressed in a multitude of other tissues and represents a multifunctional protein that also includes neurotrophic, neuroprotective, and antitumorigenic properties [3–7]. PEDF is also present in the skeletal system, in particular in regions undergoing endochondral ossification and bone remodeling [5, 8, 9]. During mouse development, PEDF is expressed in chondrocytes within epiphyseal growth plate cartilage [10]. PEDF could also be found in the growth zone of the deer antler [11]. While healthy articular cartilage typically lacks PEDF, it is detectable in osteoarthritic cartilage [9, 12]. High levels of PEDF could also be detected in osteophytes [9], which suggests a role of PEDF in terminal chondrocyte differentiation and the endochondral ossification process. Recently, mutations of the SERPINF1-gene resulting in loss-of-function of PEDF cause the recessive osteogenesis imperfecta type IV that is characterized by a severe skeletal phenotype with increased risk of fractures and skeletal deformities [13–15]. But the role of PEDF within skeletal tissues is still not clear. PEDF was suggested to support mineral deposition by induction of osteoblastic genes [16]. In osteosarcoma cells and mesenchymal progenitor cells, PEDF was shown to exert antiangiogenic and proapoptotic effects involving upregulation of

FasL [9, 17]. However, differentiated chondrocytes were not permissive to PEDF-induced apoptosis [9], which suggests further functions of PEDF within the skeletal tissue.

Thus, the aim of this study was to investigate the functional role and involved genes of PEDF in cartilaginous tissue, in particular during the process of endochondral ossification. Therefore, we focused on the distribution of PEDF within different human cartilaginous tissues of different developmental stages that are subjected to endochondral ossification, including the fetal epiphysis, the postnatal growth plate, and adult articular and osteophytic cartilage. As a reference, MMP13 expression served as marker for endochondral ossification. Transcriptome analyses using RNA sequencing (RNA-Seq) and bioinformatics pathway analyses based on PEDF-stimulated human articular chondrocytes were performed to define potential target genes and downstream-pathways activated by PEDF.

2. Methods

2.1. Tissue Samples. Human epiphyses (20-week gestation) and postnatal epiphyseal growth plate cartilage were obtained from donors at autopsy. Human adult articular cartilage as well as osteochondral osteophyte samples was obtained as matched pairs from the same knee joints of 10 patients undergoing total knee arthroplasty for osteoarthritis (mean age 75.5 years, range 70–82 years). The diagnosis of primary osteoarthritis was based on clinical and radiographic evaluations according to standard criteria. Secondary osteoarthritis or rheumatoid arthritis was excluded from this study. For mRNA expression analysis, macroscopically intact articular cartilage (AC) was isolated from osteochondral specimen originating from the resected femoral condyles with a macroscopically intact joint surface characterized by an Outerbridge score of 0 or 1. Osteophytic cartilage (OC), as well as periosteal mesenchyme (PM) and subchondral bone (B), were isolated from osteochondral samples of the same respective joints originating from the edges of the femoral condyles. Only joints with distinct osteophyte formation were used for this study. Osteophytes were distinguished from the marginal transition area of the joint surface by the existence of a concave ridge towards the joint surface.

For protein isolation and mRNA-analysis, articular cartilage, osteophytic cartilage, periosteal mesenchyme, and bone were dissected and isolated. In order to distinguish between articular cartilage and to selectively isolate the cartilaginous cap of the osteophytes and to exclude any abrasion of bone trabeculae and calcified tissue of the deepest cartilaginous zone, only minimal forces were applied with a scalpel by cutting tangentially to the surface to yield thin cartilaginous osteophyte slices of less than 1 mm thickness. Each patient gave informed consent prior to surgery, and the institutional ethics committee approved the study (Ref. number 3555).

2.2. Immunohistological Analysis. Specimens of human fetal epiphyses, human fetal epiphyseal growth plates, and human adult osteochondral section from the edges of the femoral condyles, containing periosteal mesenchyme, osteophyte cartilage, articular cartilage and subchondral bone, were used for histological analysis. All osteochondral specimens were fixed in 4% paraformaldehyde for 12 hours, followed by decalcification in 0.5 M ethylene diamine tetraacetic acid (EDTA) for 3 months. After standard processing, the samples were embedded in paraffin. Specimens were cut in serial transverse 5 μm sections and stained with toluidine blue (TB) or hematoxylin/eosin (H/E) for morphological assessment.

For immunohistochemical detection of PEDF, sections were pretreated with 0.05% trypsin and incubated with goat anti-human PEDF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). For detection of MMP13, sections were pretreated with 0.05 U/ml Chondroitinase (Sigma-Aldrich, Taufkirchen, Germany) and incubated with mouse anti-human MMP13 antibodies (Calbiochem, Darmstadt, Germany). Negative control sections were incubated with isotype normal mouse IgG (Santa Cruz Biotechnology). The sections were incubated with biotinylated anti-goat or anti-mouse secondary antibodies (Dianova, Hamburg, Germany), respectively. Bound antibodies were visualized by a complex of streptavidin and biotinylated alkaline phosphatase (Vectastain, ABC-AP, Vector Laboratories, Burlingame, CA). The sections were developed with fast red and counterstained with hematoxylin. Immunohistochemical detection of type I and type II collagen was performed as previously described in detail [18].

2.3. Immunoblot Analysis. Protein extracts were obtained from articular cartilage, osteophyte cartilage, and bone as described previously [19]. After boiling, 50 μg protein extracts were separated by sodium dodecyl sulfate (SDS) PAGE on a 10% gel and transferred on nitrocellulose transfer membranes. The efficiency of protein transfer was controlled by Ponceau S staining. After blocking, the nitrocellulose membranes were incubated with a monoclonal mouse anti-PEDF antibody diluted 1 : 1250 (Abcam, Cambridge, UK) or a rat anti-α-tubulin antibody (diluted 1 : 1000) (AbD Serotec, Kidlington, UK) overnight at 4°C. Horseradish peroxidase-conjugated goat anti-mouse antibodies (GE Healthcare, Little Chalfont, UK) or goat anti-rat antibodies (Dianova, Hamburg, Germany) (diluted 1 : 10000) were used as secondary antibodies. The immunoreactive proteins were visualized using a chemiluminescence kit (Roche, Mannheim, Germany) followed by exposure to a chemiluminescent detection film.

2.4. Cell Culture. Human primary chondrocytes were isolated from intact knee cartilage (Outerbridge score 0 or 1) from 6 different donors as described previously [19]. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Germany) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 0.1% amphotericin.

2.5. Transcriptome Analysis (RNA-Seq). Prior to RNA-Seq analysis, dose-finding studies were performed, which revealed a concentration-dependent increase in target gene expression (MMP3) from 50 to 250 ng/ml recombinant human PEDF (Biolegend, San Diego, CA) (Suppl. Figure 1 in

Supplementary Material available online at https://doi.org/10.1155/2017/7183516). Therefore, we used 250 ng/ml PEDF as the most effective concentration of PEDF for further stimulation experiments.

Articular chondrocytes from 3 different human adult donors were independently stimulated with 250 ng/ml rhPEDF. RNA from stimulated cells and nonstimulated control cells was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The quality of RNA of PEDF-stimulated cells and control cells was assessed using a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). Barcoded RNA sequencing libraries were prepared from 100 ng total RNA using TruSeq® Stranded mRNA Sample Preparation Kit, according to the manufacturer's instructions (Illumina, San Diego, CA). To control sources of variation during sample and data processing, a common set of external spike-in RNA controls from Ambion, developed by the external RNA controls consortium (ERCC), was used (Life Technologies, Carlsbad, CA).

Libraries were subjected to single-end sequencing (101 bp) on a HighSeq-2500 platform (Illumina). Quality filtering was performed using cutadapt v. 1.9.1; then reads mapping to rRNAs, tRNAs, snRNAs, and interspersed repeats were filtered out by performing alignment against a manually curated filter reference file using bwa-mem v.0.7.14-r1136 and keeping only unmapped reads. Subsequently, these reads were mapped against the hg19 reference genome using the STAR aligner v. 2.4.0j [20] and a STAR genome directory created by supplying an Ensembl gtf annotation file (genebuild 2013-09) for hg19. Absolute read counts per gene were obtained using Subread's feature Counts program v. 1.4.6-p2 [21] and the Ensembl gtf annotation file.

The following analyses were performed using R version 3.2.1. In particular, differential expression analysis was performed with the DESeq2 package v. 1.8.1 [22]. DESeq2 models count data by means of a negative binomial distribution and employs a Wald test for hypothesis testing. Differences in gene expression were considered statistically significant if their Benjamini-Hochberg adjusted p value was less than 0.1. Functional annotation analysis for genes significantly differentially expressed following rhPEDF stimulation and with fold difference in expression between the two sample groups ≥ 2 was performed using Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA) and Gene Set Enrichment Analysis (GSEA) (Broad Institute; http://www.broadinstitute.org/gsea) [23]. In order to assess robustness of our results, we have reanalyzed our data using edgeR. While DESeq2 identified 1134 differentially expressed genes (adj $p < 0.1$), edgeR identified 1240 differentially expressed genes, 946 of which overlapped between the two analyses. Comparison of the quantile ranks of fold changes revealed a very high correlation between both analytical tools, yielding Spearman's R^2 of 0.94 for all genes and 0.98 for significantly regulated genes (Suppl. Figure 2 a, b).

2.6. Quantitative Reverse-Transcription PCR (qRT-PCR). Quantitative mRNA expression was analyzed in human articular cartilage tissue, periosteal mesenchyme, osteophyte cartilage, and bone. qRT-PCR was also performed in isolated human articular chondrocytes after stimulation with 250 ng/ml rhPEDF. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Gene expression levels were quantified by qRT-PCR using the QuantStudio 12K Flex Real-time PCR System and TaqMan RNA-to-Ct 1-Step Kit (Life Technologies, Carlsbad, CA). TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used for, Serum Amyloid A1 (SAA1) (Hs00761940_s1), interleukin-6 (IL-6) (Hs00174131_m1), matrix metalloproteinase-1 (MMP1) (Hs00233958_m1), matrix metalloproteinase-3 (MMP3) (Hs00233962_m1), matrix metalloproteinase-13 (MMP13) (Hs00233992_m1), vascular endothelial growth factor-A (VEGFA) (forward primer: 5′-GGGCAGAATCATCACGAAGTG-3′; reverse primer: 5′-GGTCTCGATTGGATGGCAGTA-3′; probe 5′-TGAAGTTCATGGATGTCTATCAGCGCAG-3′), sex determining region Y-box 9 (Sox9) (Hs00165814_m1), NKX3-2 (forward primer: 5′-CCAAGAAGGTGGCCGTAAAG-3′; reverse primer: 5′-ACTTCGCCGGGCAGGTAT-3′; probe 5′-TGGTGCGCGACGACCAGAG-3′), aggrecan core protein (ACAN) (Hs00153936_m1), sex determining region Y-box 5 (Sox5) (Hs00374709_m1), chondromodulin-1 (LECT1) (Hs00170877_m1), cartilage oligomeric matrix protein (COMP) (Hs00164359_m1Hs), collagen type II, alpha1 (Col2A1) (Hs00156568_m1), and β2-microglobulin (β2M) (Hs00984230_m1). The relative quantification of gene expression was performed by the standard curve method. For each sample, the relative amount of the target mRNA was determined and normalized to β2M.

2.7. Cell Pellet Culture. After passaging, human articular chondrocytes ($4 \times 10e5$) from three different donors were cultivated independently at 1% O_2 in DMEM supplemented with 10 mM β-glycerophosphate, 1 nM dexamethasone, and 0.05 mmol/l ascorbate-P-phosphate in monolayer. After 24 h, the cells were stimulated with 250 ng/ml recombinant PEDF. Another 24 h later, the cells were released from monolayer and centrifuged at 2500 rpm for 5 min and left in conical tubes to form pellets. The cells were stimulated again with 250 ng/ml PEDF at day 3 and 7 of pellet culture. The pellets were fixed and stained at day 21 of the pellet culture.

2.8. Statistical Analysis. All data are presented as mean ± SD. Statistical analysis was done by GraphPadPrism6 software V 6.04. Quantitative gene expression was analyzed using ANOVA and Student's two-sided t-test. All statistical results were considered significant for p values < 0.05, unless stated otherwise.

3. Results

3.1. PEDF Expression in Osteophytes and Different Tissue Types within the Joint. The forming osteophyte in osteoarthritic joints represents a model for endochondral ossification within the adult organism. Osteophytes are typically characterized by a superficial fibrocartilaginous layer, followed by

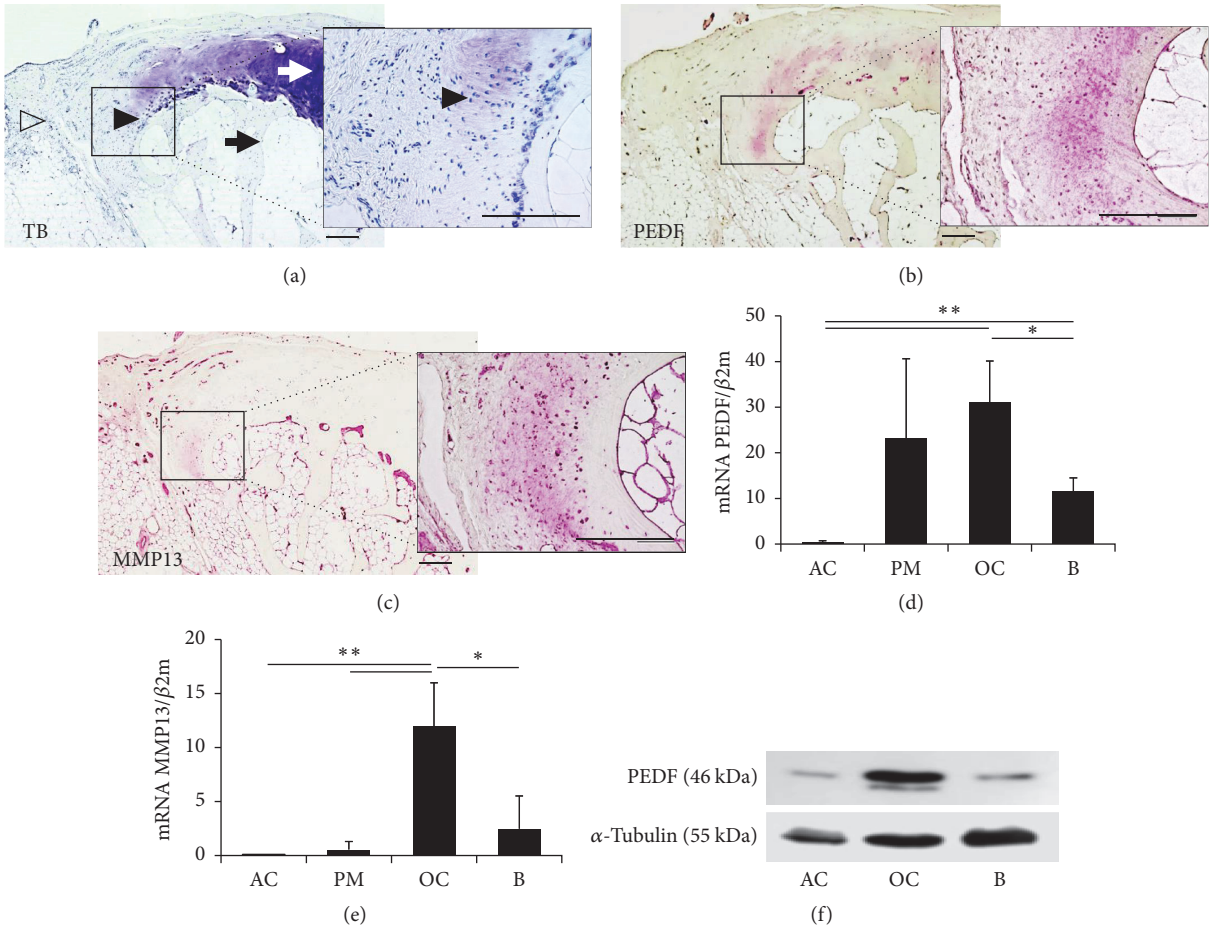

Figure 1: Detection of PEDF mRNA and protein in adult human joint tissues. Toluidine blue staining (a) depicts articular cartilage (white arrow), osteophytic cartilage (black arrowhead), subchondral bone (black arrow), and periarticular mesenchyme (open arrowhead). Inserts show higher magnifications of osteophytic cartilage. PEDF is detectable by immunostaining in osteophytic cartilage and the osteoblastic lining of subchondral bone (b). Correspondingly, MMP13 immunostaining is positive in osteophytic cartilage and subchondral bone (c). Quantitative RT-PCR analysis of PEDF (d) and MMP13 (e) in articular cartilage (AC), periarticular mesenchyme/periosteum (PM), osteophytic cartilage (OC), and subchondral bone (B). Detection of protein levels of PEDF and α-tubulin (loading control) by immunoblot analysis (f). Bars = 200 μm. $^{*}P < 0.05$; $^{**}P < 0.01$.

a cartilaginous layer and deeper osseous core (Figure 1(a)). Strong immunostaining for PEDF is detectable in the deeper cartilaginous zone of the osteophyte (Figure 1(b)). Periarticular mesenchyme and bone structures show moderate immunoreactivity for PEDF. Articular cartilage is completely negative for PEDF staining (Figure 1(b)). The quantitative gene expression analysis of the corresponding tissues reflects the pattern of immunohistochemistry, with strong PEDF mRNA expression in osteophytic cartilage (OC), bone (B), and periarticular mesenchyme, but virtually no PEDF mRNA expression in healthy articular cartilage (AC) (Figure 1(d)). As a control, the presence and expression of MMP13 were simultaneously analyzed as a reference marker for cartilage tissue undergoing endochondral ossification. The immunohistochemical detection of MMP13 and gene expression analysis revealed strong staining and high mRNA levels within the deeper cartilaginous zones of the osteophyte and moderate expression subchondral bone, but virtually no expression in healthy articular cartilage (Figures 1(c) and 1(e)). Corresponding to the mRNA expression, PEDF protein could hardly be detected by immunoblot in articular cartilage (AC), but strongly in osteophytic cartilage (OC) and moderately in bone tissue (B) (Figure 1(f)).

3.2. Detection of PEDF in Fetal Epiphyses and Growth Plate. At the fetal developmental stage, the epiphysis of the femoral condyle is composed of cartilaginous tissue that is characterized by zone-specific cellular phenotypes and still lacks the secondary center of ossification (Figure 2(a)). The superficial zone contains chondrocytes of small diameters at high cellular density. In this zone, PEDF and MMP13 were not detected by immunohistochemistry. The cartilaginous matrix of the superficial zone is typically positive for type II collagen (Col II) and negative for type I collagen (Col I), except for the very superficial cell layer in which this characteristic collagen pattern is inversed. Within the center

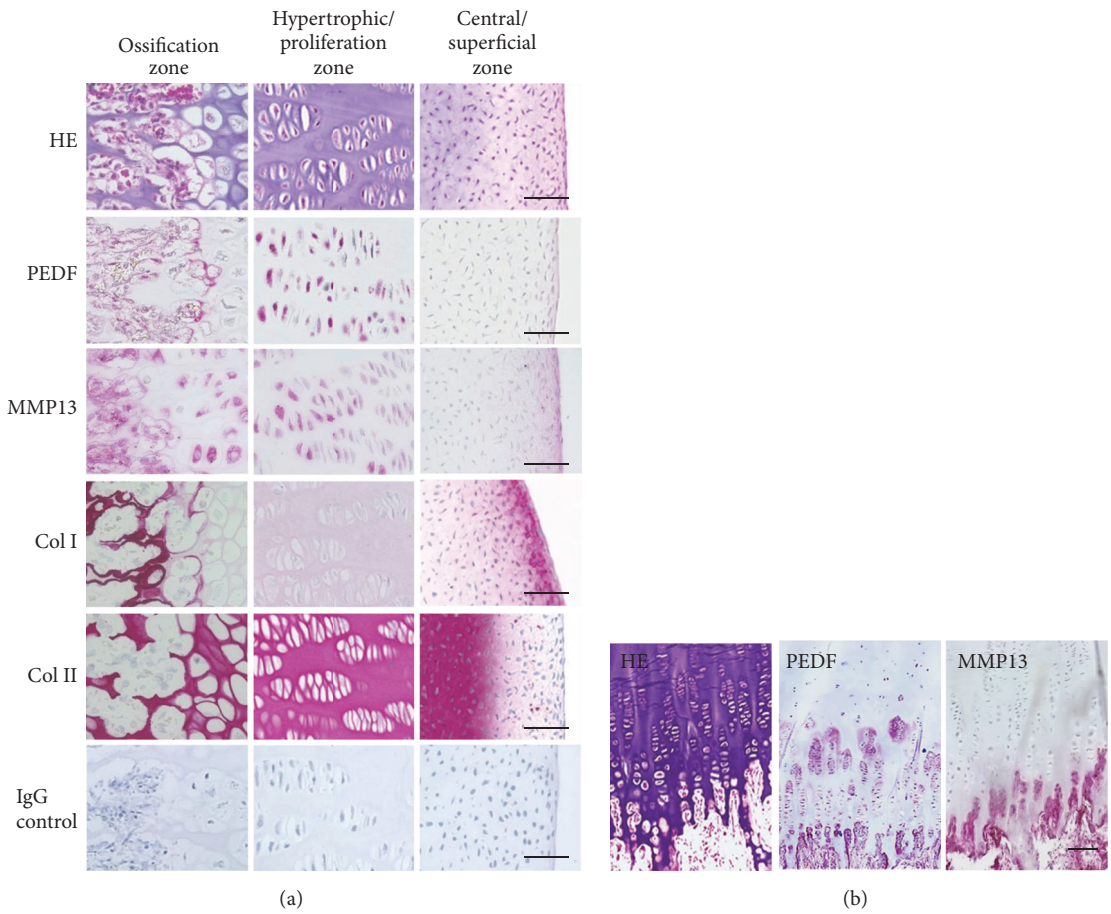

FIGURE 2: Immunohistochemical analysis of the fetal epiphysis and growth plate. The epiphyses were sectioned into the central and superficial zone, the proliferation and hypertrophic zone, and the ossification zone (a). The typical morphological characteristics of the different zones can be identified by hematoxylin eosin (HE) staining. Immunohistochemical staining for PEDF, MMP13, Col I, and Col II reveals a respective zone-specific pattern. No nonspecific staining was evident in negative controls incubated with isotype normal mouse IgG control. In the postnatal epiphyseal growth plate (b), PEDF is detectable in the proliferation zone, ossification zone, and osteoblastic lining. Correspondingly, MMP13 staining is positive in the hypertrophic zone and osteoblastic lining. Bars = 50 μm.

of the epiphysis, the chondrocytes are surrounded by a type II collagen-positive matrix negative for PEDF and type I collagen immunostaining. MMP13 is not detectable in the epiphyseal center, either. The proliferation zone and ossification zone of the fetal epiphysis resemble the respective zones of the growth plate in later postnatal stages. In the proliferation zone, the chondrocytes are aligned in a columnar pattern and are embedded in matrix positive for type II collagen, PEDF, and MMP13. In the ossification zone, the ossification front and forming bone tissue shows positive immunostaining for type I collagen. PEDF is also strongly detectable in proliferating and hypertrophic chondrocytes as well as in forming bone trabeculae, which corresponds to the immunoreactivity pattern of MMP13.

PEDF and MMP13 can also be detected in the growth plate from a newborn human donor in a zone-dependent manner (Figure 2(b)). While the resting zone was negative for both PEDF and MMP13, moderate staining for PEDF could be detected around the columnar aligned chondrocytes in the proliferation zone. Strong positive staining for both PEDF and MMP13 was found in the lower hypertrophic zone and the osteoblastic lining of forming trabeculae.

3.3. Gene Expression Profile and Biological Effects. RNA-Seq data analysis was performed to globally identify potential transcriptional targets of PEDF in primary human chondrocytes. We sequenced the transcriptome of chondrocytes isolated from the knee joints of three individuals that were either stimulated by 250 ng/ml PEDF or left as nonstimulated controls.

The expression patterns of PEDF-stimulated cells and untreated control cells were clearly separated as confirmed by principal component analysis (Figure 3(a)). The hierarchically clustered heatmap depicts a total of 1133 genes that were differentially expressed between chondrocytes stimulated by PEDF and nonstimulated cells using RNA sequencing analysis (adj. $p < 0.1$) (Figure 3(b)). The most differentially expressed genes (>3-fold change) are listed in Table 1. The most significantly upregulated and downregulated genes with roles in the skeletal system are shown in Table 2.

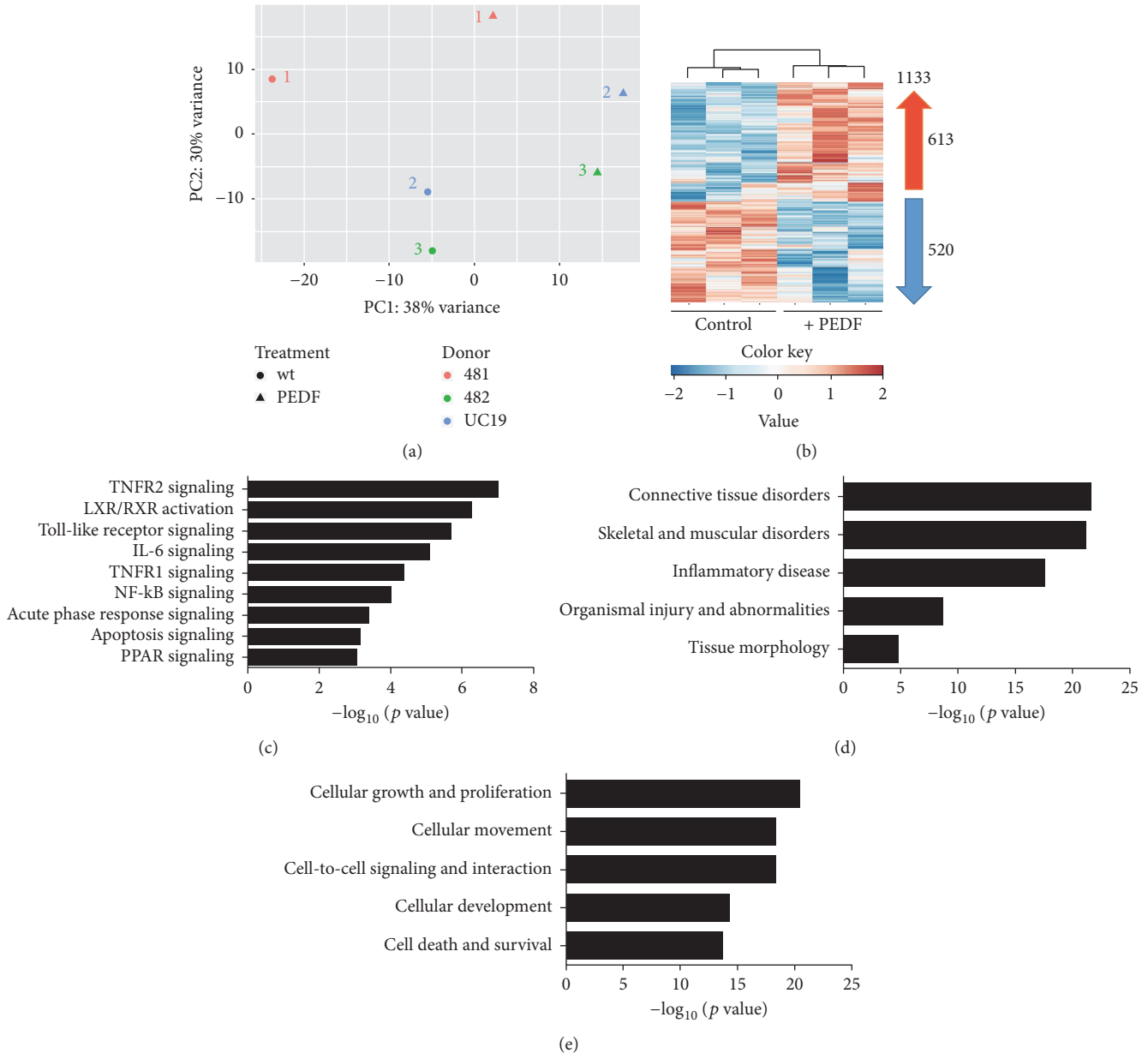

Figure 3: Analysis of PEDF target genes and pathways in chondrocytes using RNA-Seq. Principal component analysis of RNA-Seq expression data demonstrate distinct separation between PEDF-stimulated and nonstimulated chondrocytes (a). Hierarchical clustered heatmap shows differentially expressed genes for each sample. A total of 613 genes (in red) are upregulated and 520 genes (in blue) are downregulated following PEDF treatment (b). IPA-determined enriched signaling pathways (c), disorders (d), and cellular functions (e) associated with PEDF treatment.

Ingenuity Pathway Analysis (IPA) was applied for the set of genes which were more than 2-fold up- or down-regulated by PEDF (adj. $p < 0.01$), in an effort to identify canonical pathways, diseases, and biological functions that are closely related to PEDF stimulus in the context of the skeletal system (Figures 3(c)–3(e)). The top canonical pathways found to be differentially expressed between PEDF-stimulated and nonstimulated control chondrocytes belonged to catabolic, inflammatory, and matrix-degradative pathways (Figure 3(c)). Notably, the most enriched signaling pathways functionally converged to NFkB, as one of the common central elements. The disease and function analysis by IPA demonstrated that the most significantly gene sets influenced by PEDF in chondrocytes were attributable to connective tissue disorders, skeletal and muscular disorders, inflammatory diseases, organismal injury and abnormalities, and tissue morphology (Figure 3(d)). The IPA-analysis for cellular functions enriched by PEDF included cellular growth and development, cellular movement, cell-to-cell signaling, cellular development, and cell death/survival (Figure 3(e)).

Table 1: Most differentially expressed genes in PEDF-stimulated cells versus nonstimulated control cells (change > 3-fold).

Gene symbol	Description	Fold change	Adj p
SAA1	Serum amyloid A1	34.58	$<1.0E-120$
NOS2	Nitric oxide synthase 2, inducible	20.13	$4.89E-118$
LCN2	Lipocalin 2	15.97	$<1.0E-120$
C3	Complement component 3	12.00	$3.22E-171$
MMP13	Matrix metallopeptidase 13 (collagenase 3)	10.46	$5.59E-93$
MT1H	Metallothionein 1H	10.39	$1.63E-56$
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	10.02	$2.28E-90$
LTF	Lactotransferrin	8.94	$1.62E-53$
PTX3	Pentraxin 3, long	8.92	$4.89E-118$
CXCL5	Chemokine (C-X-C motif) ligand 5	8.86	$7.08E-29$
VNN2	Vanin 2	8.33	$1.31E-32$
BIRC3	Baculoviral IAP repeat containing 3	7.73	$3.05E-49$
CXCL2	Chemokine (C-X-C motif) ligand 2	7.33	$6.25E-24$
MT1F	Metallothionein 1F	7.25	$2.15E-48$
SOD2	Superoxide dismutase 2, mitochondrial	7.06	$5.50E-164$
PI3	Peptidase inhibitor 3, skin-derived	6.82	$3.28E-22$
MT1G	Metallothionein 1G	6.75	$3.79E-23$
CCL2	Chemokine (C-C motif) ligand 2	6.50	$2.98E-107$
IL32	Interleukin 32	5.85	$8.08E-19$
VCAM1	Vascular cell adhesion molecule 1	5.61	$1.31E-66$
IL4I1	Interleukin 4 induced 1	5.49	$4.38E-18$
LAMB3	Laminin, beta 3	5.46	$7.05E-99$
CX3CL1	Chemokine (C-X3-C motif) ligand 1	5.43	$7.54E-28$
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	5.31	$5.42E-51$
TLR2	Toll-like receptor 2	4.99	$8.76E-58$
CST2	Cystatin SA	4.62	$2.95E-14$
CHI3L2	Chitinase 3-like 2	4.44	$2.82E-68$
SLC7A2	Solute carrier family 7 (cationic amino acid transporter), member 2	4.37	$2.65E-72$
RELB	V-rel avian reticuloendotheliosis viral oncogene homolog B	4.05	$2.58E-24$
IL34	Interleukin 34	3.86	$3.32E-18$
CSF1	Colony stimulating factor 1 (macrophage)	3.82	$1.62E-53$
TNFAIP8	Tumor necrosis factor, alpha-induced protein 8	3.77	$1.17E-17$
WTAP	Wilms tumor 1 associated protein	3.71	$5.00E-47$
VNN3	Vanin 3	3.66	$3.56E-10$
ELF3	E74-like factor 3 (ETS domain transcription factor, epithelial-specific)	3.60	$1.61E-11$
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	3.56	$1.26E-10$
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer inhibitor, alpha	3.53	$2.20E-41$
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	3.50	$1.32E-10$
NOD2	Nucleotide-binding oligomerization domain containing 2	3.45	$1.37E-09$
LBP	Lipopolysaccharide binding protein	3.29	$2.40E-20$
LIF	Leukemia inhibitory factor	3.25	$1.40E-15$
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	3.13	$1.41E-21$
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	3.13	$1.63E-18$
MT1E	Metallothionein 1E	3.09	$7.77E-24$
IRAK2	Interleukin-1 receptor-associated kinase 2	3.07	$4.03E-24$
MSC	Masculine	3.06	$7.48E-08$
PLK2	Polo-like kinase 2	3.05	$3.82E-17$
SLC30A2	Solute carrier family 30 (zinc transporter), member 2	3.04	$2.15E-07$
G0S2	G0/G1switch 2	3.00	$3.86E-18$
OGN	Osteoglycin	−3.03	$3.96E-34$
COMP	Cartilage oligomeric matrix protein	−3.05	$9.37E-48$
ACTC1	Actin, alpha, cardiac muscle 1	−3.07	$2.01E-10$

Table 1: Continued.

