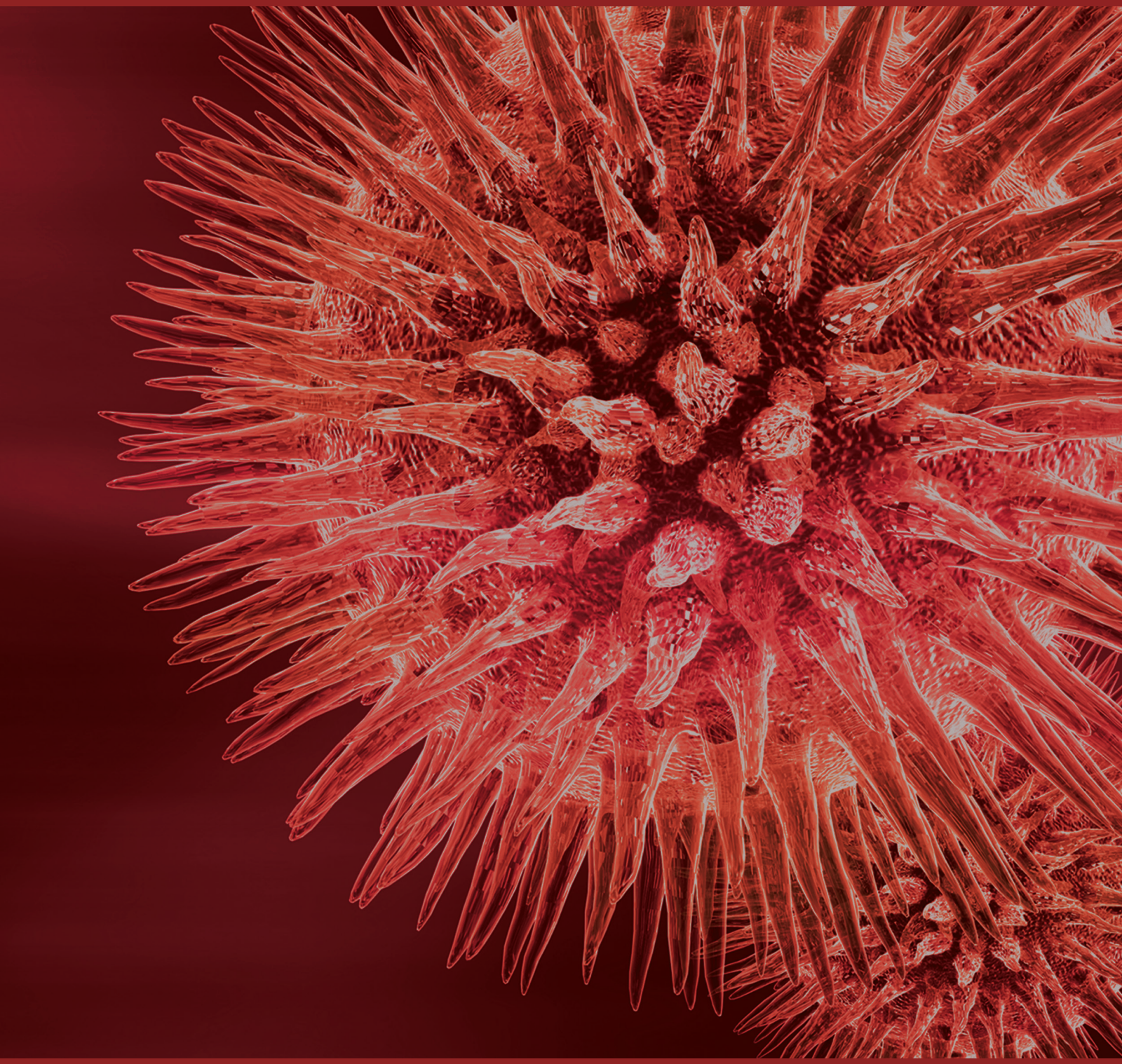
BioMed Research International

Inflammatory Joint Diseases

Guest Editors: Guixiu Shi, Julian L. Ambrus, Shuang Ye, and Long Shen



Inflammatory Joint Diseases

BioMed Research International

Inflammatory Joint Diseases

Guest Editors: Guixiu Shi, Julian L. Ambrus, Shuang Ye, and Long Shen

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 973510, 1 page
http://dx.doi.org/10.1155/2015/973510

Editorial
Inflammatory Joint Diseases

Guixiu Shi,[1] Julian L. Ambrus,[2] Shuang Ye,[3] and Long Shen[4]

[1]*Department of Rheumatology and Clinical Immunology, The First Hospital of Xiamen University, Xiamen 361003, China*
[2]*Buffalo General Medical Center, State University of New York at Buffalo School of Medicine, Buffalo, NY 14203, USA*
[3]*Department of Rheumatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200001, China*
[4]*Division of Allergy, Immunology and Rheumatology, Department of Medicine, State University of New York at Buffalo School of Medicine and Biomedical Sciences, Buffalo, NY 14203, USA*

Correspondence should be addressed to Guixiu Shi; gshi@xmu.edu.cn

Received 1 April 2015; Accepted 1 April 2015

Arthritis is a common clinical manifestation of rheumatic diseases. It can be a clinical feature in many rheumatic diseases, including rheumatoid arthritis, spondyloarthritis, crystal-induced arthritis, systemic lupus erythematosus, and Sjogren's syndrome. Joint inflammation and damage may result in disability and morbidity. Understanding the pathogenesis of inflammatory joint diseases remains a complex problem, although the level of understanding has progressed considerably in recent years. Knowledge of the pathogenesis, diagnosis, and treatment of inflammatory joint arthritis will lead to significant clinical benefit.

Based on this background, we assembled this special issue for presenting recent advances and a better understanding of inflammatory joint diseases, on aspects of pathogenesis, diagnosis, and treatment of inflammatory joint arthritis, including rheumatoid arthritis, spondyloarthritis, and osteoarthritis.

In this special issue, V. Romão compared effectiveness of tocilizumab and TNFα inhibitors in rheumatoid arthritis patients, showing that tocilizumab was associated with greater likelihood of achieving DAS28, CDAI, and SDAI remission/LDA and EULAR good response. I. Arstikyte analyzed the influence of immunogenicity on the efficacy of long-term treatment with TNFα blockers in rheumatoid arthritis and spondyloarthritis patients. H. Cho studied the effects of cyclooxygenase-2 (COX2) inhibitor and steroids on matrix metalloproteinases (MMPs) and prostaglandin E2 (PGE2) production in osteoarthritis patients, demonstrating that celecoxib and steroids exert similar effects on MMP-1 and PGE2 production in vitro and that celecoxib may demonstrate favorable effects on anabolic metabolism in vivo. X. Cen studied the association between serum 25-hydroxyvitamin D level and rheumatoid arthritis. G. Yin demonstrated that Pim-2/mTORC1 pathway shapes inflammatory capacity in rheumatoid arthritis synovial cells exposed to lipid peroxidations. X. Zhang designed a novel DKK1 multiepitope DNA vaccine and evaluated its bone protective effects on collagen-induced arthritis (CIA), which provide a potential treatment for bone erosion in RA. S. Joplin examined the effectiveness of measures to improve patient medication adherence and proposed a new approach to patient education using musculoskeletal ultrasound. M. Westergaard studied the humoral immune response against Epstein-Barr virus (EBV) in patients with rheumatoid arthritis and found that RA patients had elevated antibodies of all isotypes characteristics of latent EBV infection, notably, for IgM and IgA (but not IgG); these were associated with the presence of characteristic RA autoantibodies.

This special issue covers many important aspects in inflammatory joint diseases, which will surely provide us with a better understanding about the pathogenesis, diagnosis, and treatment of inflammatory joint diseases.

Guixiu Shi
Julian L. Ambrus
Shuang Ye
Long Shen

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 765490, 9 pages
http://dx.doi.org/10.1155/2015/765490

Research Article

Designation of a Novel DKK1 Multiepitope DNA Vaccine and Inhibition of Bone Loss in Collagen-Induced Arthritic Mice

Xiaoqing Zhang,[1,2] Sibo Liu,[1] Shentao Li,[1] Yuxuan Du,[1] Yunpeng Dou,[1] Zhanguo Li,[3] Huihui Yuan,[1] and Wenming Zhao[1]

[1]*Department of Immunology, School of Basic Medical Sciences, Capital Medical University, No. 10 Xitoutiao, You An Men, Fengtai, Beijing 100069, China*
[2]*Department of Immunology, College of Medical Sciences, China Medical University, No. 77 Puhe Road, Shenyang North Area, Shenyang, Liaoning 110122, China*
[3]*Department of Rheumatology & Immunology, Clinical Immunology Center, Peking University People's Hospital, No. 11 Xizhimen South Street, Beijing 100044, China*

Correspondence should be addressed to Huihui Yuan; huihui_yuan@163.com and Wenming Zhao; zhao-wenming@163.com

Received 28 November 2014; Accepted 5 January 2015

Academic Editor: Long Shen

Dickkopf-1 (DKK1), a secretory inhibitor of canonical Wnt signaling, plays a critical role in certain bone loss diseases. Studies have shown that serum levels of DKK1 are significantly higher in rheumatoid arthritis (RA) patients and are correlated with the severity of the disease, which indicates the possibility that bone erosion in RA may be inhibited by neutralizing the biological activity of DKK1. In this study, we selected a panel of twelve peptides using the software DNASTAR 7.1 and screened high affinity and immunogenicity epitopes *in vitro* and *in vivo* assays. Furthermore, we optimized four B cell epitopes to design a novel DKK1 multiepitope DNA vaccine and evaluated its bone protective effects in collagen-induced arthritis (CIA), a mouse model of RA. High level expression of the designed vaccine was measured in supernatant of COS7 cells. In addition, intramuscular immunization of BALB/c mice with this vaccine was also highly expressed and sufficient to induce the production of long-term IgG, which neutralized natural DKK1 *in vivo*. Importantly, this vaccine significantly attenuated bone erosion in CIA mice compared with positive control mice. These results provide evidence for the development of a DNA vaccine targeted against DKK1 to attenuate bone erosion.

1. Introduction

Rheumatoid arthritis (RA), a chronic symmetrical autoimmune disease, is characterized by synovial inflammation and proliferation accompanied by cartilage erosion and bone loss [1]. More than one-third of patients eventually experience employment disability and lower quality of life because of this disease, which is largely responsible for the high socioeconomic burden of RA [2]. Furthermore, mortality rates in RA patients are higher than in the healthy population [3].

The aim of RA treatment is the achievement of remission [4], but many RA patients who are judged by their consulting rheumatologist to be in remission after receiving conventional therapy still show structural deterioration [5]. Therefore, the long-term goals of treatment are to prevent joint destruction and the comorbidities of the disease [3]. A number of reports have suggested that modern therapy does not inhibit joint damage satisfactorily despite achieving clinical remission [6]. Therefore, the development of effective agents to inhibit bone erosion in RA patients is urgent.

The canonical Wnt signaling pathway promotes bone formation not only by stimulating the differentiation of osteoblasts, increasing the growth rate of osteoblasts and reducing their apoptosis but also by inhibiting osteoclastogenesis [7]. The canonical Wnt signaling pathway is triggered by the association of secretory Wnt with its frizzled (Fz) receptors and low density lipoprotein receptor-related

protein 5/6 (LRP5/6) on the cell surface. This stabilizes β-catenin, which is eventually translocated into the nucleus, and activates the TCF/LEF-mediated transcription of target genes that elicit a variety of effects, including the induction of osteoblast differentiation and proliferation [8]. Dickkopf-1 (DKK1), a soluble and natural inhibitor of the canonical Wnt signaling pathway, may play an active role in inhibiting osteogenesis by binding the ligands of Wnt proteins [9, 10]. In addition to LPR5/6, DKK1 was also found to bind to Kremen, another cell surface coreceptor, forming a ternary complex that is rapidly endocytosed, resulting in the depletion of cell surface LRP5/6 [11]. Studies have demonstrated that the levels of DKK1 in serum were significantly higher in RA patients and were correlated with the severity of the disease [12]. In a mouse model of RA, treatment with an anti-DKK1 antibody has attenuated bone erosion [9]. Therefore, DKK1 may be a promising therapeutic target for RA bone loss.

Over the last 20 years, great progress has been made in developing DNA vaccines [13]. DNA vaccines have many advantages over conventional chemical agents, biological agents, and protein vaccines in the treatment of diseases. First, plasmid preparation is rapid and cost effective and does not suffer from problems such as improper protein folding. Plasmid DNA is highly stable and flexible, allowing for the modification of plasmid sequences [14]. In addition, the antigen presenting cells (APCs) process and present the epitopes from antigens on MHC I and II molecules, thereby inducing both humoral immunity and cellular immunity. Active immunotherapy is feasible to reverse the disorders of autoimmune diseases. DNA vaccines have proven effective in animal models, including RA, Crohn's disease, systemic erythematosus lupus (SLE), and infectious diseases [15] and have been extensively evaluated in humans. The advancements of bioinformation software and molecular immunology promoted the development of DNA vaccine [16]. Currently, 72 Phase I, 20 Phase II, and 2 Phase III clinical trials have been identified [17].

In this study, we selected a panel of twelve peptides using the software DNASTAR 7.1 and screened high affinity and immunogenicity epitopes *in vitro* and *in vivo* assays. Then, we optimized four B cell epitopes and designed a novel DKK1 multiepitope DNA vaccine, determined its immunogenicity, and evaluated its protective effects. Our data demonstrated that this DNA vaccine ameliorated bone erosion significantly in mice with collagen-induced arthritis (CIA).

2. Materials and Methods

2.1. Cells and Mice. COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37°C.

Six-week-old female BALB/c (H-2d) mice and 5-week-old male DBA/1 mice (a CIA-susceptible mouse strain, H-2q) were both purchased from HFK Biotechnology Co. Ltd. All mice were maintained in a specific pathogen-free environment. All animal experiments were performed according to the guidelines of the Animal Care and Use Committee of Capital Medical University.

2.2. Construction and Preparation of the DNA Vaccine. B cell epitopes in the amino acid sequence of human DKK1 were analyzed using the software DNASTAR 7.1. The separated epitopes were synthesis from Invitrogen (Life Technologies, California, USA). Subsequently, the indirect ELISA was utilized to detect the affinity of separated epitopes. Simply, 96-well plates were coated with peptides (1 μg/mL) overnight at 4°C. The DKK1 polyclonal antibodies (diluted 1 : 1000, 200 μL, R&D Systems, Minneapolis, MN, USA) were added to wells and incubated overnight at 4°C. After washing, the HRP conjugate goat anti-human DKK1 secondary antibody (100 μL, diluted 1 : 2000, Southern Biotech, Birmingham, AL, USA) was added and the plates were incubated for 1 h at 37°C. Then, the peptides were immunized BALB/c mice and the immunogenicity was measured as previously described [18]. The reactions were stopped by the addition of 50 μL of Stop Solution (R&D Systems), and OD450 readings were measured with an ELISA plate reader. To reduce the bioactivity of DKK1 vaccine, the peptides (100 μg/injection, intraperitoneally) were applied for a week. The tibias were collected and an acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) was used to demonstrate osteoclasts [19].

Four fragments in the DKK1 sequence (110–144aa, 153–181aa, 182–216aa, and 228–253aa) with high affinity and immunogenicity were selected to construct the multiepitope DNA vaccine. The DNA sequences encoding four amino acids (R203, H204, K211, and R236) in DKK1 were mutated to glutamate to reduce the biological activity of DKK1 [20]. AAY spacers between two adjacent epitope fragments were used to link the four selected epitopes. A Th2 cell epitope was added at both sequence termini. Finally, the signal peptide of human DKK1 was added at the N-terminus of the sequence. The synthetic nucleotide sequence, named DP, was incorporated into the expression vector pCMV6-XL5 using a standard DNA recombination procedure, resulting in the recombinant plasmid pCMV-DP. Plasmids for immunization were extracted and purified from transformed *Escherichia coli* strain DH5α using an endotoxin-free plasmid extraction kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The purified plasmids were adjusted to a concentration of 1 mg/mL in sterile saline and stored at −80°C.

2.3. Transfection of Plasmid pCMV-DP into COS7 Cells. COS7 cells were cultured in a 6-well tissue culture plate until the cells reached approximately 60% to 80% confluence. The cells were transfected with the purified plasmid DNA using TurboFect *in vitro* Transfection Reagent (Fermentas, ThermoScientific, Pittsburgh PA, USA) according to the manufacturer's instructions. Briefly, 4 μL of plasmid DNA (1 mg/mL) was diluted in 390 μL of serum-free DMEM. TurboFect reagents (6 μL) were added to the diluted DNA. After immediate mixing and incubation for 20 min at room temperature, 400 μL of the mixture was distributed dropwise into cultured COS7 cells in a 6-well plate. The transfected cells

were cultured for 72 h. Cell lysates and culture supernatants were collected for further analyses.

2.4. Western Blotting of the Expressed Multiepitope Protein in Cell Lysates. The proteins in the COS7 cell lysates were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane by electroblotting. The membrane was blocked with 5% nonfat dry milk in TBST (TBS with 0.05% Tween-20) for 1 h at room temperature and then incubated with a polyclonal rabbit anti-DKK1 antibody (1 : 1000, Millipore) in TBST containing 0.25% bovine serum albumin overnight at 4°C. After washing three times with TBST, the membrane was incubated with horseradish peroxidase-(HRP-) conjugated goat anti-rabbit IgG (1 : 10000, ProteinTech Group, Chicago, USA) for 1 h at room temperature. After washing three times, the membrane was exposed to a SuperSignal West Pico stable peroxide solution (Pierce, ThermoScientific, Pittsburgh, PA, USA).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of the Expressed Multiepitope Protein in the Cell Culture Supernatant. The protein secreted in the cell culture supernatant was detected with ELISA. Briefly, 96-well plates were coated with a goat anti-human DKK1 antibody (200 ng/mL, R&D Systems, Minneapolis, MN, USA) overnight at 4°C. The cell culture supernatant (200 μL) was added to the wells and was incubated overnight at 4°C. After incubating with 100 μL of a goat anti-human DKK1 antibody (50 ng/mL, R&D Systems) for 2 h at 37°C, 100 μL of a working dilution of Streptavidin-HRP was added to each well. Color was developed by the addition of a substrate solution of orthophenylene diamine (OPD) for 20 min at 37°C. The reaction was stopped by the addition of 50 μL of 2 M H_2SO_4. The absorbance was read at 450 nm by a microplate reader (Thermomax Technologies).

2.6. Immunohistochemical Analysis of the Multiepitope Protein In Vivo. BALB/c mice were injected intramuscularly with 100 μg of plasmid pCMV-DP or empty vector pCMV. Seven days later, the injected muscles were surgically removed and frozen sections were prepared. The sections were dried for 45 min at room temperature, fixed with anhydrous acetone, and then incubated with 0.05% H_2O_2 for 20 min to quench the endogenous peroxidase. After blocking with 5% horse serum, the sections were incubated with a goat anti-human DKK1 antibody (5 μg/mL, R&D Systems) overnight at 4°C. The next day, the sections were incubated with a HRP-conjugated donkey anti-goat IgG (1 : 1000, ProteinTech Group) for 1 h at room temperature. The positive signals were detected with 3, 3′ diaminobenzidine (DAB, R&D Systems).

2.7. Immunization and Serum Collection. For DNA immunization experiments, 6-week-old female BALB/c mice (n = 6) were injected intramuscularly (i.m.) with plasmid pCMV-DP three times at weeks 0, 2, and 4. Mice (n = 6) immunized with empty vector pCMV served as negative controls. Intramuscular injection of plasmid DNA followed by electroporation (DNA + EP) was performed as previously described [21]. Briefly, 100 μg of plasmid pCMV-DP was injected intramuscularly in one tibialis anterior muscle using a 27-gauge needle. Immediately after the injection, electroporation with 6 electric pulses was applied through a pair of silver electrodes spaced 3 mm apart covering the i.m. injection site. The electric pulses were 50 ms in duration and 1 s apart at a voltage of 60 V (i.e., 200 V/cm). From the first injection, mouse serum samples were collected at 2-week intervals and stored at −80°C.

2.8. Antibody Assay. The antibodies against human DKK1 were evaluated using ELISA. Briefly, 96-well microtiter plates were coated with 100 μL of recombinant human DKK1 proteins (200 ng/mL, R&D Systems) and incubated overnight at 4°C. After washing three times with PBST (PBS with 0.05% Tween-20), the plates were blocked with 200 μL of 1% bovine serum albumin (BSA) in PBST for 2 h at 37°C. After washing, 100 μL of diluted serum (1 : 200) was added to each well. Then, the plates were incubated for 2 h at 37°C and washed five times with PBST. HRP-conjugated goat anti-mouse IgG secondary antibodies (100 μL) (1 : 10000, ProteinTech Group) were added to each well followed by incubation for 1 h at 37°C. Next, the plates were washed five times with PBST. After adding the substrate solution and stop solution, the absorbance was read at 450 nm by a microplate reader (Thermomax Technologies). The end-point titer of antibody was determined in the same way. The serum samples were serially diluted from 1 : 200 to 1 : 12800.

2.9. Preparation and Evaluation of Collagen-Induced Arthritis Mouse Model. DBA/1 mice (n = 6) were immunized with plasmid pCMV-DP three times at 2-week intervals as described above. Control mice (n = 6) were immunized with an equal amount of empty vector pCMV. One week after the final immunization, all mice were injected intradermally at the base of the tail with 100 μg of bovine C II emulsified with complete Freud's adjuvant (CFA) containing 4 mg/mL of heat-killed *Mycobacterium tuberculosis*. On day 21, the animals were given a booster injection with 100 μg of bovine C II dissolved in incomplete Freud's adjuvant (IFA) [22]. Animals were observed and recorded every 3 days after disease onset. Clinical scores were assigned to evaluate the disease severity as follows: 0: no signs of arthritis; 1: swelling and/or redness of one paw or one digit; 2: two joints involved; 3: three or more joints involved; and 4: severe arthritis of the entire paws and digits. Each limb was graded independently, resulting in a maximal clinical score of 16 per affected animal [23].

2.10. Microcomputer Tomography (CT) Scanning. To determine the protective effects of the DNA vaccine, 40 days after the challenge with bovine C II, a micro-CT-200 system (AlokA latheta Laboratory, Japan) was employed to detect the bone mineral density (BMD) and the degree of bone erosion after the mice were anaesthetized with chloral hydrate (10%). X-ray images were analyzed by reconstructed 3D quantitative analyses using the software VGstudio MAX 2.0.

2.11. Histopathology. Murine hind paws were removed postmortem, stored in 10% neutral formalin, decalcified in 20%

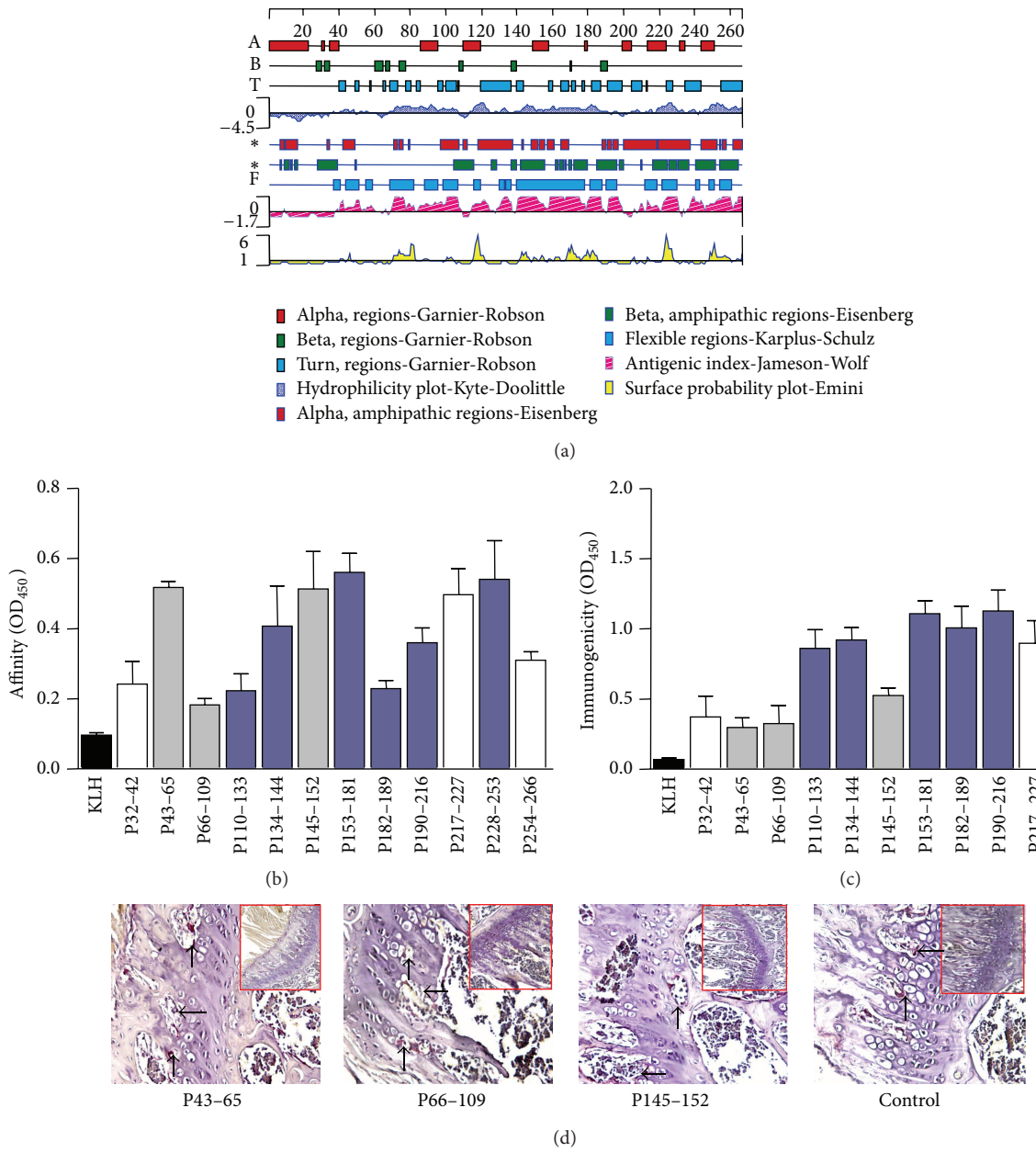


Figure 1: Designation of the DNA vaccine.(a) B cell epitope scanning of human DKK1 was performed with the software DNASTAR 7.1. (b) The affinity of epitopes was measured by indirect ELISA. (c) The separated epitopes were immunized BALB/c mice and the immunogenicity of epitopes was measured by sandwich ELISA. (d) The separated epitopes were injected to BALB/c mice for seven days. TRAP staining was performed to identify the mature osteoclasts. Magnification: 200x; data are expressed as the mean ± SEM.

ethylenediaminetetraacetic acid (EDTA) for 6 weeks, and then dehydrated and embedded in paraffin. Sections were cut along the longitudinal axis and stained with toluidine blue (TB) as previously described [18].

2.12. Statistical Analysis. Data were analyzed using the software SPSS (version 16.0) and presented as the mean ± SEM. Differences between two groups of mice were compared using Student's t-test. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Construction of the DNA Vaccine. According to the analysis of the potential B cell epitopes in human DKK1 by the epitope prediction software DNASTAR 7.1, a panel of twelve peptides fragments was synthesized (Figure 1(a)). In addition, to select the high affinity and immunogenicity of synthesis peptides, the titers of peptides were determined and the peptides of grey-blue columns were candidates to design DNA vaccine (Figures 1(b)-1(c)). Furthermore, to decrease the pathological functions of DKK1 in peptides,

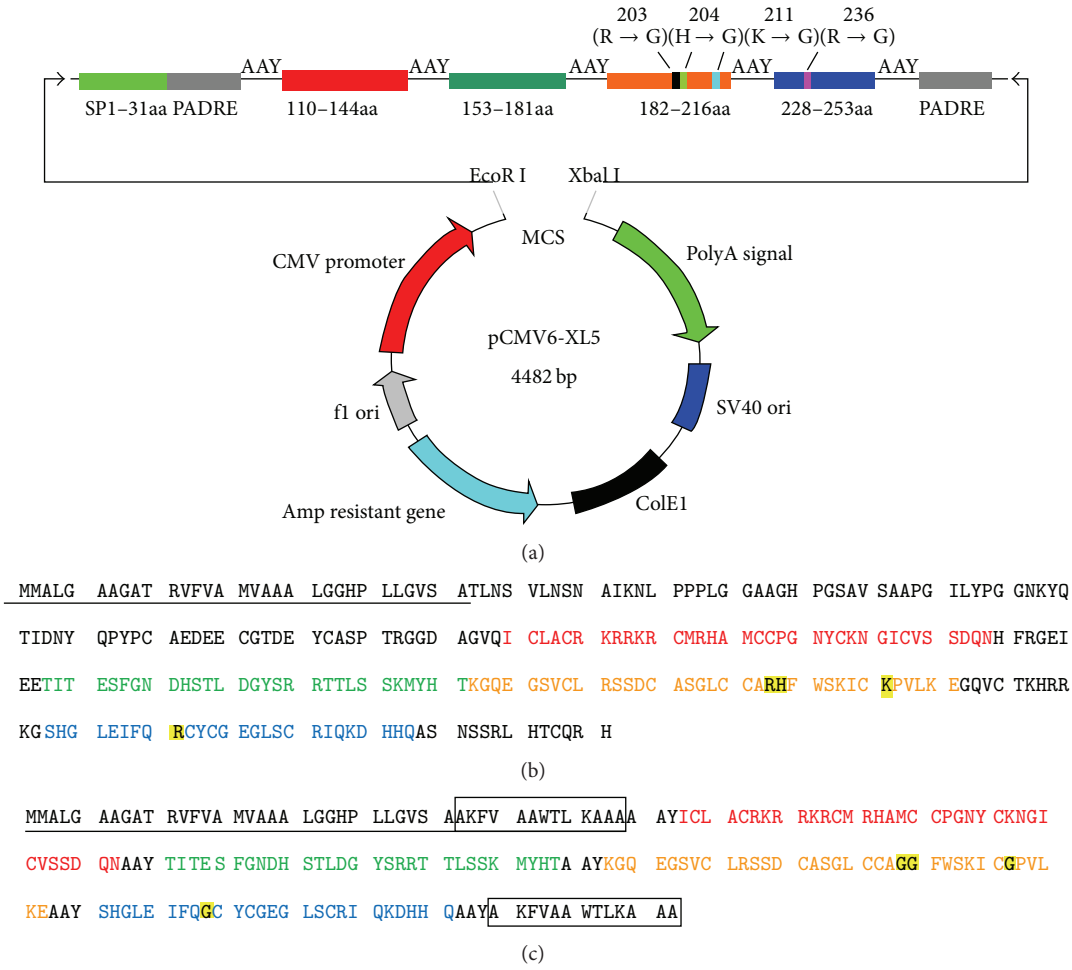


Figure 2: Construction of the DNA vaccine. (a) The maps of recombinant DKK1 DNA vaccine. (b) The amino acid sequence of human DKK1. (c) The recombinant amino acid sequence of DKK1 DNA vaccine. Red line, 110–144aa; green line, 153–181aa; orange line, 182–216aa; blue line, 228–253aa; black line, signal peptide; box, PADRE.

the osteoclast-forming assay was performed. Compared with controls, no significant osteoclastogenesis was observed in peptides-treatment group (Figure 1(d)). The synthetic nucleotide sequence with muted four amino acids encoding the DNA vaccine was cloned in the eukaryotic expression vector pCMV6-XL5 (Figure 2).

3.2. Expression of the Multiepitope Chimera Gene In Vitro and In Vivo. The expression of the multiepitope DNA vaccine *in vitro* was evaluated in COS7 cells. ELISA showed that the multiepitope DNA vaccine recombinant protein of DKK1 was secreted abundantly and was recognized by its polyclonal antibodies (Figure 3(a)). In addition, Western blotting also showed that COS7 cells transfected with plasmid pCMV-DP highly expressed the recombinant protein (Figure 3(b)). To assess the expression of the target protein *in vivo*, the injected muscles of mice were further stained with an anti-DKK1 antibody. The expression of recombinant protein in the DKK1 DNA vaccine was much higher in the muscles at the injected site than in the control group (Figure 3(c)). Due to these observations, we concluded that the multiepitope gene was expressed in eukaryotes both *in vitro* and *in vivo*.

3.3. The DNA Vaccine Induced a Specific Antibody. A specific anti-human DKK1 antibody was identified in BALB/c mice immunized with plasmid pCMV-DP. The serum IgG titer began to increase as early as 4 weeks after the primary immunization and reached its peak at 6 weeks (Figure 3(d)). The end-point titer of the specific antibody was also determined to evaluate its neutralizing effects *in vitro* (Figure 3(e)). Although the end-point titer of anti-DKK1 was only 1 : 1600, the specific antibody existed persistently in the serum up to 6 months after immunization.

3.4. Induction of Arthritis in DBA/1 Mice. DBA/1 mice were injected with bovine C II twice at 3-week intervals after immunization. The signs of arthritis appeared around day 24 after injection with bovine C II and continued to develop at later time points. The disease incidence was 83% in prevaccinated mice and 100% in the positive control mice

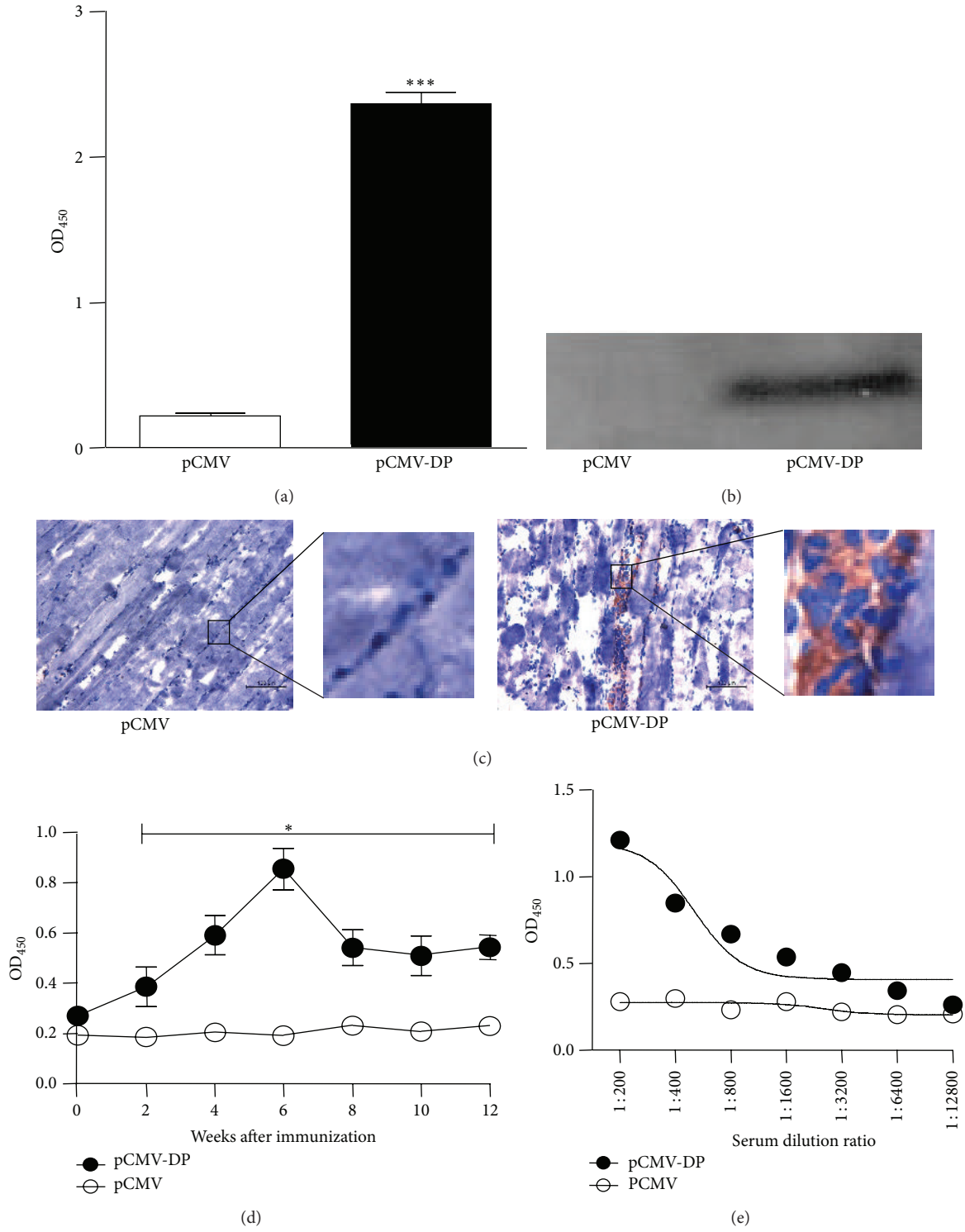


Figure 3: Expression and immunogenicity of the DNA vaccine. (a–c) Expression of the multiepitope protein *in vitro* and *in vivo* were determined in cell culture supernatants by ELISA, cell lysates by Western blotting, and the muscles of mice by immunohistochemical analysis. (d-e) The DNA vaccine induced a specific IgG antibody against human DKK1. The titer and the end-point titer of the specific antibody were tested by ELISA. Bars indicate 100 μm. Data are expressed as the mean ± SEM, $^{*}P < 0.05$, $^{***}P < 0.001$.

on day 39. Paw erythema and swelling also became more severe with time in control mice than the DKK1 vaccine group (Figures 4(a) and 4(b)). In addition, the infiltration of inflammatory cells was reduced in the vaccine-immunized mice compared with the controls (Figure 4(c)).