Gene symbol	Description	Fold change	Adj p
CFH	Complement factor H	−3.07	$1.65E-57$
COL9A3	Collagen, type IX, alpha 3	−3.14	$8.30E-65$
COL9A2	Collagen, type IX, alpha 2	−3.18	$1.32E-46$
SLC14A1	Solute carrier family 14 (urea transporter)	−3.28	$7.13E-14$
ADAMTSL2	ADAMTS-like 2	−3.34	$9.68E-17$
CHAD	Chondroadherin	−3.92	$3.85E-30$
COL2A1	Collagen, type II, alpha 1	−4.33	$3.10E-49$
CYTL1	Cytokine-like 1	−4.45	$2.11E-35$
GDF10	Growth differentiation factor 10	−4.57	$1.43E-46$

Likewise, GSEA showed significant positive correlation with TNFα-signaling via NFkB, cellular protein catabolic processes, and cellular component disassembly, but negative correlation with collagen formation, extracellular matrix organization, and glycosaminoglycan metabolism (Figure 4(a)). These, findings based on transcriptome analysis are in agreement with the biological response of human chondrocytes that were stimulated by PEDF. In three-dimensional pellet cultures, isolated human articular chondrocytes have the capability to redifferentiate and form a proteoglycan-rich matrix, as indicated by a metachromatic Toluidine blue staining and negative staining for MMP13 (Figure 4(b)). In contrast, treatment by PEDF interfered with chondrogenic redifferentiation resulting in a matrix largely lacking proteoglycans. However, the PEDF-treated cell pellets showed a distinct immunoreactivity for MMP13, particularly in the superficial layers (Figure 4(b)).

The data from RNA-Seq were confirmed by qRT-PCR resulting in a 32.5-fold increase of SAA1 mRNA ($p = 0.003$), 7.8-fold increase of MMP13 mRNA ($p = 0.001$), 4.2-fold increase of MMP1 mRNA ($p = 0.03$), 7.5-fold increase of MMP3 mRNA ($p = 0.003$), and 20.2-fold increase of IL6 mRNA ($p = 0.04$) (Table 2; Figure 4(c)). A number of genes involved in skeletal development showed significantly reduced expression as detected by RNA-Seq. These results were confirmed by qRT-PCR, which showed a 7.1-fold decrease of COL2A1 mRNA ($p = 0.00001$), 4.8-fold decrease of COMP mRNA ($p = 0.00001$), and 3.5-fold decrease of LECT1 mRNA ($p = 0.001$) (Table 2; Figure 4(c)). A number of additional cartilage-associated genes were also shown to be significantly downregulated upon PEDF stimulus, including a 2.2-fold decrease of NKX3-2 mRNA ($p = 0.00004$), 2.3-fold decrease of ACAN mRNA ($p = 0.00006$), 2.9-fold decrease of SOX5 mRNA ($p = 0.01$), and a 1.4 decrease in SOX9 mRNA ($p = 0.001$) (Table 2).

4. Discussion

This is the first study that comprehensively investigated the role of PEDF on the genome-wide gene expression profile of chondrocytes. We could demonstrate that PEDF significantly upregulates the expression of catabolic and matrix-degrading factors that promote the termination and decay of transient cartilage tissue, while simultaneously, typical cartilage-specific genes are downregulated. SAA1 was the most highly upregulated gene by PEDF. SAA1, an acute-phase protein, was shown to be expressed by chondrocytes and to promote cartilage destruction by stimulating the expression of matrix metalloproteinases [24]. SAA1 is also expressed by MSCs and osteoblasts, in which it may support the proinflammatory phase of osteogenic differentiation and enhanced mineralization [25, 26].

Interestingly, MMP13, the reference marker for terminal chondrocyte differentiation and endochondral ossification, was among the 5 most significantly upregulated genes following PEDF stimulus. Immunohistochemical analysis demonstrated a corresponding staining pattern of MMP13 to that of PEDF, which was observed in the zone of endochondral ossification in human epiphyseal cartilage, growth plate cartilage, and osteophytic tissue. MMP13 is the major collagenase expressed in the primary and secondary ossification centers and drives endochondral ossification by degrading major cartilage components, such as type II collagen and aggrecan [27]. MMP13 is known as one crucial mediator to promote the endochondral ossification process and the terminal differentiation of chondrocytes [27, 28]. MMP13 is also required for adequate bone development, in particular bone remodeling [29]. Thus, MMP13 may be one of the most relevant target genes induced by PEDF for the skeletal system, in particular for cartilage tissue that undergoes endochondral ossification.

PEDF is a pluripotency protein that is expressed in multiple tissues. Several signaling pathways have been described for PEDF, including the IP3-AKT, MEK-ERK, or PLA_2-PPARγ pathway, which all converge in the activation of the NFκB complex [30–34]. Indeed, the Ingenuity Pathway Analysis IPA of the present study revealed NFκB as one common central target upon PEDF stimulus. In fact, NFκB- and ERK-pathways are known to be the main mechanisms that induce MMP13 and MMP1 expression in chondrocytes [35–37].

The activation of the NFκB and PPARγ pathways may also explain the highly significant downregulation of many genes encoding for cartilage-specific matrix proteins. Most of these genes are under the transcriptional control of the transcriptional complex of Sox5, Sox6, and Sox9 [38]. Sox9 is a central transcription factor for a large number of cartilage-specific genes and represents stabilizing factor of the chondrocyte

Table 2: Selected genes with roles in the skeletal system significantly up- or downregulated by rhPEDF.

gene symbol	Description	Fold change	Adj p	Ranking among the upregulated genes ($n = 613$)	qRT-PCR Fold change mean (95% CI)	P value
SAA1	Serum amyloid A1	34.53	$<1.0E-120$	1	32.5 (11.5–53.5)	0.003
NOS2	Nitric oxide synthase 2, inducible	20.11	$4.89E-118$	2		
MMP13	Matrix metallopeptidase 13	10.41	$5.59E-93$	5	7.8 (4.3–11.3)	0.001
SOD2	Superoxide dismutase 2, mitochondrial	7.06	$5.50E-164$	15		
TLR2	Toll-like receptor 2	4.99	$8.76E-58$	25		
MMP1	Matrix metallopeptidase 1	3.50	$1.32E-10$	38	4.2 (0.2–8.2)	0.03
MMP3	Matrix metallopeptidase 3	2.28	$2.20E-4$	87	7.5 (3.2–11.7)	0.003
PPARG	Peroxisome proliferator-activated receptor gamma	1.65	0.031	275		
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.55	$7.48E-07$	338		
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1.53	$1.70E-4$	345		
IL6	Interleukin 6 (interferon, beta 2)	1.47	0.076	386	20.2 (−7.7–48.2)	0.04
SOX9	SRY (sex determining region Y)-box 9	−1.31	$6.30E-04$	370	−1.4 −(1.2–1.8)	0.001
SOX5	SRY (sex determining region Y)-box 5	−1.61	$1.02E-05$	143	−2.9 −(1.2–5.5)	0.01
ACAN	Aggrecan	−1, 75	$4.49E-08$	104	−2.3 −(1.7–3.2)	0.00006
COL9A1	Collagen, type IX, alpha 1	−1.82	$7.93E-08$	84		
NKX3-2	NK3 homeobox 2	−1.92	$9.43E-05$	66	−2.2 −(1.7–3.0)	0.00004
FGFR3	Fibroblast growth factor receptor 3	−1.92	$2.51E-10$	53		
S100A1	S100 calcium binding protein A1	−2.17	$1.43E-22$	41		
PRG4	Proteoglycan 4	−2.27	$2.01E-26$	29		
LECT1	Leukocyte cell derived chemotaxin 1	−2.33	$5.31E-07$	26	−3.5 −(1.6–18.5)	0.001
COL11A1	Collagen, type XI, alpha 1	−2.38	$1.68E-24$	25		
COL11A2	Collagen, type XI, alpha 2	−2.50	$1.71E-24$	20		
FRZB	Frizzled-related protein	−2.87	$6.31E-27$	14		
COMP	Cartilage oligomeric matrix protein	−3.03	$9.37E-48$	11	−4.8 −(3.2–9.2)	0.00001
COL9A2	Collagen, type IX, alpha 2	−3.12	$1.32E-46$	7		
COL9A3	Collagen, type IX, alpha 3	−3.13	$8.30E-65$	8		
CHAD	Chondroadherin	−4.00	$3.85E-30$	4		
COL2A1	Collagen, type II, alpha 1	−4.32	$3.10E-49$	3	−7.1 −(3.8–41.4)	0.00001

phenotype [38]. The only moderate downregulation of Sox5 and Sox9 gene expression by PEDF may not solely explain the highly significant downregulation of many cartilage genes. It is likely that posttranscriptional mechanisms contribute to the antichondrogenic effects of PEDF. In this context, the activated NFκB complex and PPARγ were shown to reduce the transcriptional activity of Sox9 by interfering with nuclear translocation, destabilization of Sox9 mRNA, and limiting the availability of cofactors such as p300 [39–41]. Furthermore, MMP13 itself may affect the activity of Sox9, since knockdown of MMP13 resulted in increased nuclear translocation of Sox9, [42, 43].

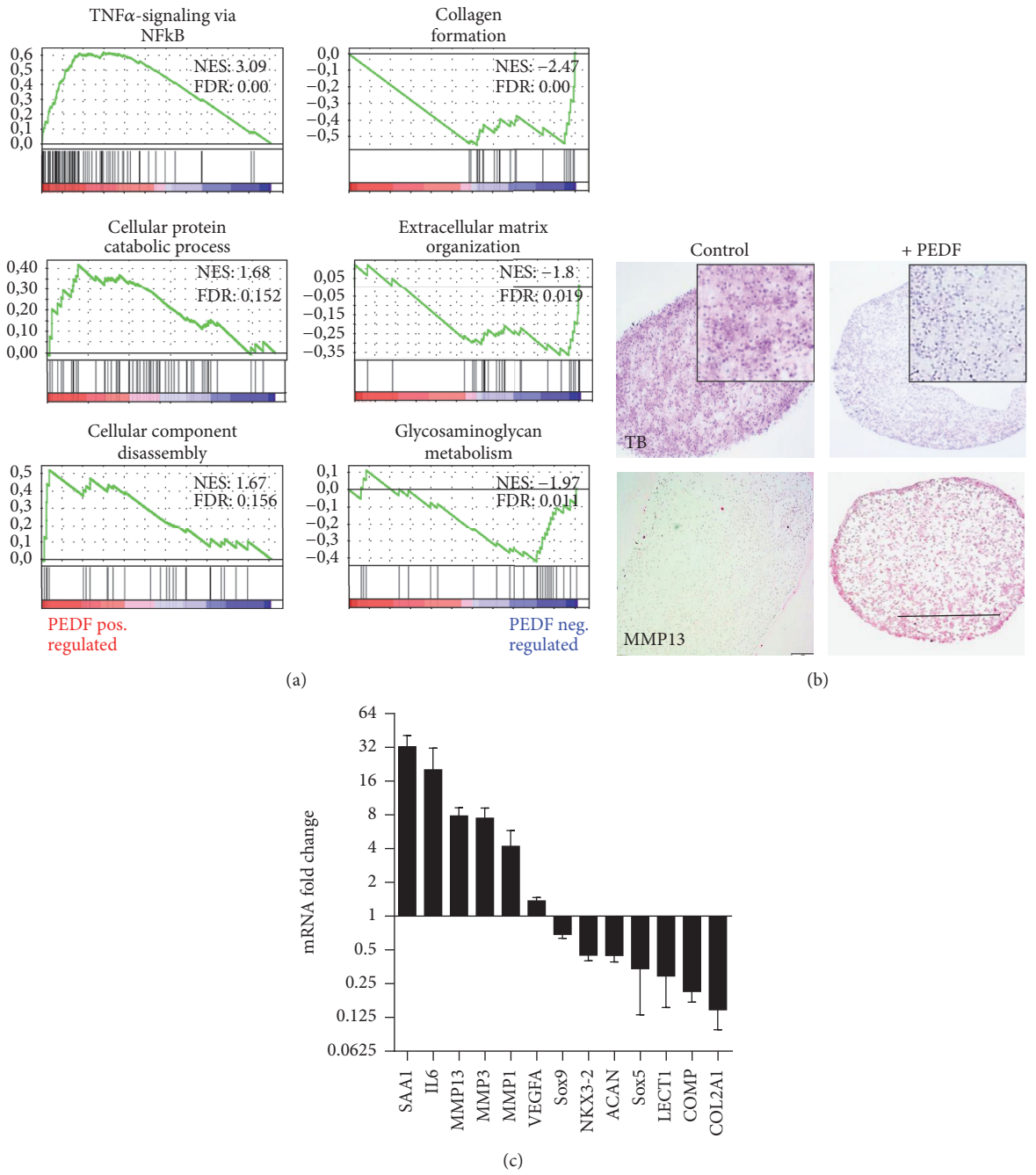

Figure 4: Biological effects exerted by PEDF in chondrocytes. GSEA shows significantly enriched biological processes and signaling pathways based on the gene sets positively and negatively correlated with PEDF treatment (a). PEDF-treated or nontreated three-dimensional cell pellets were analyzed by Toluidine blue (TB) staining and MMP13 immunohistochemistry (b). RNA-Seq data were confirmed by qRT-PCR showing the *n*-fold expression of significantly positively and negatively regulated genes with relevance for the skeletal system (c). Bars show the mean and SD. Bar = 1 mm.

Recently, the loss of PEDF function was identified to be associated with osteogenesis imperfecta type VI that is characterized by a severe skeletal phenotype. In human mesenchymal stem cells, PEDF was able to induce osteoblastic related genes such as ALP, Runx2, OCN, and BSP and increase ALP activity and support mineral deposition, albeit, this effect was much lower than the effect achieved by osteogenic medium [16]. Nevertheless, an in vivo model demonstrated that PEDF is able to mediate ectopic bone formation in muscle pockets [44]. In the present study focusing on the gene expression of chondrocytes, we could not detect significant effects of PEDF on the expression of osteogenic genes. Thus, the impact of PEDF on endochondral ossification may rather be based on degradation and remodeling processes than on

induction of mineral deposition. However, further work is needed to explore the impact of PEDF on mineral deposition in cells other than mesenchymal stem cells, such as matrix-embedded chondrocytes. Indeed, high-resolution backscattered electron imaging and synchrotron X-ray scattering of human bone biopsies of osteogenesis imperfecta type VI patients revealed even an increased calcium content of the bone matrix, as well as coexistence of highly mineralized bone matrix with seams of abnormally low mineral content atypical collagen fibril organization around osteocytes, which supports the concept of a disturbed matrix remodeling during early steps of mineralization [15]. In fact, a similar phenotype could be observed in MMP13-deficient mice which are characterized by an impaired remodeling of the perilacunar matrix, disruption of the canalicular network, abnormal collagen, and mineral organization [29]. These observations underline the assumption that PEDF exerts its functional role in endochondral ossification via MMP13.

PEDF could also be detected within cartilage repair tissue induced by bone marrow-stimulating techniques (data not shown). The repair tissue typically suffers from an instable transient chondrocyte phenotype and excessive subchondral bone formation within the defects [45–47]. This supports the concept that PEDF serves as a marker for a transient chondrogenic differentiation status and the corresponding staining pattern of MMP13 (data not shown) may serve as a trigger for inadvertent endochondral ossification within cartilage repair tissue.

In conclusion, PEDF plays an important role in the endochondral ossification process during all developmental stages. PEDF mediated the decay of transient cartilage tissue by promoting matrix degradation and suppression of cartilage-specific gene expression. PEDF represents a marker for the transient status of the chondrocyte phenotype that is subjected to terminal differentiation and endochondral ossification. The inhibition of PEDF action may be the basis for future therapeutic strategies in order to stabilize the chondrocyte phenotype of articular cartilage and to prevent its degradation.

Competing Interests

The authors did not receive any financial support or other benefits from commercial sources for the work reported on in the manuscript or any other financial interests. There are no potential competing interests with regard to the work.

Authors' Contributions

Authors P. Klinger, B. Swoboda, and K. Gelse designed the study and prepared the first draft of the paper. Authors P. Klinger, A. B. Ekici, T. Hotfiel, T. Aigner, and K. Gelse contributed to the experimental work. Authors P. Klinger, F. Ferrazzi, and S. Lukassen performed RNA-Seq data analysis. Authors P. Klinger and K. Gelse were responsible for statistical analysis of the data. All authors revised the paper critically for intellectual content and approved the final version.

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References

[1] J. Tombran-Tink and L. V. Johnson, "Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells," *Investigative Ophthalmology and Visual Science*, vol. 30, no. 8, pp. 1700–1707, 1989.

[2] D. W. Dawson, O. V. Volpert, P. Gillis et al., "Pigment epithelium-derived factor: a potent inhibitor of angiogenesis," *Science*, vol. 285, no. 5425, pp. 245–248, 1999.

[3] J. Tombran-Tink, "The neuroprotective and angiogenesis inhibitory serpin, PEDF: new insights into phylogeny, function, and signaling," *Frontiers in Bioscience*, vol. 10, no. 1, pp. 2131–2149, 2005.

[4] M. L. Tan, P. F. M. Choong, and C. R. Dass, "Anti-chondrosarcoma effects of PEDF mediated via molecules important to apoptosis, cell cycling, adhesion and invasion," *Biochemical and Biophysical Research Communications*, vol. 398, no. 4, pp. 613–618, 2010.

[5] M. L. Broadhead, T. Akiyama, P. F. M. Choong, and C. R. Dass, "The pathophysiological role of PEDF in bone diseases," *Current Molecular Medicine*, vol. 10, no. 3, pp. 296–301, 2010.

[6] E. T. H. Ek, C. R. Dass, K. G. Contreras, and P. F. M. Choong, "Inhibition of orthotopic osteosarcoma growth and metastasis by multitargeted antitumor activities of pigment epithelium-derived factor," *Clinical & Experimental Metastasis*, vol. 24, no. 2, pp. 93–106, 2007.

[7] K. Takenaka, S.-I. Yamagishi, Y. Jinnouchi, K. Nakamura, T. Matsui, and T. Imaizumi, "Pigment epithelium-derived factor (PEDF)-induced apoptosis and inhibition of vascular endothelial growth factor (VEGF) expression in MG63 human osteosarcoma cells," *Life Sciences*, vol. 77, no. 25, pp. 3231–3241, 2005.

[8] J. Tombran-Tink and C. J. Barnstable, "Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone," *Biochemical and Biophysical Research Communications*, vol. 316, no. 2, pp. 573–579, 2004.

[9] P. Klinger, C. Beyer, A. B. Ekici et al., "The transient chondrocyte phenotype in human osteophytic cartilage: a role of pigment epithelium-derived factor?" *Cartilage*, vol. 4, no. 3, pp. 249–255, 2013.

[10] G. M. Y. Quan, J. Ojaimi, Y. Li, V. Kartsogiannis, H. Zhou, and P. F. M. Choong, "Localization of pigment epithelium-derived factor in growing mouse bone," *Calcified Tissue International*, vol. 76, no. 2, pp. 146–153, 2005.

[11] E. A. Lord, S. K. Martin, J. P. Gray, C. Li, and D. E. Clark, "Cell cycle genes PEDF and CDKN1C in growing deer antlers," *Anatomical Record*, vol. 290, no. 8, pp. 994–1004, 2007.

[12] D. Pfander, C. Grimmer, T. Aigner, B. Swoboda, R. Schmidt, and T. Cramer, "Pigment epithelium derived factor—the product of the EPC-1 gene—is expressed by articular chondrocytes and up regulated in osteoarthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 7, pp. 965–967, 2006.

[13] E. P. Homan, F. Rauch, I. Grafe et al., "Mutations in SERPINF1 cause osteogenesis imperfecta type VI," *Journal of Bone and Mineral Research*, vol. 26, no. 12, pp. 2798–2803, 2011.

[14] J. Becker, O. Semler, C. Gilissen et al., "Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta," *American Journal of Human Genetics*, vol. 88, no. 3, pp. 362–371, 2011.

[15] N. Fratzl-Zelman, I. Schmidt, P. Roschger et al., "Unique micro- and nano-scale mineralization pattern of human osteogenesis imperfecta type VI bone," *Bone*, vol. 73, pp. 233–241, 2015.

[16] F. Li, N. Song, J. Tombran-Tink, and C. Niyibizi, "Pigment epithelium-derived factor enhances differentiation and mineral deposition of human mesenchymal stem cells," *Stem Cells*, vol. 31, no. 12, pp. 2714–2723, 2013.

[17] M. L. Broadhead, C. R. Dass, and P. F. M. Choong, "Systemically administered PEDF against primary and secondary tumours in a clinically relevant osteosarcoma model," *British Journal of Cancer*, vol. 105, no. 10, pp. 1503–1511, 2011.

[18] K. Gelse, C. Mühle, K. Knaup et al., "Chondrogenic differentiation of growth factor-stimulated precursor cells in cartilage repair tissue is associated with increased HIF-1α activity," *Osteoarthritis and Cartilage*, vol. 16, no. 12, pp. 1457–1465, 2008.

[19] C. Grimmer, D. Pfander, B. Swoboda et al., "Hypoxia-inducible factor 1α is involved in the prostaglandin metabolism of osteoarthritic cartilage through up-regulation of microsomal prostaglandin E synthase 1 in articular chondrocytes," *Arthritis and Rheumatism*, vol. 56, no. 12, pp. 4084–4094, 2007.

[20] A. Dobin, C. A. Davis, F. Schlesinger et al., "STAR: ultrafast universal RNA-seq aligner," *Bioinformatics*, vol. 29, no. 1, pp. 15–21, 2013.

[21] Y. Liao, G. K. Smyth, and W. Shi, "FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features," *Bioinformatics*, vol. 30, no. 7, pp. 923–930, 2014.

[22] M. I. Love, W. Huber, and S. Anders, "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2," *Genome Biology*, vol. 15, no. 12, article 550, 2014.

[23] A. Subramanian, P. Tamayo, V. K. Mootha et al., "Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 43, pp. 15545–15550, 2005.

[24] R. Vallon, F. Freuler, N. Desta-Tsedu et al., "Serum amyloid a (apoSAA) expression is up-regulated in rheumatoid arthritis and induces transcription of matrix metalloproteinases," *The Journal of Immunology*, vol. 166, no. 4, pp. 2801–2807, 2001.

[25] R. Ebert, P. Benisch, M. Krug et al., "Acute phase serum amyloid a induces proinflammatory cytokines and mineralization via toll-like receptor 4 in mesenchymal stem cells," *Stem Cell Research*, vol. 15, no. 1, pp. 231–239, 2015.

[26] A. Kovacevic, A. Hammer, E. Stadelmeyer et al., "Expression of serum amyloid A transcripts in human bone tissues, differentiated osteoblast-like stem cells and human osteosarcoma cell lines," *Journal of Cellular Biochemistry*, vol. 103, no. 3, pp. 994–1004, 2008.

[27] D. Stickens, D. J. Behonick, N. Ortega et al., "Altered endochondral bone development in matrix metalloproteinase 13-deficient mice," *Development*, vol. 131, no. 23, pp. 5883–5895, 2004.

[28] M. Inada, Y. Wang, M. H. Byrne et al., "Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 49, pp. 17192–17197, 2004.

[29] S. Y. Tang, R.-P. Herber, S. P. Ho, and T. Alliston, "Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance," *Journal of Bone and Mineral Research*, vol. 27, no. 9, pp. 1936–1950, 2012.

[30] T. Yabe, D. Wilson, and J. P. Schwartz, "NFκB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons," *The Journal of Biological Chemistry*, vol. 276, no. 46, pp. 43313–43319, 2001.

[31] I.-H. Pang, H. Zeng, D. L. Fleenor, and A. F. Clark, "Pigment epithelium-derived factor protects retinal ganglion cells," *BMC Neuroscience*, vol. 8, article 11, 2007.

[32] X. He, R. Cheng, S. Benyajati, and J.-X. Ma, "PEDF and its roles in physiological and pathological conditions: implication in diabetic and hypoxia-induced angiogenic diseases," *Clinical Science*, vol. 128, no. 11, pp. 805–823, 2016.

[33] V. Chandolu and C. R. Dass, "Cell and molecular biology underpinning the effects of PEDF on cancers in general and osteosarcoma in particular," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 740295, 9 pages, 2012.

[34] U. Sagheer, J. Gong, and C. Chung, "Pigment Epithelium-Derived Factor (PEDF) is a determinant of stem cell fate: lessons from an ultra-rare disease," *Journal of Developmental Biology*, vol. 3, no. 4, pp. 112–128, 2015.

[35] Z. Fan, H. Yang, B. Bau, S. Söder, and T. Aigner, "Role of mitogen-activated protein kinases and NFκB on IL-1β-induced effects on collagen type II, MMP-1 and 13 mRNA expression in normal articular human chondrocytes," *Rheumatology International*, vol. 26, no. 10, pp. 900–903, 2006.

[36] M. B. Goldring, M. Otero, D. A. Plumb et al., "Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis," *European Cells and Materials*, vol. 21, pp. 202–220, 2011.

[37] A. Liacini, J. Sylvester, W. Q. Li et al., "Induction of matrix metalloproteinase-13 gene expression by TNF-α is mediated by MAP kinases, AP-1, and NF-κB transcription factors in articular chondrocytes," *Experimental Cell Research*, vol. 288, no. 1, pp. 208–217, 2003.

[38] C.-F. Liu and V. Lefebvre, "The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis," *Nucleic Acids Research*, vol. 43, no. 17, pp. 8183–8203, 2015.

[39] R. Sitcheran, P. C. Cogswell, and A. S. Baldwin Jr., "NF-κB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism," *Genes and Development*, vol. 17, no. 19, pp. 2368–2373, 2003.

[40] J. S. Rockel, J. C. Kudirka, A. J. Guzi, and S. M. Bernier, "Regulation of Sox9 activity by crosstalk with nuclear factor-κB and retinoic acid receptors," *Arthritis Research and Therapy*, vol. 10, no. 1, article no. R3, 2008.

[41] X. Ren, D. Zheng, F. Guo et al., "PPARγ suppressed Wnt/β-catenin signaling pathway and its downstream effector SOX9 expression in gastric cancer cells," *Medical Oncology*, vol. 32, no. 4, article 91, 2015.

[42] R. M. Borzí, E. Olivotto, S. Pagani et al., "Matrix metalloproteinase 13 loss associated with impaired extracellular matrix remodeling disrupts chondrocyte differentiation by concerted effects on multiple regulatory factors," *Arthritis and Rheumatism*, vol. 62, no. 8, pp. 2370–2381, 2010.

[43] T. Hattori, C. Müller, S. Gebhard et al., "SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification," *Development*, vol. 137, no. 6, pp. 901–911, 2010.

[44] M. Elahy, M. Doschak, J. Hughes, S. Baindur-Hudson, and C. Dass, "Alginate bead-encapsulated PEDF induces ectopic bone formation in vivo in the absence of co-administered mesenchymal stem cells," *Current Drug Targets*, vol. 16, no. 999, pp. 1–1, 2015.

[45] K. Gelse, P. Klinger, M. Koch et al., "Thrombospondin-1 prevents excessive ossification in cartilage repair tissue induced by osteogenic protein-1," *Tissue Engineering Part A*, vol. 17, no. 15-16, pp. 2101–2112, 2011.

[46] P. Klinger, C. Surmann-Schmitt, M. Brem et al., "Chondromodulin 1 stabilizes the chondrocyte phenotype and inhibits endochondral ossification of porcine cartilage repair tissue," *Arthritis and Rheumatism*, vol. 63, no. 9, pp. 2721–2731, 2011.

[47] M. Blanke, H. D. Carl, P. Klinger, B. Swoboda, F. Hennig, and K. Gelse, "Transplanted chondrocytes inhibit endochondral ossification within cartilage repair tissue," *Calcified Tissue International*, vol. 85, no. 5, pp. 421–433, 2009.

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Review Article

Gray Matter Atrophy within the Default Mode Network of Fibromyalgia: A Meta-Analysis of Voxel-Based Morphometry Studies

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Over the years, studies have demonstrated morphological changes in the brain of fibromyalgia (FMS). We aimed to conduct a coordinate-based meta-analytic research through systemic review on voxel-based morphometry (VBM) imaging results to identify consistent gray matter (GM) difference between FMS patients and healthy subjects. We performed a comprehensive literature search in PubMed (January 2000–December 2015) and included six VBM publication on FMS. Stereotactic data were extracted from 180 patients of FMS and 123 healthy controls. By means of activation likelihood estimation (ALE) technique, regional GM reduction in left medial prefrontal cortex and right dorsal posterior cingulate cortex was identified. Both regions are within the default mode network. In conclusion, the gray matter deficit is related to the both affective and nonaffective components of pain processing. This result also provided the neuroanatomical correlates for emotional and cognitive symptoms in FMS.

1. Introduction

Fibromyalgia (FM) is characterized by chronic widespread pain with accompanying symptoms, such as fatigue, morning stiffness, insomnia, cognitive dullness, depression, and anxiety [1, 2]. Not only does FM impair life quality [3], but it also increases disability and absence from work [4]. With the evolving diagnostic criteria of FM [5], the prevalence rate is estimated about 2~4% in the general population with female predominance [6, 7]. Despite genetic, environmental, and biochemical factors proposed as the underlying pathophysiology [8, 9], the exact mechanism pertaining to FM is still under debate. Early research on peripheral neural or muscular damage in patients of FM failed to detect consistent evidence [10]. Therefore, central sensitization has been proposed to explain the features of FM, including allodynia (increased sensitivity to stimulus that does not normally provoke pain) and hyperalgesia (heightened response to painful stimulus) [11, 12].

Over the past two decade, central sensitization theory can be further exemplified in the studies using neuroimaging techniques. Single-photon-emission-computed tomography (SPECT) imaging has demonstrated regional cerebral blood flow (rCBF) decrease in thalamus and pontine region in

patients of FM [13, 14]. The ensuing PET study has not only shown rCBF decrease in several brain regions, supporting the dysfunctional cognitive processing of pain, but also higher retrosplenial rCBF, suggesting secondary hyperalgesia [15]. Since the inception of functional magnetic resonance imaging (fMRI), it became easier to infer neural activity by proxy of blood-oxygen-level-dependent (BOLD) contrast without the need of radioactive tracer or contrast agent. Multiple paradigms were applied to study the differential activation between FM patients and normal controls. With the stimuli of pressure [16–18], repetitive heat pulses [19], nociceptive injection [20], or even incision of forearm [21], compared to normal, FM patients showed increased activation in assorted pain related brain areas, such as thalamus, primary (S1) as well as secondary (S2) somatosensory cortices, insula, and cingulate cortex [16–21]. On the other hand, impaired descending inhibitory pain system was evinced from the results of decreased activation in rACC (rostral anterior cingulate cortex) and thalamus [18] and reduced resting-state functional connectivity between ACC with PAG (periaqueductal gray) [22] in FM patients.

Although tasked-related fMRI helps us extract specific cognitive function in FM patients, the variety of different paradigms impede us from compiling or comparing all these studies directly. Besides functional changes, research on structural changes in chronic pain patients has concurrently been in the limelight. Voxel-based morphometry (VBM) [23] is the most widely employed technique and has been implicated in several groups, including chronic low back pain [24, 25], tension headache [26], irritable bowel syndrome [27], and chronic pelvic pain [28]. In fibromyalgia, several articles using region-of-interest (ROI) method focusing on "pain matrix" in brain have discovered gray matter decrease in anterior [29–31] and midcingulate [29] cortices, prefrontal cortex [30], midinsula [29], amygdala [30, 31], right and left lateral orbitofrontal cortex [31], and hippocampi [32].

These studies reiterated the theoretical importance of the key brain regions. However, unbiased whole-brain studies could also be conducted by VBM without need of selected ROIs. Furthermore, by using meta-analytic approach of activation likelihood estimate (ALE) method [33, 34], we are able to pinpoint consistent structural brain changes in several illness, such as schizophrenia [35], bipolar disorder [36], posttraumatic stress disorder [37], Alzheimer's disease [38], narcolepsy [39], chronic pain [40], and headache [41]. Therefore, to provide greater insight into the structure changes of FM, current study aims to review the findings from all the whole-brain VBM research published to date and to conduct an ALE-based meta-analysis. We also hypothesize that we could observe convergent brain areas associated with the features FM encompasses.

2. Methods and Materials

2.1. Search Strategies and Selection Criteria. We conducted a comprehensive literature search in PubMed database (January 2000–December 2015) by using the key works "fibromyalgia," "voxel," "morphometry," "voxel-based morphometry," and "VBM." We did not set specific restrictions on language of the literature. Cited articles in systemic reviews related to brain structure changes in fibromyalgia were also examined to increase any possibility of unincluded papers in previous literature search. Two independent investigators (LC and WHH) evaluated the methodology and the quality of the selected studies, where demographics, name of the first author, publication year, title of the journal, age of the participants, total number of participants, sex ratio, matching method, and the methods and thresholds in SPM were also examined.

2.2. Study Selection

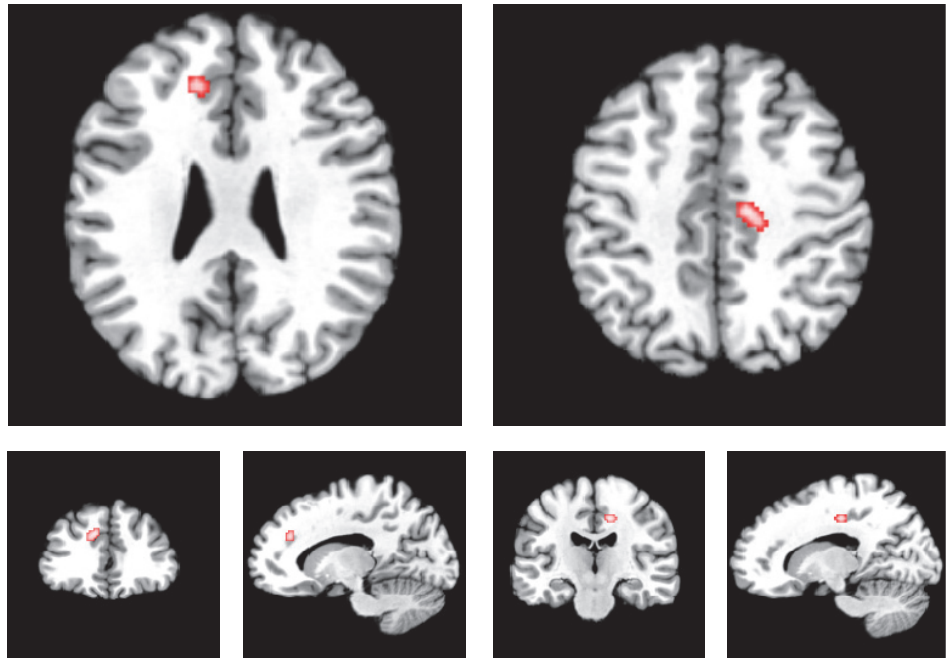
2.2.1. Inclusion Criteria. The inclusion criteria for the studies in this meta-analysis are (1) peer-reviewed articles with retrievable full-text, (2) patients were diagnosed with fibromyalgia with healthy controls, (3) VBM procedure in MR anatomical analysis done in whole-brain structure by either GMV or GMC; we excluded region-of-interest (ROI) or volume-of-interest (VOI) method, and (4) peak coordinates available in Montreal Neurologic Institute (MNI) or Talairach and Tournoux stereotactic space.

2.2.2. Data Extraction. We inputted the x, y, and z peak activation coordinates from the eligible contrasts into the meta-analysis. Coordinates reported in MNI space were converted to Talairach coordinates [42, 43]. We texted and employed the MNI coordinates from the data to GingerALE 2.1.1 (http://brainmap.org/ale/, Research Imaging Institute of the University of Texas Health Science Center, San Antonio, TX).

2.2.3. ALE Meta-Analysis. The version of current ALE approach [33, 44, 45] is the one implemented from the CBMA of neuroimaging results [34, 45]. In short, each stereotactic coordinates were represented as the peak center of the three-dimensional (3D) Gaussian probability distribution. A computed modeled activation (MA) map was derived to encapsulate the localization probabilities. The sample size of each study was used to estimate the spatial uncertainty related to the activation [46]. The overlapping of the distributions across different studies was calculated. We calculated the ALE values voxel-by-voxel by summing the MA maps derived from above. In doing so, the current analysis would not deal with foci (fixed effects), but with the concurrence from the studies (random effects). We set a 0.05 false discovery rate (FDR; q) for multiple comparisons correction [34, 47] with a minimum cluster size 200 mm^3. We created NIfTI (.nii) format in our output image and displayed result with Mango (Multi-Image Analysis Graphical User Interface [GUI]), along with MNI space Colin brain template [48]. We labeled the clusters by using SPM Anatomy Toolboxv1.8 (http://www.fz-juelich.de/inm/inm-1/DE/Forschung/_docs/SPMAnatomyToolbox/SPMAnatomyToolbox_node.html) [46, 49, 50].

3. Results

Based on the inclusion criteria, 7 VBM studies [51–57], in which 8 groups of comparison between FMS patients and



(a) (b)

Figure 1: Sagittal, coronal, and axial sections of regions of gray matter reduction in fibromyalgia compared with normal controls ((a) left medial prefrontal cortex and (b) right dorsal posterior cingulate cortex). Results are from the activation likelihood estimation. Activation is significant at $p < 0.05$ corrected for multiple comparisons using the false discovery rate.

controls, were eligible in the final analysis (Table 1). In sum, 180 fibromyalgia patients and 123 controls were included. A total of 47 peak coordinates were reported for brain structural changes, of which 31 were included for decreased gray matter and 16 were included for increased gray matter, in patients of FMS compared with NC. The ALE meta-analysis revealed gray matter decreased in patients of FMS in 2 clusters (illustrated in Figure 1 and Table 2), including left medial prefrontal cortex, extending partly to anterior cingulate area (mPFC/ACC, BA 9/32, cluster size: 432 mm^3, and coordinates of cluster maxima: x: −10, y: 34, z: 30) and right dorsal posterior cingulate cortex, extending to posterior-midcingulate cortex (dPCC/pMCC, BA 31, cluster size: 368 mm^3, and coordinates of cluster maxima: x: 12, y: −18, z: 42). We adopted the classification proposed by Vogt and Destrieux [58, 59] in the subdivisions of the cingulate cortex. No foci survived in the analysis showing gray matter increases in the reverse contrast.

4. Discussion

We employed meta-analytic analysis with ALE to pool VBM studies to assess the structural brain changes between FM patients and healthy subjects. We identified the patients of FM to have GM reduction in left mPFC and right dPCC.

mPFC has been denoted as part of the medial pain system [60, 61]. Stimulation of mPFC with transcranial magnetic stimulation (TMS) pulse may increase sensitivity [62] and decrease threshold of pain perception [63]. In another single-photon emission computed tomography (SPECT) study, the deactivation of mPFC after repetitive TMS over the primary motor cortex was correlated with pain reduction [64]. Thus, mPFC has been postulated to be involved in the descending pain modulation system [65, 66], whether inhibitory or facilitatory. Therefore, our result may be supportive to the proposed mechanism of the dysfunctional descending analgesia in FMS, rendering the chronic widespread pain [67, 68].

Besides pain, neuropsychological symptoms, including depression, anxiety [69], and cognitive complaints [70], are frequent in FMS. As catastrophizing may amplify pain processing [71], it is found that brain activity in mPFC is correlated with catastrophizing [72]. A plausible explanation is that, along with ACC, mPFC is the brain region activated by the anticipation of pain [73] and serves as motivational aspect of pain [74]. Moreover, the morphology in mPFC is negatively correlated with performance in working memory and positively correlated with pain perception [75], a finding that signifies the pain-cognition interaction. In sum, MPFC is the brain region known to modulate cognitive and emotional processing. Along the caudal-rostral axis of mPFC, objective, subject, and even meta-cognitive aspects of pain are represented [76]. It may be these varied functions of mPFC to account for the diverse symptoms in FMS.

Another area that exhibited decreased GM volume in FMS was right dorsal PCC. In the more specific breakdown of this cluster in our study, it actually comprises of dPCC and pMCC. Dorsal (rostral) PCC is associated with pain in meta-analysis [77], and pMCC is the region linked with several chronic somatic pain disorders [78]. Unlike the involvement

Table 1: Overview of the fibromyalgia VBM studies in this meta-analysis.

Name	Year	Journal	Sample/female		Mean age ± SD		Symptoms duration (years)	Foci number	Main area of foci	Scanner	Statistical threshold	Smoothing kernel (mm)	GM change	Tal or MNI
			Case	Control	Case	Control								
Kuchinad	2007	J. Neurosci	10/10	10/10	52	45	9.1 ± 6.8	5	Cingulate, insular, medial frontal cortices, parahippocampal gyri	Siemens 1.5 T	$p < 0.05$ corrected	10	↓	Tal
Schmidt-Wilcke	2007	J. Pain	20/19	22/20	53.6 ± 7.7	50.7 ± 7.3	14.4 ± 7.2	6	GM decrease in superior temporal gyrus and post. thalamus; GM increase in OFC, cerebellum, striatum	Siemens 1.5 T	$p < 0.05$ corrected	10	↑↓	Tal
Wood	2009	J. Pain	30/30	20/20	42.0 ± 8.4	40.1 ± 20.0	NA	4	GM decrease in PCC, ACC, parahippocampal gyri	GE 1.5 T	$p < 0.0005$ uncorr.	12	↓	Tal
Hsu	2009	J. Pain	29/29	29/29	42.6 ± 3.7	42.2 ± 3.8	12.8 ± 8.3	0	No change	GE 3 T	$p < 0.05$ corrected	10	N/A	MNI
Fallon	2013	Neuroimage Clin.	16/16	15/15	38.5 ± 8.5	39.4 ± 8.7	9.1 ± 6.8	4	GM decrease in pons, precuneus; GM increase in SI	Siemens 3 T	$p < 0.05$ corrected	10	↑↓	MNI
Ceko	2013	Neuroimage Clin.	14/14	13/13	55.0 ± 2.9	55.4 ± 3.7	12.1 ± 9.0	14	GM decrease in bilateral ACC, MPFC, frontal pole, right premotor cortex, VLPFC, right DLPFC, right PCC	Siemens 3 T	$p < 0.05$ corrected	8	↓	MNI
Ceko	2013	Neuroimage Clin.	14/14	15/15	42.4 ± 5.9	43.1 ± 5.3	8.8 ± 7.1	9	GM increase in right VLPFC, putamen/GP/NAc, left insula, putamen	Siemens 3 T	$p < 0.05$ corrected	8	↑	MNI
Diaz-Piedra	2015	Brain Imaging Behav	23/23	23/23	41.6 ± 4.4	39.7 ± 5.4	8.6 ± 6.3	2	GM decrease in l' Frontal Med/Sup Orb; GM increase in l' Temporal Pole	Phillips 3 T	$p < 0.05$ corrected	8	↑↓	MNI

Table 2: Regions of gray matter reduction in fibromyalgia relative to healthy controls.

Region	BA	MNI coordinates			ALE extrema value	Cluster size (mm^3)
		x	y	z		
Left medial prefrontal cortex	9/32	−10	34	30	0.0103	432
Right dorsal posterior cingulate cortex	31	12	−18	42	0.0101	368

BA: Brodmann area; MNI: Montreal Neurological Institute; ALE: activation likelihood estimation.

of mPFC and ACC in the emotional component of pain processing [79], dPCC/pMCC is related to orienting the body toward nocuous stimuli, the nonaffective component of pain processing [59], and contributes to the prepotent withdrawal reaction from pain [80]. Damage of dPCC/pMCC could lead to visuospatial damage [81].

While clock drawing test has been employed as a screening tool for cognitive decline in FMS [82], visuospatial memory impairment has been denoted as the early signal of cognitive decline in FMS, even in ahead of verbal memory decline [83, 84]. Our result may provide the neural underpinning for this domain of cognitive deficit in FMS. Besides its role in orienting motor response, dPCC/pMCC is also the initial region demonstrating damage in mild cognitive impairment [81]. Along with MPFC, PCC is involved in self-referential mental activity [85] and plays a pivotal part in default mode network (DMN) [86]. Convergent evidences have reported reduced metabolic activities in PCC in early Alzheimer's disease [87], cognitive decline [88], and ageing [89]. Hence, GM decrease in PCC is attributable to the cognitive dysfunction frequently found in FMS [54] and is also supportive to the view that FMS accelerates premature ageing of the brain [51].

The finding of concurrent GM decrease in mPFC and dPCC in our result does not come in coincidence as they are among the key brain regions constituting DMN. The implication of this result could be inferred from previous resting-state functional connectivity brain imaging studies.

dPCC has been demonstrated with greater connectivity at rest with mPFC [90, 91]. Nevertheless, this connectivity is diminished in FMS [22]. Besides, DMN abnormality is associated with FMS [92] and chronic back pain [93]. At the same time, it has been suggested that greater activation of DMN helps people mind-wander away from pain [94]. Thus, we speculate that integrity of the DMN is necessary for ongoing cognitive modulation on pain processing and that its dysfunction may jeopardize nociception.

5. Limitations

Our study is based on meta-analytic approach with several inherent limitations. First, despite rigorous literature search, we included only 7 studies in the final analysis due to exclusion of those studies using ROI method and those without providing coordinates. In addition, our results may be subject to publication bias. Those with negative finding are less often published. Second, these included VBM studies are varied in their methods, such as difference in preprocessing steps (traditional or optimized), smoothing kernels, and statistical threshold. Besides, inclusion and exclusion criteria differ across the studies. This may also influence the individual result. Third, FMS is a complex disease itself with multiple comorbidities. A recent study found that each symptom dimension in FMS was related to a specific brain morphological change [57]. Another recent database research demonstrated a bidirectional association between FMS and depression [95]. We did not exclude those comorbid with depression specifically. Indeed, our result revealed GM decreases in mPFC, a common region reported both in depression [96, 97] and FMS [54, 56] research. As most pain related brain imaging studies, we cannot ascertain this structural change related to depression or pain itself [98]. In the same vein, to answer the question whether the GM decreases is a consequence of illness or the cause of the illness is out of the scope by current study design. Chronic nociceptive stimulus is thought as a cause for GM decreases [99] as some evidences have displayed correlation between pain duration [25]. Still, more longitudinal cohort studies are needed to disentangle this problem.

6. Conclusions

In conclusion, current meta-analytic results indicate the decrease gray matter volume in left mPFC and right dPCC in patients of FMS. These two areas are related to cognitive, affective, and nonaffective components of pain processing, supporting the notion of central sensitization theory of FMS. In addition, mPFC and right dPCC are also deeply linked with the default mode network. Thus, beside the areas of the typical "pain matrix," such as insula or anterior cingulate gyrus, our results may shed light on other area of the same importance and provide insight over future research on the mechanism of fibromyalgia.

Disclosure

The funders did not play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Chemin Lin, Shwu-Hua Lee, and Hsu-Huei Weng conceived the study design, Chemin Lin and Hsu-Huei Weng conducted the experiments, Chemin Lin wrote the paper, and Hsu-Huei Weng reviewed the analysis. All authors reviewed the manuscript.

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References

[1] F. Wolfe, H. A. Smythe, M. B. Yunus et al., "The American College of Rheumatology 1990 criteria for the classification of fibromyalgia," *Arthritis & Rheumatism*, vol. 33, no. 2, pp. 160–172, 1990.

[2] F. Wolfe, D. J. Clauw, M.-A. Fitzcharles et al., "The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity," *Arthritis Care & Research*, vol. 62, no. 5, pp. 600–610, 2010.

[3] J. A. Verbunt, D. H. F. M. Pernot, and R. J. E. M. Smeets, "Disability and quality of life in patients with fibromyalgia," *Health and Quality of Life Outcomes*, vol. 6, no. 1, article 8, 2008.

[4] M. Kivimäki, P. Leino-Arjas, L. Kaila-Kangas et al., "Increased absence due to sickness among employees with fibromyalgia," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 65–69, 2007.

[5] M.-A. Fitzcharles, P. A. Ste-Marie, and J. X. Pereira, "Fibromyalgia: evolving concepts over the past 2 decades," *Canadian Medical Association Journal*, vol. 185, no. 13, pp. E645–E651, 2013.

[6] F. Wolfe, K. Ross, J. Anderson, I. J. Russell, and L. Hebert, "The prevalence and characteristics of fibromyalgia in the general population," *Arthritis and Rheumatism*, vol. 38, no. 1, pp. 19–28, 1995.

[7] F. Wolfe, E. Brähler, A. Hinz, and W. Häuser, "Fibromyalgia prevalence, somatic symptom reporting, and the dimensionality of polysymptomatic distress: results from a survey of the general population," *Arthritis Care & Research*, vol. 65, no. 5, pp. 777–785, 2013.

[8] P. Mease, "Fibromyalgia syndrome: review of clinical presentation, pathogenesis, outcome measures, and treatment," *The Journal of Rheumatology*, vol. 75, pp. 6–21, 2005.

[9] D. J. Clauw, "Fibromyalgia: an overview," *American Journal of Medicine*, vol. 122, no. 12, pp. S3–S13, 2009.

[10] R. W. Simms, S. H. Roy, M. Hrovat et al., "Lack of association between fibromyalgia syndrome and abnormalities in muscle energy metabolism," *Arthritis & Rheumatism*, vol. 37, no. 6, pp. 794–800, 1994.

[11] J. A. Desmeules, C. Cedraschi, E. Rapiti et al., "Neurophysiologic evidence for a central sensitization in patients with fibromyalgia," *Arthritis & Rheumatism*, vol. 48, no. 5, pp. 1420–1429, 2003.

[12] R. Staud, "Evidence of involvement of central neural mechanisms in generating fibromyalgia pain," *Current rheumatology reports*, vol. 4, no. 4, pp. 299–305, 2002.

[13] J. M. Mountz, L. A. Bradley, J. G. Modell et al., "Fibromyalgia in women. Abnormalities of regional cerebral blood flow in the thalamus and the caudate nucleus are associated with low pain threshold levels," *Arthritis and Rheumatism*, vol. 38, no. 7, pp. 926–938, 1995.

[14] R. Kwiatek, L. Barnden, R. Tedman et al., "Regional cerebral blood flow in fibromyalgia: single-photon-emission computed tomography evidence of reduction in the pontine tegmentum and thalami," *Arthritis and Rheumatism*, vol. 43, no. 12, pp. 2823–2833, 2000.

[15] G. Wik, H. Fischer, B. Bragée, M. Kristianson, and M. Fredrikson, "Retrosplenial cortical activation in the fibromyalgia syndrome," *NeuroReport*, vol. 14, no. 4, pp. 619–621, 2003.

[16] T. Giesecke, R. H. Gracely, M. A. B. Grant et al., "Evidence of augmented central pain processing in idiopathic chronic low back pain," *Arthritis & Rheumatism*, vol. 50, no. 2, pp. 613–623, 2004.

[17] R. H. Gracely, F. Petzke, J. M. Wolf, and D. J. Clauw, "Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia," *Arthritis & Rheumatism*, vol. 46, no. 5, pp. 1333–1343, 2002.

[18] K. B. Jensen, E. Kosek, F. Petzke et al., "Evidence of dysfunctional pain inhibition in Fibromyalgia reflected in rACC during provoked pain," *Pain*, vol. 144, no. 1-2, pp. 95–100, 2009.

[19] R. Staud, J. G. Craggs, W. M. Perlstein, M. E. Robinson, and D. D. Price, "Brain activity associated with slow temporal summation of C-fiber evoked pain in fibromyalgia patients and healthy controls," *European Journal of Pain*, vol. 12, no. 8, pp. 1078–1089, 2008.

[20] M. Diersl, M. T. Schleyl, M. Rancel et al., "Differential central pain processing following repetitive intramuscular proton/prostaglandin E2 injections in female fibromyalgia patients and healthy controls," *European Journal of Pain*, vol. 15, no. 7, pp. 716–723, 2011.

[21] M. Burgmer, E. Pogatzki-Zahn, M. Gaubitz, E. Wessoleck, G. Heuft, and B. Pfleiderer, "Altered brain activity during pain processing in fibromyalgia," *NeuroImage*, vol. 44, no. 2, pp. 502–508, 2009.

[22] I. Cifre, C. Sitges, D. Fraiman et al., "Disrupted functional connectivity of the pain network in fibromyalgia," *Psychosomatic Medicine*, vol. 74, no. 1, pp. 55–62, 2012.

[23] J. Ashburner and K. J. Friston, "Unified segmentation," *NeuroImage*, vol. 26, no. 3, pp. 839–851, 2005.

[24] T. Schmidt-Wilcke, E. Leinisch, S. Gänbauer et al., "Affective components and intensity of pain correlate with structural differences in gray matter in chronic back pain patients," *Pain*, vol. 125, no. 1-2, pp. 89–97, 2006.

[25] A. V. Apkarian, Y. Sosa, S. Sonty et al., "Chronic back pain is associated with decreased prefrontal and thalamic gray matter density," *Journal of Neuroscience*, vol. 24, no. 46, pp. 10410–10415, 2004.

[26] T. Schmidt-Wilcke, E. Leinisch, A. Straube et al., "Gray matter decrease in patients with chronic tension type headache," *Neurology*, vol. 65, no. 9, pp. 1483–1486, 2005.

[27] D. A. Seminowicz, J. S. Labus, J. A. Bueller et al., "Regional gray matter density changes in brains of patients with irritable bowel syndrome," *Gastroenterology*, vol. 139, no. 1, pp. 48.e2–57.e2, 2010.

[28] S. As-Sanie, R. E. Harris, V. Napadow et al., "Changes in regional gray matter volume in women with chronic pelvic pain: a voxel-based morphometry study," *Pain*, vol. 153, no. 5, pp. 1006–1014, 2012.

[29] M. E. Robinson, J. G. Craggs, D. D. Price, W. M. Perlstein, and R. Staud, "Gray matter volumes of pain-related brain areas are decreased in fibromyalgia syndrome," *Journal of Pain*, vol. 12, no. 4, pp. 436–443, 2011.

[30] M. Burgmer, M. Gaubitz, C. Konrad et al., "Decreased gray matter volumes in the cingulo-frontal cortex and the amygdala in patients with fibromyalgia," *Psychosomatic Medicine*, vol. 71, no. 5, pp. 566–573, 2009.

[31] K. B. Jensen, P. Srinivasan, R. Spaeth et al., "Overlapping structural and functional brain changes in patients with long-term exposure to fibromyalgia pain," *Arthritis and Rheumatism*, vol. 65, no. 12, pp. 3293–3303, 2013.

[32] J. Lutz, L. Jäger, D. de Quervain et al., "White and gray matter abnormalities in the brain of patients with fibromyalgia: a diffusion-tensor and volumetric imaging study," *Arthritis & Rheumatism*, vol. 58, no. 12, pp. 3960–3969, 2008.

[33] S. B. Eickhoff, A. R. Laird, C. Grefkes, L. E. Wang, K. Zilles, and P. T. Fox, "Coordinate-based activation likelihood estimation meta-analysis of neuroimaging data: a random-effects approach based on empirical estimates of spatial uncertainty," *Human Brain Mapping*, vol. 30, no. 9, pp. 2907–2926, 2009.

[34] A. R. Laird, P. M. Fox, C. J. Price et al., "ALE meta-analysis: controlling the false discovery rate and performing statistical contrasts," *Human Brain Mapping*, vol. 25, no. 1, pp. 155–164, 2005.

[35] R. C. K. Chan, X. Di, G. M. McAlonan, and Q.-Y. Gong, "Brain anatomical abnormalities in high-risk individuals, first-episode, and chronic schizophrenia: an activation likelihood estimation meta-analysis of illness progression," *Schizophrenia Bulletin*, vol. 37, no. 1, pp. 177–188, 2011.

[36] E. Bora, A. Fornito, M. Yücel, and C. Pantelis, "Voxelwise meta-analysis of gray matter abnormalities in bipolar disorder," *Biological Psychiatry*, vol. 67, no. 11, pp. 1097–1105, 2010.

[37] S. Kühn and J. Gallinat, "Gray matter correlates of posttraumatic stress disorder: a quantitative meta-analysis," *Biological Psychiatry*, vol. 73, no. 1, pp. 70–74, 2013.

[38] L. K. Ferreira, B. S. Diniz, O. V. Forlenza, G. F. Busatto, and M. V. Zanetti, "Neurostructural predictors of Alzheimer's disease: a meta-analysis of VBM studies," *Neurobiology of Aging*, vol. 32, no. 10, pp. 1733–1741, 2011.

[39] H.-H. Weng, C.-F. Chen, Y.-H. Tsai et al., "Gray matter atrophy in narcolepsy: an activation likelihood estimation meta-analysis," *Neuroscience & Biobehavioral Reviews*, vol. 59, pp. 53–63, 2015.

[40] R. F. Smallwood, A. R. Laird, A. E. Ramage et al., "Structural brain anomalies and chronic pain: a quantitative meta-analysis of gray matter volume," *Journal of Pain*, vol. 14, no. 7, pp. 663–675, 2013.

[41] A. May, "Morphing voxels: the hype around structural imaging of headache patients," *Brain*, vol. 132, no. 6, pp. 1419–1425, 2009.

[42] J. L. Lancaster, D. Tordesillas-Gutiérrez, M. Martinez et al., "Bias between MNI and talairach coordinates analyzed using the ICBM-152 brain template," *Human Brain Mapping*, vol. 28, no. 11, pp. 1194–1205, 2007.

[43] A. R. Laird, J. L. Robinson, K. M. McMillan et al., "Comparison of the disparity between Talairach and MNI coordinates in functional neuroimaging data: validation of the Lancaster transform," *NeuroImage*, vol. 51, no. 2, pp. 677–683, 2010.

[44] S. B. Eickhoff, D. Bzdok, A. R. Laird, F. Kurth, and P. T. Fox, "Activation likelihood estimation meta-analysis revisited," *NeuroImage*, vol. 59, no. 3, pp. 2349–2361, 2012.

[45] P. E. Turkeltaub, G. F. Eden, K. M. Jones, and T. A. Zeffiro, "Meta-analysis of the functional neuroanatomy of single-word reading: method and validation," *NeuroImage*, vol. 16, no. 3, part 1, pp. 765–780, 2002.

[46] S. B. Eickhoff, K. E. Stephan, H. Mohlberg et al., "A new SPM toolbox for combining probabilistic cytoarchitectonic maps and functional imaging data," *NeuroImage*, vol. 25, no. 4, pp. 1325–1335, 2005.

[47] C. R. Genovese, N. A. Lazar, and T. Nichols, "Thresholding of statistical maps in functional neuroimaging using the false discovery rate," *NeuroImage*, vol. 15, no. 4, pp. 870–878, 2002.

[48] C. J. Holmes, R. Hoge, L. Collins, R. Woods, A. W. Toga, and A. C. Evans, "Enhancement of MR images using registration for signal averaging," *Journal of Computer Assisted Tomography*, vol. 22, no. 2, pp. 324–333, 1998.

[49] S. B. Eickhoff, S. Heim, K. Zilles, and K. Amunts, "Testing anatomically specified hypotheses in functional imaging using cytoarchitectonic maps," *NeuroImage*, vol. 32, no. 2, pp. 570–582, 2006.

[50] S. B. Eickhoff, T. Paus, S. Caspers et al., "Assignment of functional activations to probabilistic cytoarchitectonic areas revisited," *NeuroImage*, vol. 36, no. 3, pp. 511–521, 2007.

[51] A. Kuchinad, P. Schweinhardt, D. A. Seminowicz, P. B. Wood, B. A. Chizh, and M. C. Bushnell, "Accelerated brain gray matter loss in fibromyalgia patients: premature aging of the brain?" *The Journal of Neuroscience*, vol. 27, no. 15, pp. 4004–4007, 2007.

[52] T. Schmidt-Wilcke, R. Luerding, T. Weigand et al., "Striatal grey matter increase in patients suffering from fibromyalgia—a voxel-based morphometry study," *Pain*, vol. 132, no. 1, pp. S109–S116, 2007.

[53] M. C. Hsu, R. E. Harris, P. C. Sundgren et al., "No consistent difference in gray matter volume between individuals with fibromyalgia and age-matched healthy subjects when controlling for affective disorder," *Pain*, vol. 143, no. 3, pp. 262–267, 2009.

[54] P. B. Wood, M. F. Glabus, R. Simpson, and J. C. Patterson II, "Changes in gray matter density in fibromyalgia: correlation with dopamine metabolism," *The Journal of Pain*, vol. 10, no. 6, pp. 609–618, 2009.

[55] N. Fallon, J. Alghamdi, Y. Chiu, V. Sluming, T. Nurmikko, and A. Stancak, "Structural alterations in brainstem of fibromyalgia syndrome patients correlate with sensitivity to mechanical pressure," *NeuroImage: Clinical*, vol. 3, pp. 163–170, 2013.

[56] M. Ceko, M. C. Bushnell, M.-A. Fitzcharles, and P. Schweinhardt, "Fibromyalgia interacts with age to change the brain," *NeuroImage: Clinical*, vol. 3, pp. 249–260, 2013.

[57] C. Diaz-Piedra, M. A. Guzman, G. Buela-Casal, and A. Catena, "The impact of fibromyalgia symptoms on brain morphometry," *Brain Imaging and Behavior*, pp. 1–14, 2015.

[58] C. Destrieux, B. Fischl, A. Dale, and E. Halgren, "Automatic parcellation of human cortical gyri and sulci using standard anatomical nomenclature," *NeuroImage*, vol. 53, no. 1, pp. 1–15, 2010.

[59] B. A. Vogt, "Pain and emotion interactions in subregions of the cingulate gyrus," *Nature Reviews Neuroscience*, vol. 6, no. 7, pp. 533–544, 2005.

[60] B. A. Vogt, R. W. Sikes, and L. J. Vogt, "Anterior cingulate cortex and the medial pain system," in *Neurobiology of Cingulate Cortex and Limbic Thalamus*, pp. 313–344, Springer, 1993.

[61] A. K. P. Jones, B. Kulkarni, and S. W. G. Derbyshire, "Pain mechanisms and their disorders: imaging in clinical neuroscience," *British Medical Bulletin*, vol. 65, no. 1, pp. 83–93, 2003.

[62] V. Mylius, J. Reis, M. Kunz et al., "Modulation of electrically induced pain by paired pulse transcranial magnetic stimulation of the medial frontal cortex," *Clinical Neurophysiology*, vol. 117, no. 8, pp. 1814–1820, 2006.

[63] W.-K. Yoo, Y.-H. Kim, W.-S. Doh et al., "Dissociable modulating effect of repetitive transcranial magnetic stimulation on sensory and pain perception," *NeuroReport*, vol. 17, no. 2, pp. 141–144, 2006.

[64] Y. Tamura, S. Okabe, T. Ohnishi et al., "Effects of 1-Hz repetitive transcranial magnetic stimulation on acute pain induced by capsaicin," *Pain*, vol. 107, no. 1-2, pp. 107–115, 2004.

[65] M. M. Heinricher, I. Tavares, J. L. Leith, and B. M. Lumb, "Descending control of nociception: specificity, recruitment and plasticity," *Brain Research Reviews*, vol. 60, no. 1, pp. 214–225, 2009.

[66] I. Tracey and P. W. Mantyh, "The cerebral signature for pain perception and its modulation," *Neuron*, vol. 55, no. 3, pp. 377–391, 2007.

[67] A. M. Abeles, M. H. Pillinger, B. M. Solitar, and M. Abeles, "Narrative review: the pathophysiology of fibromyalgia," *Annals of Internal Medicine*, vol. 146, no. 10, pp. 726–734, 2007.

[68] T. Schmidt-Wilcke and D. J. Clauw, "Pharmacotherapy in fibromyalgia (FM)—implications for the underlying pathophysiology," *Pharmacology & Therapeutics*, vol. 127, no. 3, pp. 283–294, 2010.

[69] L. Gormsen, R. Rosenberg, F. W. Bach, and T. S. Jensen, "Depression, anxiety, health-related quality of life and pain in patients with chronic fibromyalgia and neuropathic pain," *European Journal of Pain*, vol. 14, no. 2, pp. 127.e1–127.e8, 2010.

[70] J. M. Glass, "Fibromyalgia and cognition," *The Journal of Clinical Psychiatry*, vol. 69, no. 2, pp. 20–24, 2008.

[71] R. R. Edwards, C. O. Bingham III, J. Bathon, and J. A. Haythornthwaite, "Catastrophizing and pain in arthritis, fibromyalgia, and other rheumatic diseases," *Arthritis Care & Research*, vol. 55, no. 2, pp. 325–332, 2006.

[72] R. H. Gracely, M. E. Geisser, T. Giesecke et al., "Pain catastrophizing and neural responses to pain among persons with fibromyalgia," *Brain*, vol. 127, no. 4, pp. 835–843, 2004.

[73] A. Ploghaus, I. Tracey, J. S. Gati et al., "Dissociating pain from its anticipation in the human brain," *Science*, vol. 284, no. 5422, pp. 1979–1981, 1999.

[74] T. V. Sewards and M. A. Sewards, "Fear and power-dominance drive motivation: neural representations and pathways mediating sensory and mnemonic inputs, and outputs to premotor structures," *Neuroscience & Biobehavioral Reviews*, vol. 26, no. 5, pp. 553–579, 2002.

[75] R. Luerding, T. Weigand, U. Bogdahn, and T. Schmidt-Wilcke, "Working memory performance is correlated with local brain morphology in the medial frontal and anterior cingulate cortex in fibromyalgia patients: structural correlates of pain–cognition interaction," *Brain*, vol. 131, no. 12, pp. 3222–3231, 2008.

[76] D. M. Amodio and C. D. Frith, "Meeting of minds: the medial frontal cortex and social cognition," *Nature Reviews Neuroscience*, vol. 7, no. 4, pp. 268–277, 2006.

[77] F. Å. Nielsen, D. Balslev, and L. K. Hansen, "Mining the posterior cingulate: segregation between memory and pain components," *NeuroImage*, vol. 27, no. 3, pp. 520–532, 2005.

[78] B. A. Vogt, G. R. Berger, and S. W. G. Derbyshire, "Structural and functional dichotomy of human midcingulate cortex," *European Journal of Neuroscience*, vol. 18, no. 11, pp. 3134–3144, 2003.

[79] A. Etkin, T. Egner, and R. Kalisch, "Emotional processing in anterior cingulate and medial prefrontal cortex," *Trends in Cognitive Sciences*, vol. 15, no. 2, pp. 85–93, 2011.

[80] B. A. Vogt, "Submodalities of emotion in the context of cingulate subregions," *Cortex*, vol. 59, pp. 197–202, 2014.

[81] B. Vogt, *Cingulate Neurobiology and Disease*, Oxford University Press, 2009.

[82] S. S. Can, A. Gencay-Can, and Z. Gunendi, "Validity and reliability of the clock drawing test as a screening tool for cognitive impairment in patients with fibromyalgia," *Comprehensive Psychiatry*, vol. 53, no. 1, pp. 81–86, 2012.

[83] R. Cánovas, I. León, M. D. Roldán, R. Astur, and J. M. Cimadevilla, "Virtual reality tasks disclose spatial memory alterations in fibromyalgia," *Rheumatology*, vol. 48, no. 10, pp. 1273–1278, 2009.

[84] S.-H. Kim, S.-H. Kim, S.-K. Kim, E. J. Nam, S. W. Han, and S. J. Lee, "Spatial versus verbal memory impairments in patients with fibromyalgia," *Rheumatology International*, vol. 32, no. 5, pp. 1135–1142, 2012.

[85] D. A. Gusnard, E. Akbudak, G. L. Shulman, and M. E. Raichle, "Medial prefrontal cortex and self-referential mental activity: relation to a default mode of brain function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 4259–4264, 2001.

[86] P. Fransson and G. Marrelec, "The precuneus/posterior cingulate cortex plays a pivotal role in the default mode network: evidence from a partial correlation network analysis," *NeuroImage*, vol. 42, no. 3, pp. 1178–1184, 2008.

[87] S. Minoshima, B. Giordani, S. Berent, K. A. Frey, N. L. Foster, and D. E. Kuhl, "Metabolic reduction in the posterior cingulate cortex in very early Alzheimer's disease," *Annals of Neurology*, vol. 42, no. 1, pp. 85–94, 1997.

[88] R. Leech and D. J. Sharp, "The role of the posterior cingulate cortex in cognition and disease," *Brain*, vol. 137, no. 1, pp. 12–32, 2014.

[89] J. S. Damoiseaux, C. F. Beckmann, E. J. S. Arigita et al., "Reduced resting-state brain activity in the 'default network' in normal aging," *Cerebral Cortex*, vol. 18, no. 8, pp. 1856–1864, 2008.

[90] B. A. Vogt, L. Vogt, and S. Laureys, "Cytology and functionally correlated circuits of human posterior cingulate areas," *NeuroImage*, vol. 29, no. 2, pp. 452–466, 2006.