3.5. The DNA Vaccine Attenuated Bone Erosion in CIA Mice. We further evaluated the amelioration of arthritic bone, and the joint structure was well conserved in the vaccinated mice compared with the positive control mice as demonstrated by microcomputer tomography (CT) scanning (Figure 4(d)). The total bone mineral density (BMD) of the mice in the vaccinated groups was significantly higher than that in the positive control group (Figure 4(e)). The results of TB staining showed that the degree of overall destruction of cartilage was reduced markedly in the arthritic paws of vaccinated mice compared with the positive controls (Figure 4(f)). The results of micro-CT scanning and TB staining demonstrated that the DNA vaccine attenuated bone erosion in CIA mice.

4. Discussion

DNA vaccines, also termed nucleic acid vaccines or gene vaccines, were first described in the 1990s when Wolff found that DNA could be taken up and expressed by mouse skeletal muscle cells *in vivo* [24]. It is generally thought that the gene of interest in a eukaryotic expression vector is translated into antigen protein using the cellular expression system of the host. Subsequently, the antigen protein stimulates the host to generate an immune response. Compared with traditional vaccines, the greatest advantage of DNA vaccines is their ability to elicit both humoral immunity and cellular immunity. Simultaneously, they are simple to prepare, easy to deliver, and relatively safe to apply. Therefore, DNA vaccination is a promising new technology for the prevention and therapy of diseases, such as infectious diseases, autoimmune diseases, and cancers.

In this study, we constructed a DNA vaccine targeting human DKK1, a major contributor to bone loss in RA. We expected to use this vaccine to inhibit bone erosion in a RA mouse model through the blockade of the biological activity of DKK1. We have adopted optimization strategies to improve the antigen expression and the immunogenicity of the DNA vaccine, which are discussed below.

4.1. Construction Strategies for the DNA Vaccine. To produce specific antibodies, B cell epitopes of DKK1 were the first choice to construct the DNA vaccine. To increase the titer of specific antibodies, we combined the predictions of the software DNASTAR 7.1 and the affinity and immunogenicity assays. To reduce their potential bone resorption of natural DKK1, the synthesis epitopes were injected to BALB/c and no significant osteoclastogenesis was observed. These data demonstrated that these separated epitopes were safe as a vaccine utilized *in vivo*. To increase the immunogenicity of the DNA vaccine as expressed in the host, the key binding sites of DKK1 (R203, H204, K211, and R236) to LRP5/6 and Kremen were mutated. At the same time, the mutated protein expressed *in vivo* enhanced the immunogenicity of the vaccine [20]. To facilitate the epitope processing, the four selected epitopes were separated from one another with AAY spacers [25]. AAYs expressed inside the selected epitopes were easily recognized by proteasomes, and the full-length protein was more easily processed and presented *in vivo*. DKK1 is expressed naturally in the body. To further increase the immunogenicity of the recombinant DNA vaccine, the pan DR T helper epitope (PADRE) was added to enhance antibody responses [26]. Finally, the signal peptide for human DKK1 was added at the N-terminus of the DNA vaccine to facilitate the extracellular secretion of the protein antigen.

4.2. Immunization Strategies. The DNA vaccine delivery method is one of the most important factors that affect immunization efficiency. The methods for DNA vaccine delivery currently used include intramuscular, intraperitoneal, intravenous, intradermal, subcutaneous injection, and others. The delivery method for the plasmid directly affects the uptake of the gene and further affects the expression of the target protein. Intramuscular injection is the earliest and simplest method for DNA delivery. However, the majority of the administered plasmid DNA was blocked by perimysia of the skeletal muscles, where most of the DNA was degraded by DNase I in the plasma. Less than 1% of the DNA actually entered the nucleus [27]. Recently, electroporation has been used for DNA vaccine delivery and has proven to be a better DNA delivery method. In this study, intramuscular injection of plasmid DNA followed by electroporation (DNA + EP) was employed. The DC electric current disrupts cell membranes for a short time, allowing the plasmid DNA to cross the membranes into the nucleus [28]. In addition, the electric current causes cell destruction and a local inflammatory reaction. A large number of inflammatory cells, including macrophages and other antigen-presenting cells, infiltrated the injected muscles, which enhanced the efficiency of antigen presentation. In our study, the highly specific IgG antibodies in serum were induced as early as 4 weeks, which also demonstrated the efficiency of the electroporation of the DNA vaccine.

5. Conclusions

DKK1, a negative regulator of the canonical Wnt signaling pathway, plays an important role in RA bone destruction. Inhibition of its biological functions can attenuate bone destruction in RA [9]. Our results showed that the recombinant human DKK1 multiepitope DNA vaccine induced specific antibodies that effectively neutralized the biological activities of DKK1 and attenuated bone destruction in CIA mice. This study may provide a potential therapy for RA and other bone loss diseases.

Conflict of Interests

The authors declare no conflict of interests.

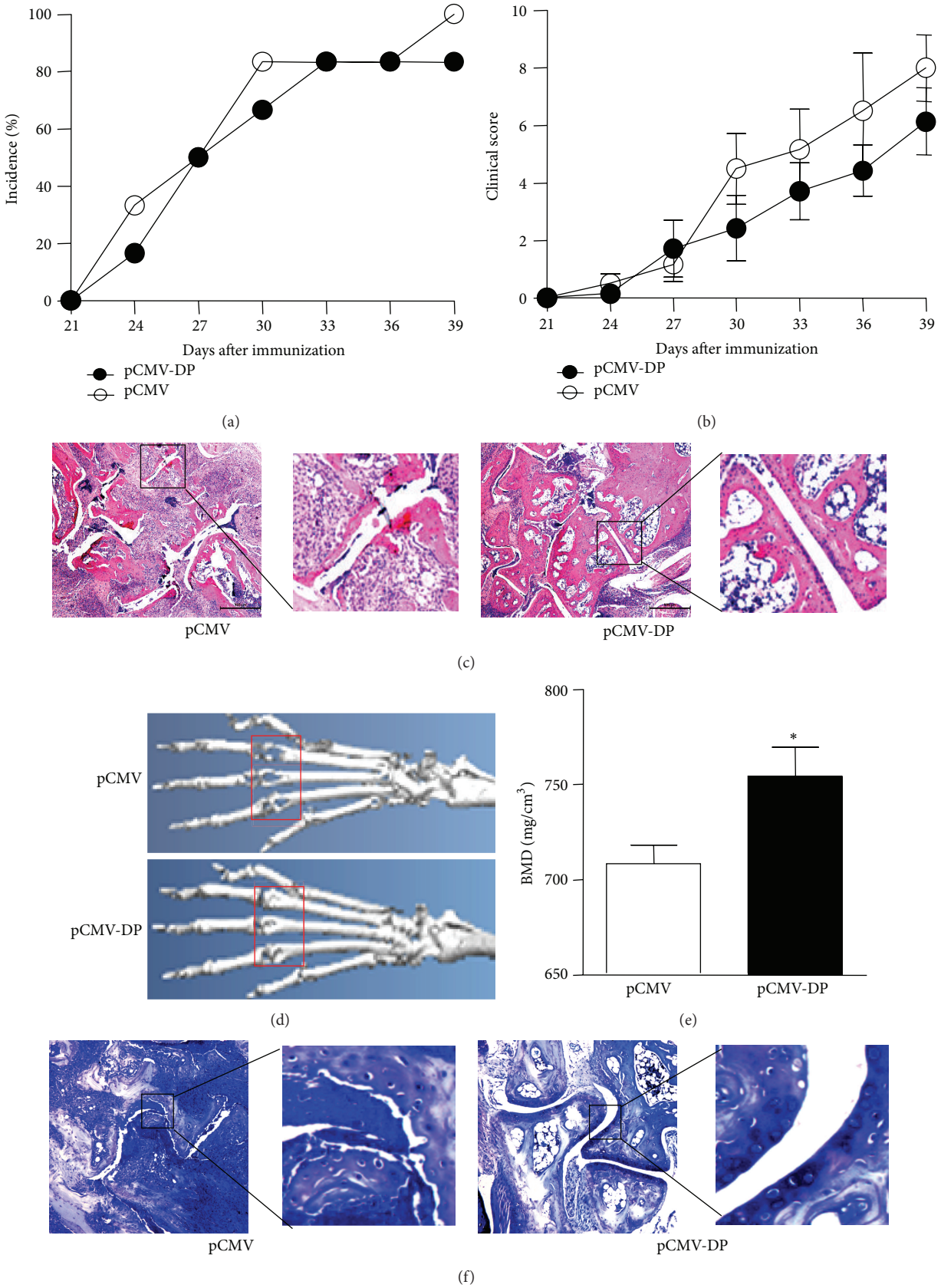


FIGURE 4: The DNA vaccine attenuated bone erosion in CIA mice. Mice were injected intradermally with bovine type II collagen to induce arthritis (n = 6/group). (a-b) The incidence of arthritis and clinical score were recorded until day 39. (c–f) Representative joints on the day of sacrifice were analyzed with HE staining, Micro-CT images, BMD analysis, and TB staining images. Bars indicate 500 μm and 200 μm. Data are expressed as the mean ± SEM. $^{*}P < 0.05$.

Authors' Contribution

Xiaoqing Zhang, Huihui Yuan, and Wenming Zhao conceived and designed the experiments; Xiaoqing Zhang and Sibo Liu performed the experiments; Yuxuan Du and Yunpeng Dou analyzed the data; Shentao Li and Zhanguo Li contributed reagents/materials/analysis tools; Xiaoqing Zhang and Huihui Yuan wrote the paper. Xiaoqing Zhang and Sibo Liu contributed equally to this work.

Acknowledgments

This study was supported by the State Key Development Program for Basic Research of China (Grant no. 2010CB529106) and the National Nature Science Foundations of China (Grant no. 31400767).

References

[1] D. L. Scott, "Biologics-based therapy for the treatment of rheumatoid arthritis," *Clinical Pharmacology and Therapeutics*, vol. 91, no. 1, pp. 30–43, 2012.

[2] J. M. Davis and E. L. Matteson, "My treatment approach to rheumatoid arthritis," *Mayo Clinic Proceedings*, vol. 87, no. 7, pp. 659–673, 2012.

[3] I. Colmegna, B. R. Ohata, and H. A. Menard, "Current understanding of rheumatoid arthritis therapy," *Clinical Pharmacology and Therapeutics*, vol. 91, no. 4, pp. 607–620, 2012.

[4] P. J. Mease, "Improving the routine management of rheumatoid arthritis: the value of tight control," *Journal of Rheumatology*, vol. 37, no. 8, pp. 1570–1578, 2010.

[5] A. K. Brown, P. G. Conaghan, Z. Karim et al., "An explanation for the apparent dissociation between clinical remission and continued structural deterioration in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 58, no. 10, pp. 2958–2967, 2008.

[6] E. T. H. Molenaar, A. E. Voskuyl, H. J. Dinant, P. D. Bezemer, M. Boers, and B. A. C. Dijkmans, "Progression of radiologic damage in patients with rheumatoid arthritis in clinical remission," *Arthritis and Rheumatism*, vol. 50, no. 1, pp. 36–42, 2004.

[7] Y. Kawano and R. Kypta, "Secreted antagonists of the wnt signalling pathway," *Journal of Cell Science*, vol. 116, no. 13, pp. 2627–2634, 2003.

[8] E. Bourhis, C. Tam, Y. Franke et al., "Reconstitution of a Frizzled8·Wnt3a·LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6," *The Journal of Biological Chemistry*, vol. 285, no. 12, pp. 9172–9179, 2010.

[9] D. Diarra, M. Stolina, K. Polzer et al., "Dickkopf-1 is a master regulator of joint remodeling," *Nature Medicine*, vol. 13, no. 2, pp. 156–163, 2007.

[10] D. Daoussis and A. P. Andonopoulos, "The emerging role of dickkopf -1 in bone biology: is it the main switch controlling bone and joint remodeling?" *Seminars in Arthritis and Rheumatism*, vol. 41, no. 2, pp. 170–177, 2011.

[11] A. Bafico, G. Liu, A. Yaniv, A. Gazit, and S. A. Aaronson, "Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow," *Nature Cell Biology*, vol. 3, no. 7, pp. 683–686, 2001.

[12] 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.

[13] L. Li, F. Saade, and N. Petrovsky, "The future of human DNA vaccines," *Journal of Biotechnology*, vol. 162, no. 2, pp. 171–182, 2012.

[14] F. Saade and N. Petrovsky, "Technologies for enhanced efficacy of DNA vaccines," *Expert Review of Vaccines*, vol. 11, no. 2, pp. 189–209, 2012.

[15] D. M. Klinman, S. Klaschik, D. Tross, H. Shirota, and F. Steinhagen, "FDA guidance on prophylactic DNA vaccines: analysis and recommendations," *Vaccine*, vol. 28, no. 16, pp. 2801–2805, 2010.

[16] M. Mooney, S. McWeeney, G. Canderan, and R.-P. Sékaly, "A systems framework for vaccine design," *Current Opinion in Immunology*, vol. 25, no. 5, pp. 551–555, 2013.

[17] M. A. Kutzler and D. B. Weiner, "DNA vaccines: ready for prime time?" *Nature Reviews Genetics*, vol. 9, no. 10, pp. 776–788, 2008.

[18] H. Yuan, H. Qian, S. Liu et al., "Therapeutic role of a vaccine targeting RANKL and TNF-α on collagen-induced arthritis," *Biomaterials*, vol. 33, no. 32, pp. 8177–8185, 2012.

[19] A.-R. Kim, H. S. Kim, J. M. Lee et al., "Arctigenin suppresses receptor activator of nuclear factor κb ligand (RANKL)-mediated osteoclast differentiation in bone marrow-derived macrophages," *European Journal of Pharmacology*, vol. 682, no. 1–3, pp. 29–36, 2012.

[20] K. Wang, Y. Zhang, X. Li et al., "Characterization of the kremen-binding site on Dkk1 and elucidation of the role of kremen in Dkk-mediated wnt antagonism," *Journal of Biological Chemistry*, vol. 283, no. 34, pp. 23371–23375, 2008.

[21] B. Peng, Y. Zhao, H. Lu, W. Pang, and Y. Xu, "In vivo plasmid DNA electroporation resulted in transfection of satellite cells and lasting transgene expression in regenerated muscle fibers," *Biochemical and Biophysical Research Communications*, vol. 338, no. 3, pp. 1490–1498, 2005.

[22] D. D. Brand, K. A. Latham, and E. F. Rosloniec, "Collagen-induced arthritis," *Nature Protocols*, vol. 2, no. 5, pp. 1269–1275, 2007.

[23] S. Cuzzocrea, E. Ayroldi, R. di Paola et al., "Role of glucocorticoid-induced TNF receptor family gene (gitr) in collagen-induced arthritis," *The FASEB Journal*, vol. 19, no. 10, pp. 1253–1265, 2005.

[24] J. A. Wolff, R. W. Malone, P. Williams, G. Acsadi, A. Jani, and P. L. Felgner, "Direct gene transfer into mouse muscle in vivo," *Science*, vol. 247, no. 4949 I, pp. 1465–1468, 1990.

[25] X. Wang, W. Xu, D. Tong et al., "A chimeric multi-epitope DNA vaccine elicited specific antibody response against severe acute respiratory syndrome-associated coronavirus which attenuated the virulence of SARS-CoV in vitro," *Immunology Letters*, vol. 119, no. 1-2, pp. 71–77, 2008.

[26] M.-F. Del Guercio, J. Alexander, R. T. Kubo et al., "Potent immunogenic short linear peptide constructs composed of B cell epitopes and Pan DR T helper epitopes (PADRE) for antibody responses in vivo," *Vaccine*, vol. 15, no. 4, pp. 441–448, 1997.

[27] M. A. Liu, W. McClements, J. B. Ulmer, J. Shiver, and J. Donnelly, "Immunization of non-human primates with DNA vaccines," *Vaccine*, vol. 15, no. 8, pp. 909–912, 1997.

[28] M. Golzio, J. Teissié, and M.-P. Rols, "Direct visualization at the single-cell level of electrically mediated gene delivery," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 3, pp. 1292–1297, 2002.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 913804, 5 pages
http://dx.doi.org/10.1155/2015/913804

Research Article

Association between Serum 25-Hydroxyvitamin D Level and Rheumatoid Arthritis

Xiaomin Cen,[1] Yuan Liu,[2] Geng Yin,[1] Min Yang,[1] and Qibing Xie[1]

[1]*Department of Rheumatology and Immunology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China*
[2]*Department of Rheumatology and Immunology, First Affiliated Hospital of Xiamen University, Xiamen, Fujian 361003, China*

Correspondence should be addressed to Qibing Xie; qibingxie@126.com

Received 3 December 2014; Revised 21 January 2015; Accepted 27 January 2015

Academic Editor: Long Shen

The objective of this study is to examine and evaluate whether serum 25(OH)D is associated with disease activity in patients with rheumatoid arthritis (RA). Our results suggested that serum 25(OH)D in RA groups has significant lower level (35.99 ± 12.59 nmol/L) than that in the normal groups (54.35 ± 8.20 nmol/L, $P < 0.05$). Based on the DAS28, patients with RA were divided into four subgroups, and no differences were found in the four groups ($P > 0.05$). The 25(OH)D levels in complete remission, low disease activity, middle disease activity, and high disease activity group were 32.86 ± 12.26, 33.97 ± 13.28, 38.41 ± 10.64, and 38.94 ± 13.35 nmol/L, respectively. Based on the serum 25(OH)D levels, patients with RA were divided into inadequate group and normal group, and there were no significant differences in baseline characteristics and disease activity in the two groups. Our results showed that serum 25(OH)D levels in the inadequate group are significantly lower than those in the normal group. However, no correlations were found between 25(OH)D levels and disease activity among 116 patients with RA. The present findings will help to understand the association between 25(OH)D and disease activity of RA.

1. Introduction

Vitamin D has important functions in physiological processes, and vitamin D deficiency may play a role in pathophysiological processes and has increasingly been recognized as an important human health problem. 25-hydroxyvitamin D [25(OH)D], the accepted measure of vitamin D status, has been linked to several pathological states, including cardiovascular diseases, inflammatory disease, and high all-cause mortality in the general population [1]. Recent studies showed that 25(OH)D not only regulates calcium and phosphorus metabolism, but also plays a role in regulating immune and anti-inflammatory activities by adjusting growth and differentiation of macrophages, dendritic cells, T lymphocytes, and B lymphocytes, inhibiting inflammatory factors such as TNF-α, and promoting generation of anti-inflammatory factors such as IL-4 and IL-10 [2, 3].

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovitis. The aetiology and pathogenesis of RA remain obscure and many factors may be associated with its pathogenesis. 25(OH)D has immunoregulatory activities, so it is an important factor that may increase the prevalence of many autoimmune diseases such as RA and systemic lupus erythematosus [4, 5]. Increasing findings also supported that low or deficient levels of 25(OH)D may be associated with an increased risk for the development of RA [5–7]. Recently, the role of vitamin D deficiency in the pathogenesis of RA, as well as the relationship between vitamin D deficiency and the activity of RA, is discussed [8]. However, several studies have demonstrated a significant negative correlation between serum 25(OH)D levels and the activity of RA [6, 9–12]. Moreover, no correlation between serum 25(OH)D levels and the activity of RA was also observed in some reports [13–15]. Although these controversies still have not shown whether 25(OH)D deficiency is a primary phenomenon or an outcome of RA, these findings will help to understand the association between 25(OH)D and disease activity of RA. Thus, the objective of this study was performed to analyze the 25(OH)D levels in RA patients and healthy people and investigate the association between the serum levels of $25(OH)D_3$ and disease activity of RA patients from Southwest China.

2. Methods

2.1. General Data. RA patients ($n = 116$) and normal people ($n = 50$) were collected and admitted to Department of Rheumatology and Immunology, West China Hospital of Sichuan University, from June 2013 to December 2013. Participants were initially screened by telephone, and full assessments were conducted only for participants who did not report any exclusion criteria during the telephone screening. All participants provided written informed consent approved by the Hospital Human Research Ethics Committee of West China Hospital. All the cases included in the research were checked in by strict diagnosis standard. They were divided into two groups, the normal ($n = 50$) and RA ($n = 116$) group. The RA group includes 93 female and 23 male patients with RA diagnosed by Department of Rheumatology and Immunology, West China Hospital of Sichuan University. The control group includes 40 females and 10 males, which has no significant differences on sex and age with the RA group.

2.2. Inclusion and Exclusion Standards. Patients with RA in the clinical trials should be up to the standard as follows: (1) those who meet the diagnosis standard and modified classification of the American College of Rheumatology (ACR, 1987) [16] and (2) those who are beyond 18 years old. Patients with any of the following phenotypes should be excluded out of the clinical trials: (1) those who do not meet the diagnostic and modified classification standard; (2) those who are not beyond 18 years old; (3) those who have systemic lupus erythematosus, Sjogren syndrome, and other connective diseases; (4) those who have a damage in cardiovascular system, lung, kidney, and/or other organs.

2.3. Baseline Analysis. Clinical data of patients in the RA group were collected, including gender, age, height, weight, course of disease, swollen joint count, tender joint count, duration of morning stiffness, patient activity scale, C reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF). Body mass index (BMI) and Disease Activity Score-28 (DAS28) of RA patients were calculated. Basic data of participants in the control group were collected, including gender, age, weight, and height, and BMI of each participant was calculated.

2.4. Analysis of Serum 25(OH)D Concentration. 3 mL fasting blood samples of each participant in the RA and normal groups were centrifuged. Serum was applied for the measurement of 25(OH)D concentration using enzyme-linked immunosorbent (ELISA) method. The vitamin D kit of the British Immunodiagnostic Systems (IDS) was used. Correlation of 25(OH)D and disease activity of RA in the RA and control groups was analyzed. Moreover, RA group was divided into four subgroups according to DAS28 score, and further explore the association of between the values of DAS28 score and 25(OH)D levels in RA patients. According to the 25(OH)D levels, patients of RA were divided into reduced group (<50 nmol/L) and normal group (>50 nmol/L). The association between clinical data and disease activity in two groups was analyzed to explore the correlation of these parameters and 25(OH)D level.

Table 1: Participant characteristics of patients in the RA and control groups.

Variable	RA	Controls	P value
Age (years)	50.1 ± 10.9	48.1 ± 10.3	0.283
Ratio of female : male	93 : 23	40 : 10	0.980
Weight (kg)	57.5 ± 9.0	58.1 ± 8.0	0.686
Height (cm)	160.9 ± 6.4	161.6 ± 6.1	0.568
BMI (kg/m^2)	22.2 ± 2.9	22.2 ± 2.1	0.970

2.5. Statistical Analysis. Statistical analysis was carried out using SPSS Version 19.0 for Windows. Data are presented as mean or median. The continuous variables that fit the Gaussian distribution were compared by using the independent samples t test, and the other parameters are compared by using the chi-square test. P values lower than 0.05 were regarded as statistically significant difference.

3. Results

3.1. Baseline Characteristics. Participant characteristics of patients in the RA and control groups were shown in Table 1. In the RA group, a total of 116 patients with the average age of 50.1 ± 10.9 years were included in this study, and the ratio of male and female was 1 : 4.04. The average height was 160.9 ± 6.4 cm, the average body weight was 57.5 ± 9 kg, and the mean body mass index (BMI) was 22.2 ± 2.9 kg/m^2. In the control group, their average age was 48.1 ± 10.3 years, the ratio of male and female was 1 : 4, their average height was 161.6 ± 6.1 cm, the average body weight was 58.1 ± 8 kg, and the mean BMI was 22.2 ± 2.1 kg/m^2. These results showed that there were no reliable differences in age, gender, height, body weight, and BMI in two groups ($P > 0.05$).

3.2. 25(OH)D Levels in the RA and Control Groups. Table 2 presents the clinical characteristics of the study subjects according to the level of 25(OH)D. As shown in Table 2, 25(OH)D levels of the peripheral blood in the RA groups were lower than those in the control groups ($P < 0.05$). The percentage of 25(OH)D less than 25 nmol/L in the RA groups was significantly higher than those in the control groups (22.4% versus 0, $P < 0.05$, Table 2). The percentage of 25(OH)D from 25 to less than 50 nmol/L was 2.13-fold higher in the RA groups than those in the control groups (63.8% versus 20%, $P < 0.05$, Table 2). However, the percentage of 25(OH)D with higher than 50 nmol/L in the RA groups was significantly lower than those in the control groups (13.8% versus 70%, $P < 0.05$, Table 2). These results indicated that the percentage of patients with vitamin D inadequate and deficiency in the RA group was significantly higher than those in the control group, and there were statistically significant differences ($P < 0.05$).

Table 2: Distribution of 25-hydroxyvitamin D level in the RA and control groups.

25(OH)D level	RA ($n = 116$)	Controls ($n = 50$)	P value
Tested level (nmol/L)	35.99 ± 12.59	54.35 ± 8.20	<0.05
Deficiency (≤25 nmol/L)	26 (22.4%)	0 (0)	<0.05
Insufficiency (25–50 nmol/L)	74 (63.8%)	15 (30%)	<0.05
Normal level (≥50 nmol/L)	16 (13.8%)	35 (70%)	<0.05

Table 3: Differences of level of 25(OH)D in peripheral blood of four subgroups in the RA group.

Subgroups	Vitamin D level (nmol/L)	P value*
DAS28 < 2.6	32.86 ± 12.26	>0.05
2.6 ≤ DAS28 < 3.2	33.97 ± 13.28	>0.05
3.2 ≤ DAS28 ≤ 5.1	38.94 ± 13.35	>0.05
DAS28 > 5.1	38.41 ± 10.64	>0.05

* P values were obtained from the comparison of 25(OH)D levels among the subgroups.

3.3. 25(OH)D Levels in the RA Subgroups. Based on the Disease Activity Score-28 (DAS28), these patients with RA were stratified into four subgroups as follows: complete remission (DSA28 < 2.6), low disease activity (2.6 ≤ DAS28 < 3.2), moderate disease activity (3.2 ≤ DAS28 ≤ 5.1), and high disease activity (DAS28 > 5.1). Table 3 shows the differences of 25(OH)D levels in peripheral blood of four subgroups patients. As shown in Table 3, the 25(OH)D levels of complete remission, low disease activity, middle disease activity, and high disease activity groups were 32.86 ± 12.26, 33.97 ± 13.28, 38.41 ± 10.64, and 38.94 ± 13.35 nmol/L, respectively. No significant differences were observed between complete remission and low disease activity group. Similar results were also found between middle disease activity and high disease activity group.

3.4. Comparison Analysis of RA Patients with 25(OH)D Deficiency and Normal Level Groups. Based on the 25(OH)D levels, these patients with RA were divided into 25(OH)D deficiency ($n = 100$) and normal ($n = 16$) groups. Table 4 showed the results of comparison analysis of RA patients with 25(OH)D deficiency and normal groups. As shown in Table 4, there were no significant differences in age, sex, height, weight, BMI, disease duration, swelling and tenderness joint count, duration of morning stiffness, visual analogue scale (VAS), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), and DAS28 score ($P > 0.05$).

4. Discussion

RA is a chronic inflammatory autoimmune disease, mainly involving joint synovial membrane causing joint deformities. RA may cause bone loss and damage around joint [4, 5]. Reports have indicated that 90% of patients with RA symptoms will develop the radiological changes of joints damage during two years [17]. Studies have also found that T cells and cytokines play an important role in the pathogenesis of RA [18]. 25(OH)D is one of essential steroid hormones, which is involved in bone metabolism and immune regulation. Vitamin D receptors exist on the T and B lymphocytes, macrophages, and dendritic cells. After 25(OH)D combined with these receptors, they will regulate cell proliferation and differentiation and further inhibit the release of inflammatory factors. Then, they will play important roles in regulation of immune responses [19]. Thus, 25(OH)D may not only improve osteoporosis symptom of patients with rheumatoid arthritis but also play an important role in the regulation of immune system.

There has been a rapid growth in interest in the role of vitamin D in health and disease. 25(OH)D levels have been studied in RA. Previous reports have shown that serum 25(OH)D deficiency and inadequate is found in about 34–84% and 12–52% of RA patients, respectively [6, 10–12, 14, 15]. Several studies have also shown that serum 25(OH)D levels of patients with RA are significantly lower than those in the control group. Other studies reported that there were no significant differences in serum levels of $25(OH)D_3$ between RA patients and healthy controls [12, 20, 21]. These findings suggested that the proportion of patients with 25(OH)D deficiency and inadequate may be variable and related to population race, region, diet, sample size, age, sex, BMI, and other factors. In the present study, our results demonstrated that the percentages of patients with 25(OH)D deficiency and inadequate in RA group are 63.8% and 22.4%, respectively. This is close to the results of Furuya et al. [11]. Moreover, our results also showed that the serum 25(OH)D levels were significantly lower in tested RA patients than those in healthy controls. There were no significant differences in age, sex, height, weight, BMI, and so forth, showing that the lower level of serum $25(OH)D_3$ may be one of risk factors in the pathogenesis of rheumatoid arthritis.

In the previous study, the association between 25(OH)D levels and disease activity of rheumatoid arthritis still remains controversial. This may stem from the fact that 25(OH)D values are easily affected by population race, region, diet, sample size, age, sex, BMI, and other factors. Some studies confirmed that the reduction of serum 25(OH)D in patients of RA was negatively correlated with clinical indexes (joint swelling, joint tenderness, ESR, etc.) and disease activity index DAS28 [6, 9–12]. However, some reports indicated that there was no relation between 25(OH)D deficiency and disease activity in RA [13–15]. The present study suggested that no statistical difference was found among four subgroups in the RA group based on the classification of DAS28 value. Moreover, our findings also indicated that no significance between 25(OH)D deficiency and normal level groups was found under the comparison analysis of clinical parameters and

Table 4: Comparison analysis of RA patients with 25(OH)D deficiency and normal levels groups (<50 nmol/L).

Characteristics	Deficiency ($n = 100$)	Normal level ($n = 16$)	P value
Age (years)	49.8 ± 10.8	52.3 ± 11.7	0.684
Female : male ratio	81 : 19	12 : 4	0.580
Weight (kg)	57.5 ± 8.7	58.0 ± 10.9	0.608
Height (cm)	160.7 ± 6.2	162.2 ± 7.8	0.503
BMI (kg/m^2)	22.2 ± 3.0	21.9 ± 2.3	0.149
Disease duration (months)	43.5 (3, 468)	36.0 (3, 316)	0.957
DAS28	3.5 ± 1.8	4.09 ± 1.6	0.779
Patient global VAS (cm)	3.3 ± 3.1	4.2 ± 3.1	0.948
Swollen joint count (0–28)	3.9 ± 5.4	5.3 ± 7.1	0.302
Tender joint count (0–28)	5.1 ± 6.5	6.9 ± 7.1	0.775
ESR (mm/H)	33.5 (4, 102)	36.5 (6, 78)	0.360
CRP (mg/L)	5.1 (1, 78)	5.7 (3.2, 17.7)	0.595
RF (IU/ml)	63.5 (20, 2220)	107.5 (20, 1900)	0.009

DAS28 scores in these RA patients. Based on the above results, the present findings indicated that serum 25(OH)D levels might be not associated with the disease activity of RA.

5. Conclusion

In summary, the present study suggested that serum 25(OH)D level might be significantly associated with the pathogenesis of RA. However, the serum values of 25(OH)D are not a good indicator of disease activity of RA patients. As serum 25(OH)D_3 levels are not independent and/or influenced by many factors, such as region, season, and gender, further research will focus on the cross-regional and large sample to understand the association between 25(OH)D and RA in Southwest China.

Conflict of Interests

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

Authors' Contribution

Xiaomin Cen and Yuan Liu contributed equally to this work.

Acknowledgments

This research was supported by National Natural Science Foundation of China (Grant no. 30901339 and Grant no. 81172869), the Application Foundation Project in Sichuan Province (no. 2010JY0011), and the Science and Technology Support Project of Sichuan, China (no. 2009SZ0173).

References

[1] Y. Arnson, H. Amital, and Y. Shoenfeld, "Vitamin D and autoimmunity: new aetiological and therapeutic considerations," *Annals of the Rheumatic Diseases*, vol. 66, no. 9, pp. 1137–1142, 2007.

[2] M. F. Holick, "Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease," *The American journal of Clinical Nutrition*, vol. 80, no. 6, pp. 1678–1688, 2004.

[3] J. Fritsche, K. Mondal, A. Ehrnsperger, R. Andreesen, and M. Kreutz, "Regulation of 25-hydroxyvitamin D_3-1α-hydroxylase and production of 1α,25-dihydroxyvitamin D_3 by human dendritic cells," *Blood*, vol. 102, no. 9, pp. 3314–3316, 2003.

[4] L. A. Merlino, J. Curtis, T. R. Mikuls, J. R. Cerhan, L. A. Criswell, and K. G. Saag, "Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study," *Arthritis & Rheumatism*, vol. 50, no. 1, pp. 72–77, 2004.

[5] K. Gopinath and D. Danda, "Supplementation of 1,25 dihydroxy vitamin D3 in patients with treatment naive early rheumatoid arthritis: a randomised controlled trial," *International Journal of Rheumatic Diseases*, vol. 14, no. 4, pp. 332–339, 2011.

[6] G. S. Kerr, I. Sabahi, J. S. Richards et al., "Prevalence of vitamin D insufficiency/deficiency in rheumatoid arthritis and associations with disease severity and activity," *The Journal of Rheumatology*, vol. 38, no. 1, pp. 53–59, 2011.

[7] A. R. Broder, J. N. Tobin, and C. Putterman, "Disease-specific definitions of vitamin D deficiency need to be established in autoimmune and non-autoimmune chronic diseases: a retrospective comparison of three chronic diseases," *Arthritis Research & Therapy*, vol. 12, no. 5, article R191, 2010.

[8] G. G. Song, S.-C. Bae, and Y. H. Lee, "Association between vitamin D intake and the risk of rheumatoid arthritis: a meta-analysis," *Clinical Rheumatology*, vol. 31, no. 12, pp. 1733–1739, 2012.

[9] U. J. Haque and S. J. Bartlett, "Relationships among vitamin D, disease activity, pain and disability in rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 28, no. 5, pp. 745–747, 2010.

[10] Q. Hong, J. Xu, S. Xu, L. Lian, M. Zhang, and C. Ding, "Associations between serum 25-hydroxyvitamin D and disease activity, inflammatory cytokines and bone loss in patients with rheumatoid arthritis," *Rheumatology*, vol. 53, no. 11, pp. 1994–2001, 2014.

[11] T. Furuya, T. Hosoi, E. Tanaka et al., "Prevalence of and factors associated with vitamin D deficiency in 4,793 Japanese patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 32, no. 7, pp. 1081–1087, 2013.

[12] M. Rossini, S. M. Bongi, G. la Montagna et al., "Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants and associations with disease activity and disability," *Arthritis Research & Therapy*, vol. 12, no. 6, article R216, 2010.

[13] T. Baykal, K. Senel, F. Alp, A. Erdal, and M. Ugur, "Is there an association between serum 25-hydroxyvitamin d concentrations and disease activity in rheumatoid arthritis?" *Bratislava Medical Journal*, vol. 113, no. 10, pp. 610–611, 2012.

[14] R. Pakchotanon, S. Chaiamnuay, P. Narongroeknawin, and P. Asavatanabodee, "The association between serum vitamin D Level and disease activity in Thai rheumatoid arthritis patients," *International Journal of Rheumatic Diseases*, 2013.

[15] H. J. Haga, A. Schmedes, Y. Naderi, A. M. Moreno, and E. Peen, "Severe deficiency of 25-hydroxyvitamin D_3(25-OH-D_3) is associated with high disease activity of rheumatoid arthritis," *Clinical Rheumatology*, vol. 32, no. 5, pp. 629–633, 2013.

[16] 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 and Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[17] M. Bukhari, B. Harrison, M. Lunt, D. G. I. Scott, D. P. M. Symmons, and A. J. Silman, "Time to first occurrence of erosions in inflammatory polyarthritis: results from a prospective community-based study," *Arthritis & Rheumatism*, vol. 44, no. 6, pp. 1248–1253, 2001.

[18] E. Zold, P. Szodoray, J. Gaal et al., "Vitamin D deficiency in undifferentiated connective tissue disease," *Arthritis Research & Therapy*, vol. 10, no. 5, article R123, 2008.

[19] M. Hewison, L. Freeman, S. V. Hughes et al., "Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells," *Journal of Immunology*, vol. 170, no. 11, pp. 5382–5390, 2003.

[20] A. D. Turhanoğlu, H. Güler, Z. Yönden, F. Aslan, A. Mansuroglu, and C. Ozer, "The relationship between vitamin D and disease activity and functional health status in rheumatoid arthritis," *Rheumatology International*, vol. 31, no. 7, pp. 911–914, 2011.

[21] M. Cutolo, K. Otsa, K. Laas et al., "Circannual vitamin D serum levels and disease activity in rheumatoid arthritis: Northern versus Southern Europe," *Clinical and Experimental Rheumatology*, vol. 24, no. 6, pp. 702–704, 2006.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 240210, 8 pages
http://dx.doi.org/10.1155/2015/240210

Research Article

Pim-2/mTORC1 Pathway Shapes Inflammatory Capacity in Rheumatoid Arthritis Synovial Cells Exposed to Lipid Peroxidations

Geng Yin,[1] Yan Li,[2] Min Yang,[1] Xiao-min Cen,[1] and Qi-bing Xie[1]

[1]*Department of Rheumatology and Immunology, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China*
[2]*Department of Rheumatology, The First Affiliated Hospital of Xiamen University, No. 55, Zhenhai Road, Xiamen, Fujian 361003, China*

Correspondence should be addressed to Qi-bing Xie; qibingxie@126.com

Received 2 December 2014; Accepted 9 January 2015

Academic Editor: Long Shen

Rheumatoid arthritis is a systemic autoimmune disease characterized by chronic inflammation of multiple joints, with disruption of joint cartilage. The proliferation of synovial fibroblasts in response to multiple inflammation factors is central to the pathogenesis of rheumatoid arthritis. Our previous studies showed that 4-HNE may induce synovial intrinsic inflammations by activating NF-κB pathways and lead to cell apoptosis. However, the molecular mechanisms of how synovial NF-κB activation is modulated are not fully understood. Here, the present findings demonstrated that 4-HNE may induce synovial intrinsic inflammations by mTORC1 inactivation. While ectopic activation of mTORC1 pathway by the overexpression of Pim-2 may disrupt the initiation of inflammatory reactions and maintain synovial homeostasis, our findings will help to uncover novel signaling pathways between inflammations and oxidative stress in rheumatoid arthritis development and imply that Pim-2/mTORC1 pathway may be critical for the initiation of inflammatory reactions in human rheumatoid arthritis synovial cells.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of synovium that can lead to severe joint damage [1]. The central pathogenesis of rheumatoid arthritis is the proliferation of fibroblast-like synoviocytes (FLSs) in response to stimulators [2, 3]. During the process of FLSs proliferation, inflammatory responses are critical for rheumatoid arthritis development [4, 5]. Synovial inflammatory responses are mainly induced by products of autocrine, but also paracrine molecules produced by infiltrating mononuclear cells, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, IL-6, and IL-17 [5]. Therefore, the initiation and proceeding of inflammatory reactions might be critical for rheumatoid arthritis development.

Since inflammatory reactions are important for synovial homeostasis, the precise regulation of inflammation must be well achieved. And one of the central regulators is nuclear factor kappa beta (NF-κB) [6, 7]. It has been long appreciated that NF-κB is a significant transcription factor that functions in immune and inflammatory responses, stress responses, apoptosis, and differentiation. For example, NF-κB plays a pivotal role in myocardial ischemia-reperfusion injury and induces many proinflammatory cytokines and chemokines which will greatly contribute to myocardial I-R injury [8]. Moreover, NF-κB is also considered to act as a redox sensitive transcription factor that has been proposed to be the sensor for oxidative stress [9]. Thus, NF-κB activity is important for inflammatory reactions.

Notably, among all the regulators of NF-κB activation, mammalian target of rapamycin complex 1 (mTORC1) is of great significance, which is involved in differentially regulating the levels of pro- and anti-inflammatory cytokines produced by innate immune cells [10]. Studies using the mTORC1 inhibitor rapamycin have reported that mTORC1 activity plays an important role in NF-κB activation and

inflammation [11–13]. For example, inhibition of mTORC1 in lipopolysaccharide- (LPS-) stimulated cells has been shown to attenuate the phosphorylation of several targets of mTORC1, including p70S6K and 4E (eIF4E) binding protein 1 (4EBP1), as well as decreasing the levels of phosphorylated STAT3 (p-STAT3) [14]. In contrast, mTORC1 inhibition potently increased NF-κB activity, leading to enhanced IL-12 production by LPS-stimulated cells [15]. Thus, these findings support the notion that mTORC1 pathway is a master regulator of NF-κB activation and inflammation. In our previous studies, we have demonstrated that products of lipid peroxidation, 4-HNE, may induce synovial inflammations by activating NF-κB pathways and lead to cell apoptosis. Pharmacological inhibition of NF-κB activation may reduce the 4-HNE-mediated inflammations and subsequent cell apoptosis (unpublished data). However, how NF-κB is activated under lipid peroxidation conditions is not well characterized. Considering that mTORC1 inhibition may promote NF-κB activation by LPS-stimulated cells, we hypothesized that lipid peroxidation may induce NF-κB activation and inflammation by inhibiting mTORC1 activity.

In the present study, we proposed to determine whether mTORC1 activity is critical for lipid peroxidation-mediated inflammation in rheumatoid arthritis synovial cells. Our findings showed that synovial mTORC1 activity was dramatically decreased by 4-HNE treatment. Moreover, we also noticed an interesting upregulation of Pim-2 kinase activity, which is a serine/threonine kinase controlling cell growth and differentiation. Overexpression of Pim-2 kinase restored the 4-HNE-mediated mTORC1 inactivation and thus led to NF-κB inactivation and inflammation reduction. Our findings will help to uncover novel signaling pathways between inflammations and oxidative stress in rheumatoid arthritis development and offer new targets to rheumatoid arthritis clinical therapy.

2. Material and Methods

2.1. Chemicals and Reagents. The 4-hydroxynonenal was obtained from Biomol (Plymouth Meeting, PA, USA). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO Invitrogen (Carlsbad, CA, USA). The following antibodies, anti-COX-2, anti-Lamin A/C, and anti-beta actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Pim-2 and myc-tagged antibodies were from Millipore (Billerica, MA, USA). The pp70S6K, p70S6K, p4EBP1, 4EBP1, pBad, Bad, NF-κB (p65), and GAPDH antibodies were from Cell Signaling Technology (Danvers, MA, USA). Other chemicals were of the highest purity available.

2.2. Cell Culture and Pharmacological Manipulations. A widely used MH7A human rheumatoid arthritis synovial cell was chosen as *in vitro* experiment system, which was obtained from Shanghai Institute of Cell Biology (Introduced from American Type Culture Collection). For Western blots and real-time PCR experiments, MH7A cells were plated in 6-well plates at 1.0×10^6 cells/mL, while immunostaining at 1.0×10^5 cells/mL. The cells were incubated in DMEM containing 10% FBS plus antibiotics for 24 h in 5% CO_2 at 37°C.

For Pim-2 overexpression, the myc-tagged Pim-2 construct in MH7A cells was generated by subcloning the PCR-amplified human Pim-2 coding sequence into pRK5-myc vectors. Following transfection was carried out when the cell confluent was 80–90% using lipofectamine 2000, and cells were harvested at 24 h after transfection with lysis buffer. For 4-HNE treatment, the final concentrations of 5 μM of 4-HNE were applied to these cells and then incubated for 0 to 12 h. Equivalent saline was used as internal controls. After culturing, the cells were harvested for subsequent examinations.

2.3. Cell Lysates Preparation and Western Blots. For Western blots, prepared cells were trypsinized and harvested, washed with PBS once, and resuspended in cell lysis buffer (PBS with 1% Triton X-100 and protease inhibitors). After brief sonication, cell lysates were centrifuged at 13,000 rpm for 5 min. Protein concentration was determined so that equivalent amounts of lysate based on protein concentration was added to an equal volume of 2x Laemmli buffer and boiled for 10 min.

As for the NF-κB nuclear examinations, MH7A nuclear lysates were prepared according to the instructions of Nuclear/Cytosol Fractionation Kit (Biovision) as previously described. Briefly, MH7A cell pellets were resuspended in nuclei isolation buffer (20 mM HEPES-KOH, 100 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin). The cells were incubated on ice 30 min and then lysed using a homogenizer. Then, cell lysates were centrifuged (1000 ×g for 5 min), and the pellet (nuclei) and supernatant (cytosol) were collected. The nuclei were washed once in nuclei isolation buffer and pelleted by centrifugation, and the resulting supernatant was added to the cytosolic extract. For Western blot analysis, the procedure was according to the standard protocols. Finally, proteins were detected by SuperSignal enhanced chemiluminescence development (ECL) (Thermo Scientific Pierce) reagent and exposed to films (Kodak). The protein level quantification was carried out by ImageJ.

2.4. Immunostaining. For pp70S6K immunostaining in MH7A cells, HNE-treated cells were fixed with 4% PFA with 4% sucrose in phosphate-buffered saline (PBS) for 30 min and permeabilized with 0.25% Triton-X 100 in PBS for 5 min at room temperature. After incubated with pp70S6K primary antibody and fluorescence secondary antibody, cells were mounted onto glass slides with antifade reagent with DAPI and visualized under fluorescence microscopy.

2.5. Quantitative Real-Time PCR. Total RNA was extracted from tissues using TRizol reagent (Invitrogen). RNA was subjected to reverse transcription with reverse transcriptase as Manufacturer's instructions (Fermentas). Quantitative real-time PCR was performed using Bio-Rad iQ5 system, and relative gene expression was normalized to internal

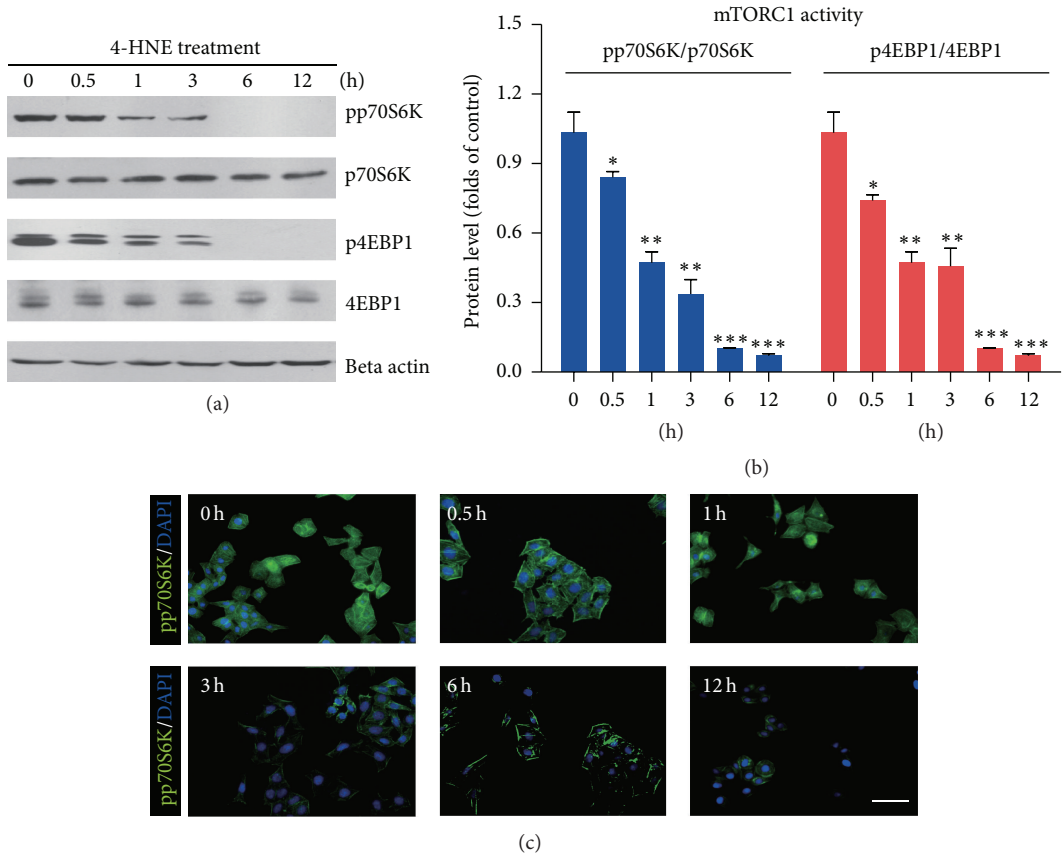


Figure 1: 4-HNE inactivates mTORC1 activity in MH7A rheumatoid arthritis synovial cells. (a-b) Western blots and histograms showing the decreased mTORC1 activity (indicated by pp70S6K/p70S6K and p4EBP1/4EBP1) by 4-HNE treatment in MH7A synovial cells. (c) Images showing that pp70S6K signals were decreased by 4-HNE treatment in MH7A synovial cells. Green fluorescence indicates pp70S6K, and blue indicates DAPI. Bar 25 μm. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

control as *Beta actin*. Primer sequences for SYBR Green probes of target genes are as follows: *Pim-2*: ACTCCAGGTGGCCATCAAAG and T CCATAGCAGTGCGACTTCG; *Tnf-α*: CATCTTCTCAAAATTCGAGTGACA and T GGGAGTAGACAAGGTACAACCC; *Beta actin*: GAGACCTTCAACACCCCAGC and ATGTCACGCACGATTTCCC.

2.6. Statistical Analysis. Data represent the mean and standard error of the mean (SEM). ANOVA tests for comparisons were performed for all statistical significance analysis using GraphPad Prism software. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Lipid Peroxidations Inactivate mTORC1 Activity in Rheumatoid Arthritis Synovial Cells. In previous studies, we have verified that products of lipid peroxidations, 4-HNE, may induce synovial intrinsic inflammations and lead to cell apoptosis (unpublished data). However, the molecular mechanisms involved in inflammatory reactions and cell apoptosis by lipid peroxidations were not fully elucidated. Considering that mTORC1 pathway is a key regulator of innate inflammatory homeostasis in several types of cells [16], we investigated mTORC1 activities by 4-HNE treatment in MH7A rheumatoid arthritis synovial cells. Biochemical results showed that, by 4-HNE treatment, the protein levels of markers of mTORC1 pathway (pp70S6K and p4EBP1) [17] were both decreased gradually as 4-HNE treatment, and the maximum folds decreased by almost 90% (6~12 h) compared to the control (Figures 1(a) and 1(b)). To confirm that reduced mTORC1 activity in MH7A cells by 4-HNE treatment, we further carried out pp70S6K immunostaining on these cells. Images showed that the pp70S6K signals (green fluorescence) also dramatically decreased by 4-HNE treatment (Figure 1(c)). Therefore, our results revealed that lipid peroxidation may inhibit mTORC1 pathway in synoviocytes, which may confer to the development of inflammations.

3.2. Lipid Peroxidation Activates Pim-2 Kinase Signaling in Rheumatoid Arthritis Synovial Cells. As for mTORC1 pathway is the master regulator cell growth, survival, and metabolism in mammalian cells [18], the decreased mTORC1

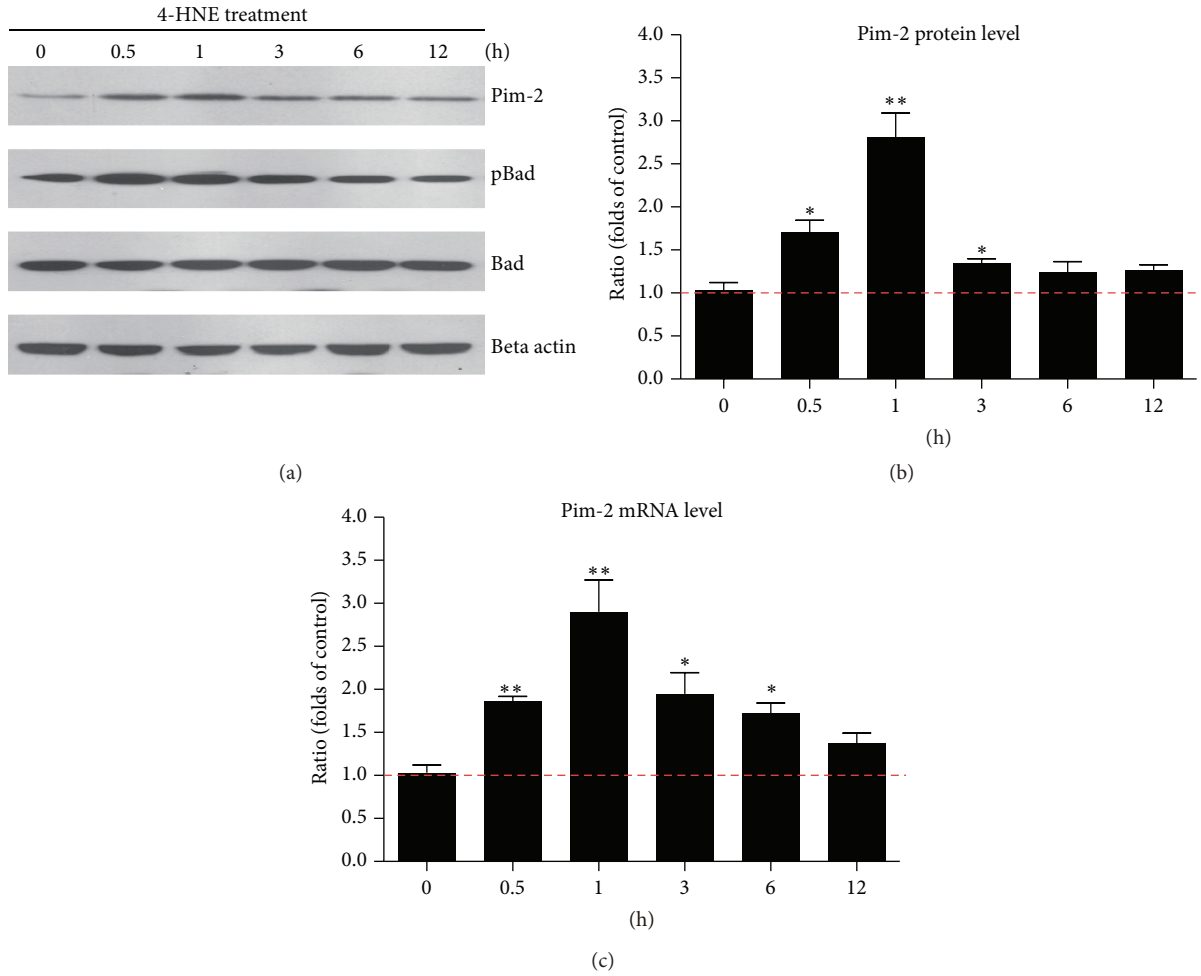


Figure 2: 4-HNE activates Pim-2 kinase signaling in MH7A synovial cells. (a-b) Western blots and histograms showing the increased Pim-2 kinase protein levels by 4-HNE treatment in MH7A synovial cells. (c) Histograms showing that the increased Pim-2 kinase mRNA levels by 4-HNE treatment in MH7A synovial cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

pathway by 4-HNE may induce adaptative alternations to compensate for the reduced mTORC1 activity. Pim kinase family, especially Pim-2, has been reported to be essential component of an endogenous pathway, activating mTORC1 signaling and regulating cell growth and survival [19, 20]. Thus, we examined whether Pim-2 kinase expression/activity was altered by 4-HNE treatment. Biochemical results showed that after short-term 4-HNE treatment, the protein level of endogenous Pim-2 kinase increased by 2.81-fold (1 h) compared to controls. As prolonged 4-HNE treatment, the Pim-2 protein level started to decrease, confirmed by the parallel reduction of BAD phosphorylation (a well-known Pim-2 substrate) [21] (Figures 2(a) and 2(b)). To investigate whether increased Pim-2 expressions were caused by upregulated transcriptions, we assessed the mRNA level of Pim-2. The results of quantitative real-time PCR showed that Pim-2 mRNA levels were indeed induced by 4-HNE treatment and highly correlated with the alternations of protein levels (Figure 2(c)). Thus, our findings showed that induced Pim-2 signaling may be cell intrinsic protective mechanisms against the toxicity of lipid peroxidations.

3.3. Pim-2 Overexpression May Partly Activate mTORC1 Pathway under 4-HNE Conditions. Since Pim-2 kinase has been reported to activate mTORC1 pathway by modulating tuberous sclerosis complex 2 (TSC2) phosphorylations [19], we proposed that upregulated Pim-2 kinase activity may partly resist 4-HNE-mediated mTORC1 inactivation. To examine how Pim-2 participates in mTORC1 activation under oxidative stress, we constructed an myc-tagged Pim-2 vector to the overexpression of Pim-2 in MH7A synovial cells and investigated the mTORC1 signaling alternations. Biochemical results showed that although 4-HNE treatment may decrease p70S6K and 4EBP1 phosphorylations, Pim-2 overexpression may constitutively maintain high phosphorylations of p70S6K and 4EBP1 under both basal and 4-HNE conditions (Figures 3(a) and 3(b)). These results clearly

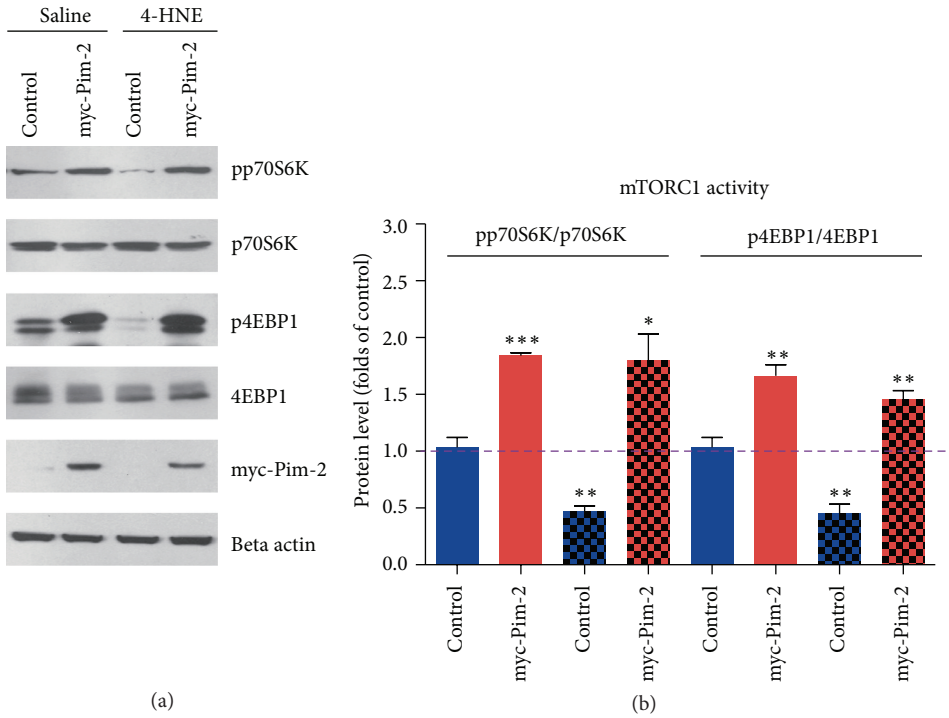


Figure 3: Pim-2 kinase overexpression may partly activate mTORC1 pathway under HNE conditions. (a-b) Western blots and histograms showing that Pim-2 overexpression activated mTORC1 activity (indicated by pp70S6K/p70S6K and p4EBP1/4EBP1) under basal and 4-HNE conditions. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

indicate that the overexpression of Pim-2 may promote constitutive mTORC1 activation under oxidative stress, which may contribute to maintenance of cell homeostasis.

3.4. Pim-2 Mediated mTORC1 Activation Inhibits 4-HNE-Induced Inflammation in Rheumatoid Arthritis Synovial Cells. Oxidative stress induced inflammation is critical for rheumatoid arthritis development [22, 23]. In our previous studies, we have shown that 4-HNE could induce synovial intrinsic inflammations by activating NF-κB and COX-2 pathways (unpublished data). Moreover, mTORC1 activity has been proved to be critical for the inflammatory balance. Therefore, we assumed that Pim-2 might mediate mTORC1 activation and may inhibit inflammation reactions caused by lipid peroxidation. Thus, we examined the inflammation markers of NF-κB and COX-2 in Pim-2 overexpressed MH7A cells. The results showed that the overexpression of Pim-2 may inhibit NF-κB nuclear localization of the p65 subunit under both basal and 4-HNE conditions, which indicated that the overexpression of Pim-2 may inhibit NF-κB signaling. Moreover, the induced COX-2 expression by 4-HNE might be also disrupted by the overexpression of Pim-2, which confirmed that the overexpression of Pim-2 may inhibit the initiation of inflammatory reactions (Figures 4(a) and 4(b)). To further confirm that the overexpression of Pim-2 may contribute to the inflammatory blocking in MH7A cells, we examined the mRNA level of canonical inflammation factor TNF-α. The results also showed that the overexpression of Pim-2 may inhibit 4-HNE induced TNF-α transcriptions (Figure 4(c)). All these findings showed that Pim-2/mTORC1 pathway may play a vital role in protection against lipid peroxidation induced inflammations.

4. Discussion

In the present study, we reveal a novel mechanism to clarify how inflammation is precisely regulated in human rheumatoid arthritis synovial cells. The present findings demonstrated that products of lipid peroxidation, 4-HNE, may induce synovial intrinsic inflammations by activating NF-κB pathways which may be mediated by mTORC1 inactivation. However, ectopic activation of mTORC1 pathway by the overexpression of Pim-2 may disrupt the initiation of inflammatory reactions and maintain synovial homeostasis (Figure 5). Our work will help to uncover novel signaling pathways between inflammations and oxidative stress in rheumatoid arthritis development and offer new targets to rheumatoid arthritis clinical therapy.

Rheumatoid arthritis (RA) is a chronic, systemic, and inflammatory autoimmune disease, targeting the synovial tissues [24]. Fibroblast-like synoviocytes (FLSs) play a central role in the formation of rheumatoid arthritis pannus [25]. Recent advances in understanding the networks that are responsible for the synovial inflammations in rheumatoid arthritis have led to the successful use of therapies [26, 27]. Yet, the regulatory mechanisms of how inflammation is precisely controlled remain incompletely understood. Here,

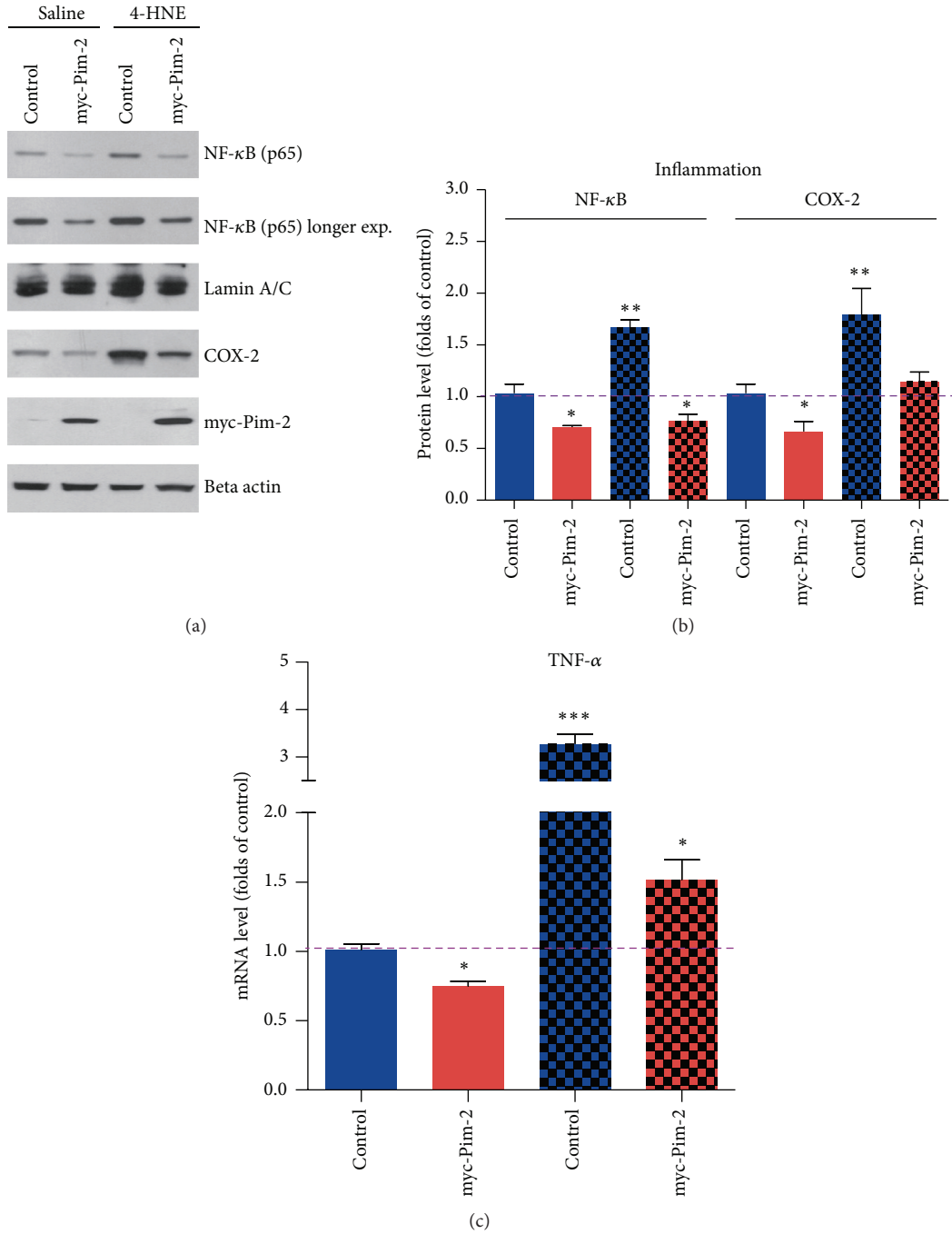


Figure 4: Pim-2 mediated mTORC1 activation inhibits HNE-induced inflammation in MH7A synovial cells. (a-b) Western blots and histograms showing that Pim-2 overexpression inhibited NF-κB nuclear localizations and COX-2 expressions under basal and 4-HNE conditions. (c) Histograms showing that Pim-2 overexpression inhibited TNF-α transcriptions under basal and 4-HNE conditions. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

we demonstrate that synovial Pim-2/mTORC1 pathway couples the oxidative stress and inflammations. Under oxidative stress conditions (lipid peroxidation), mTORC1 activity was downregulated, which may be responsible for the initiation of inflammatory reactions, such as NF-κB nuclear translocations, COX-2 expressions, and inflammation factors releases. On the other hand, to precisely modulate inflammatory reactions, oxidative stress may activate Pim-2 kinase signaling to maintain appropriate mTORC1 activity. Therefore, once Pim-2 activity is ectopic upregulated (e.g., Pim-2 overexpression, etc.), mTORC1 activity may be restored under 4-HNE conditions, which disrupts the synovial inflammations. Thus,

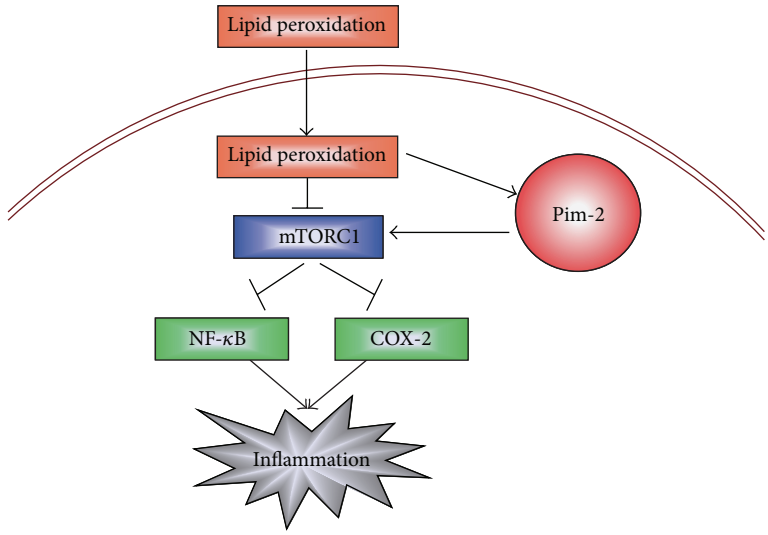


Figure 5: A model showing that Pim-2/mTORC1 pathway may be critical for the initiation of inflammatory reactions mediated by lipid peroxidation in human rheumatoid arthritis synovial cells.

Pim-2/mTORC1 pathway may be critical for the coupling of oxidative stress and synovial inflammation.

In contrast to many other kinases whose activities are tuned by phosphorylation status, the Pim-2 kinase is constitutively active and lacks regulatory domains. Instead, Pim-2 kinase is tightly regulated at both transcriptional and translational levels [19]. The signals that induce Pim-2 gene expression are diverse, including various cytokines, growth factors, and mitogenic stimuli in different cell types. Moreover, Pim-2 could modulate mTORC1 activity by directly phosphorylating TSC2 on Ser-1798 and relieves the suppression of TSC2 on mTORC1 [19]. Here, we further showed that Pim-2 expression may be induced products of lipid peroxidation, to compensate for the mTORC1 activity under oxidative stress conditions. Thus, further in-depth studies on how Pim-2 senses oxidative stress and is transcriptionally upregulated in human rheumatoid arthritis synovial cells might not only help to understand inflammatory reactions and/or synovial homeostasis, but possibly also uncover novel signaling pathways between inflammations and oxidative stress.

5. Conclusion

The present findings suggested that lipid peroxidation-mediated mTORC1 inactivation may be essential for the synovial inflammation. While ectopic activation of Pim-2 signaling may partly restore mTORC1 activity under lipid peroxidation conditions, leading to inflammation blocking, our findings imply that Pim-2/mTORC1 pathway may be critical for the initiation of inflammatory reactions and cell homeostasis in human rheumatoid arthritis synovial cells.

Conflict of Interests

The authors have declared that no conflict of interest exists.

Authors' Contribution

Geng Yin and Yan Li contributed equally to this work.

Acknowledgments

This research was supported by National Natural Science Foundation of China (Grant no. 30901339 and Grant no. 81172869), the Application Foundation Project in Sichuan Province (no. 2010JY0011), and the Science and Technology Support Project of Sichuan, China (no. 2009SZ0173).

References

[1] J. Kurkó, T. Besenyei, J. Laki, T. T. Glant, K. Mikecz, and Z. Szekanecz, "Genetics of rheumatoid arthritis—a comprehensive review," *Clinical Reviews in Allergy and Immunology*, vol. 45, no. 2, pp. 170–179, 2013.

[2] C. Perricone, F. Ceccarelli, and G. Valesini, "An overview on the genetic of rheumatoid arthritis: a never-ending story," *Autoimmunity Reviews*, vol. 10, no. 10, pp. 599–608, 2011.

[3] M. Wang, S. Zhu, W. Peng et al., "Sonic hedgehog signaling drives proliferation of synoviocytes in rheumatoid arthritis: a possible novel therapeutic target," *Journal of Immunology Research*, vol. 2014, Article ID 401903, 10 pages, 2014.

[4] A. Saxena, S. K. Raychaudhuri, and S. P. Raychaudhuri, "Interleukin-17-induced proliferation of fibroblast-like synovial cells is mTOR dependent," *Arthritis and Rheumatism*, vol. 63, no. 5, pp. 1465–1466, 2011.

[5] V. Badot, P. Durez, B. J. van den Eynde, A. Nzeusseu-Toukap, F. A. Houssiau, and B. R. Lauwerys, "Rheumatoid arthritis synovial fibroblasts produce a soluble form of the interleukin-7 receptor in response to pro-inflammatory cytokines," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 11, pp. 2335–2342, 2011.

[6] C. Giuliani, G. Napolitano, I. Bucci, V. Montani, and F. Monaco, "Nf-κB transcription factor: role in the pathogenesis of inflammatory, autoimmune, and neoplastic diseases and therapy implications," *La Clinica Terapeutica*, vol. 152, no. 4, pp. 249–253, 2001.

[7] D. Ditsworth and W. X. Zong, "NF-kappaB: Key mediator of inflammation-associated cancer," *Cancer Biology & Therapy*, vol. 3, no. 12, pp. 1214–1216, 2004.

[8] X. Liu, J. Shen, Y. Jin, M. Duan, and J. Xu, "Recombinant human erythropoietin (rhEPO) preconditioning on nuclear factor-kappa B (NF-kB) activation & proinflammatory cytokines induced by myocardial ischaemia-reperfusion," *Indian Journal of Medical Research*, vol. 124, no. 3, pp. 343–354, 2006.

[9] M. Carini, G. Aldini, and R. M. Facino, "Mass spectrometry for detection of 4-hydroxy-trans-2-nonenal (HNE) adducts with peptides and proteins," *Mass Spectrometry Reviews*, vol. 23, no. 4, pp. 281–305, 2004.