[91] D. S. Margulies, J. L. Vincent, C. Kelly et al., "Precuneus shares intrinsic functional architecture in humans and monkeys," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 47, pp. 20069–20074, 2009.

[92] V. Napadow, L. LaCount, K. Park, S. As-Sanie, D. J. Clauw, and R. E. Harris, "Intrinsic brain connectivity in fibromyalgia is associated with chronic pain intensity," *Arthritis & Rheumatism*, vol. 62, no. 8, pp. 2545–2555, 2010.

[93] M. N. Baliki, P. Y. Geha, A. V. Apkarian, and D. R. Chialvo, "Beyond feeling: chronic pain hurts the brain, disrupting the default-mode network dynamics," *Journal of Neuroscience*, vol. 28, no. 6, pp. 1398–1403, 2008.

[94] A. Kucyi, T. V. Salomons, and K. D. Davis, "Mind wandering away from pain dynamically engages antinociceptive and default mode brain networks," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 46, pp. 18692–18697, 2013.

[95] M.-H. Chang, J.-W. Hsu, K.-L. Huang et al., "Bidirectional association between depression and fibromyalgia syndrome: a nationwide longitudinal study," *The Journal of Pain*, vol. 16, no. 9, pp. 895–902, 2015.

[96] P. C. M. P. Koolschijn, N. E. M. van Haren, G. J. L. M. Lensvelt-Mulders, H. E. Hulshoff Pol, and R. S. Kahn, "Brain volume abnormalities in major depressive disorder: a meta-analysis of magnetic resonance imaging studies," *Human Brain Mapping*, vol. 30, no. 11, pp. 3719–3735, 2009.

[97] E. Bora, A. Fornito, C. Pantelis, and M. Yücel, "Gray matter abnormalities in Major Depressive Disorder: a meta-analysis of

voxel based morphometry studies," *Journal of Affective Disorders*, vol. 138, no. 1-2, pp. 9–18, 2012.

[98] R. F. Smallwood, A. R. Laird, A. E. Ramage et al., "Structural brain anomalies and chronic pain: a quantitative meta-analysis of gray matter volume," *The Journal of Pain*, vol. 14, no. 7, pp. 663–675, 2013.

[99] A. May, "Structural brain imaging: a window into chronic pain," *Neuroscientist*, vol. 17, no. 2, pp. 209–220, 2011.

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Research Article

Immunosuppressive Treatment for Lupus Nephritis: Long-Term Results in 178 Patients

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Lupus nephritis is one of the most severe Systemic Lupus Erythematosus features, defining treatment modality and prognosis. Our retrospective study, including 178 patients treated for lupus nephritis during 23 years with mostly cyclophosphamide-based initial regimens followed by azathioprine or mycophenolic acid, demonstrates 84.8% of renal response with 19.2% of flares, 15-year patient survival 78.7% and kidney survival 76.3%, and low damage accrual. Both patient and kidney survival significantly differ for subgroups that achieved complete or partial renal response and nonresponders: patient 15-year survival 95% versus 65% versus 35%; kidney 15-year survival 100% versus 58% versus 0%, respectively. 51% (24 out of 47) of patients evaluated at the end of the study period sustained complete renal response; however, only 9 of them had 0 disease activity according to SELENA SLEDAI scale, while 13 patients had scores 2–4 due to the serological abnormalities only. We conclude that (1) initial treatment with cyclophosphamide followed by azathioprine is effective and can be used in agreement with International Guidelines until the evidence for biological treatments benefits becomes available; (2) complete and even partial renal response have positive prognostic value, and failure to achieve renal response negatively influences kidney and patient survival; (3) the validity of complete renal response in SLE is questioned by the absence of conventional definition of SLE remission.

1. Introduction

Lupus nephritis (LN) is one of the most severe manifestations of Systemic Lupus Erythematosus (SLE), mainly defining treatment modality and prognosis. Approximately 50% of SLE patients develop LN, which increases the risks for renal failure, cardiovascular disease, and death. Clinical presentation of LN varies from mild asymptomatic proteinuria to severe nephrotic syndrome (NS), hematuria, and renal failure [1, 2]. The pathogenesis of LN has not been clarified so far; however, among a huge variety of autoantibodies involved in SLE tissue damage, LN retains the most extensive group and is triggered by complex autoantibody interactions. Development and progression of LN is regarded as a multistep inflammatory process which is incited by anti-DNA and antinucleosome antibodies, culminating in a self-maintaining inflammatory loop with spreading of glomerular inflammation. In the maintenance of the inflammatory process, proinflammatory antibodies are involved, among which anti-C1q is thought to play a major role [3].

Being one of the major features of SLE, renal disorder is listed in the American College of Rheumatology (ACR) Revised Criteria for Classification of SLE [4]. Pathology evaluation of LN is crucial: according to the EULAR/ERA-EDTA recommendations for the management of adult and pediatric lupus nephritis [5], immunosuppressive treatment should be guided by renal biopsy findings, assessed according to the International Society of Nephrology/Renal Pathology Society 2003 classification [6]. Initial treatment (IT) recommended for patients with class III-IV (±V) LN includes mycophenolic acid (MPA) or low-dose intravenous cyclophosphamide (CY) in combination with glucocorticoids. In patients with adverse clinical or histological features, CY can be prescribed at higher doses, while azathioprine (AZA) is an alternative for

milder cases. For patients not responding to MPA or CY, switching from MPA to CY and vice versa or introduction of rituximab should be considered. For pure class V LN, presenting with NS, IT options are MPA, CY, or calcineurin inhibitors (cyclosporine, tacrolimus) in combination with oral glucocorticoids. In patients improving after IT, subsequent treatment (ST) with MPA or AZA is recommended for at least 3 years. Calcineurin inhibitors can be considered for ST in pure class V LN. Hydroxychloroquine is currently recommended for all LN patients. KDIGO Clinical Practice Guideline for Glomerulonephritis [7] provides very similar approaches to the LN management.

According to the Treat-to-Target paradigm, the treatment target in SLE patients should be remission of systemic symptoms and organ manifestations or, if remission cannot be reached, the lowest possible disease activity, measured by a validated lupus activity index and/or by organ-specific markers. Since damage predicts subsequent death, prevention of damage accrual should be a major therapeutic goal in SLE. SELENA SLEDAI Disease Assessment Scale and SLICC/ACR Damage Index are recommended for assessment of SLE activity and damage [8].

Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), modified in the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) trial and known as SELENA SLEDAI system, is a list of 24 clinical and laboratory descriptors, scored on the basis of their presence or absence in the previous 10 days before scoring. The maximum theoretical score for the SELENA SLEDAI is 105 (all 24 descriptors present simultaneously) with 0 indicating inactive disease. The Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) Damage Index was designed and validated for SLE patients to capture nonreversible organ damage, not related to active inflammation, and lasting at least 6 months [9–11].

However, while remission was used to be described as a favourable clinical state for patients with SLE since at least 1970s, there has not yet been an agreed-upon definition of remission in SLE. There are a number of different ad hoc definitions of remission that have been used in clinical trials and observational studies. The definition of SLE remission, merging clinical disease activity, serological activity, duration, and subsequent treatment still is under discussion [12]. The recent analysis highlights important ongoing disease activity, symptom burden, and immunosuppressive medication in European patients with SLE considered by their treating physician to be "in remission," indicating that for a further improvement of outcomes there is an urgent need for an international consensus on the definitions for remission among patients with SLE [13].

On the other hand, instruments for lupus nephritis evaluation are currently developed. Although the definitions of remission for LN were controversial for more than two decades [14, 15], and the impact of decrease of proteinuria versus hematuria is not completely clear so far [16], KDIGO, based on the evaluation of published clinical trials, provides definitions for the response to therapy in LN as follows: complete response (CR)—return of serum creatinine (SCr) to previous baseline plus decline in urine protein/creatinine ratio (uPCR) to <50 mg/mmol; partial response (PR)—stabilisation or improvement of SCr but not to normal range, plus ≥50% decrease in uPCR and uPCR ≤300 mg/mmol [7]. EULAR/ERA-EDTA recommendations also point that immunosuppressive treatment targets are complete renal response (proteinuria <0.5 g/24-hr with normal or near-normal renal function) or at least partial renal response (≥50% reduction in proteinuria with decrease to subnephrotic levels and normal or near-normal GFR), which should be achieved preferably by 6 months and no later than 12 months following treatment initiation [4].

In this retrospective study, we aimed to evaluate some demographic and clinical features, pathology patterns, treatment results, and outcomes in the group of patients with lupus nephritis, receiving immunosuppressive treatment in our unit for 23 years, and asses the remission status in the cohort followed till 2015.

2. Materials and Methods

2.1. Patient's Selection and Workup. Using electronic database and specifically designed charts, we selected 185 SLE patients, treated in our centre in 1992–2015. Workup, beyond routine, included lupus serology tests (anti-DNA antibodies, antinuclear antibodies, anticardiolipin antibodies, lupus anticoagulant, and C3/C4 complement) and kidney biopsy. Diagnosis was based on ACR criteria.

2.2. Kidney Biopsy. Kidney core biopsy was taken with BARD-Magnum biopsy guidance facility. Obtained specimens were divided into two parts and processed for light microscopy and immunohistology. Formalin fixed/paraffin embedded sections for light microscopy were stained with hematoxylin and eosin, Masson's trichrome, and periodic acid-Shiff. Unfixed cryosections stained for IgA, IgG, IgM, C3, C1q, kappa and lambda light chains, and fibrinogen. Kidney biopsies were evaluated by dedicated nephropathologists according to ISN/RPS Classification; biopsies obtained before 2004 were reassessed for current analysis.

2.3. Treatment Regimens. IT regimens included high dose i.v. and oral steroids in combination with i.v. CY, MPA, cyclosporine-A (CyA), or AZA. Low-dose steroids combined with MPA, AZA, CyA, and i.v. CY quarterly were used for ST. In some patients, steroids only were used both for IT and ST. Hydroxychloroquine was added on top of any regimen since 2012. Anticoagulants and/or antiplatelet agents were used in patients with antiphospholipid syndrome and circulating antiphospholipid antibodies. Rituximab was used as a rescue therapy since 2013 in selected refractory cases.

2.4. Results Assessment. Primary efficacy end points, complete response (CR) and partial response (PR), for LN were evaluated according to the degree of proteinuria and SCr level based on KDIGO definition, plus resolving of hematuria. Failure to achieve at least PR in 12 months of IT was considered as no response (NR). "Hard" outcomes were defined as

Table 1: Clinical presentation of lupus nephritis.

Symptom	Haematuria	Proteinuria	Nephrotic syndrome	Impaired kidney function	SCr in patients with impaired kidney function, μmol/L
n	161	95	90	92	236 [121; 2097]
%	87.0	51.3	48.6	49.7	

Table 2: The distribution of lupus nephritis pathology classes.

LN classes	1st biopsy		2nd biopsy	
	n	%	*n*	%
Class I	5	4.6	1	6.6
Class II	12	11.1	2	13.3
Class III	22	20.3	3	20
Class IV	42	38.8	3	20
Class V	15	13.8	2	13.3
Class V + class III/IV	3	2.7	2	13.3
Class VI	9	8.3	2	13.3
Total	108	100	15	100

patient's death and kidney death, which was defined as the progression to end stage of renal disease (ESRD).

SELENA SLEDAI Disease Assessment Scales and SLICC/ACR Damage Index were used for SLE activity and damage accrual evaluation.

2.5. Statistics. Statistical analysis was performed using SPSS 11.5 program package. Differences significance for categorical variables was evaluated by Fisher's exact test and χ^2 test. For abnormally distributed variables, median value and interquartile range were calculated, and Mann-Whitney U test and Kruskall-Wallis test were used for comparison of these variables. p value < 0.05 was defined for statistical significance.

3. Results

3.1. Study Population. Patients with SLE constituted 1.7% (185 out of 10599) of subjects treated in our nephrology clinic over more than 20 years. Study group included 28 (15.1%) males and 157 (84.8%) females with median age of 29 [15; 70] years; 173 (93.5%) were Caucasian and 12 (6.5%) were Asian. In 89 (48.1%) cases, SLE was first diagnosed in our centre, and 96 (51.9%) patients were referred from other centres, mostly rheumatology, with previously diagnosed SLE.

3.2. Clinical Presentation. Patients presented with hematuria, proteinuria/NS, impaired kidney function, and multiple extrarenal manifestations; LN clinical features are shown in Table 1.

3.3. Pathology Presentation. 108 (58.3%) patients underwent kidney biopsy; in 15 cases (13.8% out of biopsied patients), the second kidney biopsy was performed in 6–118 months after the first biopsy (Table 2).

3.4. Immunosuppressive Treatment Regimens. Seven patients did not receive immunosuppressants and were excluded from further analysis. 165 out of 178 patients on immunosuppression were started on IT; 111 patients received ST; 96 patients were treated with both IT and ST in our centre. Treatment regimens are shown in Table 3. Hydroxychloroquine in 51 (28.6%) cases and anticoagulants and/or antiplatelet agents in 83 (46.6%) were used on the top of any regimen.

3.5. Initial Treatment Results. CR of LN in 63 (35.3%) cases and PR of LN in 88 (49.4%) cases were achieved, while in 27 (15.1%) patients treatment failed. Among those 151 who achieved remission, 122 (80.7%) sustained remission status and 29 (19.2%) patients subsequently developed renal flares.

3.6. Long-Term Outcomes. Median follow-up period comprised 12 [1; 236] months. At the end of the study period (last assessment, December 2015), 47 (26.4%) out of 178 patients on immunosuppression were alive and not on dialysis, 18 (10.1%) started dialysis, 95 (53.3%) were lost for follow-up, and 18 (10.1%) died.

In patients who did not develop ESRD and did not recover kidney function at the last evaluation, median SCr was 182 [115; 580] μmol/L. 32 patients completely recovered kidney function.

Causes of death were thrombotic complications of antiphospholipid syndrome in 7 cases, infectious complications in 5 cases, cardiac failure in 4 cases, and intracranial haemorrhage in 2 cases.

3.7. Patient and Kidney Survival. We did not find differences in the overall patient and kidney survival. 5-year patient

Table 3: Treatment regimens for initial and subsequent therapy.

	Steroids + CY		Steroids + MPA		Steroids + CyA		Steroids + AZA		Steroids only		Total
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	
IT	90	54.5	11	6.6	20	12.1	20	12.1	24	14.5	165
ST	5	4.5	27	24.3	17	15.3	30	27.0	32	28.2	111

Table 4: SELENA SLEDAI and SLICC scoring in the cohort of 47 patients with LN remission.

	SELENA SLEDAI				SLICC/ACR			
	0	2–4	6–8	10–12	0	1-2	3-4	5-6
n	19	18	8	2	14	20	11	2
%	40.4	38.2	17.0	4.2	29.7	42.5	23.4	4.2

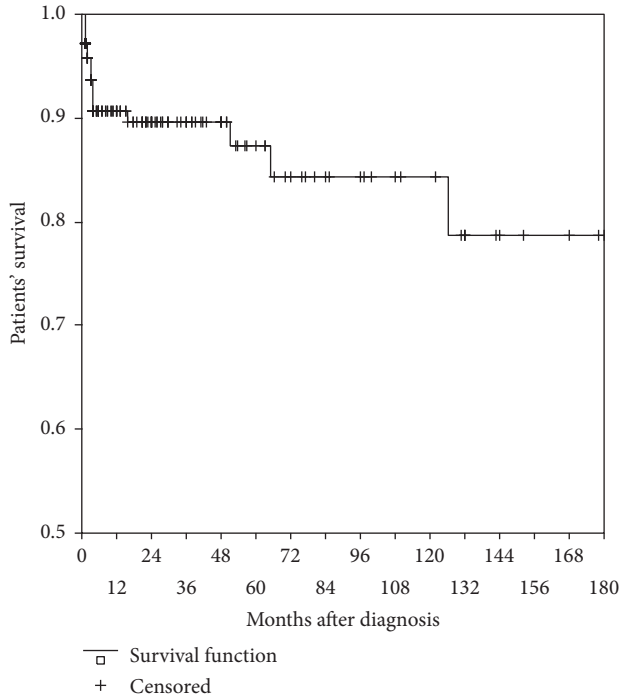

Figure 1: 15-year patient survival.

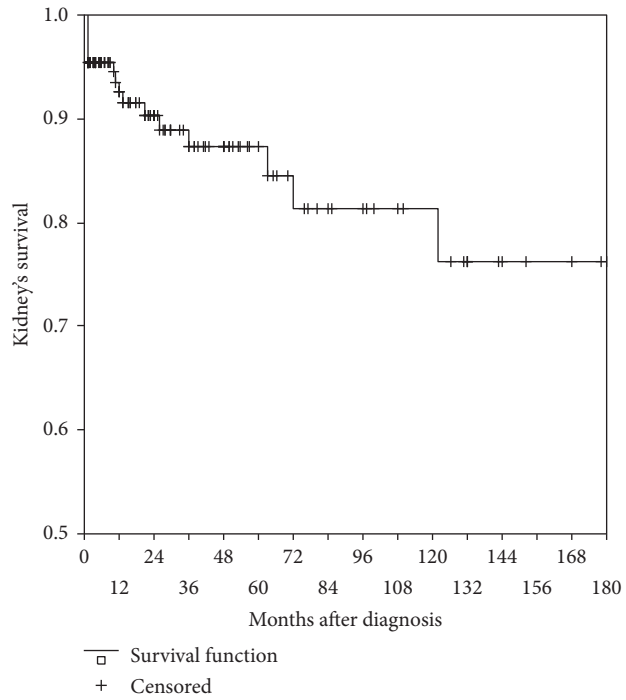

Figure 2: 15-year kidney survival.

and kidney survival were 87.2% and 87.3%, respectively, 10-year patient and kidney survival were 81.3% and 81.4%, respectively, and 15-year patient and kidney survival turned to be 78.7% and 76.3%, respectively, as shown in Figures 1 and 2.

We analysed patient and kidney survival with respect to CR and PR of LN, achieved after IT, or to NR. 15-year patient survival was 95% for CR of LN versus 65% for PR of LN. In cases with NR, 5-year patient survival was only 35% (Figure 3). All the differences were statistically significant ($p < 0.01$).

15-year kidney survival was 100% in patients with CR of LN versus 58% in patients who achieved only PR of LN. In patients with NR kidney death occurred in all cases to the 5th year of follow-up (Figure 4). All the differences were statistically significant ($p < 0.01$).

3.8. Remission Status. 47 patients were evaluated for LN remission status, SLE activity, and damage accrual at the latest follow-up visit in 2015. In this cohort, 24 (51.0%) patients achieved and sustained CR, and 21 (44.7%) had PR of LN. Only 2 (4.3%) patients, who previously achieved CR, had a nonresolved renal flare at the latest follow-up assessment. SELENA SLEDAI Disease Assessment Scales and SLICC/ACR Damage Index data for these patients are shown in Table 4.

Among 24 patients with sustained CR of LN, only 9 (37.5%) had score of 0 disease activity, 13 (54.1%) had scores of 2–4, and 2 had score of 6 according to SELENA SLEDAI Disease Assessment Scales. In all 13 cases with CR of LN and SELENA SLEDAI scores 2–4, disease activity presented only by increased anti-DNA antibodies and/or decreased complement levels.

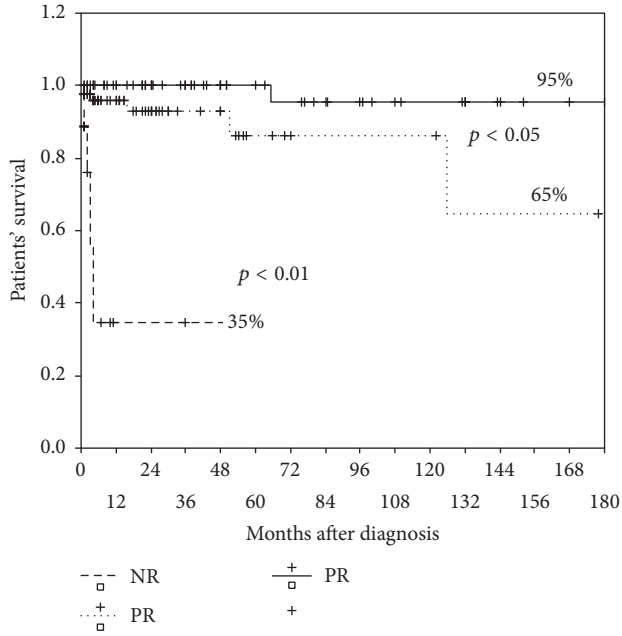

Figure 3: 15-year patient survival in patients with CR, PR, and NR.

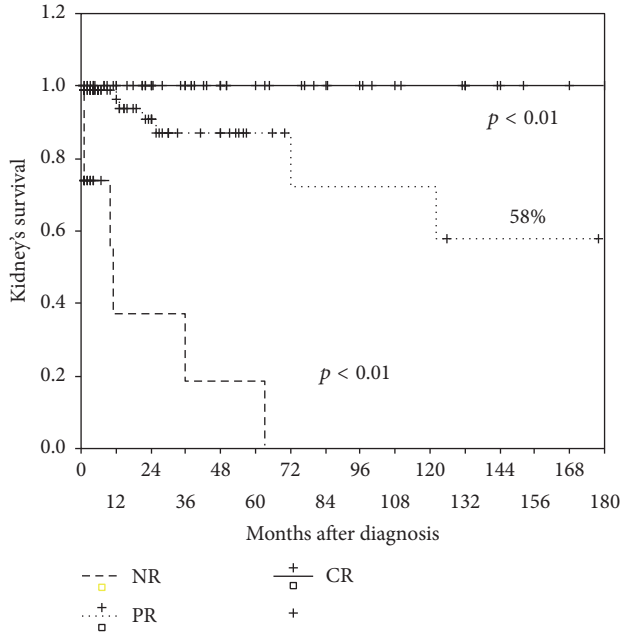

Figure 4: 15-year kidney survival in patients with CR, PR, and ND.

4. Discussion

In our LN patients population prevailed young women of Caucasian origin, almost half of them with newly diagnosed SLE, mainly presenting with NS, hematuria, impaired kidney function, and diffuse or focal proliferative LN (classes III and IV) by pathology. Our retrospective study includes patients treated long before International Guidelines, outlining the exclusively low threshold for kidney biopsy indications that were developed [5, 7]; therefore, the proportion of biopsy proven LN is only 58%, reflecting the fact that in early 90s we rarely biopsied patients with less severe clinical manifestations.

Immunosuppressive treatment regimens in our group are compatible with the current guidelines recommendations [5, 7]. Combination of steroids and CY was the dominant treatment option for IT, while MPA and AZA in equal proportion were more often used for ST. The only exception is the usage of steroids only for IT and/or ST in early 90s. That time steroids only were used in patients who did not tolerate or refused CY/AZA and could not receive MPA, which was not available for LN treatment in our country before 1999. Cyclosporine was used for IT and ST mostly in patients with class V LN, which again matches the current guidelines recommendations. We did not analyze rituximab usage results, as it was not available for LN treatment until 2013, and since that it was always second treatment option after IT failure. We also did not analyze hydroxychloroquine and anticoagulants/antiplatelets impact, as that was beyond the scope of the current research.

IT overall efficacy (CR plus PR) turned to be 84.8%, with the rate of CR 35.3%, which is similar to Chen et al. data [15] and higher than the ALMS study [17], probably because our study group included not only patients with LN lass III–V but also milder cases. Under ST, the rate of flares turned to be 19.2% during median follow-up of 12 [1; 236] months, similar to the data from the long-term follow-up of the MAINTAIN Nephritis Trial [18]. We did not specifically address the issue of different immunosuppression regimens efficacy in this study, but the general clinical assessment does not suggest benefits of MPA over CY and AZA in our group of patients, which is in agreement with the findings from ALMS study and long-term follow-up of the MAINTAIN Nephritis Trial [17, 18].

Patient and kidney overall 15-year survival were higher than 75%. Importantly, in those who achieved CR after IT, patient and kidney 15-year survival were 95% and 100%, respectively. In patients who achieved PR, patient and kidney survival were 65% and 58%, respectively, and in nonresponders they were 35% and 0%, respectively. These differences confirm the positive prognostic value of complete and even partial LN response [15, 16], associated with significantly better outcomes compared to NR, and stress that failure to achieve renal response to immunosuppression negatively influences not only kidney but also patient survival.

Almost half of biopsy proven LN cases were available for evaluation at the end of the study period. Number of remissions increased to 95.7%, confirming the higher efficacy of biopsy-guided treatment [5, 7].

In terms of remission assessment, it is important to highlight that among 24 patients with sustained CR of LN more than a half had scores 2–4 by SELENA SLEDAI Disease Assessment Scales due to the elevated anti-DNA antibodies and complement abnormalities. These data support the need for the agreed-upon definition of remission in SLE [12].

Damage accrual was relatively low; majority of patients had scores 0–2 according to SLICC/ACR Damage Index, mostly due to steroid cataract, diabetes, osteoporosis, or incomplete recovery of kidney function. Steroid therapy

complications clearly prevailed, confirming the necessity of tapering or even discontinuation of steroid usage after 3 years of sustained remission [5, 7].

5. Conclusions

Treatment results and long-term outcomes in our group of 178 lupus nephritis patients, treated during the 23-year period with mostly cyclophosphamide-based initial regimens followed by azathioprine or mycophenolic acid, demonstrate 84.8% of renal response with only 19.2% of flares during 12 [1; 236] months of follow-up, overall 15-year patient and kidney survival of 78.7% and 76.3%, respectively, and low damage accrual. We conclude that initial treatment with cyclophosphamide and subsequent treatment with azathioprine ensure high efficacy and good safety profile and can be used according to current International Guidelines until the evidence for biological treatments benefits becomes available.

Patient and kidney survival significantly differed between subgroups that achieved complete renal response, partial renal response, and nonresponders, with patient 15-year survival 95% versus 65% versus 35%, respectively ($p < 0.01$), and kidney 15-year survival 100% versus 58% versus 0%, respectively ($p < 0.01$). We conclude that complete and even partial renal response has a positive prognostic value, while failure to achieve renal response to immunosuppression negatively influences not only kidney's but also patients' survival.

In the cohort of 47 patients followed up at the end of the study period, 51% demonstrated sustained complete renal response. However, only 9 out of these 24 patients had 0 disease activity according to SELENA SLEDAI Disease Assessment Scale, while 13 patients had scores 2–4 due to the elevated anti-DNA antibodies and complement abnormalities without clinical activity features. We conclude that the validity of complete renal response in SLE is questioned by the absence of conventional definition of SLE remission and the uncertain value of serological abnormalities.

Competing Interests

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

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References

[1] A. Doria, L. Iaccarino, A. Ghirardello et al., "Long-term prognosis and causes of death in systemic lupus erythematosus," *American Journal of Medicine*, vol. 119, no. 8, pp. 700–706, 2006.

[2] G. Moroni and C. Ponticelli, "The multifaceted aspects of refractory lupus nephritis," *Expert Review of Clinical Immunology*, vol. 11, no. 2, pp. 281–288, 2015.

[3] M. Gatto, L. Iaccarino, A. Ghirardello, L. Punzi, and A. Doria, "Clinical and pathologic considerations of the qualitative and quantitative aspects of lupus nephritogenic autoantibodies: a comprehensive review," *Journal of Autoimmunity*, vol. 69, pp. 1–11, 2016.

[4] M. C. Hochberg, "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 40, no. 9, article 1725, 1997.

[5] G. K. Bertsias, M. Tektonidou, Z. Amoura et al., "Joint European League against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis," *Annals of the Rheumatic Diseases*, vol. 71, no. 11, pp. 1771–1782, 2012.

[6] J. J. Weening, V. D. D'Agati, M. M. Schwartz et al., "The classification of glomerulonephritis in systemic lupus erythematosus revisited," *Journal of the American Society of Nephrology*, vol. 15, no. 2, pp. 241–250, 2004.

[7] Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group, "KDIGO clinical practice guideline for glomerulonephritis," *Kidney International Supplements*, vol. 2, pp. 139–274, 2012.

[8] R. F. van Vollenhoven, M. Mosca, G. Bertsias et al., "Treat-to-target in systemic lupus erythematosus: recommendations from an international task force," *Annals of the Rheumatic Diseases*, vol. 73, no. 6, pp. 958–967, 2014.

[9] C. Bombardier, D. D. Gladman, M. B. Urowitz et al., "Derivation of the SLEDAI: a disease activity index for lupus patients," *Arthritis and Rheumatism*, vol. 35, no. 6, pp. 630–640, 1992.

[10] M. Petri, M. Y. Kim, K. C. Kalunian et al., "Combined oral contraceptives in women with systemic lupus erythematosus," *New England Journal of Medicine*, vol. 353, no. 24, pp. 2550–2558, 2005.

[11] D. Gladman, E. Ginzler, C. Goldsmith et al., "The development and initial validation of the systemic lupus international collaborating clinics/American college of rheumatology damage index for systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 39, no. 3, pp. 363–369, 1996.

[12] R. F. van Vollenhoven, A. Voskuyl, E. Morand, and C. Aranow, "Remission in SLE: closing in on the target," *Annals of the Rheumatic Diseases*, vol. 74, no. 12, pp. 2103–2106, 2015.

[13] M. Schneider, M. Mosca, J. M. Pego-Reigosa et al., "Understanding remission in real-world lupus patients across five European countries," *Lupus*, vol. 25, no. 5, pp. 505–512, 2016.

[14] D. T. Boumpas and J. E. Balow, "Outcome criteria for lupus nephritis trials: a critical overview," *Lupus*, vol. 7, no. 9, pp. 622–629, 1998.

[15] Y. E. Chen, S. M. Korbet, R. S. Katz et al., "Value of a complete or partial remission in severe lupus nephritis," *Clinical Journal of the American Society of Nephrology*, vol. 3, no. 1, pp. 46–53, 2008.

[16] H. S. Koo, S. Kim, and H. J. Chin, "Remission of proteinuria indicates good prognosis in patients with diffuse proliferative lupus nephritis," *Lupus*, vol. 25, no. 1, pp. 3–11, 2016.

[17] G. B. Appel, G. Contreras, M. A. Dooley et al., "Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis," *Journal of the American Society of Nephrology*, vol. 20, no. 5, pp. 1103–1112, 2009.

[18] F. Tamirou, D. D'Cruz, S. Sangle et al., "Long-term follow-up of the MAINTAIN Nephritis Trial, comparing azathioprine and mycophenolate mofetil as maintenance therapy of lupus nephritis," *Annals of the Rheumatic Diseases*, vol. 75, no. 3, pp. 526–531, 2016.

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Clinical Study

The -174G/C Interleukin-6 Gene Promoter Polymorphism as a Genetic Marker of Differences in Therapeutic Response to Methotrexate and Leflunomide in Rheumatoid Arthritis

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Objective. To evaluate the association of -174G/C *IL-6* polymorphism with failure in therapeutic response to methotrexate (MTX) or leflunomide (LEF). This prospective, observational cohort included 96 Mexican-Mestizo patients with moderate or severe rheumatoid arthritis (RA), initiating MTX or LEF, genotyped for *IL-6* -174G/C polymorphism by PCR-RFLP. Therapeutic response was strictly defined: only if patients achieved remission or low disease activity (DAS-28 < 3.2). *Results*. Patients with MTX or LEF had significant decrement in DAS-28 ($p < 0.001$); nevertheless, only 14% and 12.5% achieved DAS-28 < 3.2 at 3 and 6 months. After 6 months with any of these drugs the -174G/G genotype carriers (56%) had higher risk of therapeutic failure compared with GC (RR: 1.19, 95% CI: 1.07–1.56). By analyzing each drug separately, after 6 months with LEF, GG genotype confers higher risk of therapeutic failure than GC (RR = 1.56; 95% CI = 1.05–2.3; $p = 0.003$), or CC (RR = 1.83; 95% CI = 1.07–3.14; $p = 0.001$). This risk was also observed in the dominant model (RR = 1.33; 95% CI = 1.03–1.72; $p = 0.02$). Instead, in patients receiving MTX no genotype was predictor of therapeutic failure. We concluded that *IL-6* -174G/G genotype confers higher risk of failure in therapeutic response to LEF in Mexicans and if confirmed in other populations this can be used as promissory genetic marker to differentiate risk of therapeutic failure to LEF.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease that involves synovial joints and other organs and it is associated with impairment in physical function, higher morbidity, and premature mortality [1]. Multiple guidelines of treatment for RA recommend, as first-line treatment, the use of conventional synthetic disease-modifying antirheumatic drugs (cs-DMARDs), with methotrexate (MTX), as the cornerstone of the majority of the therapeutic schemes [2–4]. Leflunomide (LEF) was the last cs-DMARD to appear before the biologic-DMARDs era, and LEF is considered an alternative as first-line treatment in patients with RA with intolerance to or contraindication for MTX [4]. In developing countries with serious economic limitations for the utilization of biologic-DMARDs, treatment based on MTX or LEF constitutes an alternative, frequently employed as monotherapy or combined therapy. In Mexico, a multicenter study performed, with the aim of describing the drugs most frequently utilized for the treatment of RA and ankylosing spondylitis, found that around 72.3% of 1,096 patients with RA were taking MTX and 18.5% LEF [5]. However, a significant number of patients are considered nonresponders to cs-DMARDs. Several works have reported wide variability in the efficacy of MTX or LEF. Strand et al. reported that the rate of response in ACR20 for MTX is only 46% and for LEF 52%, but only 20% with LEF and 9% with MTX achieved therapeutic response in ACR70 [6]. In fact, other authors observed a higher response rate using ACR20 criteria: 62% of responders to LEF and 54% to MTX [7]. According to treat-to-target concepts, one of the main objectives in the treatment of RA is to maintain remission of the disease activity or at least the achievement of low disease activity [8]. Unfortunately, a high proportion of patients with monotherapy with cs-DMARDs do not achieve these therapeutic targets. A concern regarding the treatment of RA comprises earlier recognition of patients with factors that predict a lack of efficacy in order to modify the therapeutic strategy. Cytokines constitute important mediators of the immune and inflammatory response and play an important role in the pathophysiology of joint inflammation and destruction in RA [9]. Among these cytokines, interleukin-6 (IL-6) has a relevant role in the perpetuation of synovial joint inflammation in RA, being widely related with disease activity [10, 11] and radiologic joint damage [12]. Some authors have described that genetic differences in the expression of IL-6 can be related with more severe disease [13]. The -174G/C IL-6 gene promoter polymorphism (rs1800795), localized in the negative regulative domain of the IL-6 gene promoter, is involved in transcriptional regulation [14, 15], whereas Konenkov et al. identified that GG genotype was associated with higher IL-6 serum levels [16].

Several studies have suggested that the -174G/C *IL-6* polymorphism may constitute a genetic marker for identifying a predisposition for therapeutic response to biologic-DMARDs [17–19]. However, only a single group of authors, to our knowledge, have examined, in two separate studies, the influence of the -174G/C *IL-6* gene promoter polymorphism on therapeutic response to MTX or LEF [20, 21]. Pawlik et al. analyzing -174G/C *IL-6* gene promoter polymorphism in RA patients identified that genotype GG may confer a risk for lower response to MTX compared with genotypes GC and CC [20]. Instead, the -174G/C polymorphism may not affect therapy outcomes in patients with RA treated with LEF [21]. Although these studies presented interesting findings, two major limitations can be observed. First, these two studies investigate therapeutic response using ACR20 or ACR50 as the main outcome measures; these are well-validated scales for clinical trials, but not for cohort studies, and neither ACR20 nor ACR50 provide sufficient information of disease severity at baseline. Second, because these two studies analyzed the effects of MTX and LEF in the presence of the -174G/C *IL-6* gene promoter polymorphism separately, they were unable to evaluate the overall response to both of these cs-DMARDs and to compare if -174G/C *IL-6* gene promoter polymorphism is predictor of the therapeutic response comparing both drugs. A more strict definition of therapeutic response should be applied to achieve the aim of maintaining remission or at least low disease activity with the therapy; in this regard, it is highly recommended to use DAS-28 < 3.2 as main outcome measure. Therefore, the aim of this observational cohort study was to evaluate the association of -174G/C *IL-6* polymorphism with failure in therapeutic response to MTX or LEF in Mexican-Mestizo women with RA.

2. Materials and Methods

2.1. Study Design. This study was a prospective, observational cohort (patients were included in a period covering October 2014 to December 2015) of patients with established moderate or severely active RA who initiated MTX or LEF for the treatment of their disease.

2.2. Clinical Setting. This was a single-center study performed at an outpatient rheumatology clinic of a secondary-care hospital in Guadalajara, Mexico (Hospital General Regional 110, IMSS). These patients were referred by primary-care physicians from a primary-care clinic.

2.3. Patients. Inclusion criteria comprised patients with RA according to American College of Rheumatology (ACR) 1987 criteria [22], >18 years of age, with an active disease defined as a Disease-Activity Score (DAS-28) index of 28 joints with a score of >3.2, with Mexican-Mestizo ethnicity (defined as individuals who, for three generations including their own, were born in Mexico and who were descendants of the original autochthonous inhabitants of the region and of individuals who were mainly Spaniards) [23], and all of these with at least 6 months or more without MTX or LEF. In the case of patients with a familial history of RA, only one case by family was included. Patients were excluded if they had antecedents of or concomitant therapy with biologic-DMARD; also, we excluded patients with chronic infections including hepatitis B or C infections, human immunodeficiency virus (HIV) infections, overlapping syndrome with other rheumatic diseases such as Systemic Lupus

Erythematosus (SLE), or patients who had an increase of transaminases of >2-fold of normal values, pulmonary fibrosis, serum creatinine > 1.2, or any other contraindications for MTX or LEF or had a ≤3.2 DAS-28 score. A total of 177 patients were screened for the study; among these, 81 were excluded due to the following reasons: two patients presented a <3.2 DAS-28 score; there were 48 patients with previous history of MTX or LEF suspended because of toxicity or noncompliance, and 31 patients did not accept to be included in the 6-month follow-up with the same drug.

At the time of inclusion in the study, all patients were initiating MTX or LEF as therapy for disease control in RA and had moderate or severe disease activity defined as a score of >3.2, according to the validated modified Disease Activity Score (DAS-28) index for 28 joints [24].

2.4. Baseline Evaluation

(a) Clinical Evaluation. All patients were interviewed to assess clinical and sociodemographic characteristics. Patients were evaluated regarding their functioning, using the validated version for Mexicans of the Health Assessment Questionnaire-Disability Index (HAQ-Di) [25]. Additionally, DAS-28 was evaluated by trained evaluators and a history of RA medication was obtained. Rheumatoid Factor (RF) in IU/mL (Dade Behring, DE, USA) was quantified in serum by nephelometry. Positive RF was considered >20 IU/mL. Erythrocyte Sedimentation Rate (ESR) was determined employing the Wintrobe technique.

(b) Determination of Serum IL-6 Levels. These were measured with an Enzyme-Linked Immunosorbent Assay (ELISA) utilizing commercial kits (R&D Systems, Minneapolis, MN, USA). This kit has a detection range from 3.10 to 300 pg/mL, and the Minimal Detectable Dose (MDD) of IL-6 is <0.70 pg/mL.

(c) DNA Isolation and Genotyping. Genomic DNA was obtained by the Miller method [26] from the patients' peripheral blood that was collected in tubes containing EDTA. Genotype was screened by an approach based on Polymerase Chain Reaction-Restriction Fragment-Length Polymorphism (PCR–RFLP), and *SfaNI* restriction endonuclease was used, as described elsewhere [27]. The resulting fragments were analyzed by electrophoresis in a 6% polyacrylamide gel stained with silver nitrate. The resulting genotypes for both polymorphisms were classified in one of the following three categories: nonexcisable homozygote genotype (CC); excisable homozygote (GG), and heterozygote (CG). All sample genotyping was carried out by three researchers blinded to the clinical characteristics and evolution of the patients included in the test, which included quality-control samples with experimental samples for validation.

2.5. Follow-Up of the Cohort. All patients with RA initiating MTX or LEF were evaluated by three researchers trained in the clinical parameters of RA at the baseline and at 3 and 6 months. Differences in the DAS-28 index at 3 and 6 months regarding baseline values were obtained. We classified all patients according to therapeutic response, which was defined according to treat-to-target guidelines, as patients that have reached at least low disease activity or remission of RA. Operatively, these patients in order to be classified as responders have undergone the treatment DAS-28 ≤ 3.2.

2.6. Statistical Analysis. A comparison between these two groups at baseline was performed using the unpaired Student's t-test in order to compare differences in means, and chi-square test (or Fisher exact test) was utilized to compare differences in proportions between groups. Relative Risks (RR) for therapeutic response and their 95% Confidence Intervals (95% CI) were obtained at 3 and 6 months.

Allele and genotype frequencies of both polymorphisms were obtained by direct counting. Genotype and allele frequencies were compared using the chi-square test (or the Fisher exact test if required). We initially examine failure rates for therapeutic response for each genotype of the -174G/C *IL-6* polymorphism separately at 3 or 6 months. Thereafter, we performed the RR analysis in three forms as follows: (a) rate of therapeutic failure in patients with GG genotype divided by rate of therapeutic failure in patients with GC and CC genotypes separately; (b) after that, analyzing the risk of therapeutic failure in patients with GG genotype versus rate of therapeutic failure in patients with GG or GC genotypes (dominant model); and (c) finally, examining RR for therapeutic failure employing the rate of failure to therapy in patients with GG or GC genotype divided by rate of therapeutic failure in patients with CC genotype (recessive model) of the -174G/C polymorphism of the *IL-6* gene. A similar approach was utilized for a subanalysis of patients treated with MTX or LEF separately. The p value was set at 0.05 level. All of the statistical analyses were performed using the software SPSS software 20.0 (SPSS Inc., Chicago, IL).

3. Results

Table 1 describes the baseline characteristics of 96 patients included in the cohort. Mean age of these patients was 50.6 years and 97% were females. Patients had mean disease duration of 7.6 years, a HAQ-Di score of 0.95, a DAS-28 of 5.6, the mean titles of RF of 83.2 IU/mL, and a mean glucocorticoid dose of 2.0 mg. The cs-DMARDs used by the patients during the study were the following: 57.3% received MTX and 42.7% received LEF. All patients were genotyped for the presence of -174G/C *IL-6* promoter polymorphisms, and the following genotype frequencies were observed: GG (56%), GC (32%), and CC (12%). Low rate of response using the strict criteria of achieving low disease activity or remission was achieved in the total group independently of the drug: response rates were 14% at 3 months and 12.5% at 6 months. There was a rate of response of 12.7% at 3 and 6 months in patients receiving MTX and of 17% at 3 months and of 12% at 6 months. No statistical differences were observed in the rate of responders between LEF and MTX at 3 or 6 months.

Comparison of the clinical and genetic characteristics between patients with MTX versus LEF demonstrated a difference in disease duration (6.05 years, MTX, versus 9.2 years,

Table 1: Comparison of selected characteristics of patients receiving methotrexate (MTX) versus leflunomide (LEF).

	MTX or LEF $n = 96$	MTX $n = 55$	LEF $n = 41$	p
Sociodemographic characteristics				
Females, *n* (%)	93 (97)	53 (96)	40 (98)	1.00
Age (yr), mean ± standard deviation	50.6 ± 10.2	48.7 ± 7.2	52.8 ± 11.0	0.06
Disease characteristics				
Disease duration (yr), mean ± SD	7.6 ± 7.5	6.05 ± 7.2	9.2 ± 7.6	0.05
HAQ-Di score at baseline, mean ± SD	0.95 ± 0.58	1.01 ± 0.60	0.88 ± 0.57	0.33
DAS-28 score at baseline, mean ± SD	5.6 ± 1.1	5.6 ± 1.1	5.5 ± 1.1	0.73
Rheumatoid Factor (RF) (IU/mL), mean ± SD	83.2 ± 127.4	77.4 ± 124.8	89.6 ± 132.0	0.70
Erythrocyte Sedimentation Rate (ESR) (mm/h), mean ± SD	27.1 ± 11.4	26.2 ± 12.7	28.08 ± 10.0	0.48
Interleukin-6 (IL-6) at baseline, (pg/mL) mean ± SD	19.6 ± 55.5	23.7 ± 68.7	12.9 ± 19.9	0.40
Glucocorticoid dose (mg), mean ± SD	2.0 ± 2.2	1.9 ± 1.4	2.05 ± 2.8	0.88
Patients achieving response				
At 3 months, *n* (%)	14 (6.8)	7 (12.7)	7 (17.1)	0.38
At 6 months, *n* (%)	12 (8.0)	7 (12.7)	5 (12.2)	0.38
Genetic characteristics				
Genotype GG, *n* (%)	54 (56)	33 (60)	21 (51)	
Genotype GC, *n* (%)	31 (32)	17 (31)	14 (34)	0.58
Genotype CC, *n* (%)	11 (12)	5 (9)	6 (15)	

RA: rheumatoid arthritis; IL-6: interleukin-6; MTX: methotrexate; LEF: leflunomide; HAQ-Di: Health Assessment Questionnaire-Disability index; DAS-28: modified Disease Activity Score (28 joints).

Qualitative variables were expressed in frequencies (%); quantitative variables were expressed in means ± standard deviations (SD). Comparisons between differences in proportions were performed with the chi-square test (or Fisher exact test if applicable). Comparisons between differences in means were performed with independent samples Student's *t*-tests. * p values were obtained comparing MTX versus LEF. Response was defined as the patient achieving, at 3 or at 6 months, low disease activity or remission (DAS28, <3.2).

LEF; $p = 0.05$). Other variables evaluated did not achieve statistically significant differences. In data that are not shown in tables the response rates for MTX were 12.7% at 3 months and the same percentage at 6 months, while those of LEF were 17.1% at 3 months and 12.2% at 6 months.

In data not shown in the tables, we observed a significant decrease in the DAS-28 index score employed as the quantitative variable at 3 or 6 months independently of treatment with MTX or with LEF. At cohort onset, the DAS-28 index score was 5.6 ± 1.1 and, on being evaluated at 3 months, decreased to 4.56 ± 1.12 ($p < 0.001$), with a similar decrease at 6 months with results of 4.49 ± 0.08 ($p < 0.001$). In a separate analysis, patients with MTX obtained a DAS-28 score at baseline of 5.65 ± 1.10; at 3 months, this decreased to 4.68 ± 1.16 ($p < 0.001$) and at 6 months, to 4.44 ± 1.06 ($p < 0.001$). LEF obtained for DAS-28 at baseline was 5.57 ± 1.13; at 3 months, this decreased to 4.39 ± 1.05 ($p < 0.001$) and at 6 months, to 4.57 ± 1.12 ($p < 0.001$).

Table 2 compares sociodemographic and clinical characteristics between patients who are GG genotype carriers and patients with GC or CC genotype. GG genotype carriers exhibited significantly higher RF levels ($p = 0.009$) and ESR ($p = 0.02$) compared with GC or CC genotype. No other variables achieved statistical significance, although there was a nonsignificant trend to higher IL-6 levels in GG genotype carriers.

Table 3 shows therapeutic failure in patients with RA with MTX or LEF during follow-up at 3 or 6 months according to the -174G/C *IL-6* gene polymorphism. After 3 months of treatment, a higher percentage of GG genotype was observed in nonresponders (87.0%) as well as at 6 months (92.6%); in the comparison between genotypes associated with nonresponse at 6 months of follow-up, GG compared with GC confers significantly more risk for therapeutic failure (RR = 1.19; 95% CI = 1.07–1.56; $p = 0.03$). The remaining comparisons of genotype or allele evaluated did not achieve statistically significant differences.

In Table 4, the analysis shows the therapeutic failure in patients with MTX during follow-up at 3 or 6 months according to the -174G/C *IL-6* gene polymorphism. The number of patients carrying the GG genotype was slightly increased in the group of nonresponders compared with responders without statistical significance. After 3 months of treatment, the GG genotype was observed in 90.9% of nonresponders and at 6 months in 87.9% of the same group. All the comparisons between rates of response according to genotype or allele did not achieve statistically significant differences.

Table 5 presents therapeutic failure in patients with RA treated with LEF during follow-up at 3 or 6 months according to the -174G/C *IL-6* gene polymorphism. There were no statistical differences after 3 months of treatment; however, at

Table 2: Comparison of clinical and laboratory characteristics at the baseline between GG genotype carriers and GC or CC genotype carriers.

	GG $n = 54$	GC or CC $n = 42$	p
Sociodemographic characteristics			
Females, n (%)	51 (94.4)	42 (100)	0.25
Age (yr), mean ± SD	50.5 ± 9.7	50.7 ± 10.9	0.93
Disease characteristics			
Disease duration (yr), mean ± SD	6.9 ± 7.4	8.5 ± 7.1	0.36
DAS-28 score, mean ± SD	5.6 ± 1.1	5.5 ± 1.04	0.87
HAQ-Di score, mean ± SD	0.95 ± 0.6	0.94 ± 0.51	0.98
Rheumatoid Factor (RF) (IU/mL), mean ± SD	108.6 ± 151.9	39.1 ± 39.7	0.009
Erythrocyte Sedimentation Rate (ESR) (mm/h), mean ± SD	29.5 ± 12.3	23.8 ± 9.3	0.02
IL-6 serum levels (pg/mL), mean ± SD	25.7 ± 68.0	8.5 ± 11.8	0.08
Treatment characteristics			
Glucocorticoid dose (mg), mean ± SD	2.3 ± 2.7	1.5 ± 1.0	0.06
Methotrexate (MTX) n (%)	33 (61.1)	17 (55)	0.39
Leflunomide (LEF), n (%)	21 (39.0)	14 (45)	

GG: excisable homozygote genotype; GC: heterozygote genotype; CC: homozygote genotype; RA: rheumatoid arthritis; HAQ-Di: Health Assessment Questionnaire-Disability index; DAS-28: modified Disease Activity Score (28 joints); IL-6: interleukin-6 serum levels. Qualitative variables were expressed in frequencies (%); quantitative variables were expressed in means ± standard deviations (SD). Comparisons between differences in proportions were performed with chi-square test (or Fisher exact test if applicable). Comparisons between differences in means were performed using independent samples Student's t-tests.

6 months, the GG genotype carriers had a higher risk of therapeutic failure compared with GC genotype carriers (RR = 1.56; 95% CI = 1.05–2.30; $p = 0.003$) and compared with the CC genotype carriers (RR = 1.83; 95% CI = 1.07–3.14; $p = 0.001$); in addition, dominant model GG versus GC + CC increased the risk of nonresponse (RR = 1.33; 95% CI = 1.03–1.72; $p = 0.02$).

4. Discussion

In the overall analysis that included all patients entering into the cohort independently and if they were treated with MTX or LEF, we did not observe that the -174G/C *IL-6* gene polymorphism may confer higher risk for therapeutic failure except in the GG genotype versus GC genotype carriers at 6 months. Nevertheless, when the rates of therapeutic failure of these drugs are analyzed separately we observed that in patients treated with LEF at 6 months of follow-up, GG genotype confers more risk for failure in therapeutic response compared with patients carrying GC or CC genotypes; this higher risk for failure to respond to LEF was also observed in patients using the dominant model (GG versus GC or CC). Instead, by analyzing only patients treated exclusively with MTX, the -174G/C *IL-6* gene polymorphism may not confer differences in the rate of therapeutic failure at 3 or 6 months.

Our results reflect that the -174G/C *IL-6* gene promoter polymorphism has a low influence on the therapeutic failure in patients treated with the two main cs-DMARDs employed currently in RA. For patients treated with MTX, we observed that GG genotype or G allele does not confer a clinically relevant risk for development of therapeutic failure. These results are in disagreement with the results obtained by Pawlik et al., who described a lower remission rate in patients with GG genotype when these patients are compared with carriers of GC and CC genotype [28]. This discordance can be explained by a series of potential confounders that were not evaluated by Pawlik et al. in their study. First, it is relevant that we included patients with moderate or severe disease activity according to the DAS-28 index score instead of only patients with any level of active disease. Patients with moderate or severe disease activity have an expected lower rate of achieving low disease activity or remission compared with patients with lower scores of this index. Second, we used a stricter definition for therapeutic response that is in concordance with the current concepts that show that a major target in the therapy of RA is maintaining remission or at least low disease activity.

Contrary to our observations of noninfluence of the -174G/C *IL-6* gene polymorphism in response to MTX, we observed in patients treated with LEF that the *IL-6* -174G/C polymorphism may confer differences in the therapeutic response to this drug. We observed that GG genotype carriers had a significant higher risk for failure in therapeutic response in patients treated with LEF at 6 months. These data are in disagreement with the results observed by Pawlik et al., who did not observe an association of -174G/C polymorphisms of the *IL-6* gene with therapeutic outcomes in their patients with RA treated with LEF [21]. The reasons for these differences include that many possible confounders can affect the risk of failure in therapeutic response, including differences in the definition of therapeutic failure (we described above that we used a stricter definition compared with that used by Pawlik et al.), other variables such as disease duration, functioning, or the baseline IL-6 levels that were not evaluated. However, some other variables at the baseline are comparable with the Pawlik cohort and

Table 3: Evaluation of -174G/C *IL-6* as predictor of therapeutic response to any treatment (MTX or LEF) defining nonresponse, as DAS-28 > 3.2 at 3 or 6 months in rheumatoid arthritis (RA).

Treatment with any treatment MTX or LEF, $n = 96$	MTX and LEF nonresponse $n = 82$	MTX and LEF response $n = 14$	RR	95% CI	p
	Follow-up at 3 months				
Genotype					
GG $n = 54$ (%)	47 (87.0)	7 (13.0)	—	—	0.62
GC $n = 31$ (%)	25 (80.6)	6 (19.4)	—	—	
CC $n = 11$ (%)	10 (91)	1 (9)	—	—	
GG versus GC (as referent)	—	—	1.08	0.88 to 1.32	0.20
GG versus CC (as referent)	—	—	0.96	0.77 to 1.18	0.39
GC versus CC (as referent)	—	—	0.88	0.73 to 1.14	0.40
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	1.04	0.88 to 1.24	0.41
Recessive model (GG + GC versus CC as referent)	—	—	0.93	0.76 to 1.16	0.49
Alleles 2n = 192	*2n = 164*	*2n = 28*			
G allele, $2n = 139$ (%)	119 (85.6)	20 (14.4)	1.00	0.88 to 1.15	0.44
C allele, $2n = 53$ (%)	45 (84.9)	8 (15.1)	Referent	—	
	Follow-up at 6 months				
	MTX and LEF nonresponse $n = 84$	MTX and LEF response $n = 12$	RR	95% Cl	p
Genotype					
GG $n = 54$ (%)	50 (92.6)	4 (7.4)	—	—	0.13
GC $n = 31$ (%)	24 (77.4)	7 (22.6)	—	—	
CC $n = 11$ (%)	10 (90.9)	1 (9.1)	—	—	
GG versus GC (as referent)	—	—	1.19	1.07 to 1.56	**0.03**
GG versus CC (as referent)	—	—	1.01	0.81 to 1.24	0.61
GC versus CC (as referent)	—	—	0.85	0.65 to 1.11	0.31
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	1.19	0.96 to 1.35	**0.05**
Recessive model (GG + GC versus CC as referent)	—	—	0.93	0.78 to 1.17	0.58
Alleles 2n = 192	*2n = 168*	*2n = 24*			
G allele, $n = 139$ (%)	124 (89.2)	15 (10.8)	1.07	0.94 to 1.23	0.13
C allele, $n = 53$ (%)	44 (83.0)	9 (17.0)	Referent	—	

MTX: methotrexate; LEF: leflunomide; DAS-28: Disease Activity Score for 28 joints; GG: excisable homozygote genotype; GC: heterozygote genotype; CC: homozygote genotype. Qualitative variables were expressed in frequency (%); RR: Relative Risk; 95% CI: 95% Confidence Interval. Therapeutic failure (nonresponse) was defined if patients did not achieve remission or low disease activity (DAS-28 < 3.2).

our cohort with LEF [21]. Our patients had a mean age of 52.8 years, very similar to the mean age of 52.9 years in Pawlik's cohort [21]. Similarly, the baseline values for DAS-28 were comparable with these two cohorts (DAS-28 of 5.5 in our group with LEF versus 5.3 in the patients included by Pawlik et al.). Instead, our patients with LEF had long-disease duration of RA of 9.2, whereas Pawlik et al. did not present the disease duration of their patients [21]. This long-disease duration may influence the low rate of therapeutic response; instead, patients with short disease duration (early RA) achieve higher rates of therapeutic response when they are treated early with cs-DMARDs [29]. However, because this was an observational study, these patients were treated late with cs-DMARD; therefore, the rate of patients that we expected to achieve low disease activity or remission is lower than that expected by studies employing these drugs earlier. In contrast with our data, Pawlik et al. also did not describe other characteristics in addition to those of disease activity that may contribute to therapeutic response to cs-DMARDs, such as positivity for RF and impairment in functioning

Table 4: Evaluation of -174G/C *IL-6* as predictor of therapeutic response to methotrexate (MTX) defining nonresponse, as DAS-28 > 3.2 at 3 or 6 months in rheumatoid arthritis (RA).

Treatment with methotrexate (MTX), $n = 55$	MTX nonresponse $n = 48$	MTX response $n = 7$	RR	95% CI	p
	Follow-up at 3 months				
Genotype					
GG $n = 33$ (%)	30 (90.9)	3 (9.1)	—	—	0.60
GC $n = 17$ (%)	14 (82.4)	3 (17.6)	—	—	
CC $n = 5$ (%)	4 (80)	1 (20)	—	—	
GG versus GC (as referent)	—	—	1.10	0.86 to 1.41	0.32
GG versus CC (as referent)	—	—	1.13	0.72 to 1.78	0.44
GC versus CC (as referent)	—	—	1.02	0.63 to 1.68	0.67
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	1.12	0.88 to 1.41	0.25
Recessive model (GG + GC versus CC as referent)	—	—	1.10	0.70 to 1.72	0.50
Alleles 2n = 110	*2n = 96*	*2n = 14*			
G allele, $2n = 83$ (%)	74 (89.2)	9 (10.8)	1.09	0.90 to 1.33	0.20
C allele, $2n = 27$ (%)	22 (81.5)	5 (18.5)	Referent	—	
	Follow-up at 6 months				
	MTX nonresponse $n = 48$	MTX response $n = 7$	RR	95% CI	p
Genotype					
GG $n = 33$ (%)	29 (87.9)	4 (12.1)	—	—	0.87
GC $n = 17$ (%)	15 (88.2)	2 (11.8)	—	—	
CC $n = 5$ (%)	4 (80)	1 (20)	—	—	
GG versus GC (as referent)	—	—	0.99	0.80 to 1.23	0.67
GG versus CC (as referent)	—	—	1.09	0.68 to 1.73	0.52
GC versus CC (as referent)	—	—	1.10	0.68 to 1.76	0.55
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	1.01	0.82 to 1.25	0.58
Recessive model (GG + GC versus CC as referent)	—	—	1.10	0.70 to 1.72	0.50
Alleles, 2n = 110	*2n = 96*	*2n = 14*			
G allele, $2n = 83$ (%)	73 (88.0)	10 (12.0)	1.03	0.86 to 1.23	0.35
C allele, $2n = 27$ (%)	23 (85.2)	4 (14.8)	Referent	—	

MTX: methotrexate; GG: excisable homozygote genotype; GC: heterozygote genotype; CC: homozygote genotype. Qualitative variables were expressed in frequencies (%); RR: Relative Risk; 95% CI: 95% Confidence Interval. Therapeutic failure (nonresponse) was defined if patients did not achieve remission or low disease activity (DAS-28 < 3.2).

assessed by the HAQ-Di [21]. Therefore, it is likely that major confounders may hide in Pawlik's study on the influence of the -174G/C *IL-6* gene polymorphism on failure in therapeutic response. Additionally, at this time, the DAS-28 index constitutes a standard for defining patients who achieve low disease activity or remission; consequently, we used this index to define strictly therapeutic failure and we choose not to use ACR20 or ACR50 indices as in Pawlik's study [21].

It is interesting that although patients treated with MTX or LEF exhibited a significant decrease in mean DAS28 index at 3 and 6 months with respect to baseline values, only a very low proportion of patients achieved the therapeutic target of maintaining low disease activity or remission. This is in accordance with a number of studies pointing out that MTX or LEF used as monotherapy may achieve a low proportion of patients in remission [30, 31].

Patients carrying GG genotype demonstrate higher levels of ESR and RF compared with patients with the GC or CC genotype. A previous study reported that patients who were carriers of the GG genotype had a significantly increased

Table 5: Evaluation of -174G/C *IL-6* as predictor of therapeutic response to leflunomide (LEF) defining nonresponse, as DAS-28 > 3.2 at 3 or 6 months in rheumatoid arthritis (RA).

Treatment with leflunomide (LEF) $n = 41$	Follow-up at 3 months				
	LEF nonresponse $n = 34$	LEF response $n = 7$	RR	95% CI	p
Genotype					
GG $n = 21$ (%)	17 (81)	4 (19)	—	—	
GC $n = 14$ (%)	11 (78.6)	3 (21.4)	—	—	0.64
CC $n = 6$ (%)	6 (17.6)	0 (0)	—	—	
GG versus GC (as referent)	—	—	1.03	0.73 to 1.45	0.43
GG versus CC (as referent)	—	—	0.81	0.66 to 0.99	0.17
GC versus CC (as referent)	—	—	0.78	0.60 to 1.03	0.16
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	0.95	0.72 to 1.26	0.37
Recessive model (GG + GC versus CC as referent)	—	—	0.80	0.68 to 0.94	0.15
Alleles, 2n = 82	*2n = 68*	*2n = 14*			
G allele, $2n = 56$ (%)	45 (80.4)	11 (19.6)	0.91	0.75 to 1.10	0.19
C allele, $2n = 26$ (%)	23 (88.5)	3 (11.5)	Referent	—	
	Follow-up at 6 months				
	LEF nonresponse $n = 36$	LEF response $n = 5$	RR	95% CI	p
Genotype					
GG $n = 21$ (%)	21 (100)	0 (0)	—	—	
GC $n = 14$ (%)	9 (25)	5 (100)	—	—	**0.006**
CC $n = 6$ (%)	6 (16.7)	0 (0)	—	—	
GG versus GC (as referent)	—	—	1.56	1.05 to 2.30	**0.003**
GG versus CC (as referent)	—	—	1.83	1.07 to 3.14	**0.001**
GC versus CC (as referent)	—	—	0.64	0.43 to 0.95	0.06
Genetic models					
Dominant model (GG versus GC + CC as referent)	—	—	1.33	1.03 to 1.72	**0.02**
Recessive model (GG + GC versus CC as referent)	—	—	0.86	0.75 to 0.98	0.43
Alleles, 2n = 82	*2n = 72*	*2n = 10*			
G allele, $2n = 56$ (%)	51 (91.1)	5 (8.9)	1.13	0.92 to 1.38	0.11
C allele, $2n = 26$ (%)	21 (81.0)	5 (19.0)	Referent	—	

LEF: leflunomide; GG: excisable homozygote genotype ($n = 21$); GC: heterozygote genotype ($n = 14$); CC: homozygote genotype ($n = 6$). Qualitative variables were expressed in frequencies (%); quantitative variables were expressed as means ± standard deviations (SD). RR: Relative Risk; 95% CI: 95% Confidence Intervals. Therapeutic failure (nonresponse) was defined if patients did not achieve remission or low disease activity (DAS-28 < 3.2).

ESR rate; these findings are consistent with our results [32]; however, our results on increased titres of RF are different from those of other authors such as Pavkova Goldbergova, who found that patients who were carriers of the GC genotype had higher levels of RF without significance [33], although these findings are inconsistent with some studies suggesting that the C allele of the -174 polymorphism of *IL-6* is associated with increased levels of IL-6 in general population [34, 35]. This finding was also observed in patients with RA [36], whereas other authors have found that higher IL-6 levels are observed in patients with GG genotype or G allele [16]. Similarly, in our study, we found a trend for higher levels of IL-6 in patients with GG genotype compared with genotype GC or CC, because some of the patients included in our study had been previously treated prior to study entry with other cs-DMARDs such as sulfasalazine, chloroquine, or azathioprine,

although these patients suspended these cs-DMARDs at least 3 month before the study entry. Nevertheless, a possible delayed effect of the previous cs-DMARDs contributing to a decrement in serum IL-6 levels in these patients with RA cannot be completely excluded [37]. Therefore, these results about the lack of statistically significant relationship between GG genotype and IL-6 serum levels should be interpreted with caution and studies with patients naïve to cs-DMARDs should be made in the future.

There are several limitations in the present study. First, we were only able to assess the short-time response to these two cs-DMARDs in terms of disease activity at 3 and 6 months; however, we were unable to evaluate the influence of the -174G/C polymorphism of the *IL-6* gene on other relevant responses different from disease activity that develop in the long term and are also determinant of the prognosis, such as radiographic damage or permanent work disability. However, in order to evaluate the therapeutic response to cs-DMARD, the majority of guidelines recommend reevaluating in the short term the therapeutic response in order to adjust dosage or modifications in the therapeutic scheme. Under this concept, the results of our study are useful to describe the utility of -174G/C *IL-6* gene polymorphism to determine a subgroup of higher risk for failure in therapeutic response in patients with LEF.

We also have some strengthens in our study: we analyzed, in the same study, patients treated with LEF or MTX as monotherapy, and this aspect gave the opportunity to make comparisons between response to both drugs differently from the studies performed by Pawlik et al., who examined these groups separately [20, 21]. This strategy allowed the comparison of patients being treated with either of these two DMARDs as well as the examination of the risk that the -174G/C polymorphism of *IL-6* may confer therapeutic failure in each synthetic DMARD separately. The second and most important strength of this study is that we used a stricter definition for failure of the therapy that is according to the current concepts and goals of treatment in RA.

5. Conclusion

In conclusion we did not observe that the -174G/C *IL-6* gene promoter polymorphism conferred a significant risk for failure on MTX, whereas a significant risk for failure on LEF was observed after 6 months of treatment in patients carrying the GG. Future studies should evaluate whether the -174G/C *IL-6* gene promoter polymorphism can be associated with the long-term prognosis of other relevant outcome measures, such as radiographic structural damage and permanent disability in these patients. Therefore, further multicenter studies evaluating the impact of this polymorphism in a large cohort of patients with RA are required.

Ethical Approval

This study was approved by a Research Committee from participating center R-2012-1303-54. All of the procedures in the protocol were performed according to the guidelines of the Declaration of Helsinki.

Consent

All of the participating patients signed informed voluntary consent.

Competing Interests

The authors declare that they have no competing interests.

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References

[1] K. Walker-Bone and S. Farrow, "Rheumatoid arthritis," *BMJ Clinical Evidence*, vol. 2007, article 1124, 2007.

[2] M. H. Cardiel, A. Díaz-Borjón, M. Vázquez del Mercado Espinosa et al., "Update of the Mexican college of rheumatology guidelines for the pharmacologic treatment of rheumatoid arthritis," *Reumatologia Clinica*, vol. 10, no. 4, pp. 227–240, 2014.

[3] J. A. Singh, K. G. Saag, S. L. Bridges et al., "2015 American College of Rheumatology guideline for the treatment of rheumatoid arthritis," *Arthritis & Rheumatology*, vol. 68, no. 1, pp. 1–26, 2016.

[4] J. S. Smolen, F. C. Breedveld, G. R. Burmester et al., "Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force," *Annals of the Rheumatic Diseases*, vol. 75, no. 1, pp. 3–15, 2016.

[5] M. V. Goycochea-Robles, C. A. Arce-Salinas, S. Guzmán-Vázquez, and M. H. Cardiel-Ríos, "Prescription rheumatology practices among Mexican specialists," *Archives of Medical Research*, vol. 38, no. 3, pp. 354–359, 2007.

[6] V. Strand, S. Cohen, M. Schiff et al., "Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group," *Archives of Internal Medicine*, vol. 159, no. 21, pp. 2542–2550, 1999.

[7] P. Emery, F. C. Breedveld, E. M. Lemmel et al., "A comparison of the efficacy and safety of leflunomide and methotrexate for the treatment of rheumatoid arthritis," *Rheumatology*, vol. 39, no. 6, pp. 655–665, 2000.

[8] R. Landewé and D. van der Heijde, "New concepts of clinical trials in rheumatoid arthritis: a boom of noninferiority trials," *Current Opinion in Rheumatology*, vol. 28, no. 3, pp. 316–322, 2016.

[9] M. Feldmann, F. M. Brennan, and R. N. Maini, "Role of cytokines in rheumatoid arthritis," *Annual Review of Immunology*, vol. 14, pp. 397–440, 1996.