[10] H. C. Dan, M. J. Cooper, P. C. Cogswell, J. A. Duncan, J. P.-Y. Ting, and A. S. Baldwin, "Akt-dependent regulation of NF-κB is controlled by mTOR and Raptor in association with IKK," *Genes & Development*, vol. 22, no. 11, pp. 1490–1500, 2008.

[11] A. Giordano, R. Avellino, P. Ferraro, S. Romano, N. Corcione, and M. F. Romano, "Rapamycin antagonizes NF-kappaB nuclear translocation activated by TNF-alpha in primary vascular smooth muscle cells and enhances apoptosis," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 6, pp. H2459–H2465, 2006.

[12] T. Kakegawa, A. Nemoto, M. Matsuda, H. Saito, and H. Kobayashi, "Effect of rapamycin on NF-kappaB induction," *The Japanese Journal of Antibiotics*, vol. 56, supplement, pp. 97–99, 2003.

[13] X. Chen, S. Zeng, J. Zou et al., "Rapamycin attenuated cardiac hypertrophy induced by isoproterenol and maintained energy homeostasis via inhibiting NF-κB activation," *Mediators of Inflammation*, vol. 2014, Article ID 868753, 15 pages, 2014.

[14] H. Wang, J. Brown, Z. Gu et al., "Convergence of the mammalian target of rapamycin complex 1- and glycogen synthase kinase 3-beta-signaling pathways regulates the innate inflammatory response," *Journal of Immunology*, vol. 186, no. 9, pp. 5217–5226, 2011.

[15] T. Weichhart, G. Costantino, M. Poglitsch et al., "The TSC-mTOR signaling pathway regulates the innate inflammatory response," *Immunity*, vol. 29, no. 4, pp. 565–577, 2008.

[16] W. Wang, J. Yan, H. Wang et al., "Rapamycin ameliorates inflammation and fibrosis in the early phase of cirrhotic portal hypertension in rats through inhibition of mTORC1 but not mTORC2," *PLoS ONE*, vol. 9, no. 1, Article ID e83908, 2014.

[17] E. V. Haar, S. I. Lee, S. Bandhakavi, T. J. Griffin, and D.-H. Kim, "Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40," *Nature Cell Biology*, vol. 9, no. 3, pp. 316–323, 2007.

[18] M. Laplante and D. M. Sabatini, "MTOR signaling in growth control and disease," *Cell*, vol. 149, no. 2, pp. 274–293, 2012.

[19] J. Lu, T. Zavorotinskaya, Y. Dai et al., "Pim2 is required for maintaining multiple myeloma cell growth through modulating TSC2 phosphorylation.," *Blood*, vol. 122, no. 9, pp. 1610–1620, 2013.

[20] C. J. Fox, P. S. Hammerman, and C. B. Thompson, "The Pim kinases control rapamycin-resistant T cell survival and activation," *The Journal of Experimental Medicine*, vol. 201, no. 2, pp. 259–266, 2005.

[21] B. Yan, M. Zemskova, S. Holder et al., "The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death," *The Journal of Biological Chemistry*, vol. 278, no. 46, pp. 45358–45367, 2003.

[22] K. Bauerová and A. Bezek, "Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis," *General Physiology and Biophysics*, vol. 18, pp. 15–20, 1999.

[23] K. A. Gelderman, M. Hultqvist, L. M. Olsson et al., "Rheumatoid arthritis: the role of reactive oxygen species in disease development and therapeutic strategies," *Antioxidants & Redox Signaling*, vol. 9, no. 10, pp. 1541–1567, 2007.

[24] E. Choy, "Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis," *Rheumatology*, vol. 51, supplement 5, pp. v3–v11, 2012.

[25] L. C. Huber, O. Distler, I. Tarner, R. E. Gay, S. Gay, and T. Pap, "Synovial fibroblasts: key players in rheumatoid arthritis," *Rheumatology*, vol. 45, no. 6, pp. 669–675, 2006.

[26] S. Richardson and J. Isaacs, "Novel immunotherapies for rheumatoid arthritis," *Clinical Medicine*, vol. 13, no. 4, pp. 391–394, 2013.

[27] T. Pap, U. Müller-Ladner, R. E. Gay, and S. Gay, "Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis," *Arthritis Research*, vol. 2, no. 5, pp. 361–367, 2000.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 150658, 10 pages
http://dx.doi.org/10.1155/2015/150658

Review Article

Medication Adherence in Patients with Rheumatoid Arthritis: The Effect of Patient Education, Health Literacy, and Musculoskeletal Ultrasound

Samantha Joplin,[1] Rick van der Zwan,[2] Fredrick Joshua,[3,4] and Peter K. K. Wong[5,6]

[1] *School of Psychology, University of Sydney, Sydney, NSW 2006, Australia*
[2] *Department of Psychology, Southern Cross University, Coffs Harbour, NSW 2450, Australia*
[3] *Department of Rheumatology, Prince of Wales Hospital, Randwick, Sydney, NSW 2031, Australia*
[4] *Prince of Wales Hospital Clinical School, University of New South Wales, Randwick, Sydney, NSW 2031, Australia*
[5] *Mid-North Coast Arthritis Clinic, Coffs Harbour, NSW 2450, Australia*
[6] *Rural Clinical School, University of New South Wales, Coffs Harbour, NSW 2450, Australia*

Correspondence should be addressed to Peter K. K. Wong; pkkw12@gmail.com

Received 24 November 2014; Revised 8 February 2015; Accepted 9 February 2015

Academic Editor: Shigeru Kotake

Background. Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting <1% of the population. Incompletely controlled RA results in fatigue, joint and soft tissue pain, progressive joint damage, reduced quality of life, and increased cardiovascular mortality. Despite an increasing range of disease modifying agents which halt disease progression, poor patient adherence with medication is a significant barrier to management. *Objective*. The goal of this review was to examine the effectiveness of measures to improve patient medication adherence. *Methods*. Studies addressing treatment adherence in patients with RA were identified by trawling PsycINFO, Medline, Cochrane, Pubmed, and ProQuest for studies published between January 2000 and October 2014. Articles were independently reviewed to identify relevant studies. *Results*. Current strategies were of limited efficacy in improving patient adherence with medications used to treat RA. *Conclusion*. Poor medication adherence is a complex issue. Low educational levels and limited health literacy are contributory factors. Psychological models may assist in explaining medication nonadherence. Increasing patient knowledge of their disease seems sensible. Existing educational interventions appear ineffective at improving medication adherence, probably due to an overemphasis on provision of biomedical information. A novel approach to patient education using musculoskeletal ultrasound is proposed.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease with a prevalence of approximately 1% [1, 2]. Incompletely controlled RA results in severe progressive joint damage, functional disability, morbidity, and increased mortality [3]. Clarification of the molecular pathogenesis of RA has led to an increasing number of targeted therapies [4, 5]. Early intervention with disease-modifying antirheumatic drugs (DMARDs) and biological DMARDs (bDMARDs) improves long-term functional outcomes [6–9]. Depending upon the clinical situation, a realistic goal for every patient with RA is now low disease activity or disease remission [10].

Despite extensive evidence regarding drug efficacy and the risk of long-term harm from uncontrolled RA, medication adherence rates remain suboptimal, ranging from 30 to 80% [11–13]. Improving medication adherence with currently available DMARDs and bDMARDs would improve treatment effectiveness and reduce healthcare costs [14, 15]. Medication nonadherence is a dynamic, multifaceted issue affected by (i) patient factors, (ii) disease features, and (iii) drug characteristics. Although more effective interventions for

improving medication adherence are needed [16], medication nonadherence remains a poorly studied phenomenon [17].

Medication nonadherence can be classified as primary or secondary, both of which are influenced by different factors [18]. Primary nonadherence occurs when a patient fails to fill an initial prescription [19]. This is often influenced by socioeconomic factors, such as out-of-pocket medication costs [18, 20]. Secondary nonadherence occurs when a patient prematurely discontinues the medication [19]. This may be associated with factors such as lack of drug efficacy, slow disease response to treatment, and adverse drug reactions.

Both primary and secondary medication nonadherence may be affected by low levels of health literacy and patient education. These factors may compromise patient understanding of the adverse outcomes from poorly controlled RA. Patients with RA often lack sufficient understanding to make informed health-care decisions [21]. In contrast, those with greater appreciation of the risks and benefits of treatment were more likely to accept risk to achieve better outcomes [21]. Unfortunately, patient educational interventions usually have limited effectiveness at improving medication adherence [3, 12, 22–24].

While many factors influence drug adherence, this review will focus on the effectiveness of existing educational and health literacy interventions targeting medication adherence in patients with RA. Psychological models of medication adherence and, within this context, current and past educational interventions will be explored. The effect of health literacy and the utility of musculoskeletal ultrasound (MSKUS) as an educational tool will also be discussed.

2. Methods

We performed a computerised systematic literature search of five databases (PsycINFO, Medline, Cochrane, Pubmed and Proquest) using a wide range of search terms to identify English language, peer reviewed papers dealing with medication adherence in patients with RA published between January 2000 and October 2014 (Table 1). A similar search strategy was employed to identify relevant publications dealing with the effect of patient education and health literacy on medication adherence in patients with RA (Table 2). Citation tracking was also used to identify additional papers. The most relevant articles for inclusion in this review were identified by SJ, RVDZ, and PKKW using strict inclusion and exclusion criteria (Table 3).

This review favours the term "adherence" over "compliance" as it reflects a shift in the clinician-patient relationship since the 1950s when the term "compliance" was commonly used [25]. "Compliance" may have authoritarian connotations as it refers to concordance of patient behaviour with medical advice [11, 26]. In contrast, "adherence" implies patient-clinician collaboration rather than obedience to didactic dissemination of medical advice.

3. Cognitive Models of Medication Adherence

Many factors affect medication adherence in patients with RA. In patients commencing the "anchor" DMARD methotrexate (MTX), adherence could be explained by a strong belief in the necessity of treatment, although it was not influenced by the severity of functional impairment [27]. Higher levels of medication adherence have been associated with participation in a patient education programme [28], following provision of more information about RA treatment [29], Caucasian ethnicity [30], and less disability [31]. Older age has been associated with both increased [27, 29, 32] and decreased [31, 33] medication adherence. Concomitant medication use has been associated with both higher [34] and lower [30] levels of medication adherence. Patients with stronger beliefs about the necessity of medication and who believed medications were generally not overused were more likely to be adherent to medication [34]. Rheumatoid disease activity had a variable relationship with medication adherence [31, 32]. Higher out-of-pocket costs [20], employment [27, 29], and cognitive impairment [29] were associated with reduced adherence rates.

As outlined above, the inability to consistently identify factors accurately predicting medication nonadherence in patients with RA has spurred development of cognitive models to better explain this complex phenomenon [11, 12]. One of the most widely used models is the Health Belief Model (HBM) which is a theoretical framework postulating that adherence decisions are based on implicit cost-benefit analyses, where the extent to which a patient views the necessity of medication is evaluated against concern about potential side effects [35]. The HBM was built on the premise that the likelihood of a person actively responding to a health threat depended on four key factors: (i) perceived illness threat (determined by disease severity and susceptibility), (ii) expectation of positive outcome (anticipated benefits of treatment), (iii) barriers associated with treatment (expected costs and drawbacks), and (iv) the extent to which they intend to adhere to treatment [36, 37].

The cost-benefit assessment offered by the HBM has been quantified as a necessity-concerns differential within the Beliefs about Medications Questionnaire (BMQ) [35, 38]. This user-friendly tool comprises two five-item scales evaluating patient beliefs about the necessity of medication relative to concern about adverse effects and predicts medication adherence more robustly than clinical or sociodemographic factors [35, 36]. When applied to a population with chronic illness such as asthma, renal or cardiac failure, and cancer, it was found that patients whose concern about medication outweighed their belief about the necessity of medication were less adherent to pharmacologic treatment [35]. This may have been an adaptive strategy to minimise potential harm from side effects or may have reflected how strongly patients believed medications were essential; that is, those believing them to be less necessary were more prone to forgetting to take them. Interestingly, many of the perceived "costs" arose from erroneous beliefs, for example, concerns regarding drug dependence [35].

Patients with RA often lacked the understanding required to make informed cost-benefit analyses leading to overestimation of medication risks [21, 39]. Medication risks were often thought to be high relative to surgery possibly because surgical risks were more tangible, making patients more likely

Table 1: Databases accessed displaying search terms employed, results, access limitation, reasons for exclusion and accepted papers dealing with factors affecting medication adherence in patients with rheumatoid arthritis.

Database *Search terms*	Number of papers identified	Number of full text peer reviewed papers able to be accessed	Reasons for exclusion (number)	Number of papers identified for inclusion [ref. number]
PsycINFO via EBSCO host *Rheumatoid arthritis* [and] *Compliance* [and] *Medication*	23	5	Juvenile population (1) Review paper (1) Not relevant (2)	$n = 1$ [34]
ProQuest (general) *Adherence* [And] *rheumatoid arthritis* [and] *Relationship*	3099	1891	Already included (1) Review papers (2) Juvenile population (1) Not relevant (1886)	$n = 1$ [87]
ProQuest (general) *Rheumatoid arthritis* [and] *Compliance* [and] *Medication*	4391	2118	Already included (1) Juvenile population (1) Not relevant (2114)	$n = 2$ [27, 28]
ProQuest (general) *Adherence* [or] *compliance* [and] *rheumatoid arthritis* [and] *Factors* [or] *predictors*	10265	5486	Already included (3) Not relevant (5481)	$n = 2$ [29, 32]
PubMed *rheumatoid arthritis* [and] *factors* [and] *Medication persistence*	48	15	Already included (3) Unrepresentative population (2) Not relevant (9)	$n = 1$ [31]
PubMed *Rheumatoid arthritis* [and] *Compliance* [and] *Medication*	163	138	Review/meta-analysis (2) Juvenile population (2) Not relevant (133)	$n = 1$ [20]
PubMed *Adherence* [or] *compliance* [and] *rheumatoid arthritis* [and] *Factors* [or] *predictors*	17	9	Osteoporosis management (1) Not relevant (7)	$n = 1$ [30]

to accept surgery even when this was associated with fewer benefits [21]. This highlights the need for effective educational interventions as patients well informed about the risks and benefits of medication performed more biomedically oriented cost-benefit analyses [39]. Those with a greater understanding of the risks and benefits of treatment were more inclined to accept risk in the pursuit of successful disease outcomes [21]. Unwillingness to accept risk compounded by poor understanding of the benefit of conventional biomedical treatment may explain the large number of patients using complementary or alternative medicines (CAMs) as these are often thought to have minimal risk [27, 40, 41]. While most CAMs are tested for safety, rigorous tests of efficacy are scarce [41, 42]. This highlights the importance of explaining the benefits and not just potential side effects of conventional treatment.

Clinicians should view patients as active decision-makers with a vested interest in their health who would be more adherent to medication if they believed the necessity of medication outweighed concerns about adverse effects [35]. Alas, due to medicolegal considerations clinicians often spend more time discussing the adverse effects of treatment

Table 2: Databases accessed displaying search terms employed, quantity of results, access limitation, reasons for exclusion, and accepted papers dealing with the effect of patient education and literacy on medication adherence in patients with rheumatoid arthritis.

Database *Search terms*	Results	Reasons for exclusion	Accepted citations [ref. number]
PsycINFO via OvidSP host *Rheuma* arthritis [or] Rheuma* disease [or] RA* [and] *Complian* [or] non?compli* [or]* adherence *[or] non?adherence [or]* refusal *[or] regime** [and] *Medic* [or]* pharmac* [or] drug [or] treatment [or] therapy [or] biologic [or] ?DMARD? [or] disease?modifying* [and] Patient education* [or] intervention [or] strategy [or] knowledge [or] health liter* [or] understanding	20	$n = 2$, juvenile/paediatric population $n = 4$, review/qualitative/book $n = 13$, not relevant	$n = 1$ [58]
EBM Reviews, All-Cochrane DSR, ACP Journal Club, DARE, and CCTR OvidSP host *Rheuma* arthritis [or] RA [or] rheama* disease* [and] *Complian* [or] non?compli* [or]* adherence *[or] non?adherence [or]* refusal *[or] regime* [and] *Medic* [or]* pharmac* [or] drug [or] treatment [or] therapy [or] biologic [or] ?DMARD?	473	$n = 3$, already included $n = 469$, not relevant	$n = 1$ [75]
MedLine via OvidSP host *Rheuma* arthritis [or] RA [or] rheama* disease* [and] *Complian* [or] non?compli* [or]* adherence *[or] non?adherence [or]* refusal *[or] regime* [and] *Medic* [or]* pharmac* [or] drug [or] treatment [or] therapy [or] biologic [or] ?DMARD? [and] Medication	221	$n = 3$, already included $n = 1$, juvenile population $n = 4$, review/qualitative/book $n = 210$, not relevant $n = 1$, female-only sample	$n = 2$ [28, 59]

The asterisk star is a wildcard search character. In this instance, it represents a string of characters, for example, the ∗ matches zero or more characters, so rheum ∗ will generate rheumatoid, rheumatology, rheumatic and so forth.

Table 3: Inclusion and exclusion criteria for identification of relevant papers.

Selection criteria	Inclusion criteria	Exclusion criteria
Participants	Human Adult American College of Rheumatology criteria for the diagnosis of RA	Animal Under 18 years Gender-specific Geriatric
Research design	Experimental Longitudinal	Qualitative Observational Case studies Editorials Cross-sectional Retrospective cohort study
Measurement scales	Validated questionnaires Blood assays Records/claims (pharmaceutical/insurance) Event monitoring	Interviews
RA-specific medication	Disease modifying antirheumatic drugs (DMARDS) Biological DMARDS Slow acting antirheumatic drugs (SAARDS)	Analgesics only Nonsteroidal anti-inflammatory drugs Corticosteroids only

rather than the benefits. This has probably been influenced by landmark cases such as the 1992 decision in *Rogers v. Whitaker* (1992) 175 CLR 479, which established in Australia the standard of care required when a doctor provides information to a patient about the risk of a proposed intervention [43]. The High Court of Australia affirmed that an ophthalmic surgeon should have warned his patient of the one in 14,000 chance of a rare complication (sympathetic ophthalmia) with its associated risk of blindness arising from a procedure. This was despite evidence tendered during the hearing that many of the defendant's colleagues would not have told their patients about the risk of such a rare complication.

The BMQ has been used in a cross-sectional study to describe the tension experienced by RA patients when assessing the importance of medication versus their concern regarding side effects [36]. Most respondents of a postal survey ($n = 344$) mailed to over 600 patients with RA agreed that their medication was necessary for health. However, almost half were concerned about potential adverse effects and this was associated with nonadherence [36]. The observed similarity in disease knowledge between adherent and nonadherent patients raises doubts about the effectiveness of educational interventions which merely increase knowledge [36].

Another psychological model used to describe medication adherence is Leventhal and colleagues' Self-Regulatory Model (SRM) [44, 45]. This is a hierarchically organised model of illness adaptation based on three primary constructs: illness representations, coping responses, and appraisal of coping responses [46, 47]. Illness representations or "lay beliefs" are defined as complex schemas from personal and familial experiences influencing how patients perceive and cope with chronic illness [48]. Cognitive and emotional illness representations form the crux of the SRM as these representations are integrated into patients' preexisting lay belief schemas and help them understand symptoms while moderating coping responses [46] and thus medication adherence [44].

A qualitative study involving semistructured interviews of 30 women with RA found that a positive patient-healthcare practitioner relationship was an important factor in the decision making process [30]. Potential and perceived adverse effects were powerful factors associated with nonadherence or discontinuation of medication [30]. These findings highlighted the importance of clear, helpful patient-practitioner communication and the need for healthcare practitioners to balance patient concerns about adverse effects with the likely benefits of treatment adherence.

Exploring medication adherence through the conceptual frameworks offered by the HBM and SRM suggests some practical strategies to improve medication adherence. Clinicians should address patient concerns about adverse effects by highlighting the positive outcomes associated with treatment [44]. The lack of association between factual knowledge and medication adherence highlights the need for more effective educational interventions [39]. The SRM suggests clinicians need to consider existing lay belief schemas used by patients to evaluate and cope with medical advice [46].

4. Assessment of Adherence

Medication adherence has been assessed by Electronic Medication Event Monitoring (EMEM) [49], pharmacy records [20, 50], self-report measures (e.g., the Compliance-Questionnaire-Rheumatology (CQR)) [49, 51], and more objective but invasive measures such as serum drug assays [28, 49]. Although susceptible to recall and social desirability biases, self-report measures represent the most pragmatic method for assessing medication adherence [35, 44]. Self-report measures such as the CQR provide clinicians with valuable information regarding how closely patients follow medication advice and their beliefs regarding the necessity of treatment [28, 35, 49]. The CQR is a 19-item rheumatology-specific questionnaire assessing medication adherence which also identifies factors associated with poor adherence [49, 51]. This tool demonstrated strong predictive validity when tested against EMEM [49]. A study of 126 RA patients which used the CQR to measure adherence and the BMQ to assess medication beliefs at initiation of MTX therapy found adherence rates during the first year of treatment could be explained by medication beliefs such as the perceived necessity of MTX [27]. Interestingly, supplying the treating clinician with information regarding medication adherence patterns does not influence patients' medication beliefs, nor does it engender any changes in adherence [52].

5. Educational Interventions and Medication Adherence

There is broad consensus regarding the importance of educational interventions for patients suffering from chronic illness such as RA [22]. A cross-sectional study of 33 RA patients with disease duration less than one year and 69 with disease duration greater than 10 years found both groups desired more information about their condition [53]. Educational interventions have usually entailed provision of information to patients about the disease and possible treatments [3] on the premise that increased knowledge leads to positive attitudes and behaviours which may be associated with small reductions in pain and disability [54, 55]. Many clinicians therefore regard education as important for equipping patients with the tools and coping strategies to manage disease flares [3, 56]. However, the effectiveness of educational interventions at improving medication adherence is questionable [3, 22, 23, 48], with potentially few short-term benefits [24].

A randomised controlled trial of 100 patients with RA found that patient education was associated with increased medication adherence [28]. Participants with active RA were randomised to an experimental group which received seven 30-minute one-on-one sessions with a rheumatology nurse aimed at increasing self-efficacy or a control group which received standard treatment. At the end of six months those in the experimental group were more adherent to medication [28]. However, others have found that patients with recent onset active RA had high levels of medication adherence regardless of participation in an educational programme

which involved group meetings with an instructor who addressed erroneous patient beliefs and provided information about RA medication, the importance of physical activity, and joint conservation [23]. This suggested that educational interventions may not be needed in patients with recent onset active RA.

Although not specifically dealing with RA patients, a randomised controlled trial involving a pharmacist-delivered telephone service to patients at home led to increased medication adherence, fewer reports of medication side effects, and more positive medication beliefs [57]. This intervention appeared to shift the patient cost-benefit analysis to favour treatment benefits, thereby increasing the proportion of adherent patients [57].

A recent study tested the effect of a motivational interviewing (MI) programme on medication adherence in RA patients using a group-based format [58]. In this study, 123 participants were randomised to either the control or intervention group; the latter received two pharmacist-delivered MI sessions. These sessions aimed to resolve barriers to adherence with a practical, problem solving approach. The trial did not demonstrate any significant change to patient beliefs or medication adherence, possibly due to a "Hawthorne effect" or suboptimal integrity of the intervention [58].

In a pilot study, patients were randomly allocated to either group-based counselling or individual counselling [59]. Adherence, as measured by pill counts, was higher in patients who were counselled in a group-based format (90%) compared to those counselled individually (69%). However, probably due to lack of power, there was no statistically significant difference between treatment groups.

A systematic review found that educational interventions in patients with RA appeared to have a positive impact upon short-term outcomes but long-term improvements in health status were not clearly evident [22]. A Cochrane review of 31 randomised controlled trials found that patient education was associated with short-term benefits on disability, patient global assessment, and psychological status [60]. However, no long-term benefits were identified. Although clinical trials have demonstrated that educational interventions enhanced patient knowledge and understanding of their disease, there is conflicting evidence regarding their effects on medication adherence [3, 61]. This suggests that education alone is insufficient to increase medication adherence as medication beliefs appear to be influenced by more than information [3, 35].

Existing educational interventions may be limited by an overemphasis on improving patient knowledge [48]. This creates a power imbalance in the therapeutic interaction as the rheumatologist assumes the patient has no prior knowledge of their disease and should fulfil a passive role in the exchange and that provision of information is sufficient. Such assumptions give little credence to lay beliefs. While often exhibiting internal consistency, lay beliefs are seldom congruent with biomedical concepts but can strongly influence patient response to advice and treatment proposed by their rheumatologist [48].

When interviewed before and after a consultation with their rheumatologist, patients with RA were more accepting of and more adherent to suggested treatment if the advice provided during the consultation aligned with their lay beliefs [48]. Likewise, those who received information during the consultation incongruent with their lay beliefs were more likely to reject the management offered [48]. These findings can be interpreted through the conceptual framework of the SRM, which highlights the importance of existing patient lay belief schemas [46]. It is important to recognise that patient and clinician disease perceptions may diverge. Patient views are influenced by subjective information such as pain levels, whereas clinicians' views are usually informed by biomedical factors such as swollen and tender joint counts [62]. In light of such findings, an interactive educational process emphasising the active role of both clinician and patient might enable a more harmonious interweaving of biomedical information into preexisting lay belief systems [48].

6. Health Literacy and Medication Adherence

Health literacy refers to the ability to acquire, comprehend, and pursue health information to guide health-related decisions [55, 63, 64]. Individuals with limited health literacy have poorer health outcomes due to poor self-management, limited health responsibility, and underutilisation of healthcare resources [55]. Up to 42% of patients with chronic musculoskeletal disease may have low health literacy [65] while up to one-third of patients incorrectly followed dosing instructions for common rheumatology drugs [66]. A cross-sectional study of 110 patients with RA found that poor health literacy was associated with functional impairment as measured by the Multidimensional Health Assessment Questionnaire (MDHAQ) [67]. Analysis of data from 6052 patients with RA enrolled in a prospective observational study found that health literacy was found to predict functional status more robustly than corticosteroid and biologic use, smoking history, and education [68]. This suggests that some of the observed benefit on medication adherence following educational interventions may be due to a positive effect on patient health literacy and raises the possibility that better outcomes could be achieved by improving health literacy rather than semantic disease knowledge [67].

The multidimensional causal model of Paasche-Orlow and Wolf has been used to explain the link between low health literacy and poor patient outcomes [69]. This model attributed poor patient outcomes from low health literacy to the challenges posed by (i) communicating with clinicians, (ii) accessing and consuming health services, and (iii) effective self-care. Effective self-care requires patients to possess the knowledge and capacity to understand and implement their medication regime [69]. Low health literacy impairs patient ability to comprehend medication labels and instructions and to recall medication names [64, 69]. Accordingly, educational interventions should aim to increase self-care and patient awareness of available healthcare resources and minimise the communication barrier between patients and clinicians.

A practical suggestion is that clinicians should use visual tools such as videos and pictorial aids to assist in meaningful

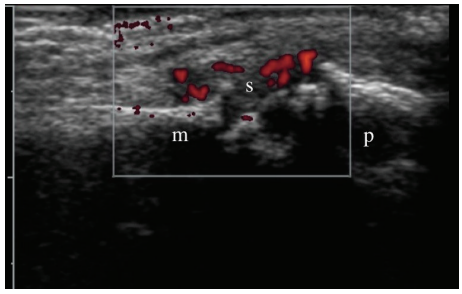


Figure 1: Longitudinal sonographic view of the right second metacarpophalangeal joint (m: metacarpal, p: proximal phalanx) in a patient with rheumatoid arthritis showing synovitis/effusion (s) with power Doppler flow (red).

delivery of key health messages [64]. The beneficial effect of visual images within the consultation is further enhanced when combined with meaningful written or verbal information and has been shown to enhance patient comprehension and, ultimately, medication adherence [70]. This further highlights the need for clinician-patient collaboration within the consultation and a multimodal approach for communicating patient information.

7. The Use of Musculoskeletal Ultrasonography as an Educational Tool

Increasing evidence suggests that health interventions are more effective when they contain visual elements and simple, comprehensible information in an accessible format [64, 71–74]. Incorporation of pictures into medication instructions improved patient understanding, recollection, and adherence to medication regimes [70]. A double blind randomised controlled trial of 111 patients with early inflammatory arthritis found that provision of visual feedback for patients in the form of charts depicting disease activity significantly increased patient adherence to DMARDs [75]. This was associated with less disease activity at 12 months. These findings suggest that incorporating visual feedback into clinical practice may have a positive effect on treatment adherence and, ultimately, disease management.

Musculoskeletal ultrasound (MSKUS) is increasingly used to assist in diagnosis and monitoring of inflammatory arthritis and to guide joint injection and aspiration [76–79]. The presence of power Doppler signal (Figure 1) in patients whose RA appeared to be in clinical remission was helpful in predicting disease flare and radiographic outcome [80]. Auto-feedback from US assessment of joints in patients with RA quickly improved joint palpation skills [81]. A 12-week MSKUS course was useful in undergraduate teaching of joint anatomy and pathology [82]. Despite these benefits, MSKUS is underutilised in clinical practice and is routinely performed by less than 50% of rheumatologists in Europe [76]. Few rheumatologists in Australia routinely use MSKUS.

Musculoskeletal ultrasound may prove a valuable educational tool in clinical practice [83]. The treating clinician is able to enhance patient understanding "real-time" through demonstration of joint structures, articular and periarticular damage, and synovial inflammation. In particular, the ability to navigate around the site of interest and to show motion information may improve patient understanding more than provision of static images [84, 85]. Careful use of the device highlighting critical features for patients using their own anatomy is important in providing visual learning cues. This type of disease visualisation, when combined with clinician-patient interaction at close quarters, may improve adaptation to the diagnosis, thereby increasing medication adherence and disease self-control. A recent study involving patients with active RA (DAS28 score > 2.6) found that showing RA patients "real-time" US images of their clinically inflamed joints resulted in a more favourable cost-benefit analysis as measured by the BMQ, that is, increased patient belief in the necessity of medication versus concern about taking medication [86]. However, this finding needs to be confirmed by larger, longer-term studies.

8. Summary

The effectiveness of commonly used educational interventions targeting poor medication adherence is questionable. Educational interventions have generally focused on provision of information [48]. While these increase patient knowledge of the disease, they have not been reliably associated with increased medication adherence or improvement in long-term health status [24, 61]. There is a tension experienced by RA patients when assessing the necessity of medication against concern regarding adverse effects [36]. The goal of education should be to provide understandable information to patients to allow them to make informed healthcare decisions.

Educational interventions should incorporate more clinician-patient interaction [27]. Clinicians should deemphasise biomedical information and give more consideration to patient lay beliefs regarding clinical management without compromising use of evidence-based treatments that halt disease progression. This should allow clinicians to allay patient concerns while highlighting treatment benefits [35, 44]. Integrating MSKUS into an evidence-based educational framework may be beneficial because of its clinical value and as an educative tool to increase patient understanding of their disease [83, 86].

Musculoskeletal US may foster increased patient-clinician interaction and disease visualization, factors which have been lacking in traditional educational frameworks. Patients will also benefit from increased appreciation of joint structures and greater understanding of the long-term implications of joint damage and inflammation. These measures may address some of the limitations of previous interventions and hopefully result in increased patient medication adherence and better disease control.

Conflict of Interests

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

References

[1] D. M. Lee and M. E. Weinblatt, "Rheumatoid arthritis," *The Lancet*, vol. 358, no. 9285, pp. 903–911, 2001.

[2] D. L. Scott, F. Wolfe, and T. W. J. Huizinga, "Rheumatoid arthritis," *The Lancet*, vol. 376, no. 9746, pp. 1094–1108, 2010.

[3] R. A. Elliott, "Poor adherence to medication in adults with rheumatoid arthritis: reasons and solutions," *Disease Management and Health Outcomes*, vol. 16, no. 1, pp. 13–29, 2008.

[4] I. B. McInnes and G. Schett, "Cytokines in the pathogenesis of rheumatoid arthritis," *Nature Reviews Immunology*, vol. 7, no. 6, pp. 429–442, 2007.

[5] K. S. Upchurch and J. Kay, "Evolution of treatment for rheumatoid arthritis," *Rheumatology*, vol. 51, supplement 6, Article ID kes278, pp. vi28–vi36, 2012.

[6] C. Escalas, M. Dalichampt, B. Combe et al., "Effect of adherence to European treatment recommendations on early arthritis outcome: data from the ESPOIR cohort," *Annals of the Rheumatic Diseases*, vol. 71, no. 11, pp. 1803–1808, 2012.

[7] B. Combe, R. Landewe, C. Lukas et al., "EULAR recommendations for the management of early arthritis: report of a task force of the European Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT)," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 34–45, 2007.

[8] Y. P. M. Goekoop-Ruiterman, J. K. de Vries-Bouwstra, C. F. Allaart et al., "Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial," *Arthritis & Rheumatism*, vol. 52, no. 11, pp. 3381–3390, 2005.

[9] S. A. Saunders, H. A. Capell, A. Stirling et al., "Triple therapy in early active rheumatoid arthritis: a randomized, single-blind, controlled trial comparing step-up and parallel treatment strategies," *Arthritis and Rheumatism*, vol. 58, no. 5, pp. 1310–1317, 2008.

[10] J. A. Singh, D. E. Furst, A. Bharat et al., "2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis," *Arthritis Care & Research*, vol. 64, no. 5, pp. 625–639, 2012.

[11] B. J. F. van den Bemt, H. E. Zwikker, and C. H. M. van den Ende, "Medication adherence in patients with rheumatoid arthritis: a critical appraisal of the existing literature," *Expert Review of Clinical Immunology*, vol. 8, no. 4, pp. 337–351, 2012.

[12] B. J. F. van den Bemt and W. G. J. M. van Lankveld, "How can we improve adherence to therapy by patients with rheumatoid arthritis?" *Nature Clinical Practice Rheumatology*, vol. 3, no. 12, p. 681, 2007.

[13] B. J. F. van den Bemt, F. H. J. van den Hoogen, B. Benraad, Y. A. Hekster, P. L. C. M. van Riel, and W. van Lankveld, "Adherence rates and associations with nonadherence in patients with rheumatoid arthritis using disease modifying antirheumatic drugs," *Journal of Rheumatology*, vol. 36, no. 10, pp. 2164–2170, 2009.

[14] K. P. Krueger, B. G. Felkey, and B. A. Berger, "Improving adherence and persistence: a review and assessment of interventions and description of steps toward a national adherence initiative," *Journal of the American Pharmacists Association*, vol. 43, no. 6, pp. 668–679, 2003.

[15] E. Sabatâe and World Health Organization, *Adherence to Long-Term Therapies: Evidence for Action*, World Health Organization, Geneva, Switzerland, 2003.

[16] M. A. Blum, D. Koo, and J. A. Doshi, "Measurement and rates of persistence with and adherence to biologics for rheumatoid arthritis: a systematic review," *Clinical Therapeutics*, vol. 33, no. 7, pp. 901–913, 2011.

[17] P. Butow and L. Sharpe, "The impact of communication on adherence in pain management," *Pain*, vol. 154, Supplement 1, pp. S101–S107, 2013.

[18] B. Hovstadius and G. Petersson, "Non-adherence to drug therapy and drug acquisition costs in a national population—a patient-based register study," *BMC Health Services Research*, vol. 11, no. 1, pp. 1–11, 2011.

[19] B. A. Fallis, I. A. Dhalla, J. Klemensberg, and C. M. Bell, "Primary medication non-adherence after discharge from a general internal medicine service," *PLoS ONE*, vol. 8, no. 5, Article ID e61735, 2013.

[20] S. Curkendall, V. Patel, M. Gleeson, R. S. Campbell, M. Zagari, and R. Dubois, "Compliance with biologic therapies for rheumatoid arthritis: do patient out-of-pocket payments matter?" *Arthritis Care and Research*, vol. 59, no. 10, pp. 1519–1526, 2008.

[21] M. Ho, B. Lavery, and T. Pullar, "The risk of treatment. A study of rheumatoid arthritis patients' attitudes," *British Journal of Rheumatology*, vol. 37, no. 4, pp. 459–460, 1998.

[22] K. Niedermann, J. Fransen, R. Knols, and D. Uebelhart, "Gap between short- and long-term effects of patient education in rheumatoid arthritis patients: a systematic review," *Arthritis Care & Research*, vol. 51, no. 3, pp. 388–398, 2004.

[23] H. L. M. Brus, M. A. F. J. Van De Laar, E. Taal, J. J. Rasker, and O. Wiegman, "Effects of patient education on compliance with basic treatment regimens and health in recent onset active rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 57, no. 3, pp. 146–151, 1998.

[24] R. P. Riemsma, E. Taal, J. R. Kirwan, and J. J. Rasker, "Patient education programmes for adults with rheumatoid arthritis: benefits are small and short lived," *British Medical Journal*, vol. 325, no. 7364, pp. 558–559, 2002.

[25] E. Salt and S. K. Frazier, "Adherence to disease-modifying antirheumatic drugs in patients with rheumatoid arthritis: a narrative review of the literature," *Orthopaedic Nursing*, vol. 29, no. 4, pp. 260–275, 2010.

[26] G. J. Treharne, A. C. Lyons, E. D. Hale, K. M. J. Douglas, and G. D. Kitas, "'Compliance' is futile but is 'concordance' between rheumatology patients and health professionals attainable?" *Rheumatology*, vol. 45, no. 1, pp. 1–5, 2006.

[27] A. de Thurah, M. Nørgaard, I. Harder, and K. Stengaard-Pedersen, "Compliance with methotrexate treatment in patients with rheumatoid arthritis: influence of patients' beliefs about the medicine. A prospective cohort study," *Rheumatology International*, vol. 30, no. 11, pp. 1441–1448, 2010.

[28] J. Hill, H. Bird, and S. Johnson, "Effect of patient education on adherence to drug treatment for rheumatoid arthritis: a randomised controlled trial," *Annals of the Rheumatic Diseases*, vol. 60, no. 9, pp. 869–875, 2001.

[29] R. Müller, R. Kallikorm, K. Põlluste, and M. Lember, "Compliance with treatment of rheumatoid arthritis," *Rheumatology International*, vol. 32, no. 10, pp. 3131–3135, 2012.

[30] E. Salt and A. Peden, "The complexity of the treatment: the decision-making process among women with rheumatoid arthritis," *Qualitative Health Research*, vol. 21, no. 2, pp. 214–222, 2011.

[31] V. Pascual-Ramos, I. Contreras-Yáñez, A. R. Villa, J. Cabiedes, and M. Rull-Gabayet, "Medication persistence over 2 years of

follow-up in a cohort of early rheumatoid arthritis patients: associated factors and relationship with disease activity and with disability," *Arthritis Research and Therapy*, vol. 11, no. 1, article R26, 2009.

[32] R. Tuncay, E. Eksioglu, B. Cakir, E. Gurcay, and A. Cakci, "Factors affecting drug treatment compliance in patients with rheumatoid arthritis," *Rheumatology International*, vol. 27, no. 8, pp. 743–746, 2007.

[33] M. Wong and D. Mulherin, "The influence of medication beliefs and other psychosocial factors on early discontinuation of disease-modifying anti-rheumatic drugs," *Musculoskeletal Care*, vol. 5, no. 3, pp. 148–159, 2007.

[34] G. J. Treharne, A. C. Lyons, and G. D. Kitas, "Medication adherence in rheumatoid arthritis: effects of psychosocial factors," *Psychology, Health and Medicine*, vol. 9, no. 3, pp. 337–349, 2004.

[35] R. Horne and J. Weinman, "Patients' beliefs about prescribed medicines and their role in adherence to treatment in chronic physical illness," *Journal of Psychosomatic Research*, vol. 47, no. 6, pp. 555–567, 1999.

[36] R. Neame and A. Hammond, "Beliefs about medications: a questionnaire survey of people with rheumatoid arthritis," *Rheumatology*, vol. 44, no. 6, pp. 762–767, 2005.

[37] K. Lorig, *Patient Education: A Practical Approach*, Sage, Thousand Oaks, Calif, USA, 2001.

[38] R. Horne, J. Weinman, and M. Hankins, "The beliefs about medicines questionnaire: the development and evaluation of a new method for assessing the cognitive representation of medication," *Psychology & Health*, vol. 14, no. 1, pp. 1–24, 1999.

[39] D. Berry, A. Bradlow, and E. Bersellini, "Perceptions of the risks and benefits of medicines in patients with rheumatoid arthritis and other painful musculoskeletal conditions," *Rheumatology*, vol. 43, no. 7, pp. 901–905, 2004.

[40] M. C. Jong, L. van de Vijver, M. Busch, J. Fritsma, and R. Seldenrijk, "Integration of complementary and alternative medicine in primary care: what do patients want?" *Patient Education and Counseling*, vol. 89, no. 3, pp. 417–422, 2012.

[41] G. J. Macfarlane, A. El-Metwally, V. de Silva, E. Ernst, G. L. Dowds, and R. J. Moots, "Evidence for the efficacy of complementary and alternative medicines in the management of rheumatoid arthritis: a systematic review," *Rheumatology*, vol. 50, no. 9, Article ID ker119, pp. 1672–1683, 2011.

[42] I. H. Kerridge and J. R. McPhee, "Ethical and legal issues at the interface of complementary and conventional medicine," *Medical Journal of Australia*, vol. 181, no. 3, pp. 164–166, 2004.

[43] J. McPhee, "Perceptions of risk—a legal perspective," *Australian Prescriber*, vol. 25, no. 5, pp. 114–115, 2002.

[44] L. B. Nicklas, M. Dunbar, and M. Wild, "Adherence to pharmacological treatment of non-malignant chronic pain: the role of illness perceptions and medication beliefs," *Psychology and Health*, vol. 25, no. 5, pp. 601–615, 2010.

[45] H. Leventhal, M. Diefenbach, and E. A. Leventhal, "Illness cognition: using common sense to understand treatment adherence and affect cognition interactions," *Cognitive Therapy and Research*, vol. 16, no. 2, pp. 143–163, 1992.

[46] E. D. Hale, G. J. Treharne, and G. D. Kitas, "The Common-Sense Model of self-regulation of health and illness: how can we use it to understand and respond to our patients' needs?" *Rheumatology*, vol. 46, no. 6, pp. 904–906, 2007.

[47] D. Nerenz and H. Leventhal, *Self-Regulation Theory in Chronic Illness*, Academic Press, New York, NY, USA, 1983.

[48] J. L. Donovan, D. R. Blake, and W. G. Fleming, "The patient is not a blank sheet: lay beliefs and their relevance to patient education," *Rheumatology*, vol. 28, no. 1, pp. 58–61, 1989.

[49] E. de Klerk, D. van der Heijde, R. Landewé, H. van der Tempel, and S. van der Linden, "The compliance-questionnaire-rheumatology compared with electronic medication event monitoring: a validation study," *The Journal of Rheumatology*, vol. 30, no. 11, pp. 2469–2475, 2003.

[50] S. de Achaval and M. E. Suarez-Almazor, "Improving treatment adherence in patients with rheumatologic disease," *The Journal of Musculoskeletal Medicine*, vol. 27, no. 10, pp. 388–394, 2010.

[51] E. de Klerk, D. van der Heijde, H. van der Tempel, and S. van der Linden, "Development of a questionnaire to investigate patient compliance with antirheumatic drug therapy," *The Journal of Rheumatology*, vol. 26, no. 12, pp. 2635–2641, 1999.

[52] B. J. F. van den Bemt, A. A. den Broeder, F. H. J. van den Hoogen et al., "Making the rheumatologist aware of patients' non-adherence does not improve medication adherence in patients with rheumatoid arthritis," *Scandinavian Journal of Rheumatology*, vol. 40, no. 3, pp. 192–196, 2011.

[53] J. H. Barlow, L. A. Cullen, and I. F. Rowe, "Comparison of knowledge and psychological well-being between patients with a short disease duration (≤1 year) and patients with more established rheumatoid arthritis (≥10 years duration)," *Patient Education and Counseling*, vol. 38, no. 3, pp. 195–203, 1999.

[54] A. Warsi, M. P. laValley, P. S. Wang, J. Avorn, and D. H. Solomon, "Arthritis self-management education programs: a meta-analysis of the effect on pain and disability," *Arthritis and Rheumatism*, vol. 48, no. 8, pp. 2207–2213, 2003.

[55] R. J. Adams, "Improving health outcomes with better patient understanding and education," *Risk Management and Healthcare Policy*, vol. 3, pp. 61–72, 2010.

[56] P. C. Hirano, D. D. Laurent, and K. Lorig, "Arthritis patient education studies, 1987–1991: a review of the literature," *Patient Education and Counseling*, vol. 24, no. 1, pp. 9–54, 1994.

[57] S. Clifford, N. Barber, R. Elliott, E. Hartley, and R. Horne, "Patient-centred advice is effective in improving adherence to medicines," *Pharmacy World and Science*, vol. 28, no. 3, pp. 165–170, 2006.

[58] H. E. Zwikker, C. H. van den Ende, W. G. van Lankveld et al., "Effectiveness of a group-based intervention to change medication beliefs and improve medication adherence in patients with rheumatoid arthritis: a randomized controlled trial," *Patient Education and Counseling*, vol. 94, no. 3, pp. 356–361, 2014.

[59] D. Homer, P. Nightingale, and P. Jobanputra, "Providing patients with information about disease-modifying anti-rheumatic drugs: individually or in groups? A pilot randomized controlled trial comparing adherence and satisfaction," *Musculoskeletal Care*, vol. 7, no. 2, pp. 78–92, 2009.

[60] R. P. Riemsma, J. R. Kirwan, E. Taal, and J. J. Rasker, "Patient education for adults with rheumatoid arthritis," *The Cochrane Database of Systematic Reviews*, no. 2, Article ID CD003688, 2003.

[61] F. J. Keefe and Y. van Horn, "Cognitive-behavioral treatment of rheumatoid arthritis pain: maintaining treatment gains," *Arthritis Care & Research*, vol. 6, no. 4, pp. 213–222, 1993.

[62] P. Studenic, H. Radner, J. S. Smolen, and D. Aletaha, "Discrepancies between patients and physicians in their perceptions of rheumatoid arthritis disease activity," *Arthritis & Rheumatism*, vol. 64, no. 9, pp. 2814–2823, 2012.

[63] D. Nutbeam, "The evolving concept of health literacy," *Social Science and Medicine*, vol. 67, no. 12, pp. 2072–2078, 2008.

[64] N. J. Roberts, R. Ghiassi, and M. R. Partridge, "Health literacy in COPD," *International Journal of Chronic Obstructive Pulmonary Disease*, vol. 3, no. 4, pp. 499–507, 2008.

[65] Y. K. Loke, I. Hinz, X. Wang, G. Rowlands, D. Scott, and C. Salter, "Impact of health literacy in patients with chronic musculoskeletal disease-systematic review," *PLoS ONE*, vol. 7, no. 7, Article ID e40210, 2012.

[66] P. K. K. Wong, L. Christie, H. Johnston et al., "How well do patients understand written instructions? Health literacy assessment in rural and urban rheumatology outpatients," *Medicine*, vol. 93, no. 25, article e129, 2014.

[67] J. M. Hirsh, D. J. Boyle, D. H. Collier et al., "Limited health literacy is a common finding in a public health hospital's rheumatology clinic and is predictive of disease severity," *Journal of Clinical Rheumatology*, vol. 17, no. 5, pp. 236–241, 2011.

[68] L. Caplan, F. Wolfe, K. Michaud, I. Quinzanos, and J. M. Hirsh, "Strong association of health literacy with functional status among rheumatoid arthritis patients: a cross-sectional study," *Arthritis Care & Research*, vol. 66, no. 4, pp. 508–514, 2014.

[69] M. K. Paasche-Orlow and M. S. Wolf, "The causal pathways linking health literacy to health outcomes," *The American Journal of Health Behavior*, vol. 31, supplement 1, pp. S19–S26, 2007.

[70] M. G. Katz, S. Kripalani, and B. D. Weiss, "Use of pictorial aids in medication instructions: a review of the literature," *American Journal of Health-System Pharmacy*, vol. 63, no. 23, pp. 2391–2397, 2006.

[71] M. H. Eckman, R. Wise, A. C. Leonard et al., "Impact of health literacy on outcomes and effectiveness of an educational intervention in patients with chronic diseases," *Patient Education and Counseling*, vol. 87, no. 2, pp. 143–151, 2012.

[72] S. Kripalani, R. Robertson, M. H. Love-Ghaffari et al., "Development of an illustrated medication schedule as a low-literacy patient education tool," *Patient Education and Counseling*, vol. 66, no. 3, pp. 368–377, 2007.

[73] D. H. Frøisland, E. Årsand, and F. Skårderud, "Improving diabetes care for young people with type 1 diabetes through visual learning on mobile phones: mixed-methods study," *Journal of Medical Internet Research*, vol. 14, no. 4, article e111, 2012.

[74] A. V. Mohan, M. B. Riley, D. R. Boyington, and S. Kripalani, "Illustrated medication instructions as a strategy to improve medication management among Latinos: a qualitative analysis," *Journal of Health Psychology*, vol. 18, no. 2, pp. 187–197, 2013.

[75] Y. El Miedany, M. El Gaafary, and D. Palmer, "Assessment of the utility of visual feedback in the treatment of early rheumatoid arthritis patients: a pilot study," *Rheumatology International*, vol. 32, no. 10, pp. 3061–3068, 2012.

[76] E. Naredo, M. A. D'Agostino, P. G. Conaghan et al., "Current state of musculoskeletal ultrasound training and implementation in Europe: results of a survey of experts and scientific societies," *Rheumatology*, vol. 49, no. 12, pp. 2438–2443, 2010.

[77] A. Iagnocco, E. Filippucci, C. Perella et al., "Clinical and ultrasonographic monitoring of response to adalimumab treatment in rheumatoid arthritis," *The Journal of Rheumatology*, vol. 35, no. 1, pp. 35–40, 2008.

[78] A. K. Brown, "Using ultrasonography to facilitate best practice in diagnosis and management of RA," *Nature Reviews Rheumatology*, vol. 5, no. 12, pp. 698–706, 2009.

[79] P. P. Cheung, A. Maxime Dougados, and L. Gossec, "Reliability of ultrasonography to detect synovitis in rheumatoid arthritis: a systematic literature review of 35 studies (1,415 patients)," *Arthritis Care & Research*, vol. 62, no. 3, pp. 323–334, 2010.

[80] E. Naredo, P. Collado, A. Cruz et al., "Longitudinal power Doppler ultrasonographic assessment of joint inflammatory activity in early rheumatoid arthritis: predictive value in disease activity and radiologic progression," *Arthritis Care and Research*, vol. 57, no. 1, pp. 116–124, 2007.

[81] M. Ogasawara, G. Murayama, Y. Yamada et al., "Autofeedback from ultrasound images provides rapid improvement in palpation skills for identifying joint swelling in rheumatoid arthritis," *The Journal of Rheumatology*, vol. 39, no. 6, pp. 1207–1214, 2012.

[82] S. A. Wright and A. L. Bell, "Enhancement of undergraduate rheumatology teaching through the use of musculoskeletal ultrasound," *Rheumatology*, vol. 47, no. 10, pp. 1564–1566, 2008.

[83] F. Joshua, "Ultrasound applications for the practicing rheumatologist," *Best Practice and Research: Clinical Rheumatology*, vol. 26, no. 6, pp. 853–867, 2012.

[84] A. L. Yuille and H. H. Bülthoff, "Action as an innate bias for visual learning," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 44, pp. 17736–17737, 2012.

[85] A. de Angeli, L. Coventry, G. Johnson, and K. Renaud, "Is a picture really worth a thousand words? Exploring the feasibility of graphical authentication systems," *International Journal of Human Computer Studies*, vol. 63, no. 1-2, pp. 128–152, 2005.

[86] S. K. Joplin, R. van der Zwan, H. Bagga, F. Joshua, and P. K. K. Wong, "Pilot study assessing the novel use of musculoskeletal ultrasound in patients with rheumatoid arthritis to improve patient attitudes and adherence to medication," *International Journal of Rheumatic Diseases*, 2014.

[87] P. Quinlan, K. O. Price, S. K. Magid, S. Lyman, L. A. Mandl, and P. W. Stone, "The relationship among health literacy, health knowledge, and adherence to treatment in patients with rheumatoid arthritis," *HSS Journal*, vol. 9, no. 1, pp. 42–49, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 604872, 10 pages
http://dx.doi.org/10.1155/2015/604872

Research Article

Influence of Immunogenicity on the Efficacy of Long-Term Treatment with TNFα Blockers in Rheumatoid Arthritis and Spondyloarthritis Patients

Inesa Arstikyte,[1,2] Giedre Kapleryte,[1] Irena Butrimiene,[1,2] and Algirdas Venalis[1,2]

[1]*Centre of Rheumatology, Vilnius University, Santariskiu Street 2, LT-08661 Vilnius, Lithuania*
[2]*State Research Institute, Centre for Innovative Medicine, Zygimantu Street 9, LT-01102 Vilnius, Lithuania*

Correspondence should be addressed to Inesa Arstikyte; inesa.arstikyte@santa.lt

Received 4 December 2014; Accepted 19 February 2015

Academic Editor: Guixiu Shi

Objective. To analyze the clinical relevance of the levels of TNFα blockers and anti-drug antibodies (anti-drug Ab) in patients with rheumatoid arthritis (RA) and spondyloarthritis (SpA) treated with adalimumab (ADA), etanercept (ETA), or infliximab (INF) for a prolonged period of time. *Methods*. Clinical characteristics (disease activity, and adverse events), serum TNFα blockers, and anti-drug Ab levels were evaluated in 62 RA and 81 SpA patients treated with TNFα blockers for a median of 28 months. *Results*. Anti-ADA Ab were detected in 1 (4.0%) and anti-INF Ab in 14 out of 57 (24.6%) RA and SpA patients. Patient with anti-ADA Ab and 57.1% patients with anti-INF Ab were considered nonresponders to treatment. Anti-ETA Ab were not found in any of 61 ETA treated patients. Anti-ADA and anti-INF Ab levels differ between responders and nonresponders ($P > 0.05$). Three (5.3%) patients with high serum anti-INF Ab levels developed infusion related reactions. Patients with anti-INF Ab more often required changing to another biologic drug (OR 11.43 (95% CI 1.08–120.93)) and treatment discontinuation (OR 9.28 (95% CI 1.64–52.52)). *Conclusion*. Patients not responding to treatment had higher serum anti-ADA and anti-INF Ab concentrations. Anti-INF Ab formation is related to increased risk of infusion related reactions, changing to another biologic drug, and treatment discontinuation.

1. Introduction

Tumor necrosis factor alpha (TNFα) blockers, such as adalimumab (ADA), etanercept (ETA), and infliximab (IFX), are playing a significant role in the treatment of autoimmune inflammatory diseases such as rheumatoid arthritis (RA) and spondyloarthritis (SpA). Unfortunately, about one-third of patients do not respond to treatment with TNFα blockers. For some patients it is due to primary treatment failure (medication is ineffective or serious side effects appear) or due to secondary treatment failure when TNFα blocker loses its effectiveness after an initial good response. Previous studies have shown that clinical response in RA patients is related to ADA, ETA, and INF serum levels; while in ankylosing spondylitis (AS) the literature reports controversial data [1–4]. Antibody (Ab) formation leads to a lower TNFα blocker concentration [5]. This is explained by immune complex formation between biologic medication and Ab with neutralization of the functional part of the drug and an increased clearance of the drug [5]. It is proved in previous studies that anti-drug antibody (anti-drug Ab) levels inversely correlate with therapeutic response and drug levels (one of the reasons for secondary treatment failure) [4–6]. It was demonstrated that only 4% of patients with anti-adalimumab antibodies (anti-ADA Abs) achieve clinical remission compared with 34% anti-ADA Abs negative ones [6]. In many studies anti-etanercept antibodies (anti-ETA Abs) were not detectable or only in a low number of patients and did not impact the clinical response, indicating that ETA is less immunogenic [4, 7–9]. The appearance of antibodies (Abs) against the drug has been described in about half of the patients receiving repeated TNFα monotherapy; as a consequence, immune suppression by concomitant administration of methotrexate (MTX) is recommended both in RA and SpA patients [10–18]. Previous studies show that detectable Abs decrease TNFα blockers response as much as 80% [19]. ADA,

Table 1: Patient's characteristics before initiation of treatment with TNFα blockers.

	RA, n = 62 (42.9%)	SpA, n = 81 (57.1%)
Median disease duration before initiation of TNFα blocker, years, median (IQR)	8.0 (4.0–20.0)	6.0 (2.0–11.75)
CRP, mg/L, mean ± SD	31.71 ± 20.86	35.87 ± 23.30
ESR, mm/h, mean ± SD	42.1 ± 25.17	44.6 ± 26.61
DAS-28, mean ± SD	5.76 ± 1.35	Na
HAQ, mean ± SD	1.37 ± 0.78	1.3 ± 0.63
ASDAS, mean ± SD	Na	15.41 ± 6.13*
BASDAI, cm, mean ± SD	Na	5.5 ± 2.78*
BASFI, cm, mean ± SD	Na	4.78 ± 2.62*
MASES index, mean ± SD	Na	4 ± 2
Patient's global VAS, mm, mean ± SD	64.19 ± 21.45	67.66 ± 20.5
Patient's pain VAS, mm, mean ± SD	63.43 ± 22.29	68.97 ± 20.33
Doctor's global VAS, mm, mean ± SD	57.61 ± 18.16	58.32 ± 11.36
Swollen joints, mean ± SD	17 ± 8	10 ± 8
28 swollen joints, mean ± SD	10 ± 7	Na
Tender joints, mean ± SD	22 ± 15	20 ± 14
28 tender joints, mean ± SD	10 ± 9	Na

Notes: data presented mean ± standard deviation (SD) or median and interquartile range (IQR); *axial forms of SpA.
Abbreviations: RA: rheumatoid arthritis, SpA: spondyloarthritis, CRP: C reactive protein, ESR: erythrocyte sedimentation rate, DAS-28: disease activity score in 28 joints, HAQ: Health Assessment Questionnaire, ASDAS: ankylosing spondylitis disease activity score, BASDAI: *bath* ankylosing spondylitis disease activity score, BASFI: *bath* ankylosing spondylitis functional index, MASES: *Maastricht* ankylosing spondylitis entesitis score, VAS: visual analogue scale, and Na: not applicable.

ETA, and INF can induce the formation of Abs, resulting in loss of efficacy and appearance of side effects such as infusion or injection related reactions [8, 20–22].

Most of the studies were made with only one or two biologic medications without comparing differences in patients suffering from different inflammatory diseases. The aim of our study was to assess the relationship between clinical response, adverse events, and TNFα blockers serum levels and antidrug Ab concentrations in RA and SpA (AS and psoriatic arthritis (PsA)) patients treated with ADA, ETA, and INF for a long period of time. We present data on 143 RA and SpA patients whose blood samples were collected once during treatment with ADA, ETA, or INF in Centre of Rheumatology from January 2012 to December 2013.

2. Patients and Methods

143 patients (62 with RA and 81 with SpA (49 AS and 32 PsA patients), 69 (48.3%) males), receiving treatment with one of TNFα blockers (ADA, n = 25 (17.4%), ETA, n = 61 (42.7%), or INF, n = 57 (39.9%)), were included in this analysis. Patient's mean age (±SD) was 44.98 (±13.38) years at the beginning of treatment with TNFα blockers. This was a retrospective observational study approved by the local Ethics Committee. Patients signed an informed consent form according to the Declaration of Helsinki. All patients before initiation treatment with one of TNFα blocker fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for RA and the Assessment of SpondyloArthritis international Society (ASAS) 2010 criteria for axial and peripheral SpA. Before initiation of TNFα blocker treatment all patients had evidence of active disease, as indicated by a Disease Activity score in 28 joints (DAS-28), 5.76 ± 1.35 (mean ± SD) for RA; swollen (10 ± 8) and tender (20 ± 14) joints for SpA (peripheral forms); and ankylosing spondylitis disease activity score (ASDAS) 15.41 ± 6.13 for axial SpA (see Table 1 for patient's characteristics before initiation of TNFα blocker therapy). Blood samples were taken from all patients treated with ADA and INF in the centre. The biggest group of patients with TNFα blockers in our centre is treated with ETA. In order to have approximately the same number of patients with ETA comparing with ADA and INF, every third patient was selected to analyze blood samples.

Tables 2 and 3 present patient's characteristics at the time the blood samples were collected. At the beginning of treatment with INF all patients received 2.7 (±1.67) mg/kg (2.86 (±1.67) for RA and 2.59 (±1.67) for SPA patients). However, due to an inadequate response to the initial dose in 28 (49.1%) patients a gradual escalation of INF dose to 3.98 (±1.74) mg/kg (3.97 (±0.72) for RA and 3.98 (±2.4) for SpA patients) was given. All patients were treated with ADA 40 mg every 2 weeks and ETA 50 mg/week subcutaneously during the study.

Lower INF doses were given to all TNFα blockers naive RA and SpA patients in our centre because previous studies have shown that in part of the patients these doses were effective [23, 24]. In addition, our center obtained similar results in a retrospective study of RA and SpA patients treated with TNFα blockers [25]. This allowed us as a country with a comparatively lower gross domestic product to treat those patients with lower doses of INF [26].

We divided patients into those responding to treatment with TNFα blockers (responders) and those not responding (nonresponders). RA patients, whose DAS28 was <3.2 or

Table 2: RA patient's characteristics when serum samples were collected.

Total RA patients, n = 62 (42.9%)	
Gender: female, n (%)	51 (82.3)
Median age, years, mean ± SD	50.65 ± 13.47
Months of treatment, when blood samples were collected, median (IQR) (interval):	
ADA	6 (3–9) (3–84)
ETA	30 (3–54) (3–66)
INF	15 (12–51) (6–102)
Number of performed tests, n (%)	
ADA	9 (14.5)
ETA	29 (46.8)
INF	24 (38.7)
RF positive, n (%)	58 (93.5)
ACCP positive, n (%)	26 (41.9)
Erosive disease, n (%)	62 (100.0)
TJC-60, median (IQR)	4 (2–12)
SJC-60, median (IQR)	3 (1–8)
Doctor's GDA, mm, mean ± SD	26.48 ± 17.8
Patient's GDA, mm, median (IQR)	36.0 (13.0–56.0)
DAS28, mean ± SD	3.44 ± 1.69
SDAI, median (IQR)	12.5 (6.0–24.6)
HAQ, median (IQR)	1.0 (0.373–1.38)
ESR, mm/h, median (IQR)	15 (8–27)
CRP, mg/L, median (IQR)	2.5 (1.2–5.7)
DMARDs use, n (%)	50 (80.6)
MTX use, n (%)	43 (69.4)
Medium MTX dose, mg/week, mean ± SD	11.22 ± 4.38
GK use, n (%)	45 (72.6)
Prednisolone equivalent dose, mg/d, mean ± SD	7.49 ± 3.35

Notes: data presented mean ± standard deviation or median and interquartile range (IQR) or number (n) and percent of total number of patients;
Abbreviations: RA: rheumatoid arthritis; IQR: interquartile range; SD: standard deviation, TJC-60: 60 tender joint count, SJC-60: 60 swollen joint count, GDA:global disease activity visual analogue scale (0–100 mm), RF: IgM rheumatoid factor; ACCP: cyclic citrullinated peptide antibody, DAS28: disease activity score in 28 joints; SDAI: simplified disease activity index; HAQ: Health Assessment Questionnaire, ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DMARDs: disease-modifying antirheumatic drugs (e.g., methotrexate, sulfasalazine, leflunomide, hydrochloroquine, and azathioprine); MTX: methotrexate, and na: data is not applicable.

Table 3: SpA patient's characteristics when serum samples were collected.

Total SpA patients, n = 81 (57.1%) AS patients, n = 49 PsA patients, n = 32	
Gender: male, n (%)	58 (71.6)
Median age, years, mean ± SD	41.85 ± 11.23
Months of treatment, when blood samples were collected, median (IQR) (interval):	
ADA	6 (3–18) (3–78)
ETA	30 (12–54) (6–72)
INF	54 (21–66) (3–108)
Number of performed tests, n (%)	
ADA	16 (19.8)
ETA	32 (39.5)
INF	33 (40.7)
Erosive disease, n (%)	43 (53.1)
HLA-B27 positive, n (%)	60 (74.1)
Sacroiliitis, n (%)	64 (79.0)
TJC-60, median (IQR)	2 (0–4)
SJC-60, median (IQR)	1 (0–2)
Doctor's GDA, mm, mean ± SD	22.45 ± 14.0
Patient's GDA, mm, median (IQR)	21 (8.25–45.75)
DAS28, mean ± SD	2.37 ± 1.36*
HAQ, median (IQR)	0.38 (0–0.88)
BASDAI, mm, median (IQR)	2.24 (1.02–4.66)**
ASDAS-CRB, mean ± SD (interval)	4.77 ± 3.2
ESR, mm/h, median (IQR)	8 (4–21)
CRP, mg/L, median (IQR)	3.6 (1.05–7.58)
DMARDs use, n (%)	66 (81.56)
MTX use, n (%)	63 (77.8)
Medium MTX dose, mg/week, mean ± SD	10.93 ± 5.07
GK use, n (%)	35 (43.2)
Prednisolone equivalent dose, mg/d, mean ± SD	8.07 ± 5.77

Notes: data presented mean ± standard deviation or median and interquartile range (IQR) or number (n) and percent of total number of patients; *peripheral forms of SpA, **axial form of SpA.
Abbreviations: SpA: spondyloarthritis, AS: ankylosing spondylitis, PsA: psoriatic arthritis, IQR: interquartile range; SD: standard deviation, TJC-60: 60 tender joint count, SJC-60: 60 swollen joint count, GDA: global disease activity visual analogue scale (0–100 mm), DAS28: disease activity score in 28 joints; HAQ: Health Assessment Questionnaire, BASDAI: *Bath* ankylosing spondylitis disease activity index (0–100 mm), ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DMARDs: disease-modifying antirheumatic drugs (e.g., methotrexate, sulfasalazine, leflunomide, hydrochloroquine, and azathioprine); MTX: methotrexate, and na: data is not applicable.

decreased >1.2 since the initial value, were considered as having good EULAR response; DAS28 ≥3.2 but ≤5.1 or decreased ≤1.2 but ≥0.6, moderate EULAR response; DAS28 >5.1 or decreased <0.6, no response to treatment [27]. RA patients with good or moderate EULAR response were considered as responders, others as nonresponders. SpA patients with ASDAS (calculated with CRP) <1.3 were considered as having inactive disease; whose ASDAS was >1.3 but <2.1, moderate disease activity; >2.1 but <3.5, high disease activity; and >3.5, very high disease activity [28]. SpA patients with inactive disease or moderate disease activity were attributed to responders while patients with high or very high disease activity were considered as nonresponders.

Serum samples were collected once (from January 2012 to December 2013) during the treatment course and were stored at −80°C until TNFα blocker and anti-drug Ab were

Table 4: Detectable serum levels of TNFα blockers and anti-drug Abs.

	All, $n = 143$	RA, $n = 62$	SpA, $n = 81$
ADA tests total, n	**25**	**9**	**16**
ADA trough levels, patients n (%)	25 (100.0)	8 (88.9)	16 (100.0)
ADA concentration, mean ± SD	8.04 ± 4.2	8.24 ± 3.8	7.1 ± 4.48
Anti-ADA Ab positive, patients n (%)	1 (4.0)	1 (11.1)	0
Anti-ADA Ab concentration (one sample)	2000	2000	na
ETA tests total, n	**61**	**29**	**32**
ETA trough levels, patients n (%)	57 (93.4)	28 (96.6)	29 (90.6)
ETA concentration, mean ± SD	6.54 ± 2.34	6.06 ± 1.18	6.83 ± 3.5
Anti-ETA Ab positive, patients n (%)	0	0	0
INF tests total, n	**57**	**24**	**33**
INF trough levels, patients n (%)	41 (71.9)	14 (58.3)	27 (81.8)
INF concentration, median (IQR) (range)	2.36 (1.95–4.26) (1.52–14.3)	3.77 (1.88–9.4) (1.52–14.3)	2.33 (1.96–4.48) (1.69–35.0)
Anti-INF Ab positive, patients n (%)	14 (24.6)	8 (33.3)	6 (18.2)
Anti-INF Ab concentration, median (IQR) (range)	130 (7.97–289.9) (4.89–1440)	136.0 (21.17–313.03) (9.2–527)	74.4 (5.4–489) (4.89–1440)

Notes: calculations for drug and anti-drug Abs concentrations was done only for trough serum levels. ADA ≤0.024 μg/mL, ETA and INF concentrations ≤0.035 μg/mL were considered as not detectable for drugs. Anti-ADA Ab ≤3.5 AU/mL, anti-ETA Ab ≤142.0 AU/mL, and anti-INF Ab ≤2.0 AU/mL concentrations were considered as negative.
Abbreviations: SD: standard deviation, na: data is not applicable.

measured. Patient's clinical and laboratory data, diagnosis, disease duration, start of the biologic therapy, adverse events, erythrocyte sedimentation rate (ESR) and C reactive protein (CRP), disease activity information, such as visual analogue scales, disease activity score in 28 joints (DAS28), ankylosing spondylitis disease activity score (ASDAS), and health assessment questionnaire (HAQ) were assessed at the same time blood samples for immunogenicity were collected. Patient's blood samples were collected at least after 3 months of treatment with one of the TNFα blockers, before dosing the next scheduled dose (ADA and ETA, before scheduled injection and INF, 8 weeks after last dose, just before next scheduled infusion). Blood samples of ADA treated patients were collected at medium 6 months (interquartile range (IQR) 3–18), ETA, 30 months (IQR 12–54) and INF, medium 42 months (IQR 12–66) after the treatment initiation (Table 2).

Blood samples were analyzed in Centre of Laboratory Medicine of Vilnius University, using Promonitor ADA, Promonitor ETA, and Promonitor INF test kits (Progenika, Derio, Spain) [29].

2.1. Serum ADA and ETA Assay Principle. Promonitor ADA and Promonitor ETA are a sandwich enzyme-linked immunosorbent assay (ELISA) [21]. The microwell strips are provided precoated with an anti-ADA and anti-ETA human $F(ab')_2$ fragment. Diluted calibrators, controls, and diluted patient samples are added to separate wells, allowing TNFα blocker present to bind to preimmobilized anti-drug Ab. Unbound sample is washed away and a second enzyme horseradish peroxidase- (HRP-) labeled anti-drug monoclonal Ab is added to each well. A second incubation step allows the HRP-labeled anti-drug monoclonal Ab to bind to the TNFα blocker that has become attached to the microwells. After washing away the excess of unbound HRP-labeled anti-drug Ab, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the color that develops in a spectrophotometer. The signal obtained is proportional to the amount of the drug in the patient sample. ADA concentration ≤0.024 μg/mL and ETA concentration ≤0.035 μg/mL were considered as negative.

2.2. Serum INF Assay Principle. Promonitor INF is a capture ELISA [21]. The microwell strips are provided precoated with an anti-INF human $F(ab')_2$ fragment bound to human recombinant TNFα. This format ensures that TNFα structure is not disrupted and is available to bind to INF. Diluted calibrators, controls, and diluted patient samples are added to separate wells, allowing INF present to bind to preimmobilized TNFα. Unbound sample is washed away and a specific HRP-labeled anti-INF monoclonal Ab is added to each well. A second incubation step allows the anti-INF Ab to bind to the INF that has become attached to the microwells. After washing away the excess of unbound HRP-labeled anti-INF Ab, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the color that develops in a spectrophotometer. The signal obtained is proportional to the amount of the drug in the patient sample. INF concentration ≤0.035 μg/mL was considered as negative.