[10] B. Dasgupta, M. Corkill, B. Kirkham, T. Gibson, and G. Panayi, "Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis," *The Journal of Rheumatology*, vol. 19, no. 1, pp. 22–25, 1992.

[11] R. Madhok, A. Crilly, J. Watson, and H. A. Capell, "Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity," *Annals of the Rheumatic Diseases*, vol. 52, no. 3, pp. 232–234, 1993.

[12] S. Kotake, K. Sato, K. J. Kim et al., "Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid

arthritis patients are responsible for osteoclast-like cell formation," *Journal of Bone and Mineral Research*, vol. 11, no. 1, pp. 88–95, 1996.

[13] S.-T. Wei, Y.-H. Sun, S.-H. Zong, and Y.-B. Xiang, "Serum levels of IL-6 and TNF-α may correlate with activity and severity of rheumatoid arthritis," *Medical Science Monitor*, vol. 21, pp. 4030–4038, 2015.

[14] D. Fishman, G. Faulds, R. Jeffey et al., "The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis," *The Journal of Clinical Investigation*, vol. 102, no. 7, pp. 1369–1376, 1998.

[15] M. Pascual, A. Nieto, L. Matarán, A. Balsa, D. Pascual-Salcedo, and J. Martín, "IL-6 promoter polymorphisms in rheumatoid arthritis," *Genes and Immunity*, vol. 1, no. 5, pp. 338–340, 2000.

[16] V. I. Konenkov, E. V. Zonova, M. A. Korolev et al., "Pharmacogenetic criteria for the efficacy of basic anti-inflammatory therapy for rheumatoid arthritis," *Terapevticheskii Arkhiv*, vol. 82, no. 12, pp. 56–61, 2010.

[17] I. Jančić, N. Arsenović-Ranin, M. Šefik-Bukilica et al., "–174G/C interleukin-6 gene promoter polymorphism predicts therapeutic response to etanercept in rheumatoid arthritis," *Rheumatology International*, vol. 33, no. 6, pp. 1481–1486, 2013.

[18] C. L. Dávila-Fajardo, A. Márquez, D. Pascual-Salcedo et al., "Confirmation of -174G/C interleukin-6 gene promoter polymorphism as a genetic marker predicting antitumor necrosis factor treatment outcome," *Pharmacogenetics and Genomics*, vol. 24, no. 1, pp. 1–5, 2014.

[19] M. Fabris, L. Quartuccio, S. Lombardi et al., "The CC homozygosis of the -174G>C IL-6 polymorphism predicts a lower efficacy of rituximab therapy in rheumatoid arthritis," *Autoimmunity Reviews*, vol. 11, no. 5, pp. 315–320, 2012.

[20] A. Pawlik, B. Czerny, E. Dabrowska-Zamojcin et al., "The influence of IL-6 polymorphism on efficacy of treatment of rheumatoid arthritis patients with methotrexate and prednisone," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 114, no. 3, pp. 843–847, 2005.

[21] A. Pawlik, M. Herczyńska, M. Kurzawski et al., "IL-1β, IL-6, and TNF gene polymorphisms do not affect the treatment outcome of rheumatoid arthritis patients with leflunomide," *Pharmacological Reports*, vol. 61, no. 2, pp. 281–287, 2009.

[22] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[23] C. Sanchez, *Mestizaje e Historia de la Población en México*, Real Academia de Ciencias Exactas, Físicas y Naturales, Madrid, Spain, 1996.

[24] M. L. L. Prevoo, M. A. van 't Hof, H. H. Kuper, M. A. van Leeuwen, L. B. A. van de Putte, and P. L. C. M. van Riel, "Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 38, no. 1, pp. 44–48, 1995.

[25] M. H. Cardiel, M. Abello-Banfi, R. Ruiz-Mercado, and D. Alarcon-Segovia, "How to measure health status in rheumatoid arthritis in non-English speaking patients: validation of a Spanish version of the Health Assessment Questionnaire Disability Index (Spanish HAQ-DI)," *Clinical and Experimental Rheumatology*, vol. 11, no. 2, pp. 117–121, 1993.

[26] S. A. Miller, D. D. Dykes, and H. F. Polesky, "A simple salting out procedure for extracting DNA from human nucleated cells," *Nucleic Acids Research*, vol. 16, no. 3, p. 1215, 1988.

[27] S. A. Zavaleta-Muñiz, B. T. Martín-Márquez, L. Gonzalez-Lopez et al., "The –174G/C and –572G/C interleukin 6 promoter gene polymorphisms in Mexican patients with rheumatoid arthritis: a case-control study," *Clinical & Developmental Immunology*, vol. 2013, Article ID 959084, 5 pages, 2013.

[28] A. Pawlik, J. Wrzesniewska, M. Florczak, B. Gawronska-Szklarz, and M. Herczynska, "IL-6 promoter polymorphism in patients with rheumatoid arthritis," *Scandinavian Journal of Rheumatology*, vol. 34, no. 2, pp. 109–113, 2005.

[29] S. Monti, C. Montecucco, S. Bugatti, and R. Caporali, "Rheumatoid arthritis treatment: the earlier the better to prevent joint damage," *Rheumatic & Musculoskeletal Diseases Open*, vol. 1, supplement 1, Article ID e000057, 2015.

[30] D. Golicki, M. Niewada, J. Lis, K. Pol, T. Hermanowski, and M. Tłustochowicz, "Leflunomide in monotherapy of rheumatoid arthritis: meta-analysis of randomized trials," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 122, no. 1-2, pp. 22–32, 2012.

[31] D.-Y. Chen, S.-J. Chou, T.-Y. Hsieh et al., "Randomized, double-blind, placebo-controlled, comparative study of human anti-TNF antibody adalimumab in combination with methotrexate and methotrexate alone in Taiwanese patients with active rheumatoid arthritis," *Journal of the Formosan Medical Association*, vol. 108, no. 4, pp. 310–319, 2009.

[32] S. Shafia, Dilafroze, F. A. Sofi, R. Rasool, S. Javeed, and Z. A. Shah, "Rheumatoid arthritis and genetic variations in cytokine genes: a population-based study in Kashmir Valley," *Immunological Investigations*, vol. 43, no. 4, pp. 349–359, 2014.

[33] M. Pavkova Goldbergova, P. Nemec, J. Lipkova et al., "Relation of IL-6, IL-13 and IL-15 gene polymorphisms to the rheumatoid factors, anti-CCP and other measures of rheumatoid arthritis activity," *International Journal of Immunogenetics*, vol. 41, no. 1, pp. 34–40, 2014.

[34] P. Jerrard-Dunne, M. Sitzer, P. Risley et al., "Interleukin-6 promoter polymorphism modulates the effects of heavy alcohol consumption on early carotid artery atherosclerosis: the Carotid Atherosclerosis Progression Study (CAPS)," *Stroke*, vol. 34, no. 2, pp. 402–407, 2003.

[35] K. G. Jones, D. J. Brull, L. C. Brown et al., "Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms," *Circulation*, vol. 103, no. 18, pp. 2260–2265, 2001.

[36] V. F. Panoulas, A. Stavropoulos-Kalinoglou, G. S. Metsios et al., "Association of interleukin-6 (IL-6)-174G/C gene polymorphism with cardiovascular disease in patients with rheumatoid arthritis: the role of obesity and smoking," *Atherosclerosis*, vol. 204, no. 1, pp. 178–183, 2009.

[37] P. Barrera, A. M. T. Boerbooms, E. M. Janssen et al., "Circulating soluble tumor necrosis factor receptors, interleukin-2 receptors, tumor necrosis factor α, and interleukin-6 levels in rheumatoid arthritis: longitudinal evaluation during methotrexate and azathioprine therapy," *Arthritis and Rheumatism*, vol. 36, no. 8, pp. 1070–1079, 1993.

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Research Article

Effects of Berberine on NLRP3 and IL-1β Expressions in Monocytic THP-1 Cells with Monosodium Urate Crystals-Induced Inflammation

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Background. Urate crystals-induced inflammation is a critical factor during the initiation of gouty arthritis. Berberine is well known for its anti-inflammatory activity. However, the underlying effects of berberine on monosodium urate crystals-induced inflammation remain obscure. *Objectives.* This study is set to explore the protective effect and mechanism of berberine on monosodium urate crystals-induced inflammation in human monocytic THP-1 cells. *Methods.* The mRNA levels of NLRP3 and IL-1β were measured by Real-Time PCR, and the protein levels of NLRP3 and IL-1β were determined by ELISA, Western blot, and immunofluorescence. *Results.* The NLRP3 and IL-1β expressions were significantly increased in model group compared to that in normal group ($P < 0.05$). Meanwhile, there was significant reduction in the expressions of NLRP3 and IL-1β mRNA in groups 6.25 μM berberine and 25 μM berberine when compared with model group ($P < 0.05$). *Conclusions.* Therefore, berberine alleviates monosodium urate crystals-induced inflammation by downregulating NLRP3 and IL-1β expressions. The regulatory effects of berberine may be related to the inactivation of NLRP3 inflammasome.

1. Introduction

Gout is characterized by hyperuricemia and triggered by the deposition of monosodium urate (MSU) crystals. The distribution of gout is imbalanced across the world, with prevalence being highest in Pacific countries. Developed countries incline to have a higher load of gout than developing countries and seem to have growing prevalence and incidence of gout [1]. The prevalence of both gout and hyperuricemia remains substantial, which is likely associated with increasing frequencies of adiposity and hypertension [2].

Gouty arthritis has self-resolution, with resolution within several days or 1-2 weeks. However, the pain is commonly unbearable. Urate-lowering therapies play a minor role in painful episodes. The IL-1β plays a key role in the acute gouty inflammation triggered by MSU crystals [3, 4]. Recent study showed that MSU crystals involved the activation of NALP3 inflammasome, bringing about the production of IL-1β [5]. Meanwhile, IL-1 inhibitors (rilonacept or anakinra) have been applied in patients with acute gouty arthritis [6, 7]. However, the relatively high medical care costs of IL-1 inhibitors restrict the application of these drugs in the developing countries.

Plant-based medicines are widely applied in treating gout and its complications in a number of hospitals in China. Among effective prescriptions, Simiao pill, which is derived from Ermiao powder and described in a famous traditional Chinese medicine monograph Chengfang Biandu in Qing Dynasty of China, has been wildly employed for treatment of gout and inflammatory arthritis [8]. Our previous study also demonstrated that modified Simiao decoction significantly decreased IL-1β release in THP-1 cells with MSU

crystals-induced inflammation [9]. The ingredients of modified Simiao decoction are complicated, including alkaloids, flavones, and organic acids. Yin and colleagues have screened the active compounds of modified Simiao decoction with antigout effects [10, 11]. However, the specific ingredients and mechanisms of modified Simiao decoction functioning as antigout effects are still unknown.

Cortex phellodendri, referred to as "ruler drug," plays a pivotal role in modified Simiao decoction. Berberine, the major component of *cortex phellodendri*, is able to lessen the expression of IL-1β in monocytes and macrophages [12, 13]. Based on the anti-inflammatory action of berberine, the study is to investigate the effects of berberine on NLRP3 and IL-1β in THP-1 cells with MSU crystals-induced inflammation on the basis of our previous research and to provide evidence for its application in gouty arthritis.

2. Materials and Methods

2.1. Reagents. Human IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Beijing Neo-Bioscience Technology Co. Ltd., China. The RNAiso Plus (Code: D9108B), PrimeScript RT reagent Kit Perfect Real Time (Code: DRR036S), and SYBR Premix Ex Taq (Code: DRR420A) were purchased from TaKaRa Biotechnology Co. Ltd., Dalian, China. Fetal bovine serum was purchased from Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). Berberine was obtained as a gift of Department of Integrated Traditional Chinese and Western Medicine (Tongji hospital, Huazhong University of Science and Technology). Tissue protein extraction kit, bicinchoninic acid (BCA) protein assay kit, and protease inhibitor cocktail were purchased from Wuhan Gugeshengwu Technology Co. Ltd. China. Rabbit polyclonal to IL-1β (Lot#: AP8531c), and Nlrp3 (Lot#: AP8564a) were purchased from Abgent Inc., USA. Rabbit anti-rat β-actin polyclonal antibody (Cat#: GB13001) was purchased from Wuhan Gugeshengwu Technology Co. Ltd., China. Fluorescent secondary antibody (Lot#: C30815-02) was obtained as a gift of Li-COR Inc., USA. FITC-labeled goat anti-rabbit secondary antibody and diamidino-phenylindole (DAPI) were purchased from Feiyi Technology Co. Ltd., Wuhan, and Boster Company, Wuhan, respectively. Uric acid sodium salt (product number: U2875) and phorbol 12-myristate 13-acetate (PMA) (product number: 79346) were ordered from Sigma-Aldrich Co., St. Louis, USA.

2.2. Main Devices. The devices used include Mastercycler gradient PCR apparatus (Eppendorf Company, Germany); Nikon microimaging system (TE2000-U, Tokyo, Japan); Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems, California, USA); microplate reader (BioTek Synergy2, Vermont, USA); inverted microscope (CKX-31, Olympus Corporation, Tokyo, Japan); CO_2 incubator (New Brunswick Scientific Co. Inc., New Jersey, USA); Odyssey infrared imaging system (Li-COR Inc., USA); Nano Drop 2000 Spectrophotometer (Thermo Scientific Inc., Beijing, China).

Table 1: Primer sequence.

Gene	Primer	Sequence
β-actin	Forward	5′-TGGCACCCAGCACAATGAA-3′
	Reverse	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′
IL-1β	Forward	5′-GCTGATGGCCCTAAACAGATGAA-3′
	Reverse	5′-TCCATGGCCACAACAACTGAC-3′
TNF-α	Forward	5′-TGCTTGTTCCTCAGCCTCTT-3′
	Reverse	5′-CAGAGGGCTGATTAGAGAGAGGT-3′
NLRP3	Forward	5′-GTCATCGGGTGGAGTCACTGTC-3′
	Reverse	5′-AAGTGAGGTGGCTGTTCACCAA-3′

NLRP3: domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator, leucine rich repeat, and pyrin domains-containing protein 3.

2.3. Cell Culture. Monocytic THP-1 cells, human monocyte line, obtained as a gift of Department of Immunology (Tongji Medical College, Huazhong University of Science and Technology), were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C and 5% CO_2. THP-1 cells were plated at the density of 1.0–1.5 $\times$ 10^6/mL in 6-well plates. THP-1 cells were stimulated for 3 h with 100 ng/mL PMA the day before stimulation. This treatment enhanced the phagocytic properties of the cells and prompted a constitutive production of pro-IL-1β [14]. THP-1 cells were stimulated with 100 μg/mL MSU in the presence or absence of berberine. The prepared MSU solution should be kept at 4°C about one week before forming MSU crystals. THP-1 cells were randomized into normal group (N), model group (M), and berberine group (Ber) of different concentrations.

2.4. Real-Time PCR for IL-1β and NLRP3 mRNA Expressions. Total RNA was extracted from THP-1 cells with TRIzol reagent in accordance with the manufacturer's instructions. RNA concentration and purity were assessed by Nano Drop 2000 Spectrophotometer. One μg of extracted total RNA was reverse transcribed with PrimeScript RT reagent Kit according to the manufacturer's instructions. The cDNA was stored at −20°C prior to PCR amplification. Real-Time PCR reactions were performed in 48-well optical PCR plates by employing an Applied Biosystems StepOne Real-Time PCR System in accordance with the manufacturer's instructions. $2^{-\Delta\Delta CT}$ method was applied for analyzing the data. The primers were designed in accordance with published sequences (Table 1).

2.5. ELISA for IL-1β Protein Expression in Supernatants. The production of IL-1β was detected by quantitative sandwich enzyme immunoassay technique according to the manufacturer's standard protocols. The sensitivity of the ELISA kits of IL-1β was 4 pg/mL. None of the samples examined had a cytokine level >1000 pg/mL. The interassay and intra-assay

coefficients of variation of the ELISA kits for IL-1β were less than 10%.

2.6. Western Blot for NLRP3 and IL-1β Protein Measurement. Total protein was extracted from THP-1 cells which were lysed in mammal tissue protein extraction reagent supplemented with protease inhibitor cocktail and then centrifuged at 12000 ×g for 10 min at 4°C. The BCA protein assay kit was applied to quantify the protein concentration of the supernatants. The total protein (μg) was mixed with sample buffer, boiled for 10 min, and ran on a 12% or 10% SDS-PAGE gel. Separated proteins on the gel were transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat-dry milk for 1 h at room temperature and incubated overnight at 4°C with antibodies to β-actin, NLRP3, and IL-1β diluted 1 : 500, 1 : 150, and 1 : 100, respectively. The membranes were incubated in a lucifugous state with the fluorescent secondary antibody to rabbit diluted 1 : 5000 at room temperature for 1 h. Then, the membranes were detected by near infrared double-color laser imaging system. The pictures were analyzed with Odyssey software to calculate the gray scale ratio of β-actin.

2.7. Immunofluorescence for NLRP3. THP-1 cells were fixed on the coverslips. The samples were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 15 min at room temperature. The cells were washed three times with ice cold PBS. The samples were incubated for 15 min with PBS containing 0.1% Triton X-100. Cells were washed in PBS three times for 5 min. Cells were incubated with 10% goat serum for 20 min at room temperature to block unspecific binding of the antibodies. Cells were incubated in the diluted NLRP3 antibody (1 : 100) in a humidified chamber overnight at 4°C. The solution was decanted and the cells were washed three times in PBS, 5 min each wash. Cells were incubated with the diluted FITC-labeled secondary antibody (1 : 50) for 45 min at room temperature in the dark. The secondary antibody solution was decanted and the cells were washed three times with PBS in the dark, 5 min each wash. Cells were incubated with DAPI (DNA stain) for 3 min at room temperature in the dark. Coverslips were mounted with a drop of neutral balsam.

2.8. Statistical Analysis. All data with a normal distribution were presented as mean ± standard deviation (SD) and analyzed with aid of SPSS17.0 Statistical Software. Statistical significance was determined by one-way analysis of variance (ANOVA). For data with equal variances assumed, ANOVA followed by LSD test was applied. For data with equal variances not assumed, ANOVA followed by Dunnett's T3 test was used. A probability of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Outcome of ELISA. Compared with group N, the IL-1β level was significantly elevated in group M ($P < 0.05$). The IL-1β expression was significantly lowered in group 3.125 μM Ber compared to that in group M ($P < 0.05$) (Figure 1). However, other concentrations of Ber did not lower the expression of IL-1β ($P > 0.05$).

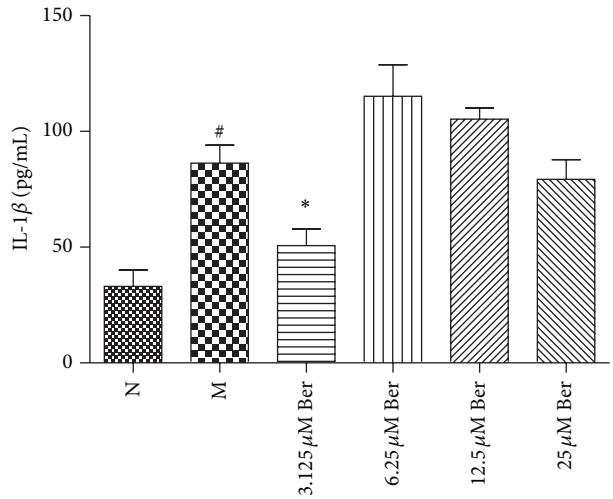

Figure 1: Expression of IL-1β in THP-1 cells. Values are mean ± SD. N: normal group; M: model group; 3.125 μM Ber, 6.25 μM Ber, 12.5 μM Ber, and 25 μM Ber: berberine group (Ber) of different concentrations. $^{\#}P < 0.05$ compared with group N; $^{*}P < 0.05$ compared with group M.

3.2. Expression of IL-1β and NLRP3 mRNA in Lysates of THP-1 Cells. As shown in Figure 2(a), IL-1β mRNA expression was significantly higher in group M compared to that in group N ($P < 0.05$). Compared with group M, there was a significant reduction in the expression of IL-1β in groups 6.25 μM Ber and 25 μM Ber. However, there was no significant reduction in the expression of IL-1β mRNA in groups 3.125 μM Ber and 12.5 μM Ber ($P > 0.05$).

As shown in Figure 2(b), NLRP3 mRNA expression was significantly higher in group M compared to that in group N ($P < 0.05$). Meanwhile, there was significant reduction in the expression of NLRP3 mRNA in groups 6.25 μM Ber and 25 μM Ber when compared with group M ($P < 0.05$). However, there was no significant reduction in the expression of NLRP3 mRNA in groups 3.125 μM Ber and 12.5 μM Ber ($P > 0.05$).

3.3. Outcome of Western Blot. Compared with group N, the IL-1β expression was decreased in group M; however, there was no significant difference between the two groups ($P > 0.05$). Compared with group M, the IL-1β expression was increased in groups 3.125 μM Ber and 12.5 μM Ber; however, there was no significant difference between the three groups ($P > 0.05$) (Figures 3(a) and 3(b)).

Compared with group N, the NLRP3 expression was nonsignificant alteration ($P > 0.05$). Compared with group M, the NLRP3 expression was blunted in groups 3.125 μM Ber and 25 μM Ber; however, there was no significant difference between the three groups ($P > 0.05$) (Figures 3(a) and 3(c)).

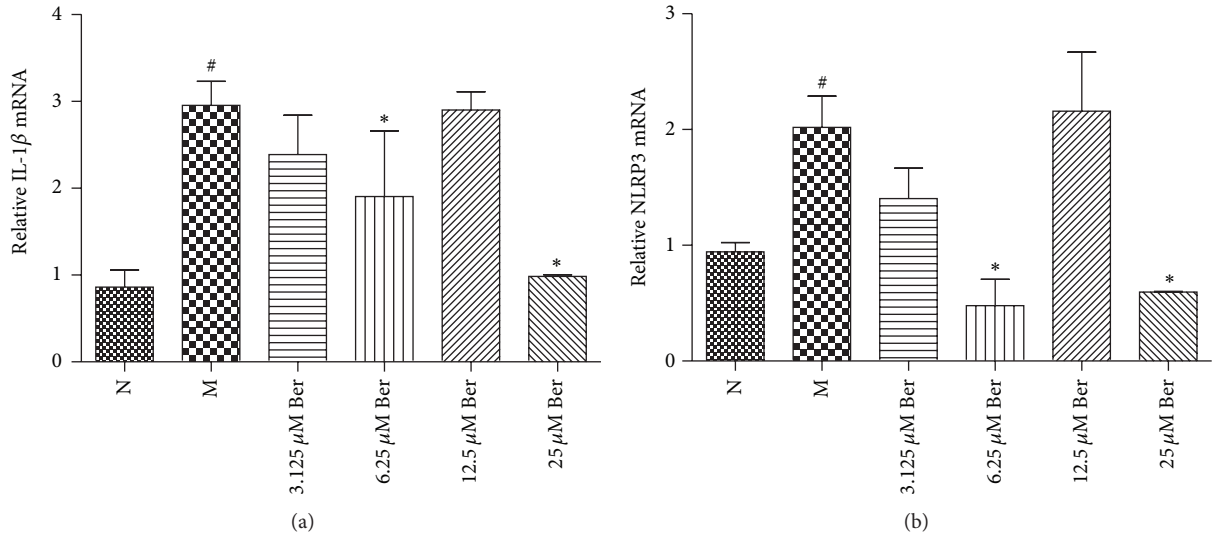

Figure 2: (a) Expression of IL-1β mRNA in THP-1 cells. (b) Expression of NLRP3 mRNA in THP-1 cells. Values are mean ± SD. N: normal group; M: model group; 3.125 μM Ber, 6.25 μM Ber, 12.5 μM Ber, and 25 μM Ber: berberine group (Ber) of different concentrations. NLRP3: domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator, leucine rich repeat, and pyrin domains-containing protein 3. $^{\#}P < 0.05$ compared with group N; $^{*}P < 0.05$ compared with group M.

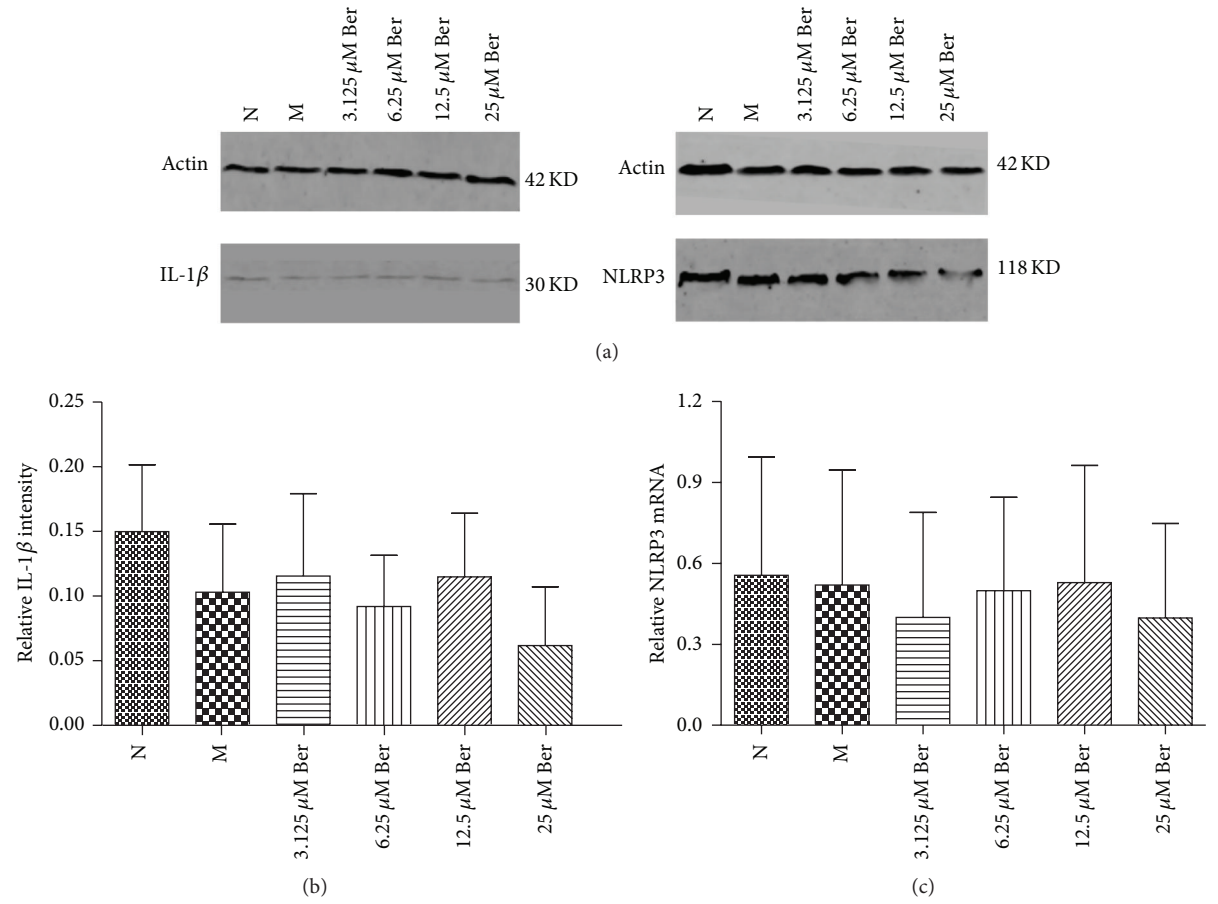

Figure 3: Outcome of Western blot ($n = 4$). (a) The representative bands of Western blot, (b) optical density of IL-1β, and (c) optical density of NLRP3. Values are mean ± SD. N: normal group; M: model group; 3.125 μM Ber, 6.25 μM Ber, 12.5 μM Ber, and 25 μM Ber: berberine group (Ber) of different concentrations. NLRP3: domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator, leucine rich repeat, and pyrin domains-containing protein 3.

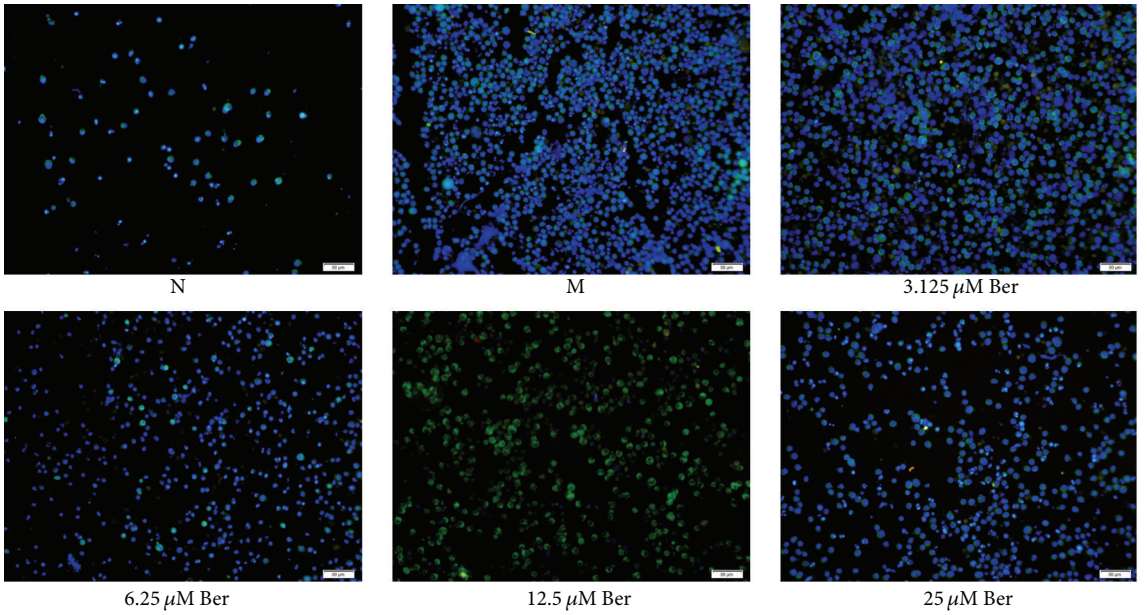

Figure 4: Immunofluorescence for NLRP3. N: normal group; M: model group; 3.125 μM Ber, 6.25 μM Ber, 12.5 μM Ber, and 25 μM Ber: berberine group (Ber) of different concentrations. NLRP3: domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator, leucine rich repeat, and pyrin domains-containing protein 3. The protein expression of NLRP3 was mainly located in the cytoplasm (green). The nuclei were stained with blue by DAPI.

3.4. Immunofluorescence for NLRP3. NLRP3 was localized in the cytoplasm of THP-1 cells, stained with green. The nuclei were stained with blue by DAPI. The expression of NLRP3 in THP-1 cells was increased in group M compared to that in group N. Compared with group M, there was an decrease in the expression of NLRP3 in groups 6.25 μM Ber and 25 μM Ber (Figure 4).

4. Discussion

Traditional Chinese medicine is based on cumulative empirical experience of previous practitioners. A systematic review validated that Chinese herbal decoctions had similar clinical efficacy for the treatment of gout, but the Chinese herbal decoctions were superior to Western medicine in regard to attenuating adverse drug reactions [15]. Previous evidences from clinical practice and experimental studies confirmed that modified Simiao decoction had the potential to treat hyperuricemia and gouty arthritis [14, 16, 17]. Ultrafiltration liquid chromatography combined with high-speed countercurrent chromatography screened and isolated α-glucosidase and xanthine oxidase inhibitors from *Cortex phellodendri* for the prevention and treatment of gout [18]. However, previous studies focused on hypouricemic effects of modified Simiao decoction. To our knowledge, gouty arthritis could attack patients with a serum uric acid concentration in the normal range.

Therefore, it is necessary to inhibit the cytokines release in acute phase of gouty arthritis. Ankle joint urate arthritis provided a useful tool for the evaluation of anti-inflammatory and antigout agents [19, 20]. However, MSU crystals-induced inflammation model in monocytes was rarely reported [5]. In our previous study, the MSU crystals-induced inflammation model in THP-1 cells was successfully established by the stimulation of PMA and MSU [9].

Innate immunity provides the first line of defense against pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) via primitive responses that are nonspecific and broad in spectrum. MSU crystals may act as PAMP or DAMP and are recognized by the pattern recognition receptors of the innate immune system. Unlike adaptive immunity, innate immune responses do not directly generate immunologic memory or lasting protective immunity, in line with the nature of recurrent acute gouty attack [21]. NLRP3 inflammasome plays an important role in MSU crystals-induced innate immune responses.

NLRP3 inflammasome is composed of leucine rich repeat- (LRR-) containing Nlrp3, an adaptor protein such as apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and an effector caspase-1 which activates proinflammatory cytokines. Intracellular MSU crystals are recognized by NLRP3 inflammasome, giving rise to oligomerization of NLRP3 and cleavage of procaspase-1 to caspase-1. Further, caspase-1 cleaves inactive pro-IL-1β to produce active IL-1β [22] (Figure 5).

Our results were consistent with Liu et al. in terms of berberine in reducing IL-1β release with lipopolysaccharide-induced inflammation [12]. Unlike the dose-effect relationship of Liu et al., high dose of berberine was unable to diminish the IL-1β expression. The difference may result from berberine purity and diverse inflammation models. In accordance with the outcome of ELISA, there was a significant increase in the expression of IL-1β mRNA in

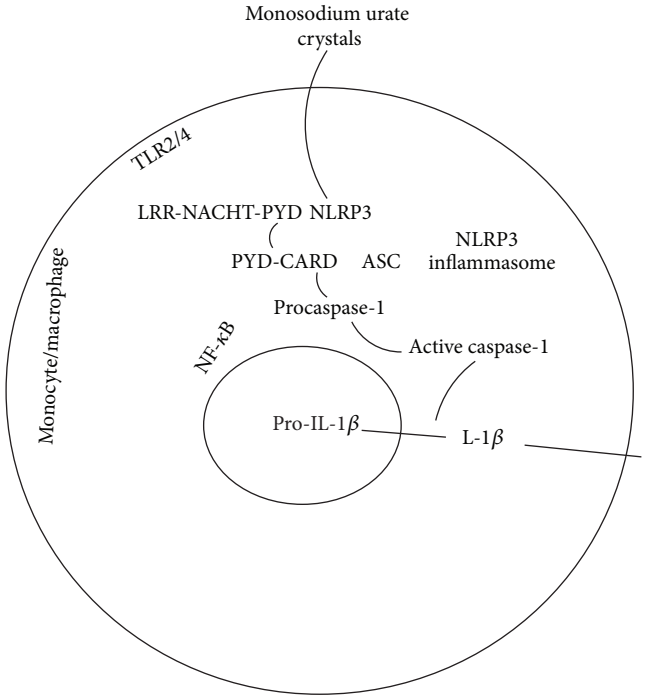

Figure 5: NLRP3 and IL-1β signalling in gout. Intracellular monosodium urate crystals are recognized by NLRP3 inflammasome, leading to oligomerization of NLRP3 and cleavage of procaspase-1 to caspase-1. Further, caspase-1 cleaves inactive pro-IL-1β to produce active IL-1β. NLRP3, domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator, leucine rich repeat, and pyrin domains-containing protein 3; TLR, Toll-like receptor; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain [CARD]; NLR, NOD-like receptor; PYD, pyrin domain; LRR, leucine rich repeat; NACHT, domain present in neuronal apoptosis inhibitor protein major histocompatibility complex class II transactivator; NF-κB, nuclear factor-κB.

group M when compared with group N. However, different concentrations of berberine had different effects on transcription and translation level of IL-1β mRNA. Posttranscriptional processing and degradation and posttranslational processing and modification may contribute to the discrepancies.

With regard to the expression of NLRP3 mRNA, our findings were consistent with Dang et al. who found that mRNA expression of NLRP3-2, NLRP3-3, and NLRP3-4 increased significantly in the patients with acute gouty arthritis and nonacute gouty arthritis compared with healthy controls [23]. However, Yang et al. showed that the expression of NLRP3 mRNA was significantly lower in patients with acute gouty arthritis compared with healthy controls [24]. The differences may result from different models *in vivo* and *in vitro*.

IL-1β is a secretive protein, and hence Western blot via cell lysates may determine the pro-IL-1β. Our results showed that different concentrations of berberine had no effects on IL-1β, which was different from the result of ELISA. The differences may result from determination of inactive or active IL-1β.

The outcomes of Western blot showed that different concentrations of berberine had no effects on NLRP3 expression, which was different from the result of Real-Time PCR. Semiquantitative method of Western blot, processing, and modification of transcription and translation may result in the divergences. NLRP3 was localized in the cytoplasm of THP-1 cells from immunofluorescence. The result of immunofluorescence was consistent with NLRP3 mRNA in terms of lowering the expression of NLRP3.

The different concentrations of berberine may regulate the expressions of mRNA and protein of NLRP3 and IL-1β through NLRP3 inflammasome and downstream signalling molecules, resulting in attenuating the MSU crystals-induced inflammation.

5. Conclusions

Berberine may influence the NLRP3 inflammasome which is involved in MSU crystals-induced innate immune responses, attenuate the expression of NLRP3, further inhibit downstream signalling molecular IL-1β, and eventually play a role in treatment of gouty arthritis. The effects and mechanisms of berberine on NLRP3 inflammasome related LRR, ASC, and caspase-1 need to be further validated.

Competing Interests

The authors have no competing interests.

Authors' Contributions

Ya-Fei Liu and Cai-Yu-Zhu Wen carried out all the assays and drafted the manuscript. Ya-Fei Liu and Cai-Yu-Zhu Wen contributed equally to this work and should be considered co-first authors. Ya-Fei Liu, Zhe Chen, Yu Wang, and Ying Huang participated in the design of the study and carried out the statistical analysis. Sheng-Hao Tu conceived the study and was responsible for its design and helped in revising the manuscript. All authors read and approved the final manuscript.

References

[1] C.-F. Kuo, M. J. Grainge, W. Zhang, and M. Doherty, "Global epidemiology of gout: prevalence, incidence and risk factors," *Nature Reviews Rheumatology*, vol. 11, no. 11, pp. 649–662, 2015.

[2] Y. Zhu, B. J. Pandya, and H. K. Choi, "Prevalence of gout and hyperuricemia in the US general population: the National Health and Nutrition Examination Survey 2007-2008," *Arthritis & Rheumatism*, vol. 63, no. 10, pp. 3136–3141, 2011.

[3] R. C. Landis and D. O. Haskard, "Pathogenesis of crystal-induced inflammation," *Current Rheumatology Reports*, vol. 3, no. 1, pp. 36–41, 2001.

[4] P. T. Chapman, H. Yarwood, A. A. Harrison et al., "Endothelial activation in monosodium urate monohydrate crystal-induced inflammation. *In vitro* and *in vivo* studies on the roles of tumor necrosis factor α and interleukin-1," *Arthritis & Rheumatism*, vol. 40, no. 5, pp. 955–965, 1997.

[5] F. Martinon, V. Pétrilli, A. Mayor, A. Tardivel, and J. Tschopp, "Gout-associated uric acid crystals activate the NALP3 inflammasome," *Nature*, vol. 440, no. 7081, pp. 237–241, 2006.

[6] R. Terkeltaub, J. S. Sundy, H. R. Schumacher et al., "The interleukin 1 inhibitor rilonacept in treatment of chronic gouty arthritis: results of a placebo-controlled, monosequence crossover, non-randomised, single-blind pilot study," *Annals of the Rheumatic Diseases*, vol. 68, no. 10, pp. 1613–1617, 2009.

[7] C. Selmi, E. Generali, M. Massarotti, G. Bianchi, and C. A. Sciré, "New treatments for inflammatory rheumatic disease," *Immunologic Research*, vol. 60, no. 2-3, pp. 277–288, 2014.

[8] L. Shi, L. Xu, Y. Yang, H. Song, H. Pan, and L. Yin, "Suppressive effect of modified Simiaowan on experimental gouty arthritis: an *in vivo* and *in vitro* study," *Journal of Ethnopharmacology*, vol. 150, no. 3, pp. 1038–1044, 2013.

[9] Y.-F. Liu, S.-H. Tu, Z. Chen, Y. Wang, Y.-H. Hu, and H. Dong, "Effects of modified Simiao decoction on IL-1β and TNFα secretion in monocytic THP-1 cells with monosodium urate crystals-induced inflammation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 406816, 7 pages, 2014.

[10] L. Shi, L. Xu, and L. Yin, "Exploring the anti-gout effective utility parts of modified simiao pill," *Journal of Nanjing TCM University*, vol. 24, no. 6, pp. 386–387, 2008.

[11] L. Yin, X.-D. Shi, and Z.-X. Qian, "Ideas and methods of screening jiaweisimiaowan for treatment of gout and principles of diagnosis," *World Science and Technology—Modernization of Traditional Chinese Medicine and Materia Medica*, vol. 8, no. 6, pp. 27–30, 2006.

[12] S.-X. Liu, Y.-F. Liu, J.-H. Yin, Y. Zhang, L.-X. Xu, and J. Yang, "Effects of berberine on inflammatory cytokines induced by lipopolysaccharide in THP-1 cells," *International Journal of Endocrinology and Metabolism*, vol. 34, no. 4, pp. 233–236, 2014.

[13] H. W. Jeong, K. C. Hsu, J.-W. Lee et al., "Berberine suppresses proinflammatory responses through AMPK activation in macrophages," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 296, no. 4, pp. E955–E964, 2009.

[14] Y.-F. Liu and S.-H. Tu, "The review of traditional Chinese medicine in the treatment of gout," *Rsearch of Integrated Traditional Chinese and Western Medicine*, vol. 4, no. 1, pp. 44–47, 2012.

[15] L. Zhou, L. Liu, X. Liu et al., "Systematic review and meta-analysis of the clinical efficacy and adverse effects of Chinese herbal decoction for the treatment of gout," *PLoS ONE*, vol. 9, no. 1, Article ID e85008, 2014.

[16] J. Hua, P. Huang, C.-M. Zhu, X. Yuan, and C.-H. Yu, "Anti-hyperuricemic and nephroprotective effects of Modified Simiao Decoction in hyperuricemic mice," *Journal of Ethnopharmacology*, vol. 142, no. 1, pp. 248–252, 2012.

[17] Q.-H. Hu, R.-Q. Jiao, X. Wang, Y.-Z. Lv, and L.-D. Kong, "Simiao pill ameliorates urate underexcretion and renal dysfunction in hyperuricemic mice," *Journal of Ethnopharmacology*, vol. 128, no. 3, pp. 685–692, 2010.

[18] S. Li, C. Liu, L. Guo et al., "Ultrafiltration liquid chromatography combined with high-speed countercurrent chromatography for screening and isolating potential α-glucosidase and xanthine oxidase inhibitors from *Cortex Phellodendri*," *Journal of Separation Science*, vol. 37, no. 18, pp. 2504–2512, 2014.

[19] T. J. Coderre and P. D. Wall, "Ankle joint urate arthritis in rats provides a useful tool for the evaluation of analgesic and anti-arthritic agents," *Pharmacology, Biochemistry and Behavior*, vol. 29, no. 3, pp. 461–466, 1988.

[20] T. J. Coderre and P. D. Wall, "Ankle joint urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produced by Freund's adjuvant," *Pain*, vol. 28, no. 3, pp. 379–393, 1987.

[21] D. M. Rose and R. Liu-Bryan, "Innate immunity in triggering and resolution of acute gouty inflammation," *Current Rheumatology Reports*, vol. 8, no. 3, pp. 209–214, 2006.

[22] S. R. Kingsbury, P.-G. Conaghan, and M.-F. McDermott, "The role of the NLRP3 inflammasome in gout," *Journal of Inflammation Research*, vol. 4, no. 1, pp. 39–49, 2011.

[23] W.-T. Dang, W.-G. Xie, M.-C. Zhao et al., "Correlation analysis of expression of NLRP3 gene transcript variant mRNA and clinical examination indexes in patients with gouty arthritis," *Journal of Third Military Medical University*, vol. 36, no. 16, pp. 1713–1719, 2014.

[24] Q.-B. Yang, J.-G. Zhou, Y.-F. Qing, W.-G. Xie, M.-C. Zhao, and M. Li, "Expression of NLRP3 inflammttsome mRNA in peripheral blood monocytes of patients with gouty arthritis," *Chinese Journal of Rheumatology*, vol. 14, no. 10, pp. 686–689, 2010.

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Review Article

Association between Toll-Like Receptor 4 Polymorphisms and Systemic Lupus Erythematosus Susceptibility: A Meta-Analysis

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Family aggregation was observed among systemic lupus erythematosus (SLE) cases, suggesting the genetic factor may contribute to the susceptibility. Toll-like receptors (TLR) play key role in human immune system; in order to gain better insight on the association between TLR4 polymorphisms and SLE risk, a meta-analysis was conducted. In total 4 case-control studies have been included, involving 503 SLE cases and 636 healthy controls. The association between TLR4 polymorphisms and SLE risk was evaluated by calculating pooled odd ratio (OR) and its 95% confidential interval (CI). The Q-test and I^2 statistic were used to estimate the degree of heterogeneity. Publication bias among enrolled studies was examined by using Egger's test and Begg's test. Overall, there was no evidence of positive association between SLE risk and D299G and T399I polymorphisms in TLR4. The meta-analysis reported a null association between TLR4 polymorphisms and SLE risk in included study populations, but the role of TLR4 polymorphisms in developing SLE among other populations remains undetermined. Moreover, some laboratory studies still discovered the involvement of TLR4 in SLE process. Therefore, the association between TLR4 polymorphisms and SLE risk requires further investigation both in laboratory and in epidemiological efforts.

1. Introduction

Systemic lupus erythematosus is an autoimmune disease that causes a chronic inflammatory condition for life-long time, and the inflammation triggered by SLE can affect multiple organs in human body, including joints, skin, lung, kidneys, and blood vessels. Particularly the impairment of kidneys may lead to lupus nephritis and possibly develop to acute or end-stage renal failure. Although some of SLE complications could be fatal, the development and application of immunosuppressive drugs aiming to control the autoimmune process helped to improve the prognosis of SLE cases; approximately 80–90% of SLE cases can expect to have a normal life expectancy [1]. However, due to the wide spectrum of clinical manifestation SLE caused, it may be very difficult for diagnosis unless with professional opinion of rheumatologist. Therefore, the early diagnosis of SLE would be critical to improve the prognosis and life quality of SLE cases. Besides, SLE has a unique pattern of incidence, it is more common in female population than male counterparts, and the gender ratio ranges from 4 to 12 : 1. The SLE could occur in all ages, but the incidence peak appears in childbearing years, according to statistics [2]. With years of endeavor in surveillance programs and SLE registry, the prevalence of SLE in some states of United States has been well documented. According to the data released by the Michigan Lupus Epidemiology and Surveillance Program, the incidence by American College of Rheumatology (ACR) definition among black female was the highest when comparing with other races, reaching 12.8 per 100,000 (95% CI: 11.1–14.8), and the

incidence of white female was approximately half of the corresponding indicator of black female; to be more accurate, the figure was 6.3 (95% CI: 5.3–7.5) [3]. Consistent with the previous findings, the data reflects that race would affect the incidence and prevalence of SLE, and the prevalence of SLE is higher among African descendants, Asians, and Hispanic descendants, when comparing with white population. The family history of SLE has also been recognized as the risk factor of developing SLE; an epidemiological study conducted among 265 SLE cases and 355 healthy controls demonstrated that family history of SLE would confer an elevated risk with an OR of 3.3 (95% CI: 1.2–8.6) [4].

Although many studies have been done to investigate the risk factors of SLE, the cause of SLE has not yet been fully understood so far. Based on the family aggregation observed, it can be assumed that genetic factor plays a critical role in developing SLE. No single causal gene has been identified so far, but several genes have been proved to influence individual's chance of developing SLE. A study conducted in Caucasian population demonstrated that the human leukocyte antigen classes I, II, and III are associated with SLE risk [5]. Similar with HLA regions, toll-like receptors are a class of proteins that play a fundamental role in the early innate immune response to pathogens by sensing microorganism and are involved in detecting endogenous danger signals. The animal experiment showed that, among the mice with TLR2 and TLR4 deficiency, the immunological alteration and autoantibody production have been significantly suppressed, suggesting the TLR4 is involved with the autoimmune process [6]. Therefore, the polymorphisms in TLR4 could possibly affect the SLE risk among population; however, there is no conclusion reached in the association between TLR4 polymorphisms and SLE risk. In order to analyze the impact of TLR4 polymorphisms and gain comprehensive insight on this issue, we conducted a meta-analysis.

2. Material and Methods

We performed the meta-analysis in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [7].

2.1. Identification and Eligibility of Relevant Studies. Literature search was independently conducted by two investigators for genetic studies on TLR4 in PUBMED. All relevant studies reported up to January 2015 and following key words were searched: "systemic lupus erythematosus," or "SLE", "polymorphism" or "SNP", "toll-like receptor 4," or "TLR4". The search was performed in February 2016 and no date and language limits were applied. In order to further expand the dataset, we also reviewed the reference lists of all retrieved studies to identify eligible studies.

2.2. Inclusion and Exclusion Criteria. The eligibility of study was defined as follows: (1) case-control study design; (2) investigating the association between TLR4 polymorphisms and the risk of developing SLE; (3) genotype distribution of TLR4 polymorphisms in both cases and controls available; reviews, cases reports, editorial comment, communications, and reports without sufficient data were excluded in our meta-analysis.

2.3. Data Extraction and Quality Assessment. The following information was extracted from each eligible study: name of first author, year of publication, ethnicity of study participants, number of cases and controls, the frequency of TLR4 genotypes in SLE cases and controls, and Hardy-Weinberg equilibrium (HWE) test results in controls. In order to assess the quality of enrolled studies, we rated the methodological quality of each included study by using the Newcastle-Ottawa quality assessment scale. This scale contains 9 items in total (1 point for each) in three parts: selection (4 items), comparability (2 items), and exposure (3 items).

2.4. Statistical Analysis. Statistical analysis was performed by using STATA version 12 (StataCorp, College Station, TX, USA). Chi-squared test was employed to determine the HWE of controls if the P value of HWE was not provided in the original study. A P value less than 0.05 was considered as the deviation of HWE. The association between TLR4 polymorphisms and SLE risk was evaluated by calculating pooled odd ratio and its 95% confidential interval and forest plot was created to demonstrate the overall effect. With respect to heterogeneity, the Q-test and I^2 statistic were used to estimate the degree of heterogeneity among the enrolled studies. In the absence of heterogeneity, fixed-effects model was applied to estimate the pooled OR and 95% CI. Otherwise, random-effect model was used to yield more conservative overall effects. Moreover, publication bias among enrolled studies was examined by using Egger's test and Begg's test, where a P value of less than 0.10 was considered statistically significant.

3. Results

3.1. Characteristics of Enrolled Studies. As shown in Figure 1, a detailed flow diagram demonstrated the process of inclusion and exclusion in this meta-analysis. Based on our search strategy, a total of 45 studies were identified in the initial search. After studying the abstracts, 39 publications have been removed. After reviewing the full text of the remaining 6 studies, 2 publications have been excluded due to the absence of genotype distribution data. Finally, a total of 4 studies were included in our meta-analysis [8–11], involving 503 SLE cases and 636 healthy controls. The characteristics of enrolled studies were demonstrated in Table 1. Of 4 studies enrolled, 2 were conducted in Caucasian population, 1 in Arabian, and 1 in Indian. The genotype distribution of both D299G and T399I polymorphisms agreed on HWE in control group in every study. The average of quality score evaluated by Newcastle-Ottawa scale was 7.2 combined with all enrolled studies together, while a score greater than 5 was considered appropriate to be included in meta-analysis.

3.2. Meta-Analysis of TLR4 Polymorphisms and SLE Risk. The main outcomes of this meta-analysis were presented in Table 3 and the genotype distribution in cases and controls

Table 1: Characteristics of enrolled studies in the meta-analysis on the association between TLR4 polymorphisms and SLE risk.

Author and year	Ethnicity	Quality score	P_{HWE} in control (D299G)	P_{HWE} in control (T399I)
Sánchez, 2004 [8]	Caucasian	8	0.78	0.27
Bogaczewicz, 2013 [9]	Caucasian	6	N/A	0.42
Dhaouadi, 2013 [10]	Arabian	8	0.55	N/A
Rupasree, 2015 [11]	Indian	7	0.28	0.22

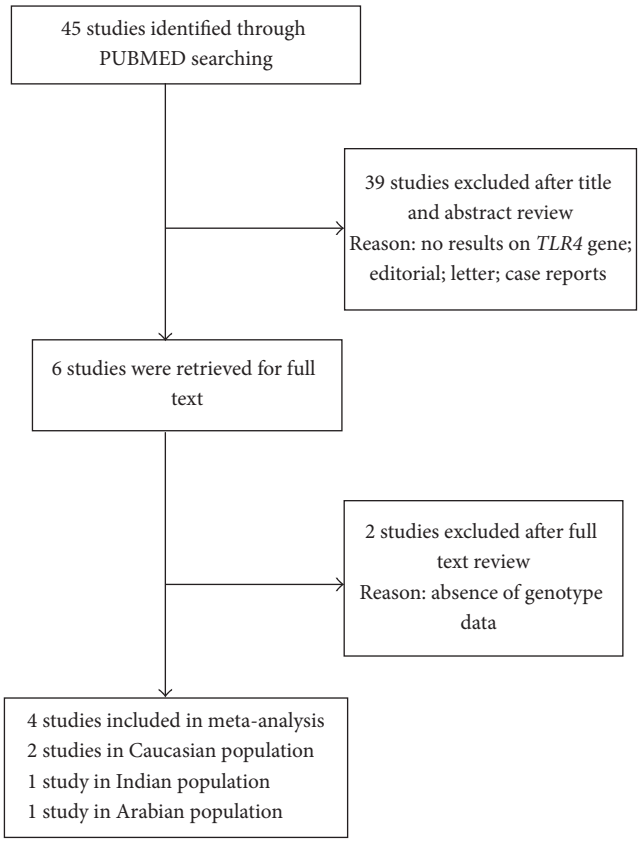

Figure 1: PRISMA flow diagram for inclusion of the studies investigating the relationship between *TLR4* polymorphisms and SLE risk.

from enrolled studies was presented in Table 2. Overall, there was no evidence of positive association between SLE risk and TLR4 D299G and T399I polymorphisms. As for D299G, the pooled ORs in dominant model and recessive model were 1.31 (95% CI: 0.96–1.80) and 2.18 (95% CI: 0.65–7.24), respectively. The pooled OR for T399I was 1.18 (95% CI: 0.85–1.64) in dominant model, and the corresponding figure was 2.52 (95% CI: 0.66–9.63) in recessive model. We further analyzed the association between alleles of these two TLR4 polymorphisms and SLE risk by pooling all subjects; the pooled ORs for D299G and T399I allele were 1.30 (95% CI: 0.98–1.73) and 1.20 (95% CI: 0.89–1.62), respectively (see Figures 2–4).

3.3. Evaluation of Heterogeneity. According to the I^2 statistics we calculated by using STATA software, there was no interstudy heterogeneity among all enrolled studies of TLR4 polymorphisms for all 3 genetic models (D299G dominant model: $I^2 = 0.0\%$, $P = 0.432$; recessive model: $I^2 = 53.3\%$, $P = 0.143$; allele: $I^2 = 16.6\%$, $P = 0.301$; T399I dominant model: $I^2 = 0.0\%$, $P = 0.710$; recessive model: $I^2 = 62.7\%$, $P = 0.102$; allele: $I^2 = 0.0\%$, $P = 0.538$). Due to the absence of heterogeneity, the pooled ORs were estimated by using fixed-effect model.

3.4. Publication Bias. Due to the limited number of enrolled studies, it was inappropriate to evaluate the publication bias by using funnel plot. Therefore, the publication bias in our meta-analysis was estimated by using Begg and Egger's test. As shown in Table 4, the P values for all genetic models were all greater than 0.05, suggesting no evidence of publication bias.

4. Discussion

Toll-like receptors were firstly discovered in *Drosophila* in 1985 and they have been proved to play fundamental role in innate immune system. After that, Nomura et al. identified the first toll-like receptor in cDNA clones of human in 1994 [12]. By comparing the sequence, it has been discovered that TLRs are highly conservative from *Drosophila* to human and share structural and functional similarities. The main function of TLR4 is recognizing lipopolysaccharide (LPS) and activating immune response; previous findings also revealed that TLR4 is capable of binding a variety of endogenous proteins such as low-density lipoprotein and heat shock protein [13]. With the initiation of LPS, TLR4 is capable of inducing the expression of multiple cytokines, including IL-1, IL-6, and IL8 via signaling pathway. The relationship between TLR4 and infection has been widely investigated; after initial TLR mediated immune response triggered by LPS, it is possible for secondary response such as activation of endothelial cells that promotes the production of adhesion molecules to occur [14]. Eventually it may lead to systemic septic syndrome including tissue perfusions and organ failure [15]. Given the great importance of TLR4 in immune response, growing evidence implicates the association between TLR4 and autoimmune condition [16].

The mutation occurring in TLR4 sequence may also alter individual's susceptibility to some diseases. In detail, two nonsynonymous polymorphisms in TLR4 gene have been well investigated; an A/G transition causes an Asp/Gly polymorphism at amino acid 299 and a C/T transition causes a Thr/Ile polymorphism at amino acid 399. Notably, the amino acid change caused by two above-mentioned polymorphisms

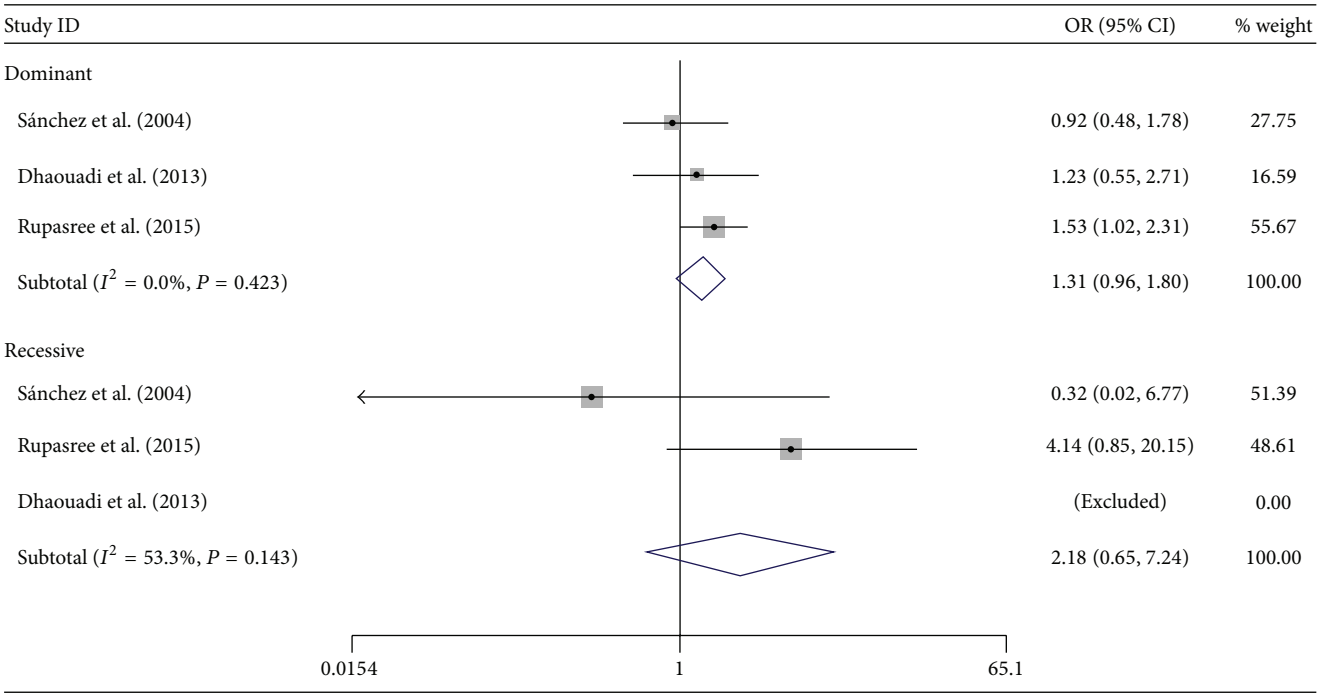

Figure 2: Forest plot of the association between *TLR4* polymorphism D299G and SLE risk.

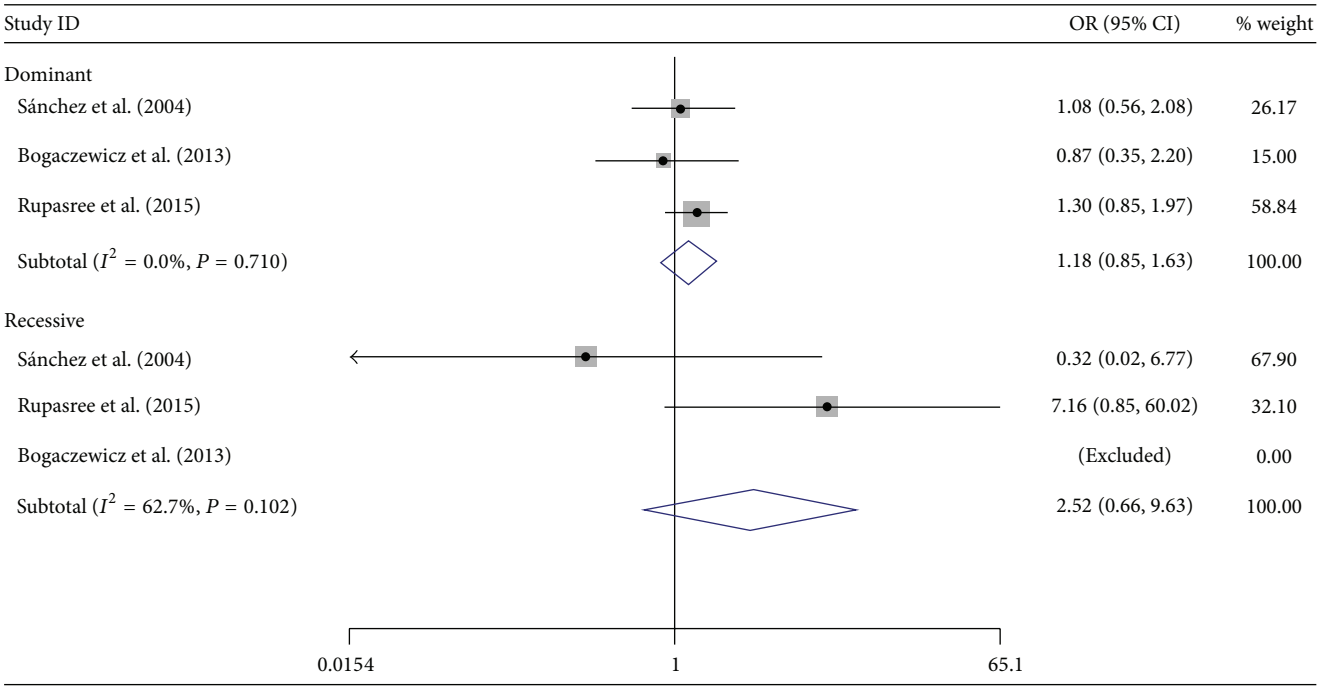

Figure 3: Forest plot of the association between *TLR4* polymorphism T399I and SLE risk.

would lead to changing the ligand-binding site of the receptor [17] and consequently contributes to the alternation of susceptibility to diseases. One of the underlying mechanisms is that the above-mentioned two polymorphisms may have impact on the responsiveness to LPS. To be more specific, the extracellular domain of TLR4 can bind with a secreted protein named MD-2 [18] and a soluble or GPI-anchored glycoprotein coreceptor CD14 which are essential for optimal TLR-4 mediated LPS response [19]. According to the results of sequencing, both D299G and T399I are located in the extracellular domain of TLR4, and these two variants have been associated with LPS hyporesponsiveness *in vitro* and

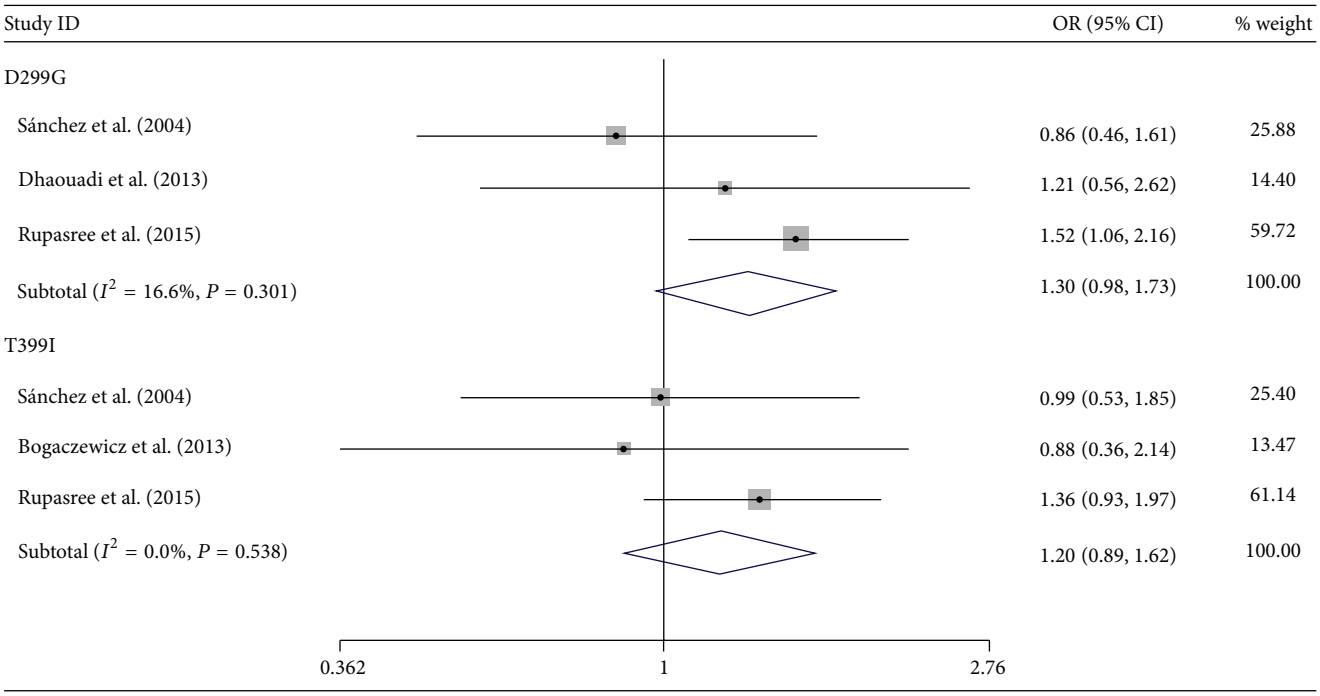

Figure 4: Forest plot of the association between D299G, T399I allele, and SLE risk.

Table 2: Genotype distribution of enrolled studies on *TLR4* polymorphisms.

(a) D299G

Author and year	Case				Control			
	Asp/Asp	Asp/Gly	Gly/Gly	Total	Asp/Asp	Asp/Gly	Gly/Gly	Total
Sánchez, 2004 [8]	106	16	0	122	171	26	2	199
Bogaczewicz, 2013 [9]	—	—	—	—	—	—	—	—
Dhaouadi, 2013 [10]	111	16	0	127	102	12	0	114
Rupasree, 2015 [11]	119	68	7	194	158	63	2	223

(b) T399I

Author and year	Case				Control			
	Thr/Thr	Thr/Ile	Ile/Ile	Total	Thr/Thr	Thr/Ile	Ile/Ile	Total
Sánchez, 2004 [8]	105	17	0	122	173	24	2	199
Bogaczewicz, 2013 [9]	52	8	0	60	85	15	0	100
Dhaouadi, 2013 [10]	—	—	—	—	—	—	—	—
Rupasree, 2015 [11]	128	58	6	192	161	61	1	223

Table 3: Meta-analysis of *TLR4* polymorphism with the risk of SLE.