2.3. Serum Anti-Drug Ab Assay Principle. Promonitor anti-ADA, Promonitor anti-ETA, and Promonitor anti-INF are bridging ELISA tests [21]. The microwell strips are provided precoated with TNFα blocker. The bridging ELISA takes advantage of the two arms of IgG subclasses 1, 2, and 3, to

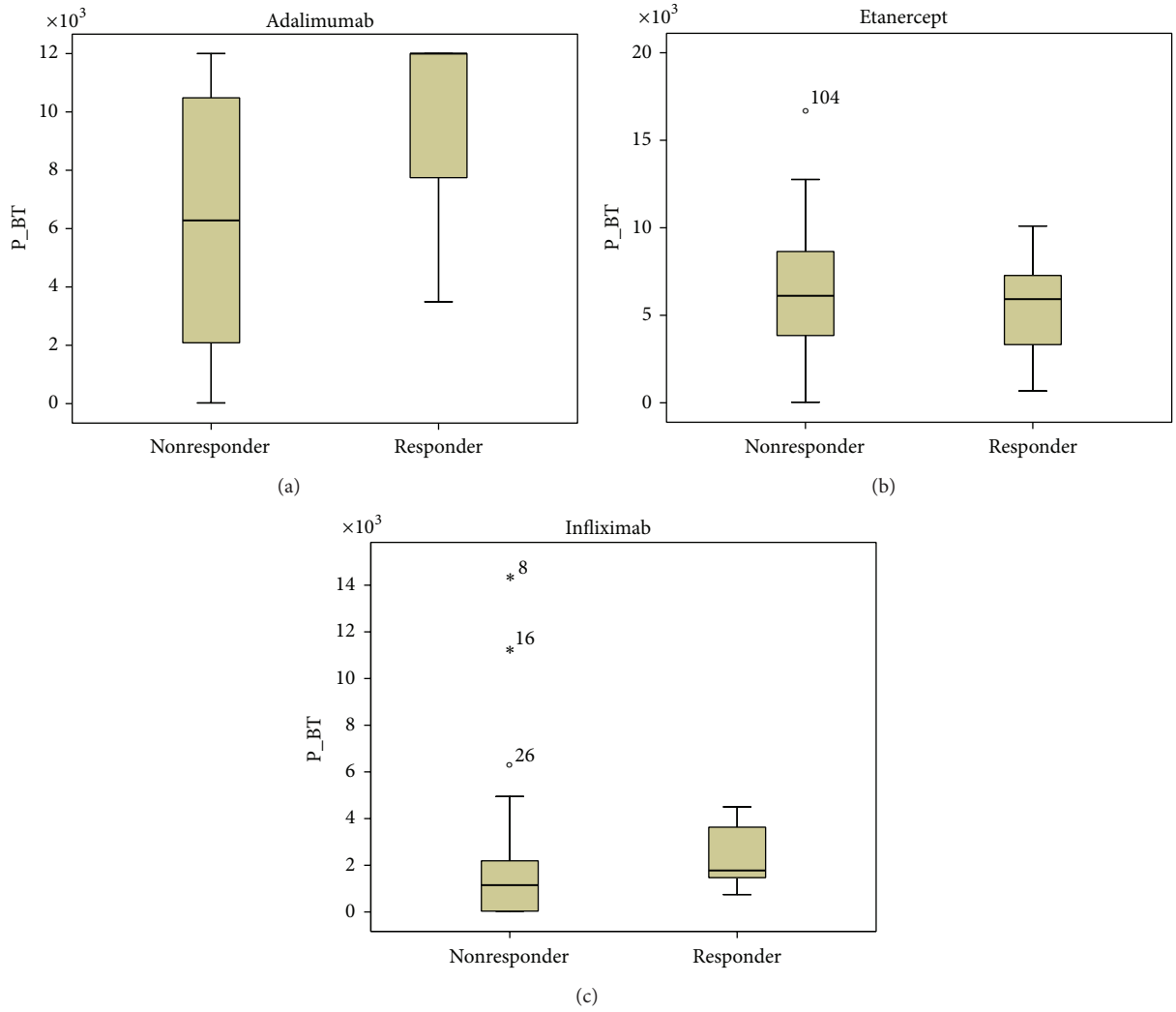


Figure 1: ADA (a), ETA (b), and INF (c) levels (μg/mL) in responders versus nonresponders RA and SpA patients ($P = 0.142$, $P = 0.488$, and $P = 0.093$, resp.). Data presented as interquartile ranges (75th centile, upper edge of the box; 25th centile, lower edge of the box, and 50th centile, midline of the box).

crosslink the TNFα blocker coated on the plane. Calibrators, controls, and diluted patient samples are added to separate wells, allowing anti-TNFα blocker Ab present to bind to preimmobilized TNFα blocker. Unbound sample is washed away and HRP-labeled TNFα blocker is added to each well. A second incubation allows the HRP-labeled TNFα blocker to bind to the Ab that has become attached to the microwells. After washing away unbound HRP conjugate, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the color that develops in a spectrophotometer. The signal obtained is proportional to the amount of anti-TNFα blocker Ab in the patient sample. Anti-ADA Ab concentration $\leq$3.5 AU/mL, anti-ETA Ab concentration $\leq$142.0 AU/mL, and anti-INF Ab concentration $\leq$2.0 AU/mL were considered as negative.

2.4. Statistical Analysis. Descriptive statistics were provided using the mean, standard deviation (SD), median (Md), and interquartile range (IQR). Frequency data were compared by the Pearson's chi-square and Fisher's exact tests. Differences in quantitative values between groups were analysed using Mann-Whitney U nonparametric test and $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, IL, USA).

3. Results

Anti-ADA Ab were detected in one patient (4.0%) with undetectable serum ADA levels. In evaluated patients we did not find anti-ETA Ab, although in 4 cases (6.6%) ETA levels were undetectable. Anti-INF Ab were detected in serum samples from 14 (24.6%) patients, in 13 cases with undetectable serum trough INF levels (Table 4).

At baseline all RA patients had active disease as indicated by a mean (±SD) DAS-28 of 5.76 (±1.35) with no differences

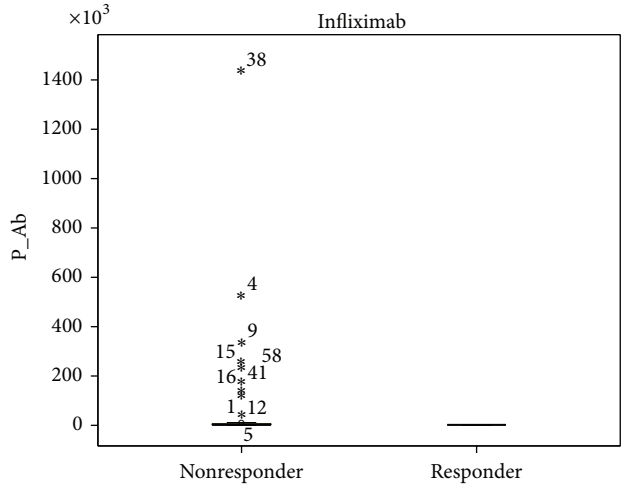


Figure 2: Anti-INF Ab concentration (AU/mL) in responders ($n = 9$) versus nonresponders ($n = 48$) RA and SpA patients ($P < 0.0001$).

in DAS-28 values between patients that subsequently did (3.3 ± 1.55) or did not (3.46 ± 1.73) develop anti-INF or anti-ADA Ab ($P = 0.727$). At baseline all SpA patients had active disease as indicated by a mean (±SD) DAS-28 of 4.48 (±1.19) for peripheral forms, ASDAS 15.41 (±6.13), and BASDAI 5.5 (±2.78) with no differences in those values between patients that subsequently did (2.91 ± 1.32, 5.73 ± 2.1, and 2.2 ± 0.9, resp.) or did not (2.3 ± 1.39, 4.82 ± 3.25, and 2.83 ± 2.31, resp.) develop anti-INF or anti-ADA Ab ($P = 0.326$, $P = 0.564$, and $P = 0.718$, resp.).

Our results showed a tendency toward higher ADA and INF levels in all patients responding to treatment, but the data was not statistically significant (Figure 1). Patients not responding to treatment had statistically significant higher anti-ADA ($P < 0.0001$) and anti-INF Ab ($P < 0.0001$) concentrations (Figure 2). When analyzing the same data separately in RA and SpA patients results did not differ statistically significant between responders and nonresponders (Figures 3–6). All RA and SpA patients, which were responding to treatment, had no detectable anti-drug Ab levels versus nonresponder patients: 1 patient with anti-ADA Ab and 14 patients with anti-INF Ab ($P < 0.0001$).

One RA patient developed anti-ADA Ab (concentration 2000 AU/mL) with no detectable levels of ADA. For this reason ADA was stopped and treatment was changed to rituximab with success. In 4 patients with good treatment response ETA levels were undetectable, although anti-ETA Ab were not found of all ETA treated patients.

We found 14 patients with anti-INF Ab and 13 of them had no detectable levels of INF. Three patients (5,3%) with anti-INF Ab had infusion related reactions, 8 (57.1%) had insufficient treatment effect; 3 patients had good clinical response. In 3 patients (5,3%) with anti-INF Ab, treatment was discontinued, in 3 cases dose was escalated, in 3 biologic drug was changed, and in 5 cases (8,8%) treatment was not changed (patient's decision). In 3 patients (5,3%) INF and anti-INF Ab levels were undetectable.

In order to know the odds of developing infusion related reactions and TNFα blockers treatment emendation in patients with anti-INF Ab, we calculated odds ratio (OR) in 143 evaluated patients. Our data shows that patients with anti-INF Ab have higher odds to have infusion related reaction (OR 5.88 (95% CI 1.04–33.28)), to change to another TNFα blocker (OR 11.43 (95% CI 1.08–120.93)), to stop treatment with INF (OR 9.28 (95% CI 1.64–52.52)), although 95% CI for these results are wide suggesting low statistical value of these results. Odds to increase INF dose were not statistically significant (OR 2.07 (95% CI 0.43–9.96)). Nevertheless, patients with anti-INF Ab have lower odds of response to treatment (OR 0.8 (95% CI 0.19–3.38), not significant) and to continue INF with the same dose (OR 0.2 (95% CI 0.05–0.69)).

We found negative correlation between MTX use and presence of anti-drug Ab in ADA patients (*Kendall's tau* correlation coefficient −0.686 ($P = 0.005$); *Spearman's rho* correlation coefficient −0.686 ($P = 0.002$)), although in INF patients group correlation was not found (*Kendall's tau* correlation coefficient −0.167 ($P = 0.220$); *Spearman's rho* correlation coefficient −0.142 ($P = 0.320$), resp.).

There was medium negative correlation between INF and anti-INF Ab concentrations (*Kendall's tau* correlation coefficient −0.473 ($P < 0.0001$); *Spearman's rho* correlation coefficient −0.590 ($P < 0.0001$)); low negative correlation between ADA and anti-ADA Ab was found (−0.302 ($P = 0.088$) and −0.348 ($P = 0.088$), resp.).

4. Discussion

Our study showed influence of anti-ADA Ab and anti-INF Ab on clinical response and odds to have infusion related reactions or treatment emendation in patients with anti-INF Ab (although it has low statistical value).

In the literature the percentage of patients who develop anti-drug Ab varies among different autoimmune inflammatory diseases. Anti-drug Ab have been seen in up to one third of RA and abour 25% SpA patients [2, 3, 30–32]. Studies have demonstrated that chimeric (mouse-human) drugs, such as INF, have a greater likelihood of inducing anti-drug Ab development than do fully human antibodies [33]. As not all patients treated with anti-TNF agents develop anti-drug Ab, immunogenicity seems to be the result of several factors associated with the treatment, the patient, and the external factors [32]. We have found similar anti-INF Ab formation levels as seen in past studies, 33.3% of patients with RA and 18.2% of patients with SpA. We did not find statistically significant differences between serum TNFα blockers concentrations in those responding and not responding to treatment, although previous studies show that serum drug levels strongly correlate with clinical response [2, 3, 6, 19–21, 32]. These results could be explained by small amount of patients in each group and low initial INF doses. However, we have found increased odds in changing treatment from INF to another TNFα blocker, stopping treatment or having insufficient treatment effect in patients with anti-INF Abs.

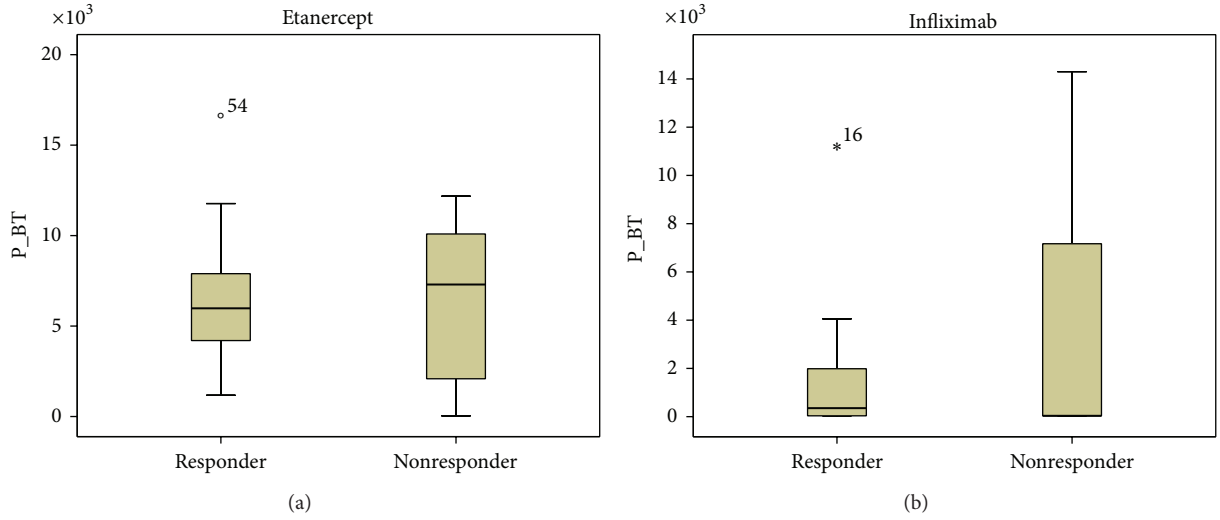


Figure 3: ETA (a) and INF (b) levels (μg/mL) in responders versus nonresponders RA patients ($P = 0.956$ for ETA and $P = 0.880$ for INF). Data presented as interquartile ranges (75th centile, upper edge of the box; 25th centile, lower edge of the box, and 50th centile, midline of the box).

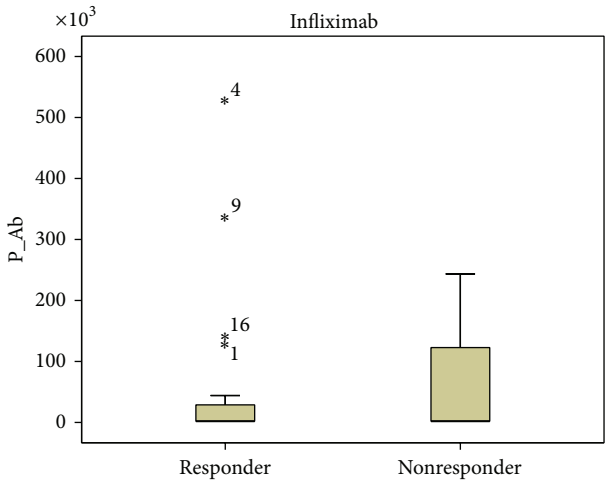


Figure 4: Anti-INF Ab concentration (AU/mL) in responders versus nonresponders RA patients ($P = 0.956$). Data presented as interquartile ranges (75th centile, upper edge of the box; 25th centile, lower edge of the box, and 50th centile, midline of the box).

As reported in previous studies patients receiving treatment with INF show a high rate of infusion-related reactions. We found 5,3% patients treated with anti-INF Ab who developed infusion reactions [20]. These data support the view that detectable titers of anti-INF Ab are associated with increased risk of infusion reactions, probably because of the formation of immune complexes and also treatment with low initial INF doses [33]. Our findings indicate that the appearance of anti-INF and anti-ADA Ab is associated with a poor clinical treatment effect, the development of infusion related reactions, and TNFα blockers treatment emendation. Detectable levels of TNFα blockers in sera of 143 patients did not correlate with clinical response to treatment with one of the TNFα blockers (the differences were not found between those who responded or not to the treatment).

In our clinic, for all INF patients, treatment was started with low doses (mean 2.7 (±1.67) mg/kg). Almost half of patients needed dose escalation due to insufficient clinical treatment effect; however only 5.3% of patients with anti-INF Ab dose were escalated due to this reason. This was also shown in previous publications [34–37]. Although patients in our clinic started treatment with lower than recommended INF dosage, the percentage of patients, requiring dose escalation or rate of infusion reactions, is similar as reported in the studies with adequate INF doses [18]. Levels of low TNFα blockers concentrations in sera did not correlate with clinical response in our patients. However it seems that low INF trough levels could influence formation of anti-INF Ab in RA and SpA patients. On the contrary, our data on amount of patients with positive anti-INF Ab titers who were treated with low INF doses did not differ from the data of the studies when the adequate dosage of INF were used [30–32, 35].

As known from the literature ETA has the lowest immunogenicity [4, 7–9]. Accordingly, in our study none of the patients were positive for anti-ETA Ab, complementary, ETA serum drug concentrations were not different in patients responding or not responding to the treatment.

We found one RA patient with positive anti-ADA Ab levels and undetectable concentration of ADA who had poor clinical response. Overall, ADA and anti-ADA Ab serum levels did not correlate with clinical response in RA and SpA patients.

Long disease duration and high disease activity (DAS28 5.76 ± 1.35 in RA patients, ASDAS 15.41 ± 6.13 in axial SpA) before treatment with TNFα blockers can be the factors also responsible for the fact that we did not find correlation between detectable levels of TNFα blockers and anti-drug Abs with clinical efficacy.

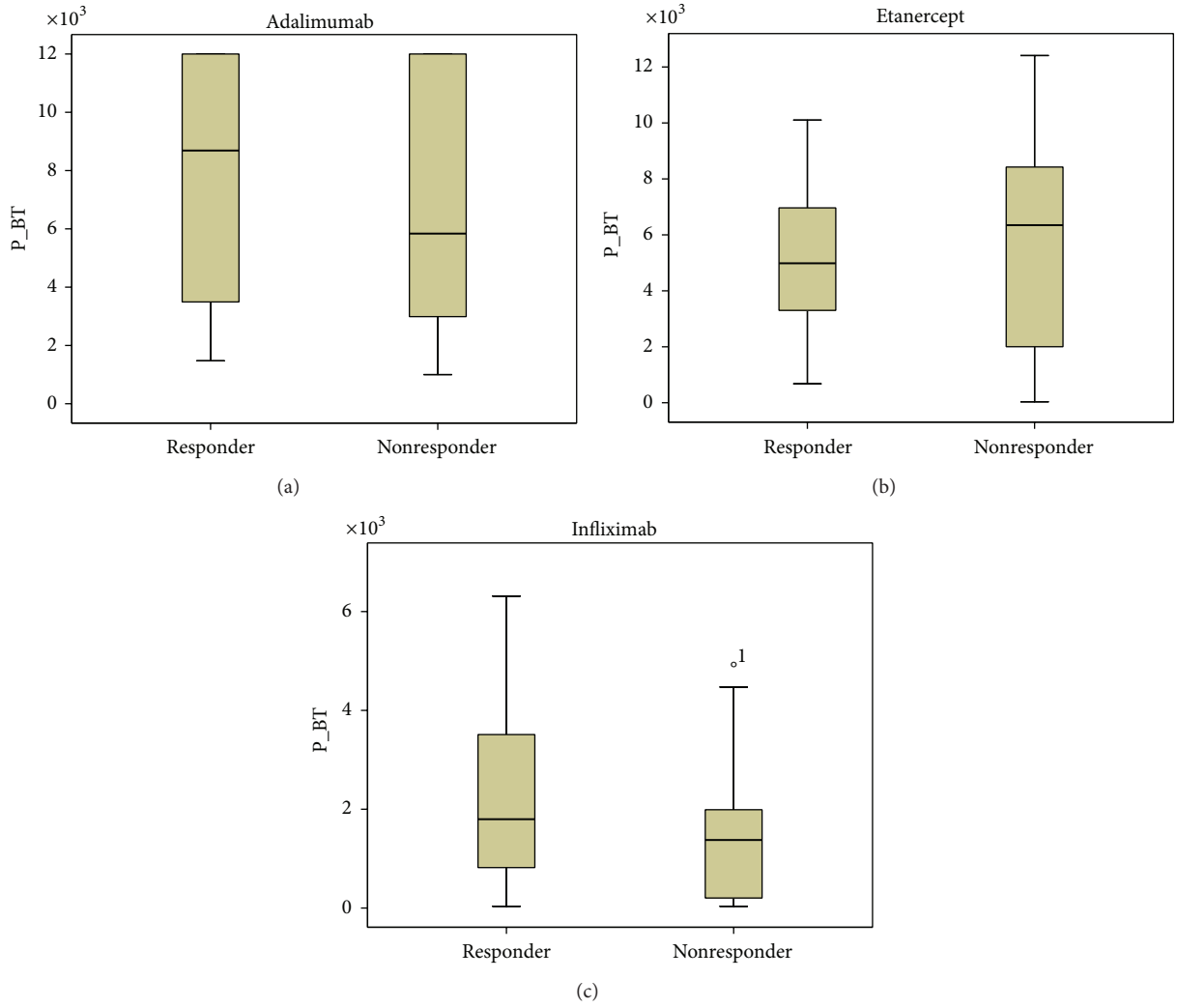


Figure 5: ADA (a), ETA (b), and INF (c) levels (μg/mL) in responders versus nonresponders SpA patients ($P = 0.861$, $P = 0.618$, and $P = 0.293$, resp.). Data presented as interquartile ranges (75th centile, upper edge of the box; and 25th centile, lower edge of the box, 50th centile, midline of the box).

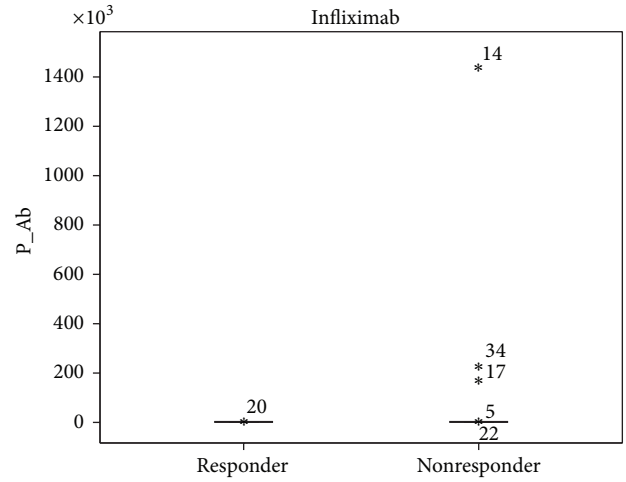


Figure 6: Anti-INF Ab concentration (AU/mL) in responders versus nonresponders SpA patients ($P = 0.243$).

Our study had its weaknesses. We had low numbers of patients in groups, and blood samples from the patients were collected in various time intervals (the longest treatment period with INF), and so detection of anti-drug Abs could be influenced by heterogeneity of time periods of treatment.

5. Conclusions

Anti-INF Ab are associated with loss in clinical response, an increase incidence of infusion reactions, probable secondary treatment inefficacy, and treatment emendation. The detection of anti-drug Ab could be helpful in order to understand the reason of treatment inefficacy when choosing an appropriate medication. Testing for immunogenicity could become a part of a patient's everyday clinical management.

Conflict of Interests

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

Auhors' Contribution

Inesa Arstikyte, Irena Butrimiene, and Algirdas Venalis have received speaker honoraria or consultancies from pharmaceutical companies producing TNFα blockers or distributing them in Lithuania (Abbvie, Phizer, MSD).

Acknowledgments

Thanks to the physicians and patients who participated in this study and Alina Prodan for performing the ELISA tests. This study was supported by a research grant from the Lithuanian Arthritis Foundation. Promonitor kits were provided by Pfizer Luxembourg SARL, Lithuania.

References

[1] E. L. Kneepkens, C. L. M. Krieckaert, D. van der Kleij et al., "Lower etanercept levels are associated with high disease activity in ankylosing spondylitis patients at 24 weeks of follow-up," *Annals of the Rheumatic Diseases*, 2014.

[2] E. L. Kneepkens, J. C.-C. Wei, M. T. Nurmohamed et al., "Immunogenicity, adalimumab levels and clinical response in ankylosing spondylitis patients during 24 weeks of follow-up," *Annals of the Rheumatic Diseases*, vol. 74, no. 2, pp. 396–401, 2015.

[3] M. K. de Vries, G. J. Wolbink, S. O. Stapel et al., "Inefficacy of infliximab in ankylosing spondylitis is correlated with antibody formation," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 133–134, 2007.

[4] D. Mazilu, D. Opriş, C. Gainaru et al., "Monitoring drug and antidrug levels: a rational approach in rheumatoid arthritis patients treated with biologic agents who experience inadequate response while being on a stable biologic treatment," *BioMed Research International*, vol. 2014, Article ID 702701, 8 pages, 2014.

[5] C. C. Mok, D. van der Kleij, and G. J. Wolbink, "Drug levels, anti-drug antibodies, and clinical efficacy of the anti-TNFα biologics in rheumatic diseases," *Clinical Rheumatology*, vol. 32, no. 10, pp. 1429–1435, 2013.

[6] G. M. Bartelds, C. L. M. Krieckaert, M. T. Nurmohamed et al., "Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during longterm follow-up," *The Journal of the American Medical Association*, vol. 305, no. 14, pp. 1460–1468, 2011.

[7] M. Hoshino, T. Yoshio, S. Onishi, and S. Minota, "Influence of antibodies against infliximab and etanercept on the treatment effectiveness of these agents in Japanese patients with rheumatoid arthritis," *Modern Rheumatology*, vol. 22, no. 4, pp. 532–540, 2012.

[8] M. K. de Vries, I. E. van der Horst-Bruinsma, M. T. Nurmohamed et al., "Immunogenicity does not influence treatment with etanercept in Patients with ankylosing spondylitis," *Annals of the Rheumatic Diseases*, vol. 68, no. 4, pp. 531–535, 2009.

[9] R. K. Dore, S. Mathews, J. Schechtman et al., "The immunogenicity, safety, and efficacy of etanercept liquid administered once weekly in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 25, no. 1, pp. 40–46, 2007.

[10] V. C. Pérez-Guijo, A. R. Cravo, M. D. C. Castro, P. Font, E. Muñoz-Gomariz, and E. Collantes-Estevez, "Increased efficacy of infliximab associated with methotrexate in ankylosing spondylitis," *Joint Bone Spine*, vol. 74, no. 3, pp. 254–258, 2007.

[11] D. Mulleman, F. Lauféron, D. Wendling et al., "Infliximab in ankylosing spondylitis: alone or in combination with methotrexate? A pharmacokinetic comparative study," *Arthritis Research and Therapy*, vol. 13, no. 3, article R82, 2011.

[12] M. Breban, P. Ravaud, P. Claudepierre et al., "Maintenance of infliximab treatment in ankylosing spondylitis: results of a one-year randomized controlled trial comparing systematic versus on-demand treatment," *Arthritis and Rheumatism*, vol. 58, no. 1, pp. 88–97, 2008.

[13] P. D. Sampaio-Barros, L. T. L. Costallat, M. B. Bertolo, J. F. M. Neto, and A. M. Samara, "Methotrexate in the treatment of ankylosing spondylitis," *Scandinavian Journal of Rheumatology*, vol. 29, no. 3, pp. 160–162, 2000.

[14] R. N. Maini, F. C. Breedveld, J. R. Kalden et al., "Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor α monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 41, no. 9, pp. 1552–1563, 1998.

[15] P. E. Lipsky, D. M. F. M. van der Heijde, E. W. St. Clair et al., "Infliximab and methotrexate in the treatment of rheumatoid arthritis," *The New England Journal of Medicine*, vol. 343, no. 22, pp. 1594–1602, 2000.

[16] E. W. St.Clair, C. L. Wagner, A. A. Fasanmade et al., "The relationship of serum infliximab concentrations to clinical improvement in rheumatoid arthritis: results from ATTRACT, a multicenter, randomized, double-blind, placebo-controlled trial," *Arthritis and Rheumatism*, vol. 46, no. 6, pp. 1451–1459, 2002.

[17] A. Arora, A. Mahajan, D. Spurden, H. Boyd, and D. Porter, "Long-term drug survival of tnf inhibitor therapy in RA patients: a systematic review of European national drug registers," *International Journal of Rheumatology*, vol. 2013, Article ID 764518, 9 pages, 2013.

[18] M. Fabbroni, L. Cantarini, F. Caso et al., "Drug retention rates and treatment discontinuation among anti-TNF-α agents in psoriatic arthritis and ankylosing spondylitis in clinical

practice," *Mediators of Inflammation*, vol. 2014, Article ID 862969, 7 pages, 2014.

[19] S. Garcês, J. Demengeot, and E. Benito-Garcia, "The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis," *Annals of the Rheumatic Diseases*, vol. 72, no. 12, pp. 1947–1955, 2013.

[20] G. J. Wolbink, M. Vis, W. Lems et al., "Development of anti-infliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 3, pp. 711–715, 2006.

[21] N. K. Bender, C. E. Heilig, B. Dröll, J. Wohlgemuth, F.-P. Armbruster, and B. Heilig, "Immunogenicity, efficacy and adverse events of adalimumab in RA patients," *Rheumatology International*, vol. 27, no. 3, pp. 269–274, 2007.

[22] S. M. D. Pan, S. Dehler, A. Ciurea, Z. Hans-Rudolf, C. Gabay, and A. Finckh, "Comparison of drug retention rates and causes of drug discontinuation between anti-tumor necrosis factor agents in rheumatoid arthritis," *Arthritis Care and Research*, vol. 61, no. 5, pp. 560–568, 2009.

[23] R. N. Jois, J. Leeder, A. Gibb et al., "Low-dose infliximab treatment for ankylosing spondylitis—clinically- and cost-effective," *Rheumatology*, vol. 45, no. 12, pp. 1566–1569, 2006.

[24] I. De La Torre, L. Valor, J. C. Nieto, M. Montoro, and L. Varreno, "Minimum effective dosages of anti-TNF in rheumatoid arthritis: a cross-sctional study," *Reumatología Clínica*, vol. 10, no. 2, pp. 101–104, 2014.

[25] I. Arštikytė and I. Butrimienė, "Lietuvos reumatinių ligų biologinės terapijos duomenų bazė: 2007–2013 metų veiklos ataskaita," *Vilniaus Reumatologijos Seminarai*, vol. 1, no. 8, pp. 46–53, 2014.

[26] P. Putrik, S. Ramiro, T. K. Kvien et al., "Inequities in access to biologic and synthetic DMARDs across 46 European countries," *Annals of the Rheumatic Diseases*, vol. 73, no. 1, pp. 198–206, 2014.

[27] A. M. van Gestel, M. L. L. Prevoo, M. A. van't Hof, M. H. van Rijswijk, L. B. A. van de Putte, and P. L. C. M. van Riel, "Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary american college of rheumatology and the world health organization/international league against rheumatism criteria," *Arthritis & Rheumatism*, vol. 39, no. 1, pp. 34–40, 1996.

[28] C. Castillo-Gallego, S. Z. Aydin, and H. Marzo-Ortega, "Clinical utility of the new ASAS criteria for spondyloarthritis and the disease activity score," *Current Rheumatology Reports*, vol. 13, no. 5, pp. 395–401, 2011.

[29] Promonitor-ADA, Promonitor-ETA, Promonitor-INF description of performing assays, provided by Proteomica company with kits.

[30] D. Pascual-Salcedo, C. Plasencia, S. Ramiro et al., "Influence of immunogenicity on the efficacy of long-term treatment with infliximab in rheumatoid arthritis," *Rheumatology*, vol. 50, no. 8, pp. 1445–1452, 2011.

[31] C. Plasencia, D. Pascual-Salcedo, S. García-Carazo et al., "The immunogenicity to the first anti-TNF therapy determines the outcome of switching to a second anti-TNF therapy in spondyloarthritis patients," *Arthritis Research and Therapy*, vol. 15, no. 4, article R79, 2013.

[32] C. Plasencia, D. Pascual-Salcedo, L. Nuño et al., "Influence of immunogenicity on the efficacy of longterm treatment of spondyloarthritis with infliximab," *Annals of the Rheumatic Diseases*, vol. 71, no. 12, pp. 1955–1960, 2012.

[33] P. J. Anderson, "Tumor necrosis factor inhibitors: clinical implications of their different immunogenicity profiles," *Seminars in Arthritis and Rheumatism*, vol. 34, no. 5, pp. 19–22, 2005.

[34] A. van der Maas, W. Kievit, B. J. F. van den Bemt, F. H. J. van den Hoogen, P. L. van Riel, and A. A. den Broeder, "Down-titration and discontinuation of infliximab in rheumatoid arthritis patients with stable low disease activity and stable treatment: an observational cohort study," *Annals of the Rheumatic Diseases*, vol. 71, no. 11, pp. 1849–1854, 2012.

[35] G. Tenga, V. Goëb, T. Lequerré et al., "A 3 mg/kg starting dose of infliximab in active spondyloarthritis resistant to conventional treatments is efficient, safe and lowers costs," *Joint Bone Spine*, vol. 78, no. 1, pp. 50–55, 2011.

[36] R. D. Inman and W. P. Maksymowych, "A double-blind, placebo-controlled trial of low dose infliximab in ankylosing spondylitis," *Journal of Rheumatology*, vol. 37, no. 6, pp. 1203–1210, 2010.

[37] B. Mörck, R. Pullerits, M. Geijer, T. Bremell, and H. Forsblad-D'Elia, "Infliximab dose reduction sustains the clinical treatment effect in active HLAB27 positive ankylosing spondylitis: a two-year pilot study," *Mediators of Inflammation*, vol. 2013, Article ID 289845, 9 pages, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 472174, 9 pages
http://dx.doi.org/10.1155/2015/472174

Research Article

Isotypes of Epstein-Barr Virus Antibodies in Rheumatoid Arthritis: Association with Rheumatoid Factors and Citrulline-Dependent Antibodies

Marie Wulff Westergaard,[1] Anette Holck Draborg,[1] Lone Troelsen,[2] Søren Jacobsen,[3] and Gunnar Houen[1]

[1]*Department of Autoimmunology and Biomarkers, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen, Denmark*
[2]*Department of Clinical Immunology, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen, Denmark*
[3]*Department of Rheumatology, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen, Denmark*

Correspondence should be addressed to Gunnar Houen; gh@ssi.dk

Received 11 November 2014; Revised 28 January 2015; Accepted 23 February 2015

Academic Editor: Hai-Feng Pan

In order to study the humoral immune response against Epstein-Barr virus (EBV) in patients with rheumatoid arthritis (RA) and to compare it with the two major autoantibody types in RA, plasma samples from 77 RA patients, 28 patients with systemic lupus erythematosus (SLE), and 28 healthy controls (HCs) were investigated by enzyme-linked immunosorbent assays (ELISA). Increased percentages of positives and concentrations of IgG/IgA/IgM antibodies against the latent EBV nuclear antigen-1 (EBNA-1) were observed in RA patients compared to SLE patients and HCs. Increased concentrations and percentages of positives of IgG/IgA/IgM against the early lytic EBV antigen diffuse (EAD) were also found in RA patients compared to HCs but were highest in SLE patients. Furthermore, associations between the elevated EBNA-1 IgA and EBNA-1 IgM levels and the presence of IgM and IgA rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs, IgG) and between elevated IgA concentrations against EAD and the presence of RFs and ACPAs in RA patients were found. Thus, RA patients had elevated antibodies of all isotypes characteristic of latent EBV infection (whereas SLE patients had elevated antibodies characteristic of lytic EBV infection). Notably, for IgM and IgA (but not IgG), these were associated with the presence of characteristic RA autoantibodies.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory systemic autoimmune disease. Worldwide, the prevalence is estimated to be about 0.5%–1%, but the incidence and prevalence vary geographically and are 2-3-fold higher in women than in men. The disease is characterised by inflamed joints and the production of autoantibodies, for example, rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs). The etiology of the disease is suggested to be a combination of environmental exposures and gene-environment interactions, but the exact cause is still unknown [1, 2]. One environmental factor may be the human herpesvirus, Epstein-Barr virus (EBV).

EBV is one of the most common viruses found in humans and is believed to infect approximately 95% of the worldwide population before an age of 40 years [3]. EBV is transmitted through saliva and infects and replicates in epithelial cells and B cells. The primary infection with EBV is mostly asymptomatic during childhood, but during adolescence it can cause infectious mononucleosis [4].

After primary infection EBV persists latently in memory B cells, where the only protein expressed is the Epstein-Barr virus nuclear antigen 1 (EBNA-1), which is responsible for maintaining viral DNA during the cell cycle and has a characteristic Gly-Ala repeat region with a presumed role in immune evasion by EBV. Occasionally, the virus reactivates and enters the lytic stage expressing genes promoting viral replication and release of virions [4, 5].

The EBV protein, early antigen diffuse (EAD) is expressed during the early lytic stage of EBV's lifecycle. It is a DNA polymerase accessory protein and is required for initiating lytic viral replication. The presence of EAD antibodies indicates initiation of viral replication [6, 7].

Cellular immunity is essential for controlling EBV infection, but the humoral immune response is also activated during EBV infection and different serological profiles can reflect the infection status/history. Viral-capsid antigen (VCA) and EAD IgM and IgG antibodies are produced during primary infection and EBNA-1 IgG antibodies are produced later in the infection. VCA IgM antibodies disappear after convalescence while VCA IgG antibodies and EBNA-1 IgG have lifelong persistence [8, 9]. IgA against EBV antigens have to our knowledge not been investigated before in RA patients.

Several studies have shown an elevated humoral and cellular anti-EBV immune response in RA patients, indicating that the virus may be associated with the autoimmune dysfunction in patients with RA [10–14]. Elevated antibody levels have been found against EBV proteins, such as VCA, EAD, early antigen restricted (EAR), and EBNA-1, in RA patients compared to healthy controls and disease controls [10–13]. In addition, RF positive RA patients have elevated EBNA-1 antibody concentrations compared to RF negative RA patients [10]. These studies have mainly focused on EBV IgG antibodies.

To obtain a detailed picture of the immune response to antigens representing the latent and lytic phases of the EBV life cycle and in order to investigate possible epithelial involvement we studied the occurrence of EBNA-1 and EAD antibodies (IgM, IgG, and IgA) in RA patients and control groups. Moreover, we looked for a possible association between EBV antibodies and the RA-characteristic autoantibodies RFs and ACPAs. Such an association would strengthen a theory of EBV as a major etiological agent in RA.

2. Patients and Methods

2.1. Patients and Controls. All patients fulfilled internationally accepted classification criteria for the autoimmune diseases investigated [15, 16]. Consents for the studies were obtained from all patients in accordance with the protocol approved by the Scientific-Ethical Committee of the Capital Region of Denmark (number HA-2007-0114).