Polymorphism	Genetic model	Test of association			Test of heterogeneity		
		OR	95% CI	P	Model	I^2	P
D299G	Dominant	1.31	0.96, 1.80	0.09	Fixed	0.0%	0.432
	Recessive	2.18	0.65, 7.24	0.21	Fixed	53.3%	0.143
	Allele	1.30	0.98, 1.73	0.07	Fixed	16.6%	0.301
T399I	Dominant	1.18	0.85, 1.64	0.33	Fixed	0.0%	0.710
	Recessive	2.52	0.66, 9.63	0.18	Fixed	62.7%	0.102
	Allele	1.20	0.89, 1.62	0.24	Fixed	0.0%	0.538

Table 4: The results of publication bias in dominant and recessive model and allele analysis.

Polymorphism	Genetic model	Begg's test		Egger's test	
		Z	P	Coefficient	P
D299G	Dominant	−0.52	0.60	−2.03	0.45
	Recessive	−1.00	0.32	−3.42	0.24
	Allele	−0.52	0.60	−2.09	0.45
T399I	Dominant	−1.57	0.12	−1.15	0.56
	Recessive	−1.00	0.32	−2.15	0.32
	Allele	−1.57	0.12	−1.83	0.15

in vivo [20]. A much debated question is how D299G and T399I affect the LPS responsiveness and the result remains inconsistent. A study which enrolled 200 plus pediatric subjects and quantification of pro- and anti-inflammatory cytokines response by fresh peripheral blood mononuclear cell upon acute exposure to LPS were conducted, and the results showed no significant difference between subjects with different genotype of TLR4 polymorphisms, indicating a null association [21]. Although the previous study reported that the polymorphisms in TLR4 have no impact on the production of cytokines, a more recent cell assay suggested that the polymorphisms in TLR4 did not alter LPS-binding to soluble TLR4/MD2; instead, the native-PAGE revealed that D299G and T399I could impair the ligand-dependent dimerization and consequently lead to the hyporesponsiveness to LPS [22]. In a case-control study involving 108 Legionnaire's disease cases and two independent control groups, both polymorphisms were inversely associated with the risk of Legionnaire's disease [23]. However, the issue of TLR4 polymorphisms and disease risk still remains controversial; another study revealed that the mutation of TLR4 is positively associated with the risk of developing leprosy [24]. So far there is only a limited number of studies that investigated the association between TLR4 polymorphisms and SLE risk; null association was reported in the case-control study in Spanish and Polish population [8, 9], while a significant association was observed in Indian population [11]. Therefore, we conducted a comprehensive literature search and meta-analysis to address this issue. According to the I^2 statistics among all genetic models, it can be assumed that the heterogeneity was insignificant in our meta-analysis; therefore, fix-effect model was employed to estimate the pooled OR. Both D299G and T399I were not significantly associated with SLE risk in dominant and recessive model. We further analyzed the overall impact of TLR4 polymorphisms allele on SLE risk, and the results were similar with the genotype analysis. Therefore, we can conclude that, based on the current evidence we obtained, the TLR4 polymorphisms are not associated with SLE risk.

The major limitation of our meta-analysis is that the number of enrolled studies was small; despite expanded search applied by reviewing the reference of enroll studies, still only 4 studies were available in our final analysis. The reason behind this problem is the unique distribution pattern of TLR4 polymorphisms between different races. According to the data of a study involving study subjects from all over the world, the D299G and T399I double mutation haplotype were of high frequency in Caucasians in Europe, and D299G and 399 wildtype can be found in African population; however, no mutations in both D299G and T399I were observed in East Asian countries, including China, Japan, and Korea [25]. Therefore, the range of study population was limited by the natural genotype distribution. Despite the limitation of the above-mentioned, the pooled outcome of this meta-analysis is solid, due to the absence of both heterogeneity and publication bias.

Although the meta-analysis we conducted reported a null association between TLR4 polymorphisms and SLE risk, some laboratory studies still discovered the involvement of TLR4 in SLE process. Flow cytometry was applied to detect the expression of TLR4 in blood monocytes from SLE cases and healthy control; the statistical comparison revealed that the TLR4 expression was significantly suppressed in SLE cases [26]. Moreover, the present meta-analysis only included Caucasian, Arabian, and Indian due to the data availability; therefore, the conclusion does not apply to all populations. Recently, TLR4 has been recognized as the potential target of novel therapy of SLE, and progress has been made. Therefore, the association between TLR4 polymorphisms and SLE risk requires further investigation both in laboratory and in epidemiological efforts.

Competing Interests

The authors declare that they have no competing interests.

References

[1] Z. Wang, Y. Wang, R. Zhu et al., "Long-term survival and death causes of systemic lupus erythematosus in China: a systemic review of observational studies," *Medicine*, vol. 94, article e794, 2015.

[2] J. Choi, S. T. Kim, and J. Craft, "The pathogenesis of systemic lupus erythematosus-an update," *Current Opinion in Immunology*, vol. 24, no. 6, pp. 651–657, 2012.

[3] E. C. Somers, W. Marder, P. Cagnoli et al., "Population-based incidence and prevalence of systemic lupus erythematosus: the michigan lupus epidemiology and surveillance program," *Arthritis and Rheumatology*, vol. 66, no. 2, pp. 369–378, 2014.

[4] G. S. Cooper, M. A. Dooley, E. L. Treadwell, E. W. St. Clair, and G. S. Gilkeson, "Risk factors for development of systemic lupus erythematosus: allergies, infections, and family history," *Journal of Clinical Epidemiology*, vol. 55, no. 10, pp. 982–989, 2002.

[5] H. A. Martens, I. M. Nolte, G. Van Der Steege et al., "An extensive screen of the HLA region reveals an independent association of HLA class I and class II with susceptibility for systemic lupus erythematosus," *Scandinavian Journal of Rheumatology*, vol. 38, no. 4, pp. 256–262, 2009.

[6] A. Lartigue, N. Colliou, S. Calbo et al., "Critical role of TLR2 and TLR4 in autoantibody production and glomerulonephritis in lpr mutation-induced mouse lupus," *The Journal of Immunology*, vol. 183, no. 10, pp. 6207–6216, 2009.

[7] D. Moher, A. Liberati, J. Tetzlaff, and D. G. Altman, "Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement," *International Journal of Surgery*, vol. 8, no. 5, pp. 336–341, 2010.

[8] E. Sánchez, G. Orozco, M. Á. López-Nevot, J. Jiménez-Alonso, and J. Martín, "Polymorphisms of toll-like receptor 2 and 4 genes in rheumatoid arthritis and systemic lupus erythematosus," *Tissue Antigens*, vol. 63, no. 1, pp. 54–57, 2004.

[9] A. Bogaczewicz, T. Sobow, J. Bogaczewicz et al., "Toll-like receptor 4 gene polymorphism 1196 C/T does not influence the risk of neuropsychiatric systemic lupus erythematosus in Polish population—a preliminary report," *Lupus*, vol. 22, no. 14, pp. 1504–1508, 2013.

[10] T. Dhaouadi, I. Sfar, Y. Haouami et al., "Polymorphisms of Toll-like receptor-4 and CD14 in systemic lupus erythematosus and rheumatoid arthritis," *Biomarker Research*, vol. 1, article 20, 2013.

[11] Y. Rupasree, S. M. Naushad, L. Rajasekhar, A. Uma, and V. K. Kutala, "Association of TLR4 (D299G, T399I), TLR9 -1486T>C, TIRAP S180L and TNF-α promoter (−1031, −863, −857) polymorphisms with risk for systemic lupus erythematosus among South Indians," *Lupus*, vol. 24, no. 1, pp. 50–57, 2015.

[12] N. Nomura, N. Miyajima, T. Sazuka et al., "Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1," *DNA Research*, vol. 1, no. 1, pp. 27–35, 1994.

[13] S. W. Brubaker, K. S. Bonham, I. Zanoni, and J. C. Kagan, "Innate immune pattern recognition: a cell biological perspective," *Annual Review of Immunology*, vol. 33, pp. 257–290, 2015.

[14] H. Bosshart and M. Heinzelmann, "Targeting bacterial endotoxin: two sides of a coin," *Annals of the New York Academy of Sciences*, vol. 1096, pp. 1–17, 2007.

[15] M. Wendel, R. Paul, and A. R. Heller, "Lipoproteins in inflammation and sepsis. II. Clinical aspects," *Intensive Care Medicine*, vol. 33, no. 1, pp. 25–35, 2007.

[16] A. Sadanaga, H. Nakashima, M. Akahoshi et al., "Protection against autoimmune nephritis in MyD88-deficient MRL/lpr mice," *Arthritis & Rheumatism*, vol. 56, no. 5, pp. 1618–1628, 2007.

[17] P. Rallabhandi, J. Bell, M. S. Boukhvalova et al., "Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling," *Journal of Immunology*, vol. 177, no. 1, pp. 322–332, 2006.

[18] R. Shimazu, S. Akashi, H. Ogata et al., "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4," *Journal of Experimental Medicine*, vol. 189, no. 11, pp. 1777–1782, 1999.

[19] T. Glück, J. Silver, M. Epstein, P. Cao, B. Farber, and S. M. Goyert, "Parameters influencing membrane CD14 expression and soluble CD14 levels in sepsis," *European Journal of Medical Research*, vol. 6, no. 8, pp. 351–358, 2001.

[20] N. C. Arbour, E. Lorenz, B. C. Schutte et al., "TLR4 mutations are associated with endotoxin hyporesponsiveness in humans," *Nature Genetics*, vol. 25, no. 2, pp. 187–191, 2000.

[21] R. N. Douville, Y. Lissitsyn, A. F. Hirschfeld et al., "TLR4 Asp299Gly and Thr399ile polymorphisms: no impact on human immune responsiveness to LPS or respiratory syncytial virus," *PLoS ONE*, vol. 5, no. 8, Article ID e12087, 2010.

[22] N. Yamakawa, U. Ohto, S. Akashi-Takamura et al., "Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A," *International Immunology*, vol. 25, no. 1, pp. 45–52, 2013.

[23] T. R. Hawn, A. Verbon, M. Janer, L. P. Zhao, B. Beutler, and A. Aderem, "Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 7, pp. 2487–2489, 2005.

[24] N. C. Suryadevara, V. S. K. Neela, S. Kovvali et al., "Genetic association of G896A polymorphism of TLR4 gene in leprosy through family-based and case-control study designs," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 107, no. 12, Article ID trt084, pp. 777–782, 2013.

[25] B. Ferwerda, M. B. McCall, S. Alonso et al., "TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 42, pp. 16645–16650, 2007.

[26] M. Kirchner, A. Sonnenschein, S. Schoofs, P. Schmidtke, V. N. Umlauf, and W. Mannhardt-Laakmann, "Surface expression and genotypes of Toll-like receptors 2 and 4 in patients with juvenile idiopathic arthritis and systemic lupus erythematosus," *Pediatric Rheumatology*, vol. 11, article 9, 2013.

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Research Article

Elevated Ratio of Th17 Cell-Derived Th1 Cells ($CD161^+$Th1 Cells) to $CD161^+$Th17 Cells in Peripheral Blood of Early-Onset Rheumatoid Arthritis Patients

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of articular cartilage and bone with elevated levels of proinflammatory cytokines. It has been reported that IL-17 and Th17 cells play important roles in the pathogenesis of RA. Recently, plasticity in helper T cells has been demonstrated; Th17 cells can convert to Th1 cells. It remains to be elucidated whether this conversion occurs in the early phase of RA. Here, we tried to identify Th17 cells, Th1 cells, and Th17 cell-derived Th1 cells ($CD161^+$Th1 cells) in the peripheral blood of early-onset RA patients. We also evaluated the effect of methotrexate on the ratio of Th17 cells in early-onset RA patients. The ratio of Th17 cell-derived Th1 cells to $CD161^+$Th17 cells was elevated in the peripheral blood of early-onset RA patients. In addition, MTX reduced the ratio of Th17 cells but not Th1 cells. These findings suggest that IL-17 and Th17 play important roles in the early phase of RA; thus, anti-IL-17 antibodies should be administered to patients with RA in the early phase.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of articular cartilage and bone with elevated levels of proinflammatory cytokines, such as TNFα and IL-6, produced from the synovial tissue [1]. We previously reported that IL-17 from activated human T cells in the synovial tissues of patients with rheumatoid arthritis (RA) is a potent stimulator of osteoclast formation [2]. In 2005, human helper T-17 type cells (Th17 cells) were identified as helper T cells, distinct from Th1 or Th2 cells [3]. Since this identification of Th17 cells, it has been reported that they play important roles in the pathogenesis of RA [4, 5].

Several reports confirm that IL-17 is an important cytokine in the early phase or the disease-onset phase of RA. In 2005, Raza et al. reported that the peripheral level of IL-17 is significantly high, analyzing the patients with RA whose disease durations were less than nine weeks [6]. Kokkonen et al. reported that the concentration of IL-17 in individuals before disease onset is significantly higher than that in patients after disease onset [7]. In addition, Kochi et al. [8] demonstrated that a regulatory variant in CCR6, which is a specific marker for Th17 cells distinguishing them from other helper T cells [9, 10], is associated with RA susceptibility. The CCR6 dinucleotide polymorphism genotype is correlated with the expression level of CCR6 and is associated with the presence of IL-17 in the sera of subjects with RA [8]. Thus, it is speculated that IL-17 plays an important role in the disease-onset or the early phase of RA.

Recently, plasticity in helper T cells has been demonstrated [11]. It has been reported that Th17 cells can convert to Th1 cells [12]. In 2008, Cosmi et al. reported that CD161 is a marker of human Th17 cells [13]. In addition, Th17 cell-derived Th1 cells express CD161, which is detected in the synovial fluid from patients with juvenile idiopathic arthritis; thus, these cells are clearly distinct from Th1 cells [14–16]. Th17 cell-derived Th1 cells are also named "non-classic Th1 cells" [16]. In contrast, Th1 cells rather than Th17 cells

were reported to be predominant in the peripheral blood of patients with late phase of RA whose average disease duration was 13 years [17]. We hypothesized that Th17 cells convert to Th1 cells in the early phase of RA and that methotrexate has an effect on the ratio of peripheral Th cells.

In the current study, we first evaluated the effect of methotrexate (MTX) on the ratio of Th cells in early-onset RA patients and then tried to identify Th17 cells, Th1 cells, and Th17 cell-derived Th1 cells in the peripheral blood of these early-onset RA patients. We report that MTX reduced the ratio of Th17 cells but not Th1 cells and that the ratio of Th17 cell-derived Th1 cells to Th17 cells was elevated in peripheral blood of early-onset RA patients.

2. Patients and Methods

2.1. Profiles of Patients. We analyzed two groups of patients with early-onset rheumatoid arthritis (RA). The RA patients met the ACR 1987 revised classification criteria. The 1st group comprised 5 patients (4 females and 1 male) whose disease durations were less than 18 months (Table 1). All patients were treated with methotrexate (MTX). The duration between first and second analysis was 1 to 6 months. RA patients were not treated by DMARDs or corticosteroids when peripheral blood was obtained. The peripheral helper T cells of these patients were analyzed according to the expressions of cytokines, interferon-γ (IFN-γ) and interleukin-17 (IL-17).

The 2nd group of patients comprised 6 patients (5 RA and 1 reactive arthritis [ReA]) (Table 2). ReA was a *Chlamydia*-associated arthritis. All patients were female. Six female, age-matched osteoarthritis (OA) patients were also analyzed as controls (data not shown). The disease durations of RA patients were less than 5 months. RA and ReA patients were not treated by DMARDs or corticosteroids when peripheral blood was obtained. The peripheral helper T cells of these RA, ReA, and OA patients were analyzed according to the expression of both CD161 and cytokines, interferon-γ (IFN-γ) and interleukin-17 (IL-17).

The current study was approved by the ethical committee of Tokyo Women's Medical University. Informed consent was obtained from each patient.

2.2. Flow Cytometry Analysis for CD4, CD161, and Intracellular IFN-γ and IL-17. After separating peripheral blood mononuclear cells (PBMCs), these cells were stimulated with 25 ng/mL PMA (Sigma) and 2 μg/mL ionomycin (Sigma) in the presence of 10 mg/mL brefeldin-A (BFA, Sigma) for 4 h at 37°C in 7% CO_2. T cells (400 μL) were incubated with 2 mL of 1x FACS lysing solution (Becton Dickinson, Mountain View, CA) for 10 min at room temperature. PBMCs were washed and incubated with 500 μL of 1x FACS permeabilizing solution (Becton Dickinson) for 10 min at room temperature. PBMCs were washed again and further incubated with PC5-conjugated anti-CD4 antibodies (Beckman Coulter), FITC-conjugated anti-human IFN-γ antibodies (Becton Dickinson), and Alexa Fluor 647-conjugated anti-human IL-17 antibodies (BD Bioscience) for 30 min at room temperature in the dark. In the analysis of the 2nd group of RA patients, PE-conjugated anti-CD161 antibodies (Becton Dickinson) were added to this incubation. The stained cells were analyzed using FACScan (BD Bioscience).

2.3. Statistical Analysis. Data were analyzed using the Wilcoxon signed-rank test and Mann-Whitney's U test (StatView®; Abacus Concepts Inc., Berkeley, CA). Data are presented as the mean ± SD. Significant difference was defined as $p < 0.05$.

3. Results

3.1. MTX Significantly Reduced the Ratio of Th17 Cells to Th Cells, but Not Those of Th1 Cells, "Both IFN-γ and IL-17 Positive Cells" ("Th17•Th1 Cells"). In the current study, we identified $CD4^+$ cells as Th cells, IL-17^+•IFN-γ^- $CD4^+$ T cells as Th17 cells, IL-17^-•IFN-γ^+ $CD4^+$ T cells as Th1 cells, and both positive IL-17^+•IFN-γ^+ $CD4^+$ T cells as "Th17•Th1 cells." [We do not use "Th17/Th1 cells" because the expression may be confused with the ratio of Th1 to Th17.] In the 1st group of RA patients, the ratio of Th17 cells to helper T cells (Th cells) was significantly reduced by MTX treatment ($p = 0.03$, Figure 1(a) left). Th1 cells were not reduced by MTX treatment (Figure 1(a) right); the ratio increased in 3 out of 5 patients. The ratio of Th1•Th17 cells was not reduced by MTX treatment (Figure 1(b)).

3.2. The Ratio of Th17 Cells to Helper T Cells. Figure 2(a) shows the ratio of Th17 cells to Th cells in the 2nd group of RA and reactive arthritis (ReA) patients. There was no significant difference among OA, RA, and ReA.

3.3. The Ratio of $CD161^+$ Helper T Cells to Helper T Cells. CD161 has been reported as a marker of human Th17 cells [13]; however, Th1 cells derived from Th17 cells also express CD161 [14]. We examined the ratio of $CD161^+CD4^+$ T cells to $CD4^+$ T cells (Figure 2(b)). There was a tendency for the ratio to be higher in RA and ReA than in OA; the ratios in 3 of 6 patients with RA or ReA were higher than the highest ratio in OA patients (a red dotted line) although the difference was not statistically significant.

3.4. The Ratio of $CD161^+$ Th1 Cells to $CD161^+$ Th17 Cells. We then examined the ratio of $CD161^+$Th1 cells to $CD161^+$Th17 cells (Figure 2(c)). The ratio of RA was significantly higher than that of OA ($p = 0.04$, Figure 2(c)). The ratio of ReA was highest among all of the data.

4. Discussion

In the current study, we clearly demonstrated that in the early-onset RA patients MTX reduced the ratio of Th17 cells among helper T cells but not those of Th1 cells or Th17•Th1 cells (Th cells producing both IFN-γ and IL-17). In addition, we also showed that the ratio of Th17 cell-derived Th1 cells to Th17 cells increased in the peripheral blood of the early-onset RA patients, compared with those of OA patients.

It has been reported that the anti-IL-17 antibody secukinumab significantly reduces signs and symptoms of RA compared with placebo, based on an analysis of biologic-naïve patients (mean disease durations: 6.0 years) [18]. In that

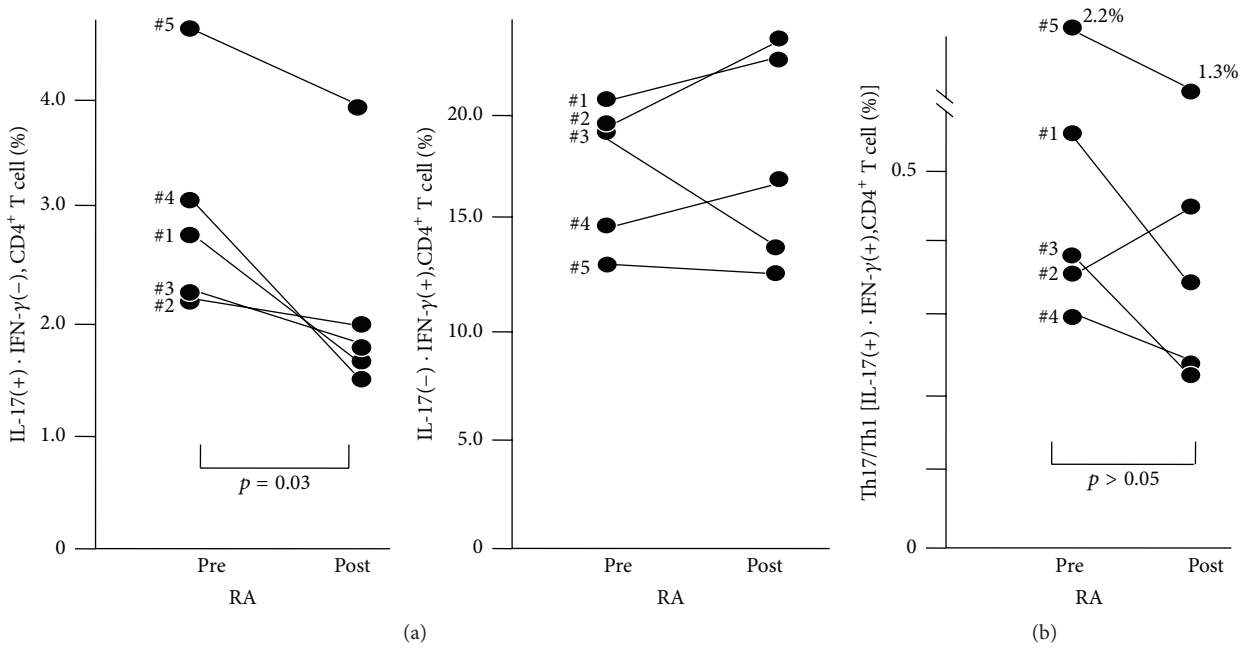

Figure 1: (a) Effect of MTX on the ratio of Th17 cells (left) or Th1 cells (right) to helper T cells. Number by the dot shows the patient number in Table 1. (b) Effect of MTX on the ratio of Th1-Th17 cells to helper T cells. Number by the dot shows the patient number in Table 1.

Table 1: Patient profile.

Patient #	Sex	Age (year)	Disease duration (m)	Anti-CCP U/mL	RF IU/mL		CRP mg/d		Treatment MTX mg/w	Duration between 1st and 2nd analysis (m)
					Pre	Post	Pre	Post		
1	F	30	12	121	1269	96	0.12	0.02	6	6
2	F	63	3	>300	68	56	0.01	0.02	6	1.5
3	F	22	12	>300	327	153	2.70	1.82	4	2
4	M	67	6	251	774	198	2.64	0.36	4	1.5
5	F	40	18	<0.6	6	3	3.66	4.65	4	1

clinical study, the RA patients were in the late phase of RA, not the early phase. As mentioned in Section 1, IL-17 plays an important role in the preonset or early-onset phase of the pathogenesis of RA [6, 7]. In addition, in the current study, we showed an elevated ratio of Th17 cell-derived Th1 cells in the early phase of RA, suggesting that Th17 cells are converted to Th1 cells in early RA. Thus, anti-IL-17 antibodies should be used in the preonset or the early-onset phase of RA to obtain more effective therapeutic results. Recently, Schett's group reported the combination of anti-TNFα antibodies and anti-IL-17 antibodies at the Fc, which had a measurable effect in a mouse model of RA [19, 20]. Combined antibodies are expected to yield an effective response in patients with early phase RA.

In the current study, MTX, which is "the gold standard" oral medication for RA, significantly reduced the ratio of Th17 cells but not Th1 or Th1•Th17 cells (Figures 1(a) and 1(b)). In addition, in the 1st group of RA patients, 4 of 5 patients with RA showed improved CRP levels (Table 1). Thus, our findings suggest that Th17 plays an important role in the pathogenesis of early-onset RA. In addition, surprisingly, the ratio of Th1 cells in 3 of 5 patients increased (Figure 1(a) right). These findings may demonstrate the pharmacological effect of MTX; however, they also suggest that Th17 cells are more important than Th1 cells in early-onset patients with RA.

We are now analyzing Th17 cells and Th1 cells using a third group of RA patients with early-onset RA. In the ongoing study, we are identifying Th17 cells and Th1 cells using only cell surface markers, as recommended in the Human Immunology Project of the Human Immunology Study Group [10]. In addition, we are trying to confirm the precision of this method of the Human Immunology Project by measuring the actual productions of cytokines IL-17 and IFN-γ and the expression of CD161.

In conclusion, through analyzing the peripheral blood of early-onset RA patients we demonstrated that MTX reduced the ratio of Th17 cells in helper T cells but not that of Th1 cells and that the ratio of Th17 cell-derived Th1 cells to Th17 cells increased. These findings suggest that Th17 cells play an important role in the pathogenesis of early phase RA,

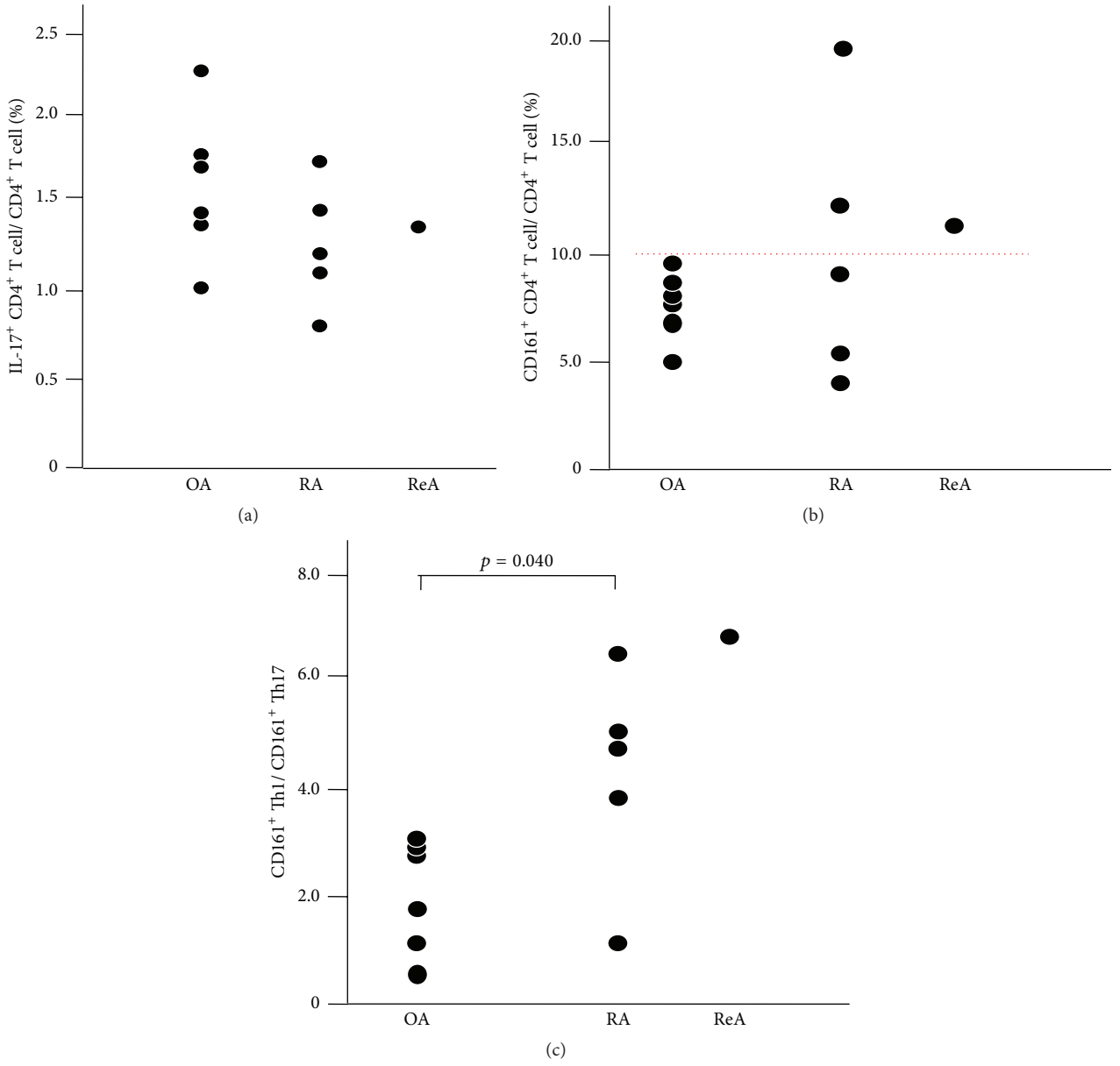

Figure 2: (a) Ratio of Th17 cells to helper T cells. (b) Ratio of $CD161^+$ helper T cells to helper T cells. The red dotted line shows the highest ratio of OA patients. (c) Ratio of $CD161^+$Th1 cells to $CD161^+$Th17 cells.

Table 2: Profiles of patients.

Patient #	Sex	Age (year)	Diagnosis 2010 ACR/EULAR	Disease duration (m)	CCP U/mL	RF IU/mL	CRP mg/d	Treatment after the analysis
6	F	43	ReA	9	—	—	0.02	—
7	F	57	RA	3	—	—	0.02	MTX 4 mg/w → SASP 500
8	F	71	RA	5	356	923	2.47	Bu 100
9	F	34	RA	1.5	—	—	0.04	—
10	F	51	RA	3	18.9	—	0.01	—
11	F	42	RA	4	280	28	0.09	MTX 6 mg/w

indicating the usefulness of anti-IL-17 antibodies in the early phase of RA but not in the late phase of RA or in patients resistant to other biologics such as anti-TNF antibodies.

5. Conclusions

In the early-onset RA patients MTX reduced the ratio of Th17 cells among helper T cells but not those of Th1 cells or Th17•Th1 cells (Th cells producing both IFN-γ and IL-17). In addition, the ratio of Th17 cell-derived Th1 cells to Th17 cells increased in the peripheral blood of the early-onset RA patients, compared with those of OA patients.

Competing Interests

The authors declare that they have no competing interests.

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References

[1] S. Kotake, K. Sato, K. J. Kim et al., "Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation," *Journal of Bone and Mineral Research*, vol. 11, no. 1, pp. 88–95, 1996.

[2] S. Kotake, N. Udagawa, N. Takahashi et al., "IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis," *The Journal of Clinical Investigation*, vol. 103, no. 9, pp. 1345–1352, 1999.

[3] Z. Yao, S. L. Painter, W. C. Fanslow et al., "Human IL-17: a novel cytokine derived from T cells," *Journal of Immunology*, vol. 155, no. 12, pp. 5483–5486, 1995.

[4] K. Sato, A. Suematsu, K. Okamoto et al., "Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction," *Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2673–2682, 2006.

[5] N. Komatsu, K. Okamoto, S. Sawa et al., "Pathogenic conversion of $Foxp^{3+}$ T cells into T_H17 cells in autoimmune arthritis," *Nature Medicine*, vol. 20, no. 1, pp. 62–68, 2014.

[6] K. Raza, F. Falciani, S. J. Curnow et al., "Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin," *Arthritis Research & Therapy*, vol. 7, no. 4, pp. R784–R795, 2005.

[7] H. Kokkonen, I. Söderström, J. Rocklöv, G. Hallmans, K. Lejon, and S. R. Dahlqvist, "Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 62, no. 2, pp. 383–391, 2010.

[8] Y. Kochi, Y. Okada, A. Suzuki et al., "A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility," *Nature Genetics*, vol. 42, no. 6, pp. 515–519, 2010.

[9] K. Hirota, H. Yoshitomi, M. Hashimoto et al., "Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model," *Journal of Experimental Medicine*, vol. 204, no. 12, pp. 2803–2812, 2007.

[10] H. T. Maecker, J. P. McCoy, and R. Nussenblatt, "Standardizing immunophenotyping for the Human Immunology Project," *Nature Reviews Immunology*, vol. 12, no. 3, pp. 191–200, 2012.

[11] K. Hirahara, A. Poholek, G. Vahedi et al., "Mechanisms underlying helper T-cell plasticity: implications for immune-mediated disease," *Journal of Allergy and Clinical Immunology*, vol. 131, no. 5, pp. 1276–1287, 2013.

[12] S. Nakayamada, H. Takahashi, Y. Kanno, and J. J. O'Shea, "Helper T cell diversity and plasticity," *Current Opinion in Immunology*, vol. 24, no. 3, pp. 297–302, 2012.

[13] L. Cosmi, R. De Palma, V. Santarlasci et al., "Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor," *Journal of Experimental Medicine*, vol. 205, no. 8, pp. 1903–1916, 2008.

[14] L. Cosmi, R. Cimaz, L. Maggi et al., "Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis," *Arthritis and Rheumatism*, vol. 63, no. 8, pp. 2504–2515, 2011.

[15] K. Nistala, S. Adams, H. Cambrook et al., "Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 33, pp. 14751–14756, 2010.

[16] F. Annunziato, V. Santarlasci, L. Maggi, L. Cosmi, F. Liotta, and S. Romagnani, "Reasons for rarity of Th17 cells in inflammatory sites of human disorders," *Seminars in Immunology*, vol. 25, no. 4, pp. 299–304, 2013.

[17] H. Yamada, Y. Nakashima, K. Okazaki et al., "Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 67, no. 9, pp. 1299–1304, 2008.

[18] G. R. Burmester, P. Durez, G. Shestakova et al., "Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis," *Rheumatology*, vol. 55, no. 1, pp. 49–55, 2016.

[19] J. A. A. Fischer, A. J. Hueber, S. Wilson et al., "Combined inhibition of tumor necrosis factor α and interleukin-17 as a therapeutic opportunity in rheumatoid arthritis: development and characterization of a novel bispecific antibody," *Arthritis and Rheumatology*, vol. 67, no. 1, pp. 51–62, 2015.

[20] J. Buckland, "Anti-TNF and anti-IL-17 antibodies—better together!," *Nature Reviews Rheumatology*, vol. 10, no. 12, p. 699, 2014.