Plasma samples were obtained from 77 RA patients and 28 SLE patients attending the Department of Rheumatology at Copenhagen University Hospital in Denmark.

Plasma samples from 28 healthy controls (HCs) were obtained with consent from volunteers at SSI, Copenhagen, Denmark.

Clinical characteristics of all patients and controls are outlined in Table 1. SLE patients and HCs have previously been reported on in the study by Draborg et al. [17] as well as RA patients in the study by Troelsen et al. [18].

2.2. ELISA

2.2.1. EBV Antibodies. ELISA was performed as previously described by Draborg et al. [6]. Briefly, dilutions and washes were performed in TTN (0.025 M Tris, 0.5% Tween 20, 0.15 M NaCl, pH 7.5). EBV EAD (Prospec protein specialist, Ness-Ziona, Israel) and EBV EBNA-1 ((whole protein) MyBioSource, San Diego, California, USA) diluted in carbonate buffer to a concentration at 1 μg/mL and 0.5 ug/mL, respectively, were applied to a 96-well microtiter polysorp plate (Nunc, Fisher Scientific Biotech Line A/S). The antigen was incubated overnight (O/N) at 4°C followed by 30 min blocking of unoccupied sites with TTN. Plasma (1 : 100 or 1 : 20 for detecting IgG antibodies or IgA/IgM antibodies, resp.) was incubated for one hour. The wells were washed before incubation with AP-conjugated goat anti-human IgG/IgA/IgM secondary antibodies (Sigma) (1 : 2000). The wells were washed again before adding *p*-nitrophenylphosphate (*p*-NPP) (1 mg/mL) diluted in AP substrate buffer. The absorbance was measured at wavelength 405–650 nm (Thermo-Max Microplate Reader, Molecular Devices).

Each sample was tested in duplicates in both coated wells and noncoated wells. For each plate, a standard curve was included using a high titre sample for normalisation of the absorbance. A sample was considered positive for EAD or EBNA-1 antibodies if the antibody binding was higher than the cut-off value (14.53, 2.98 and 22.78 U/mL, regarding EAD IgG, EAD IgA, and EAD IgM antibodies, resp., and 1.50, 7.70, and 8.27 U/mL, regarding EBNA-1 IgG, EBNA-1 IgA, and EBNA-1 IgM antibodies, resp.). The cut-off values were determined from the HCs (without outliers (determined with Grubb's test)) using the mean + 2 × SD.

For some experiments, a mosaic EBNA-1 protein without the Gly-Ala repeat region was used (results not shown).

2.2.2. Autoantibodies. ACPAs were determined by a commercial ELISA kit (Euro-Diagnostica, Malmö, Sweden), measuring IgG antibodies against cyclic citrullinated peptides (CCPs). Instructions by the manufacturer were followed.

IgM and IgA RFs were determined by ELISA using polystyrene plates (Maxisorp) coated with purified IgG. Peroxidase-conjugated F(ab)2 rabbit immunoglobulin against human IgM and IgA (DAKO, Copenhagen, Denmark), respectively, was used as detecting antibodies [19].

2.3. Statistics. Statistical analyses were carried out using GraphPad Prism software 5 (GraphPad Prism software, Inc., La Jolla, CA, USA). Comparison of antibody concentrations between patients and controls was performed using a Mann-Whitney test. Comparison of paired samples was performed using a Wilcoxon test. Statistical significant differences are indicated with $*$, $**$, $***$, or $****$ for P values less than 0.05, 0.01, 0.001, or 0.0001, respectively. Data are presented with the mean ± SEM.

3. Results

3.1. Early Antigen Diffuse (EAD). An indirect ELISA was used to measure the amount of EAD IgM/IgG/IgA in plasma samples from 77 RA patients, 28 SLE patients (disease

Table 1: Clinical characteristics of the patients and controls.

	RA	SLE	HC
Number of individuals	77	28	28
Average age (years) (range)	55.5 (27–78)	41.3 (20–81)	36.6 (22–61)
Average disease duration (years) (range)	14.8 (2–41)	—	—
Female (%)	76.6	96.4	96.4
CCP antibody positive (%)	63.6	—	—
RF IgM positive (%)	70.1	7.1	—
RF IgA positive (%)	57.1	25	—
Average hsCRP (mg/L) (range)	11.6 (0.3–69.9)	5.1 (1–22)	—
HAQ score (range)	0.82 (0–2.9)	—	—
TSS (range)	86.1 (0–321)	—	—
Methotrexate (%)	64.9	7.1	—
Glucocorticoids (%)	29.9	60.7	—
NSAID (%)	33.8	14.3	—
Anti-TNF-alfa (%)	36.4	—	—

Table 2: EAD antibody positivity rate and EBNA-1 antibody positivity rate. Table of percentage antibody (Ab) positives of EAD (a) or EBNA-1 (b) IgG/IgA/IgM, respectively. Determined by use of cut-off values. RA patients ($n = 77$), SLE patients ($n = 28$), and HCs ($n = 28$).

(a)

	EAD Ab positive (%)		
	IgG	IgA	IgM
RA ($n = 77$)	19.5	37.7	27.3
SLE ($n = 28$)	64.3	64.3	28.6
HC ($n = 28$)	10.7	7.1	7.1

(b)

	EBNA-1 Ab positive (%)		
	IgG	IgA	IgM
RA ($n = 77$)	97.4	93.5	97.4
SLE ($n = 28$)	89.3	17.6	32.1
HC ($n = 28$)	96.4	7.1	7.1

controls), and 28 HCs (Figure 1(a)). No significant differences were observed between the groups regarding EAD IgM levels, although RA and SLE patients appeared to have slightly higher levels. However, significant differences in EAD IgG and EAD IgA levels were seen between RA patients and HCs, between SLE patients and HCs, and between RA patients and SLE patients (P values of 0.0011, <0.0001, and <0.0001, resp., for EAD IgG and P values of 0.0016, <0.0001, and 0.0056, resp., for EAD IgA) (Figure 1(a)). The levels of EAD IgG and IgA were highest in SLE patients and lowest in HCs.

By use of cut-off values determined from HCs, the percentages of EAD positives were calculated. RA patients had a higher percentage of antibody positive of EAD IgM/IgG/IgA than HCs (Table 2). SLE patients had a higher percentage of positives of all 3 EAD antibody isotypes than both RA patients and HCs.

To examine a possible correlation between the two types of RA-characteristic autoantibodies and the lytic EBV protein EAD antibodies, the results for RA patients were sorted in CCP antibody positives and negatives (Figure 1(b)) and RF IgM/IgA positives and negatives (Figure 1(c)). When sorted by CCP antibody status, a significant difference was seen for EAD IgA but not for IgM or IgG (Figure 1(b)). EAD IgA levels were higher in the group with CCP IgG ($P = 0.048$). When sorted by RF status, significant differences were seen for EAD IgA and IgG (Figure 1(c)). EAD IgA levels were highest in the group with RF IgA ($P = 0.001$) and EAD IgG levels were highest in the RF positive group (RF IgM and/or IgA) ($P = 0.032$). No other significant differences were seen, when these results were sorted by RF status (IgM and/or IgA).

3.2. Epstein-Barr Virus Nuclear Antigen-1 (EBNA-1). Since EBNA-1 is the only protein expressed by EBV in the latency I stage of its life cycle [4, 20], it was decided to investigate EBNA-1 antibodies in plasma from RA patients. Plasma from 77 RA patients, 28 SLE patients, and 28 HCs was tested for EBNA-1 IgM/IgG/IgA by ELISA (Figure 2(a)). A large and highly significant difference in EBNA-1 IgM/IgG/IgA levels between RA patients and HCs and between RA patients and SLE patients ($P < 0.0001$ for all 3 antibody isotypes) was observed. In contrast, no significant difference was observed between SLE patients and HCs for EBNA-1 IgM/IgG/IgA levels (Figure 2(a)).

Cut-off values, determined from HCs, were used to calculate the percentages of positives for EBNA-1 antibodies. RA patients had noticeably higher percentages of positives for EBNA-1 IgA and EBNA-1 IgM levels than both disease controls and HCs but not for EBNA-1 IgG levels (Table 2).

To examine a possible relation between RA-characteristic autoantibodies and the latent EBV-protein EBNA-1 antibodies, the 77 RA patients were sorted into CCP antibody positives and negatives (Figure 2(b)) and IgA/IgM RF positives and negatives (Figure 2(c)).

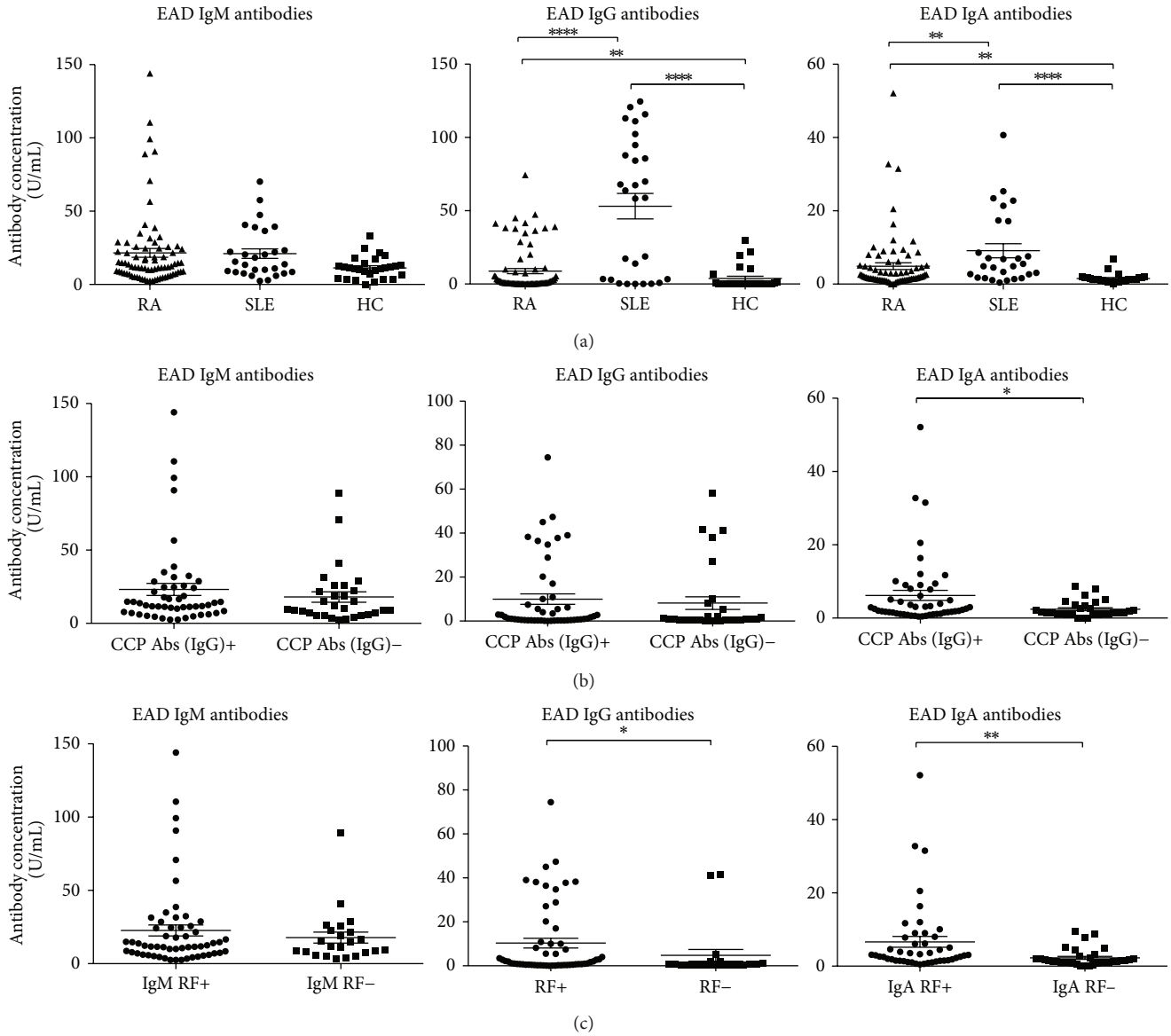


Figure 1: Scattergrams showing individual levels of EAD IgG/IgM/IgA in plasma from RA patients, SLE patients, and HCs and individual levels of EAD IgG/IgM/IgA from RA patients divided into CCP IgG positives and negatives and divided into IgA RF and IgM RF positives and negatives. (a) EAD IgM/IgG/IgA levels. RA patients (n = 77), SLE patients (n = 28), and HCs (n = 28). (b) EAD IgM/IgG/IgA levels sorted into CCP IgG positives (n = 49) and CCP IgG negatives (n = 28). RA patients total (n = 77). (c) EAD IgA levels sorted in IgA RF states, IgA RF positives (n = 44) and IgA RF negatives (n = 33). EAD IgG levels sorted in RF states, RF positives (n = 56) and RF negatives (n = 21). EAD IgM levels sorted in IgM RF states, IgM RF positives (n = 54) and IgM RF negatives (n = 23). RA patients total (n = 77). Groups were compared using the Mann-Whitney test. Data are presented as mean ± SEM. Concentrations of antibodies are presented in arbitrary units. Statistical significant differences are indicated with ∗, ∗∗, ∗∗∗, or ∗∗∗∗ for P values less than 0.05, 0.01, 0.001, or 0.0001, respectively.

A significant coherence between the EBNA-1 IgM levels and the CCP antibody state was observed ($P < 0.0001$) (Figure 2(b)). However, there was no difference for EBNA-1 IgG and EBNA-1 IgA levels with regard to CCP antibodies (Figure 2(b)).

Comparison of the EBNA-1 IgA and EBNA-1 IgM levels in the 77 RA patients sorted into IgM and IgA RF positives and negatives, respectively, revealed a significant difference between the two groups for both EBNA-1 IgM and IgA ($P < 0.0001$ and $P = 0.0005$, resp.) (Figure 2(c)). No correlation was seen when dividing RA patients' EBNA-1 IgG levels by any RF isotype positives and negatives (Figure 2(c)) or between IgM or IgA RF (not shown).

3.3. Relationships between RFs and ACPAs. Coherence between RF levels and CCP antibody state was also tested and a highly significant difference was observed in CCP antibody levels when stratified by either IgA or IgM RF state ($P < 0.0001$ for both) (Figures 3(a) and 3(b)).

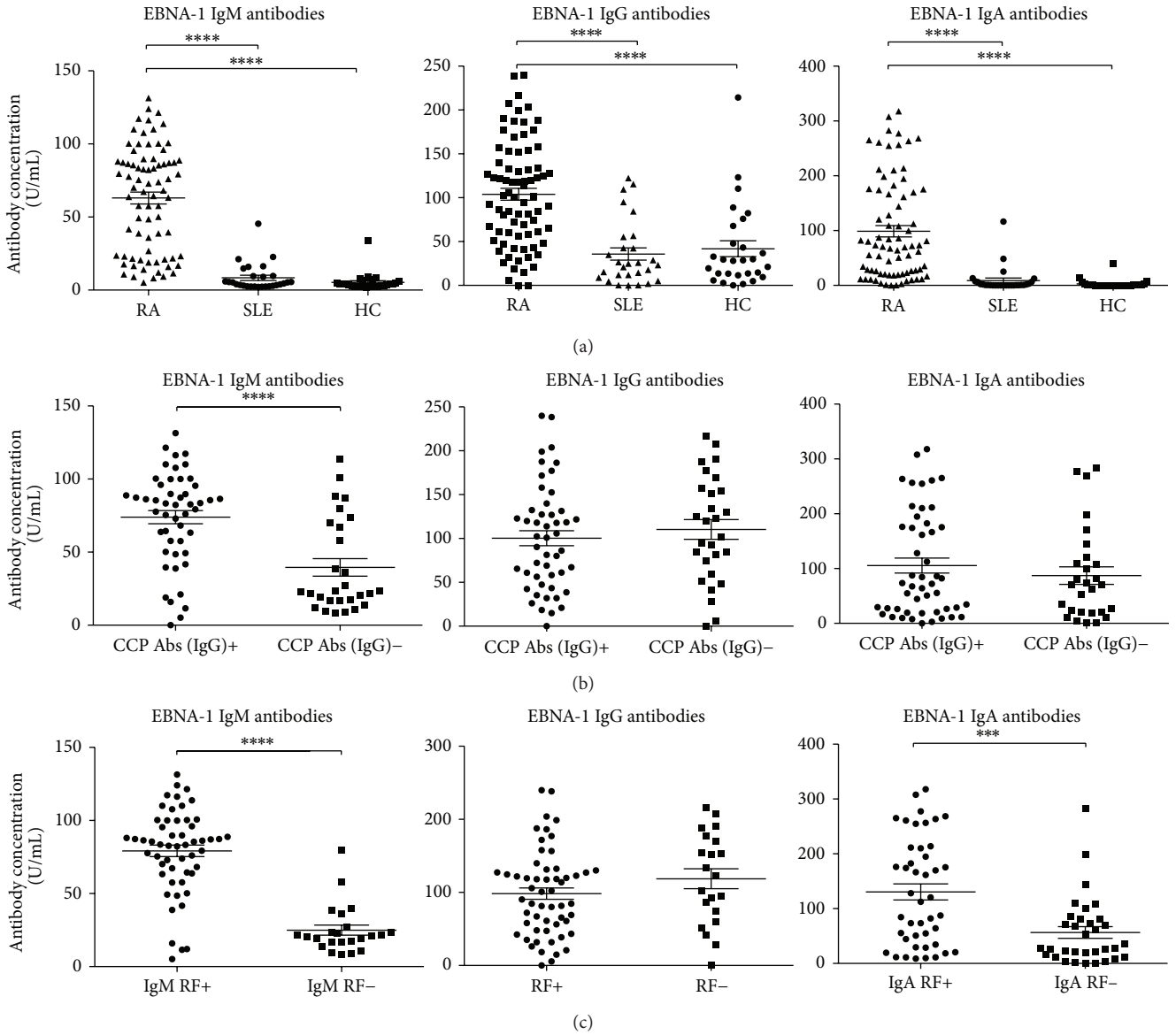


Figure 2: Scattergrams showing individual levels of EBNA-1 IgG/IgM/IgA in plasma from RA patients, SLE patients, and HCs and individual levels of EBNA-1 IgG/IgM/IgA from RA patients divided into CCP IgG positives and negatives and divided into IgA RF and IgM RF positives and negatives. (a) EBNA-1 IgM/IgG/IgA levels. RA patients (n = 77), SLE patients (n = 28), and HCs (n = 28). (b) EBNA-1 IgM/IgG/IgA levels sorted into CCP IgG positives (n = 49) and CCP IgG negatives (n = 28). RA patients total (n = 77). (c) EBNA-1 IgA levels sorted in IgA RF states, IgA RF positives (n = 44) and IgA RF negatives (n = 33). EBNA-1 IgG levels sorted in RF states, IgM or IgA RF positives (n = 56) and RF negatives (n = 21). EBNA-1 IgM levels sorted in IgM RF states, IgM RF positives (n = 54) and IgM RF negatives (n = 23). RA patients total (n = 77). Groups were compared using the Mann-Whitney test. Data are presented as mean ± SEM. Concentrations of antibodies are presented in arbitrary units. Statistical significant differences are indicated with ∗, ∗∗, ∗∗∗, or ∗∗∗∗ for P values less than 0.05, 0.01, 0.001, or 0.0001, respectively.

3.4. Relation to Treatments. Since immunosuppressive treatments may influence the results of antibody measurements, the influence of immunosuppressive medications such as Methotrexate (MTX), glucocorticoids, NSAIDs, TNF-α inhibitors, and DMARDs on both EAD and EBNA-1 antibody levels was investigated. A small but significant (P = 0.010) correlation was only observed between elevated EAD IgM levels and the intake of MTX but not for EAD IgG or IgA (Figure 4). No correlations were seen for any drug treatment and EBNA-1 IgM/IgG/IgA (results not shown).

4. Discussion

The current study examined the possible relation between EBV and RA. SLE patients were chosen as disease controls, as SLE is a related connective tissue rheumatic disease with

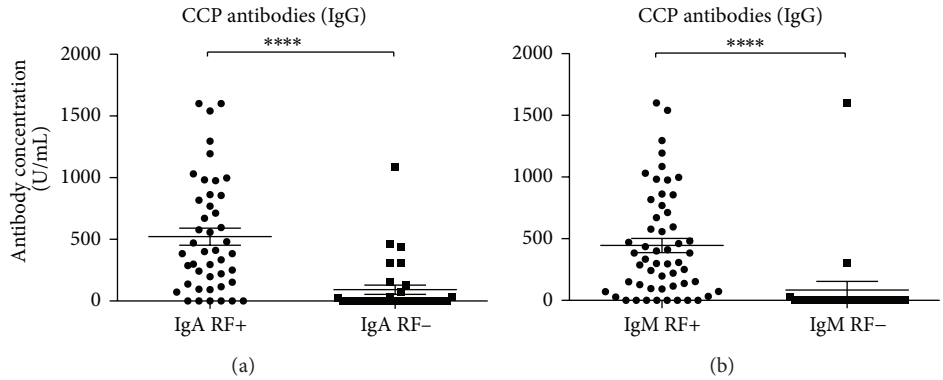


Figure 3: Scattergrams showing CCP antibodies from RA patients divided into IgA or IgM RF positives and negatives. (a) CCP antibodies divided into IgA RF states, IgA RF positives (n = 44) and IgA RF negatives (n = 33). (b) CCP antibodies divided into IgM RF states, IgM RF positives (n = 54) and IgM RF negatives (n = 23). RA patients total (n = 77). The RF positives and negatives were compared using the Mann-Whitney test. Data are presented as mean ± SEM. Concentrations of antibodies are presented in arbitrary units. Statistical significant differences are indicated with ∗, ∗∗, ∗∗∗, or ∗∗∗∗ for P values less than 0.05, 0.01, 0.001, or 0.0001, respectively.

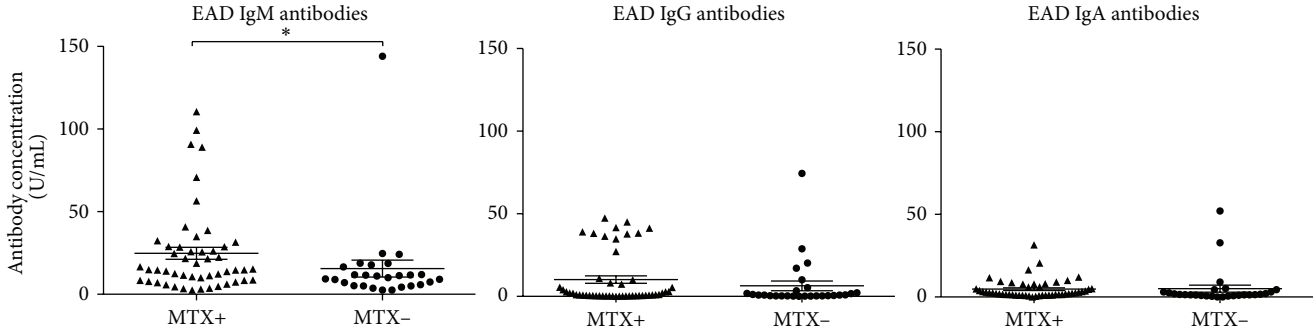


Figure 4: Scattergrams showing individual levels of EAD IgG/IgM/IgA in plasma from RA patients divided into the two groups according to acute Methotrexate (MTX) treatment. EAD IgM/IgG/IgA levels divided into patients on MTX treatment and patients not on MTX treatment. RA patients on MTX (n = 50), RA patients not on MTX (n = 27), and RA patients total (n = 77). The two groups were compared using the Mann-Whitney test. Data are presented as mean ± SEM. Concentrations of antibodies are presented in arbitrary units. Statistical significant differences are indicated with ∗, ∗∗, ∗∗∗, or ∗∗∗∗ for P values less than 0.05, 0.01, 0.001, or 0.0001, respectively.

distinct clinical and serological characteristics (rather than Sjögren syndrome which has features overlapping with both). The study focused on two antigens characteristic of EBVs different life cycles: the latent EBNA-1 and the early lytic EAD. EBNA-1 is the only essential protein for maintaining the EBV genome during the latency stage of the EBV lifecycle [21] and it is the only protein expressed in the latency stage I.

Elevated antibody concentrations (U/ml) and higher percentages of antibody positives were observed in RA patients, compared to control groups, together with an association between high EBNA-1 antibody concentrations and the presence of the characteristic RA autoantibodies, RFs, and ACPA. More specifically, the percentages of EBNA-1 IgM and IgA were remarkably higher in RA patients compared to SLE patients (disease controls) and HCs and EBNA-1 IgM, IgG, and IgA levels (U/ml) were strongly elevated in RA patients. This indicates an increased humoral response towards EBV in the latency stage in RA patients.

Noticeably, if an individual has been exposed to EBV, EBNA-1 IgG will be measurable throughout the lifetime [22]. Because of the high infection effectiveness of EBV of about 95% of the world population (>40 years old) [3], a similar percentage of EBNA-1 IgG positives were expected in all examined groups consistent with the current observations (Table 2).

In general, IgA and IgM have half-lives in blood of approximately 1 week after production [23, 24]. Therefore, high levels of these isotypes may imply an ongoing humoral immune response [25]. EBNA-1 is expressed at relatively high levels in EBV-infected cells, but the endogenous pathway of presentation of EBNA-1-derived peptides in complex with MHC I on the surface of the cells is restricted due to the Gly-Ala repeat domain in EBNA-1 that prevents degradation in the proteasomes [26]. However, the exogenous pathway presentation of EBNA-1 via MHC II is still effective [27]. This could be a part of the explanation for the present results, indicating a persistent humoral immune response against EBNA-1 released from dying infected cells.

The elevated antibody levels and higher percentages of antibody positives seen in RA patients suggest that the

immune surveillance of EBV in RA patients is dysfunctional. This is supported by previous reports that CD8+ T cells specific for lytic EBV proteins are dysfunctional in RA patients [28] and, consequently, an elevated humoral response to EBV has been induced.

The present observations that IgG and IgA levels as well as percentage of antibody positives against the lytic EBV protein EAD are elevated in RA patients compared to HCs also indicate some degree of reactivation of the EBV infection in B cells and epithelial cells.

In agreement with Draborg et al. [6], SLE patients had noticeably higher levels and percentages of antibody positives for both EAD IgG and EAD IgA compared to RA patients, which indicates that reactivation of EBV is more frequent in SLE patients than in RA patients (especially in epithelial cells).

Recent studies have shown that the intake of the immunosuppressive medical Methotrexate (MTX) can lead to increased reactivation of EBV [29]. The elevated levels of EAD IgG and EAD IgA in RA patients did not correlate with patients' intake of MTX in this study. However, a small but significant correlation was observed with elevated EAD IgM levels in patients currently taking MTX at the time where the blood sample was taken, indicating a low degree of EBV reactivation by MTX. Similarly, comparing EAD antibody levels in patients taking other immunosuppressive medications such as glucocorticoids, NSAIDs, TNF-α inhibitors, and DMARDs, no correlation was observed (data not shown). Also EBNA-1 antibody levels and the intake of immunosuppressive medications were investigated and again no correlation was observed (data not shown). Altogether, this shows that the present results cannot be explained by drug treatment.

The correlation between RFs and CCP antibodies and the EAD antibody levels was also examined. No correlation between EAD IgM or EAD IgG and the characteristic RA autoantibodies in RA patients was found, while a correlation was observed between elevated EAD IgA levels and IgA RF positivity in RA patients as well as elevated EAD IgA levels in CCP antibody positive RA patients.

No relation between EBNA-1 IgG or IgA levels and CCP antibodies was found. However, an association between the EBNA-1 IgM response and the presence of ACPA was found.

An interesting hypothesis is that EBNA-1 in a citrullinated form could act as a target for ACPAs and that this could be a direct link between EBV infection and the onset or just progression of production of ACPAs. Combined with the observation that citrullinated proteins are found to be abundant in the synovial joints [30, 31], this leads to a theory that ACPA production could play a role in the induction of the inflammation found in the synovial joints of RA patients as suggested by Klareskog et al. [32].

The involvement of exogenous antigens in the production of ACPAs has also been suggested by Pratesi et al. [33]. Observations of the presence of antibodies against citrullinated peptides from EBNA-1 and EBNA-2 in RA patients, while these are almost absent in HCs and disease controls [33, 34], led to speculations of the EBV involvement in the production of ACPA.

This indicates that an antibody response against cells with reactivated EBV infection could have an influence on autoantibody production. In accordance with the observation that several of the ACPAs react with epithelial cells due to their content of a number of the target citrullinated proteins [35], an association could be possible.

No correlation was observed between EBNA-1 IgG levels and the presence of any RF isotype. However, a significant correlation between elevated EBNA-1 IgA levels and the presence of IgA RF as well as association between elevated EBNA-1 IgM levels and the presence of IgM RF in the RA cohort was revealed. The same pattern of coherence between the EBNA-1 antibody levels and the presence of autoantibodies was observed when investigating mosaic EBNA-1 (without the Gly-Ala repeats) antibodies and EBNA-1 antibodies (results not shown). This suggests that the Gly-Ala repeat region does not have a crucial effect on the amount of EBNA-1 antibodies.

The present results are in accordance with a previous study, which observed elevated EBNA antibodies in RF-seropositive RA patients compared to RF-seronegative RA patients [10]. It may be hypothesised from these findings that simultaneous processes are responsible for induction of RFs and EBNA-1 antibodies. These processes could be induced by the recognition and destruction of EBV-infected cells, for example, infected memory B cells, where EBV is in its latency stage I only expressing EBNA-1. Together with the observations that alteration in the glycosylation of IgG from EBV-infected B cells in RA patients can impair the functionality of the IgG [36, 37], a destructed EBV-infected cell releasing EBNA-1 and IgG could be the antigen source responsible for the production of both EBNA-1 antibodies and RFs.

The increased IgM/IgA concentrations against especially the latent EBV protein, EBNA-1, but also IgG/IgA against the lytic EBV protein, EAD, in RA patients compared to HCs are in accordance with previous studies showing elevated levels of IgG against the EBV proteins, EBNA-1, VCA, and EAD in sera from RA patients relative to HCs [10, 11]. Later studies have mainly investigated antibodies against the two lytic EBV proteins VCA and EAD and found them to be elevated in RA patients compared to HCs [12, 13]. Although the present results indicate that the latent EBV infection has a major influence on RA, these previous observations suggested that lytic reactivation of the EBV infection also has an influence on RA.

Finally, the results presented here are in agreement with the polyclonal B cell activation seen in RA (possibly caused by EBV) and the clinical efficacy of B cell-depleting agents in RA [38–41] and are in agreement with previous investigations [42–46]; RFs and ACPAs were found also in this RA cohort to be correlated.

To summarise, we found an association between EBNA-1 antibodies and characteristic autoantibodies in RA patients. As expected, a significant association between anti-CCP antibody levels and presence of IgA RF and IgM RF was observed. Even though the occurrence of antibodies against EBNA-1 in this study was highly associated with occurrence of ACPA and RF antibodies, this study was cross-sectional and further

investigations are needed to elucidate the interplay between these factors in the pathogenesis of RA.

Conflict of Interests

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

References

[1] E. Myasoedova, J. M. Davis III, C. S. Crowson, and S. E. Gabriel, "Epidemiology of rheumatoid arthritis: rheumatoid arthritis and mortality," *Current Rheumatology Reports*, vol. 12, no. 5, pp. 379–385, 2010.

[2] M. Pedersen, S. Jacobsen, P. Garred et al., "Strong combined gene-environment effects in anti-cyclic citrullinated peptide-positive rheumatoid arthritis: a nationwide case-control study in Denmark," *Arthritis & Rheumatism*, vol. 56, no. 5, pp. 1446–1453, 2007.

[3] K. H. Costenbader and E. W. Karlson, "Epstein-Barr virus and rheumatoid arthritis: is there a link?" *Arthritis Research and Therapy*, vol. 8, article 204, 2006.

[4] H. H. Niller, H. Wolf, and J. Minarovits, "Regulation and dysregulation of Epstein-Barr virus latency: implications for the development of autoimmune diseases," *Autoimmunity*, vol. 41, no. 4, pp. 298–328, 2008.

[5] W. Amon and P. J. Farrell, "Reactivation of Epstein-Barr virus from latency," *Reviews in Medical Virology*, vol. 15, no. 3, pp. 149–156, 2005.

[6] A. H. Draborg, J. M. Jørgensen, H. Müller et al., "Epstein-Barr virus early antigen diffuse (EBV-EA/D)-directed immunoglobulin A antibodies in systemic lupus erythematosus patients," *Scandinavian Journal of Rheumatology*, vol. 41, no. 4, pp. 280–289, 2012.

[7] Q. Zhang, E. Holley-Guthrie, D. Dorsky, and S. Kenney, "Identification of transactivator and nuclear localization domains in the Epstein-Barr virus DNA polymerase accessory protein, BMRF1," *The Journal of General Virology*, vol. 80, no. 1, pp. 69–74, 1999.

[8] W. Hinderer, D. Lang, M. Rothe, R. Vornhagen, H. H. Sonneborn, and H. Wolf, "Serodiagnosis of Epstein-Barr virus infection by using recombinant viral capsid antigen fragments and autologous gene fusion," *Journal of Clinical Microbiology*, vol. 37, no. 10, pp. 3239–3244, 1999.

[9] É. Toussirot and J. Roudier, "Epstein-Barr virus in autoimmune diseases," *Best Practice and Research: Clinical Rheumatology*, vol. 22, no. 5, pp. 883–896, 2008.

[10] M. A. Alspaugh, G. Henle, E. T. Lennette, and W. Henle, "Elevated levels of antibodies to Epstein-Barr virus antigens in sera and synovial fluids of patients with rheumatoid arthritis," *The Journal of Clinical Investigation*, vol. 67, no. 4, pp. 1134–1140, 1981.

[11] P. B. Ferrell, C. T. Aitcheson, G. R. Pearson, and E. M. Tan, "Seroepidemiological study of relationships between Epstein-Barr virus and rheumatoid arthritis," *The Journal of Clinical Investigation*, vol. 67, no. 3, pp. 681–687, 1981.

[12] M. McDermott, M. Molloy, J. Buckley, and J. Greally, "Antibodies to Epstein-Barr viral antigens in familial rheumatoid arthritis," *Irish Journal of Medical Science*, vol. 158, no. 8, pp. 203–205, 1989.

[13] M. Mousavi-Jazi, L. Boström, C. Lövmark, A. Linde, M. Brytting, and V.-A. Sundqvist, "Infrequent detection of cytomegalovirus and Epstein-Barr virus DNA in synovial membrane of patients with rheumatoid arthritis," *Journal of Rheumatology*, vol. 25, no. 4, pp. 623–628, 1998.

[14] J. D. Lünemann, O. Frey, T. Eidner et al., "Increased frequency of EBV-specific effector memory $CD8^+$ T cells correlates with higher viral load in rheumatoid arthritis," *Journal of Immunology*, vol. 181, no. 2, pp. 991–1000, 2008.

[15] 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 & Rheumatology*, vol. 62, pp. 2569–2581, 2010.

[16] M. C. Hochberg, "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus," *Arthritis and rheumatism*, vol. 40, article 1725, 1997.

[17] A. H. Draborg, S. Jacobsen, M. Westergaard et al., "Reduced response to Epstein-Barr virus antigens by T-cells in systemic lupus erythematosus patients," *Lupus Science & Medicine*, vol. 1, no. 1, Article ID e000015, 2014.

[18] L. N. Troelsen, P. Garred, B. Christiansen et al., "Double role of mannose-binding lectin in relation to carotid intima-media thickness in patients with rheumatoid arthritis," *Molecular Immunology*, vol. 47, no. 4, pp. 713–718, 2010.

[19] M. Høier-Madsen, L. P. Nielsen, and S. Møller, "Determination of IgM rheumatoid factors by enzyme-linked immunosorbent assay (ELISA)," *Danish Medical Bulletin*, vol. 148, pp. 2018–2021, 1986.

[20] L. Frappier, "The Epstein-Barr virus EBNA1 protein," *Scientifica*, vol. 2012, Article ID 438204, 15 pages, 2012.

[21] F. Chen, J.-Z. Zou, L. Di Renzo et al., "A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1," *Journal of Virology*, vol. 69, no. 6, pp. 3752–3758, 1995.

[22] R. D. Hess, "Routine Epstein-Barr virus diagnostics from the laboratory perspective: still challenging after 35 years," *Journal of Clinical Microbiology*, vol. 42, no. 8, pp. 3381–3387, 2004.

[23] T. J. Kindt, R. A. Goldsby, B. A. Osborne, and J. Kuby, *Immunology*, W.H. Freeman, New York, NY, USA, 6th edition, 2007.

[24] O. H. Brekke and I. Sandlie, "Therapeutic antibodies for human diseases at the dawn of the twenty-first century," *Nature Reviews Drug Discovery*, vol. 2, no. 1, pp. 52–62, 2003.

[25] K. P. Murphy, C. A. Janeway, P. Travers, M. Walport, A. Mowat, and C. T. Weaver, *Janeway's Immunobiology*, Garland Science, London, UK, 8th edition, 2012.

[26] M. Zhang and P. Coffino, "Repeat sequence of Epstein-Barr virus-encoded nuclear antigen 1 protein interrupts proteasome substrate processing," *Journal of Biological Chemistry*, vol. 279, no. 10, pp. 8635–8641, 2004.

[27] R. Fåhraeus, "Do peptides control their own birth and death?" *Nature Reviews Molecular Cell Biology*, vol. 6, no. 3, pp. 263–267, 2005.

[28] T. Klatt, Q. Ouyang, T. Flad et al., "Expansion of peripheral $CD8^+$ $CD28^-$ T cells in response to Epstein-Barr virus in patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 32, no. 2, pp. 239–251, 2005.

[29] A. Ichikawa, F. Arakawa, J. Kiyasu et al., "Methotrexate/iatrogenic lymphoproliferative disorders in rheumatoid arthritis: histology, Epstein-Barr virus, and clonality are important predictors of disease progression and regression," *European Journal of Haematology*, vol. 91, no. 1, pp. 20–28, 2013.

[30] K. Matsuo, Y. Xiang, H. Nakamura et al., "Identification of novel citrullinated autoantigens of synovium in rheumatoid arthritis using a proteomic approach," *Arthritis Research and Therapy*, vol. 8, article R175, 2006.

[31] Y. Takizawa, A. Suzuki, T. Sawada et al., "Citrullinated fibrinogen detected as a soluble citrullinated autoantigen in rheumatoid arthritis synovial fluids," *Annals of the Rheumatic Diseases*, vol. 65, no. 8, pp. 1013–1020, 2006.

[32] L. Klareskog, K. Amara, and V. Malmström, "Adaptive immunity in rheumatoid arthritis: anticitrulline and other antibodies in the pathogenesis of rheumatoid arthritis," *Current Opinion in Rheumatology*, vol. 26, no. 1, pp. 72–79, 2014.

[33] F. Pratesi, C. Tommasi, C. Anzilotti, D. Chimenti, and P. Migliorini, "Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 3, pp. 733–741, 2006.

[34] F. Pratesi, C. Tommasi, C. Anzilotti et al., "Antibodies to a new viral citrullinated peptide, VCP2: fine specificity and correlation with anti-cyclic citrullinated peptide (CCP) and anti-VCP1 antibodies," *Clinical and Experimental Immunology*, vol. 164, no. 3, pp. 337–345, 2011.

[35] Z. Szekanecz, L. Soós, Z. Szabó et al., "Anti-citrullinated protein antibodies in rheumatoid arthritis: as good as it gets?" *Clinical Reviews in Allergy and Immunology*, vol. 34, no. 1, pp. 26–31, 2008.

[36] R. B. Parekh, D. A. Isenberg, B. M. Ansell, I. M. Roitt, R. A. Dwek, and T. W. Rademacher, "Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity," *The Lancet*, vol. 1, no. 8592, pp. 966–969, 1988.

[37] J. Keusch, P. M. Lydyard, and P. J. Delves, "The effect on IgG glycosylation of altering β1,4-galactosyltransferase-1 activity in B cells," *Glycobiology*, vol. 8, no. 12, pp. 1215–1220, 1998.

[38] S. P. Moyes, C. M. Brown, B. B. Scott, R. N. Maini, and R. A. Mageed, "Analysis of V_κ genes in rheumatoid arthritis (RA) synovial B lymphocytes provides evidence for both polyclonal activation and antigen- driven selection," *Clinical and Experimental Immunology*, vol. 105, no. 1, pp. 89–98, 1996.

[39] T. Dörner and G. R. Burmester, "The role of B cells in rheumatoid arthritis: mechanisms and therapeutic targets," *Current Opinion in Rheumatology*, vol. 15, no. 3, pp. 246–252, 2003.

[40] G. Cambridge, H. C. Perry, L. Nogueira et al., "The effect of B-cell depletion therapy on serological evidence of B-cell and plasmablast activation in patients with rheumatoid arthritis over multiple cycles of rituximab treatment," *Journal of Autoimmunity*, vol. 50, pp. 67–76, 2014.

[41] M. Scarsi, L. Paolini, D. Ricotta et al., "Abatacept reduces levels of switched memory B cells, autoantibodies, and immunoglobulins in patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 41, no. 4, pp. 666–672, 2014.

[42] M. G. Zavala-Cerna, A. Nava, E. García-Castañeda, J. Durán-González, M. J. Arias-Merino, and M. Salazar-Páramo, "Serum IgG activity against cyclic citrullinated peptide in patients evaluated for rheumatoid factor correlates with the IgM isotype," *Rheumatology International*, vol. 28, no. 9, pp. 851–857, 2008.

[43] A. Greiner, H. Plischke, H. Kellner, and R. Gruber, "Association of anti-cyclic citrullinated peptide antibodies, anti-citrullin antibodies, and IgM and IgA rheumatoid factors with serological parameters of disease activity in rheumatoid arthritis," *Annals of the New York Academy of Sciences*, vol. 1050, pp. 295–303, 2005.

[44] T. D. Jaskowski, H. R. Hill, K. L. Russo, G. Lakos, Z. Szekanecz, and M. Teodorescu, "Relationship between rheumatoid factor isotypes and IgG anti-cyclic citrullinated peptide antibodies," *Journal of Rheumatology*, vol. 37, no. 8, pp. 1582–1588, 2010.

[45] G. Lakos, L. Soós, A. Fekete et al., "Anti-cyclic citrullinated peptide antibody isotypes in rheumatoid arthritis: association with disease duration, rheumatoid factor production and the presence of shared epitope," *Clinical and Experimental Rheumatology*, vol. 26, no. 2, pp. 253–260, 2008.

[46] G. A. Schellekens, H. Visser, B. A. de Jong et al., "The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide," *Arthritis & Rheumatism*, vol. 43, no. 1, pp. 155–163, 2000.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 595273, 10 pages
http://dx.doi.org/10.1155/2015/595273

Research Article

Study of Osteoarthritis Treatment with Anti-Inflammatory Drugs: Cyclooxygenase-2 Inhibitor and Steroids

Hongsik Cho,[1,2] Andrew Walker,[1] Jeb Williams,[1] and Karen A. Hasty[1,2]

[1] *Department of Orthopaedic Surgery and Biomedical Engineering, University of Tennessee Health Science Center and Campbell Clinic, Memphis, TN, USA*
[2] *Veterans Affairs Medical Center, Memphis, TN, USA*

Correspondence should be addressed to Hongsik Cho; hcho4@uthsc.edu and Karen A. Hasty; khasty@uthsc.edu

Received 4 December 2014; Revised 6 February 2015; Accepted 12 March 2015

Academic Editor: Ye Shuang

Patients with osteoarthritis (OA), a condition characterized by cartilage degradation, are often treated with steroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and cyclooxygenase-2 (COX-2) selective NSAIDs. Due to their inhibition of the inflammatory cascade, the drugs affect the balance of matrix metalloproteinases (MMPs) and inflammatory cytokines, resulting in preservation of extracellular matrix (ECM). To compare the effects of these treatments on chondrocyte metabolism, TNF-α was incubated with cultured chondrocytes to mimic a proinflammatory environment with increasing production of MMP-1 and prostaglandin E2 (PGE2). The chondrocytes were then treated with either a steroid (prednisone), a nonspecific COX inhibitor NSAID (piroxicam), or a COX-2 selective NSAID (celecoxib). Both prednisone and celecoxib decreased MMP-1 and PGE-2 production while the nonspecific piroxicam decreased only the latter. Both prednisone and celecoxib decreased gene expression of MMP-1 and increased expression of aggrecan. Increased gene expression of type II collagen was also noted with celecoxib. The nonspecific piroxicam did not show these effects. The efficacy of celecoxib *in vivo* was investigated using a posttraumatic OA (PTOA) mouse model. *In vivo*, celecoxib increases aggrecan synthesis and suppresses MMP-1. In conclusion, this study demonstrates that celecoxib and steroids exert similar effects on MMP-1 and PGE2 production *in vitro* and that celecoxib may demonstrate beneficial effects on anabolic metabolism *in vivo*.

1. Introduction

Osteoarthritis (OA) is the leading cause of pain and disability in older individuals in the US. Currently, there is no cure for OA, and the standards of treatment are primarily limited to pain management, steroids and other anti-inflammatory drugs, physical therapy, and eventual joint replacement [1]. Posttraumatic OA (PTOA) occurs after joint, ligament, or bone injury or surgery. In all types of OA, mechanical stress and overuse result in stimulation of proinflammatory cytokines like TNF-α (tumor necrosis factor) and matrix metalloproteinases (MMPs) [2–4]. These MMPs (especially 1 and 13) degrade type II collagen (CII) resulting in focal lesions in the articular surface [5–7]. In this mechanism, TNF-α plays a key role in the degradation process by stimulating expression and release of proteases, such as collagenases, aggrecanases, and MMPs, which degrade collagen and aggrecan. Additionally, these proinflammatory cytokines stimulate synthesis and release of nitric oxide (NO) and prostaglandin E2 (PGE2) [8].

The anti-inflammatory effects of NSAIDs are mainly due to their ability to inhibit cyclooxygenase (COX), impairing production of prostaglandins, which are important mediators of both pain and the inflammatory response. COX enzymes metabolize arachidonic acid, forming prostaglandin H2, which is subsequently metabolized by prostaglandin E synthase into prostaglandin E2 (PGE2) [9, 10]. There are two isoforms of the COX enzyme: COX-1, found in most tissues and constitutively expressed in normal cells, and COX-2, which is not expressed in healthy tissue but is induced by various catabolic mediators, such as cytokines, growth factors, and mechanical stress [11]. Beneficial effects of NSAIDs on inflammation are mediated by COX-2 inhibition, whereas unwanted gastrointestinal effects are caused by primarily inhibition of COX-1 [12]. This data initially popularized

the use of selective COX-2 inhibitors. Celecoxib (SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfon-amide), the only FDA approved COX-2 inhibitor, has been used in the treatment of OA [13, 14].

Previous studies show that nonsteroidal anti-inflammatory drugs (NSAIDs) may have beneficial effects on cartilage damage through their inhibition of PGE2 production [15, 16]. PGE2, derived from the activity of IL-1β and TNF-α, results in decreased proteoglycan content in cartilage explants. [17]. Cartilage from joint replacement surgery patients treated with celecoxib, a COX-2 selective inhibitor, showed a higher rate of proteoglycan synthesis and a better retention of the newly formed proteoglycans, effects which preserve the articular surface and delay OA. Nonspecific COX inhibitors did not demonstrate these findings but instead showed a tendency towards a lower synthesis rate of proteoglycans [18]. A better understanding of mechanisms and the timing of significant biologic events in the development of OA and PTOA will allow investigators to determine optimal timing for biologic interventions in the future [4].

Current minimalist therapies are only palliative and little is done to prevent cartilage damage. Because treatment does not occur until after painful symptoms present, minimalist therapies (rest, ice, short term NSAIDS, elastic support) for traumatic or sport injuries whether or not they are surgically treated may be missing an opportunity to prevent or slow OA development with early pharmacological intervention.

In previous studies, we have accomplished early OA detection using a fluorescent selectively binding monoclonal antibody (Mab) which binds exposed CII in the articular cartilage. Binding is measured with a near-infrared imaging system. The amount of binding is observed to be proportional to the extent of the damage to the cartilage [19].

In this study, we subject chondrocytes *in vitro* to a proinflammatory dose of TNF-α (thus mimicking an activated chondrocyte) and subsequently compare the efficacy of a steroid (prednisone), a COX-2 selective inhibitor (celecoxib), and a nonselective COX inhibitor (piroxicam) for reducing catabolic MMP and PGE2 production and stimulating anabolic CII and aggrecan production [20]. We have also investigated the effect of the COX-2 inhibitor on the progression of PTOA *in vivo*. This is accomplished using a mechanically loaded PTOA mouse model and this lab's previously studied monoclonal antibody damage detection system. Mechanical loading induces inflammatory change through the nuclear factor-κB (NF-κB) pathway, thus initiating proinflammatory action [21]. Use of a mechanically loaded PTOA model will allow future longitudinal studies of drug efficacy without the need for sacrificing animals during the study. This preliminary study with the PTOA model establishes a noninvasive, easily reproducible method for initiating inflammatory PTOA changes in murine joints. Fluorescently tagged antibodies bind to exposed articular cartilage [19]. This binding is measured as radiant efficiency using IVIS scanning of the intact murine joint [19]. Modifications to the disease state over the course of treatment with drugs such as celecoxib can be continuously followed. Results from this preliminary study will guide future research in this direction.

2. Methods

2.1. Cell Culture. The primary chondrocytes used in this investigation were aseptically harvested from the articular cartilage of the femoral condyles of domestic pigs ranging from 25 to 35 kg. Articular cartilage was removed from the condyles in thin sections by cutting just beneath the surface in a direction that paralleled the natural curvature of the condyles. All tissues were taken from the knees of healthy pigs freshly sacrificed for other experiments according to approved protocols and experimental procedures of the University of Tennessee Health Science Center.

The chondrocytes were isolated by 1-2-hour digestion at 37°C in 0.05% Pronase (Boehringer Mannheim, Mannheim, Germany), followed by overnight digestion in at 37°C in 0.2% collagenase (Worthington Biologicals, Lakewood, NJ) using modified F-12K medium (Invitrogen, Grand Island, NY) with 5% fetal calf serum (FCS, Atlanta Biologicals, GA). The cells were then plated at 15,000 cells/cm^2. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and in F-12K medium supplemented with 10% FCS, streptomycin (50 μg/mL; Invitrogen), penicillin G (50 IU/mL; Invitrogen), L-glutamine (2 mM; Invitrogen), and ascorbic acid (50 μg/mL; Invitrogen). The medium was changed every other day until the cells were confluent.

2.2. Drug Preparation and Treatment. Piroxicam, celecoxib, and prednisone were purchased in powdered form from Sigma-Aldrich (St. Louis, MO). Drugs were prepared for three treatment groups: celecoxib (10 μM), piroxicam (5 μM), and prednisone (5 μM). All drugs had minimal solubility in water; thus 10 μL of DMSO was used to fully dissolve 1 mg of each drug. The drug/DMSO mixture was then diluted to the desired treatment molarity with serum free F12K media containing 5 mL penicillin/streptomycin and ascorbic acid (50 μg/mL). To activate chondrocytes and stimulate an inflammatory response, all groups were dosed with 5 ng/mL of TNF-α. After 24 hours, the supernatant media were removed for Western blot analysis for MMP-1 and ELISA for PGE2.

2.3. PTOA Mouse Model and In Vivo Drug Treatment. Twenty-four C57BL/6 male mice (age of 10 weeks at time of mechanical loading; body weight of ~20 g with <10% variance) were obtained from Jackson Laboratory (Maine, USA). Mice were randomly divided into two test groups: a control to which a mechanical load was applied to the left knee without drug treatment and a mechanically loaded group treated with celecoxib. All procedures, in this study, were performed according to approved protocols and experimental procedures of IACUC at the University of Tennessee Health Science Center. In order to prepare for mechanical loading, the mice were placed in an anesthetic induction chamber and anesthetized continuously with 2% isoflurane. The left leg of each mouse was positioned into a custom made loading apparatus within the calibrated ElectroForce 3200 (Bose Corp., MN, USA) biomaterials test instrument. The distal femur rested in the upper cup and the dorsiflexed ankle was inserted into the bottom cup of the apparatus (Figure 7,

schematic diagram). In order to execute the loading protocol, the left knee joint of each mouse received 40 cycles of compressive loading at 9 N, three times weekly for two weeks. These methods were adapted from Poulet's protocol [22]. The mice were allowed to have normal activity in between and after load applications. The loading data was collected for each mouse using WinTest software (Bose Corp., MN, USA). All mice were treated with 5 weeks of either celecoxib or saline with 0.1% DMSO by daily gavaging beginning on day 1 of mechanical loading. Celecoxib was dissolved in saline with 0.1% DMSO and administered at a dosage of 10 mg/kg/day (0.268 mg in 100 μL) per Cottrell's previous study [23]. Each dose was equal and administered according to the same therapeutic regimen based on the average initial weight of all experimental mice. The mice were allowed to ingest oral food and water ad libitum.

2.4. Cell Viability. For counting and general microscopic observations, isolated chondrocytes were stained with 0.4% trypan blue dye and counted under light microscopy using a 0.1 mm deep hemocytometer (Reichert, Buffalo, NY).

2.5. PGE2 Enzyme-Linked Immunosorbent Assay (ELISA). After 24 hours of treatment, supernatants were collected from each well and analyzed for PGE2 concentration with an Enzyme Immunoassay kit (Item number 514010, Cayman Chemical Co., Inc., Ann Arbor, MI). Finally, the microtiter plate was read at a wavelength of 405 nm using a plate reader (SPECTRAmaxTM, Molecular Devices Corp., CA).

2.6. Western Blot for MMP-1. After 24 hours in culture, the supernatants were collected and proteins isolated. Protein concentrations were normalized by cell number and then mixed with Laemmli sample buffer, separated by SDS/PAGE, and electrophoretically transferred to PVDF membranes (GE Healthcare, PA). Blots were blocked for 1 h in Tris-buffered saline with 5% milk and incubated overnight at 4°C with primary antibodies to MMP-1. Membranes were washed in Tris-buffered saline, incubated with HRP conjugated secondary antibodies, and washed. Immunoreactive bands were visualized by incubation with ECF substrate (Amersham, PA).

2.7. Optical and Histopathological Analysis. Cartilage damage in early OA was quantitated using a fluorescent monoclonal antibody that binds exposed CII (MabCII) in the articular cartilage. Binding of the antibody to the damaged cartilage *in vivo* is measured with an optical imaging system. We have shown that the amount of binding is observed to be proportional to the extent of the damage to the cartilage [19]. To determine the amount of cartilage damage *in vivo*, mice were injected retroorbitally with 80 μL of solution containing near-infrared fluorescent dye (NIF) conjugated to type II collagen antibody using a XenoLight CF680 Labeling kit (Caliper Life Science, MA). After 24 hours, the mice were anesthetized, depilated, and scanned using the *in vivo* imaging system (IVIS Lumina XR System, Perkin Elmer, Hopkinton, MA) with a mid-high range filter set (excitation 675 nm, emission 720 nm). The fluorescence remaining in each knee joint was quantified using Living Image 4.0 software to calculate the flux radiating omnidirectionally from the region of interest (ROI) and graphed as radiant efficiency (photons/sec/cm^2/str)/(μW/cm^2). To yield a standardized ROI for measurement of the knee fluorescence, the same area of capture was used for each mouse. Fluorescence from a null or background capture area (consisting of muscle and skin tissue) was measured and subtracted from each articular reading [19]. After IVIS imaging, the mice were sacrificed, and their knees were dissected and the femoral tibial and patella portions were reimaged separately by IVIS. The knees were dissected to get cartilage tissues and then were put into RNAlator in order to isolate RNA for rtPCR. Also, some of knees were then fixed in 10% formalin solution for histopathological analysis and decalcified with Decalcifying Solution (Thermo Scientific, MA) before embedding in paraffin. Twenty histological sections, each taken 200 μm apart, were analyzed for arthritic joint damage across the entire joint. The sections were stained with H&E.

2.8. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RNA was extracted from the cells with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. To measure target gene expression, we used an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) for RT-PCR with custom designed primers and fluorescently labeled oligonucleotide probes specific for porcine CII (Ss03373343_g1), aggrecan (Ss03373377_m1), MMP-1 (Ss04245659_m1), MMP-13 (Ss03373279_m1), and the housekeeping gene, β-actin (Ss03375629_u1) for *in vitro* test. *In vivo*, we used the probe CII (Mm00491889_m1), aggrecan (Mm00545794_m1), MMP-13 (Mm00439491_m1), and β-actin (Mm00607939_s1) but not MMP-1 as this gene is not highly expressed in mice. All primer and probe sets were purchased from Applied Biosystems. According to the manufacturer's protocol, the cycle threshold (Ct) values were measured and the relative transcription levels were calculated. The data was plotted as a relevant expression calculated as $2^{-\Delta\Delta Ct}$, where the cycle threshold is the beginning of the logarithmic amplification of the probe set, and ΔCt is the difference of the target gene Ct subtracted from the housekeeping gene Ct. Data was then calculated as 2 (the increase in probe signal generated with each cycle) to the negative exponential value of ΔCt and plotted as a relative change to either controls or the TNF-α stimulated group [24–26].

2.9. Statistics. All experiments were performed independently at least three times. Microsoft Excel with Student's *t*-test and analysis of variance were used to determine statistical significance. A P value of less than 0.05 was considered statistically significant. Also, a one-way ANOVA test was performed for analysis of cell viability.

3. Results

In order to show the efficacy of the COX-2 inhibitor in reducing cartilage damage, a catabolic state was induced *in vitro* with articular chondrocytes. This was accomplished with the

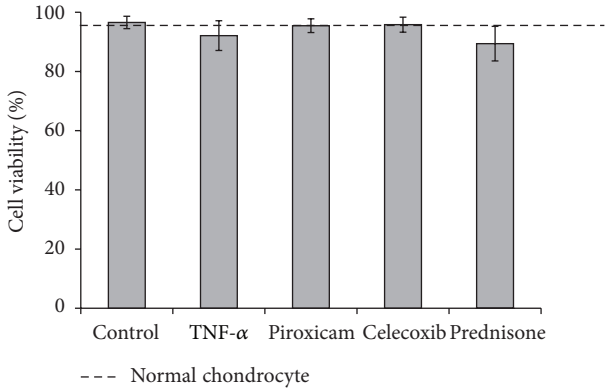


Figure 1: Cell viability. Chondrocytes viabilities were measured using trypan blue method after 24 hours of treatment with the experimental drugs. As the figure indicates, none of the drugs had an effect on cell viability as compared to the control group. The dotted line denotes normal chondrocytes untreated with TNF-α or drugs.

introduction of the cytokine TNF-α at an optimized concentration of 5 ng/mL, determined experimentally by RT-PCR for MMP-1 gene expression as shown in Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/595273. These results were previously reported [27].

Pig chondrocytes showed no change in cell viability after treatment with the various drugs, nor was there any difference between the cells stimulated with only TNF-α (5 ng/mL) compared to cells receiving different drugs. There were no statistically significant differences in cell viability between groups, and the average cell viability among the groups was greater than 90% (Figure 1).

Figure 2 shows gene expression of CII, aggrecan, and MMP-13 after treatment with the various drugs and TNF-α. All drug treatments significantly increased anabolic activity as evidenced by CII and aggrecan expression. Treatment with celecoxib resulted in a 6-fold increase in CII expression over the control. Piroxicam and prednisone increased CII expression by 2- and 3-fold, respectively. Celecoxib increased aggrecan expression by 3.2-fold, whereas piroxicam and prednisone increased aggrecan by 1.8- and 2-fold, respectively. Treatments with either celecoxib or prednisone were shown to decrease expression of the proteinase MMP-13 in a statistically equivalent manner, about 70% below the TNF stimulated level. Piroxicam had no effect on MMP-13 expression.

Western blot analyses show the effect of pharmacologic treatment on MMP-1 production (Figure 3(a)) by chondrocytes stimulated with TNF-α *in vitro*. The proinflammatory cytokine TNF-α increases both the gene expression of MMP-1 (Supplemental Figure 1) and concentration of the MMP-1 protein as measured by Western blot. Similarly, the PGE2 ELISA shows the effects that TNF-α and pharmacologic treatments have on PGE2 production by porcine chondrocytes (Figure 3(b)). Both assays showed comparable trends. Chondrocytes treated with TNF-α alone showed increased production of both MMP-1 and PGE2. However, when the TNF-α was combined with pharmacologic treatment, the production of MMP-1 and PGE2 was reduced. Celecoxib, the COX-2 selective inhibitor, proved to be the most effective at decreasing the production of the inflammatory cytokines, decreasing PGE2 concentration by 90%. Piroxicam, the nonselective COX inhibitor, and prednisone, the steroid, had a substantial effect, but they were less effective than celecoxib.

In vitro data shows celecoxib to be an effective agent both increasing anabolic activity of chondrocytes and decreasing protease activity. To more closely approximate a clinical scenario, celecoxib was administered to a murine PTOA model in mice where a knee joint was compressively loaded in a repetitive manner. These mice were subjected to mechanical loading and simultaneously treated for 5 weeks with either celecoxib or saline. At the end of this time the expression of CII, aggrecan, and MMP-13 was measured in cartilage samples taken from the mechanically loaded knee joint. Celecoxib treatment significantly increased production of aggrecan and decreased MMP-13 over that seen in mechanically loaded mice receiving no treatment. CII production was not significantly increased by celecoxib treatment but tended to trend upward (Figure 4).

In previous studies, IVIS scanning has been used to quantify the degree of cartilage damage due to PTOA [28]. In this study, we adapt this method to show how treatment with celecoxib alters damage severity. This effect is further confirmed by histological evaluation. Figure 5 shows florescence intensity in both celecoxib and saline treated PTOA mice. Due to the selectively binding nature of MabCII-NIF, this intensity (ROI) directly correlates with cartilage damage. The MAbCII-NIF showed selective binding to the damaged cartilage in the mechanically loaded left knee (Figure 5(a)). The loaded left knee of the celecoxib treated mouse shows a lower signal intensity and ROI (Figure 5(b)) as compared to the loaded left knee of the saline treated mouse, suggesting less damage to the articular surface after treatment with celecoxib. These results correlate with histology of the loaded joints. The loaded joint treated with saline (Figures 6(a) and 6(b)) shows extensive damage to the articular surface. The loaded knee of celecoxib treated mouse (Figures 6(c) and 6(d)) shows a more intact articular surface. The superficial articular layer in the celecoxib-treated knees demonstrates a more linear, intact structure than that of the saline treated knees. Chondrocytes in the deeper zone of the celecoxib-treated knee (Figure 6(d)) do not show the increased cell proliferation within the isogenous groups seen in the nontreated mechanically loaded knee (Figure 6(b)).

4. Discussion

In OA, chondrocyte homeostasis becomes imbalanced for synthesis and degradation of the extracellular matrix, resulting in progressive disruption of articular cartilage. TNF-α and other cytokines may play key roles in the destructive process by triggering release of proteases, such as matrix metalloproteinases (MMPs), which degrade collagen. As shown in this study, we used TNF-α (5 ng/mL) to stimulate cultured chondrocytes and to mimic a catabolic environment *in vitro* [27]. This proinflammatory cytokine stimulated synthesis and release of PGE2 [8]. The function of PGE2 in OA is not

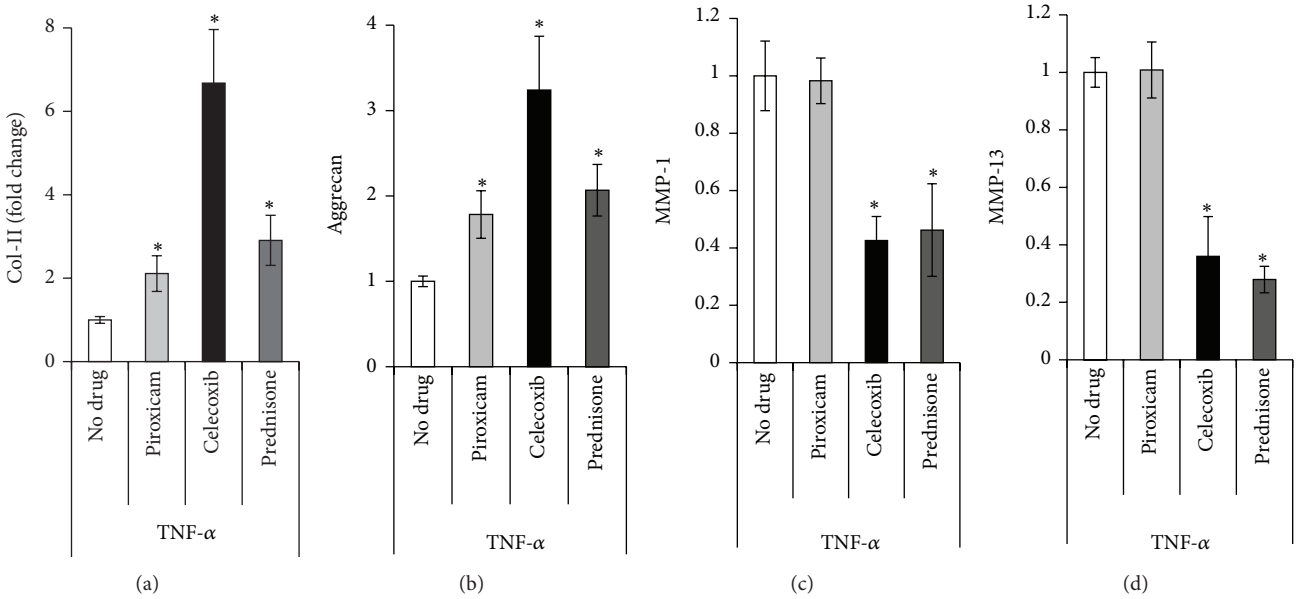


Figure 2: Gene expression *in vitro* after treatment with TNF-α with or without additional drugs. Changes in CII (a), aggrecan (b), and MMP-13 (c) gene expression after treatment with TNF-α (5 ng/mL) with or without drug treatment. Gene expression after treatment with TNF-α only is set as the base case (equal to 1). Gene expression of the drug treated chondrocytes is relative to the "no drug" case which contains TNF-α but no drug treatment. ($*$ indicates $P < 0.05$).

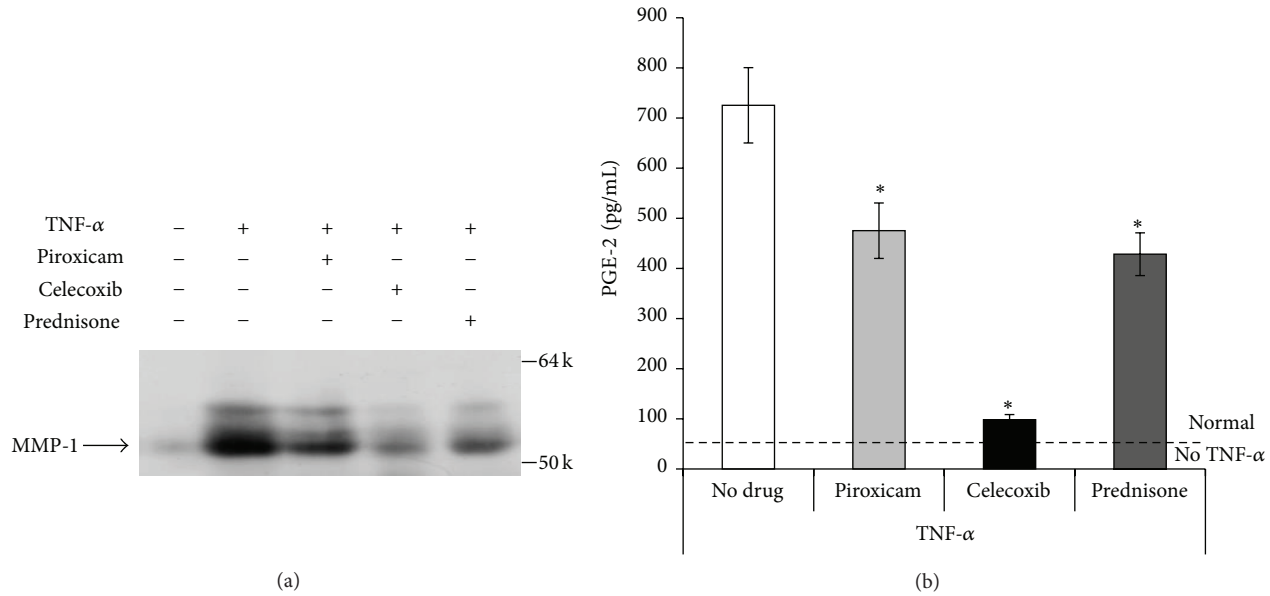


Figure 3: Protein production assays for MMP-1 and PGE2 *in vitro* after treatment with TNF-α with or without additional drugs. MMP-1 and PGE2 after treatment with TNF-α (5 ng/mL) with or without drug treatment. (a) shows that TNF-α induced MMP-1 production (compare columns 1 and 2). All drug/TNF-α combinations result in less MMP-1 production, with celecoxib reducing production of MMP-1 most dramatically. (b) shows that TNF-α alone induces PGE2 production. All drugs significantly reduce PGE-2 production, celecoxib being the most effective. The dotted line represents PGE-2 production with no TNF-α and no drug treatment.

entirely clear as it has both catabolic and anabolic effects on cartilage [15, 16]. In OA, however, chondrocyte expression of COX-2 increases, thereby increasing PGE2 concentration to nano- to micromolar concentrations [29, 30]. Hardy et al. demonstrate that both COX-2 and PGE2 effect proteoglycan production in OA chondrocytes in a concentration dependent manner; namely, high concentrations of both reduce proteoglycan synthesis [31]. Several studies suggest that at nanomolar concentrations PGE2 exerts a catabolic influence. Specifically, they have found that, in OA cultures treated with

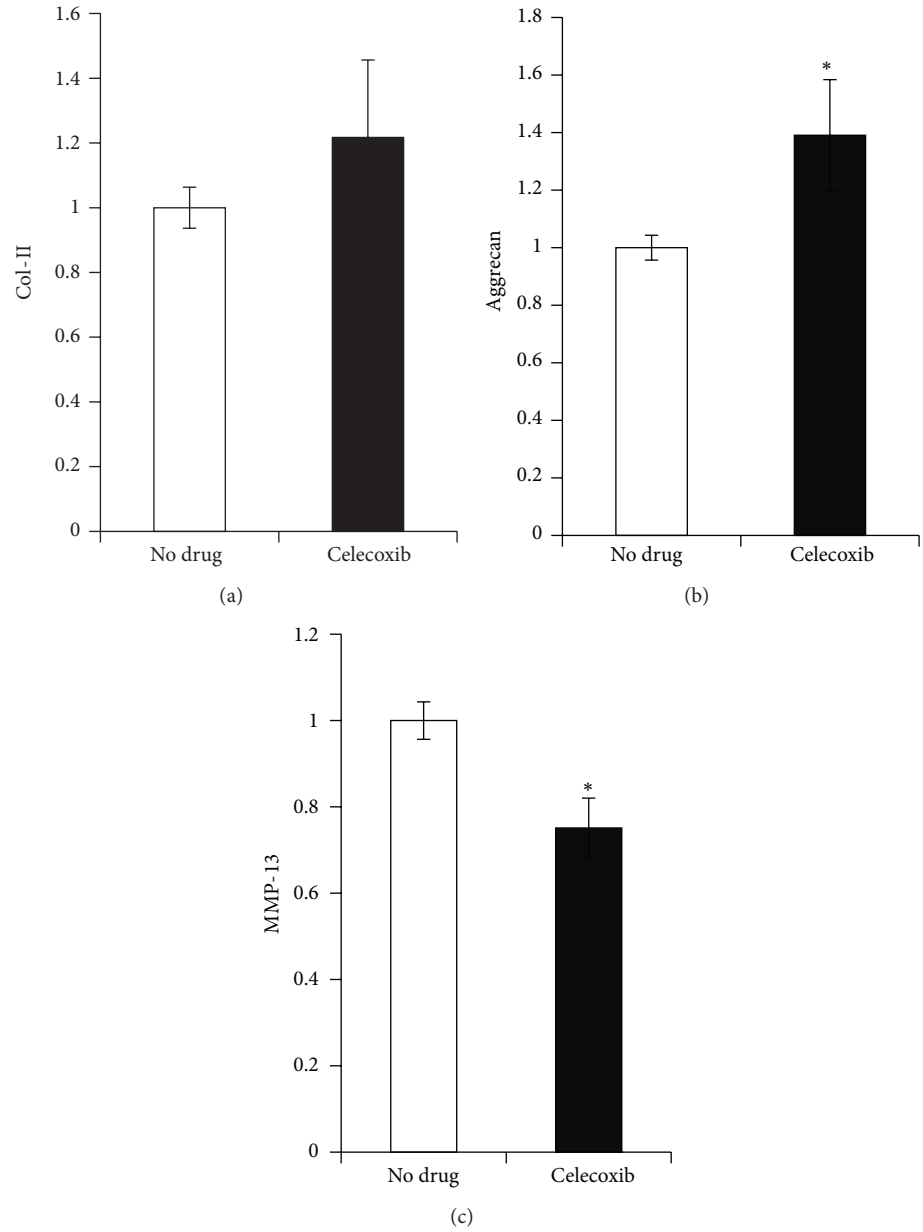


Figure 4: *In vivo* gene expression of cartilage from mechanically loaded mouse knees. Cartilage of mechanically loaded knees after treatment with celecoxib (10 mg/kg) by gavage for 5 weeks demonstrated increased gene expression of anabolic markers CII and aggrecan as compared to a mechanically loaded knees of mice treated with saline alone "no drug" (a). The increase in aggrecan gene expression reached statistical significance (b). Treatment with celecoxib significantly decreased MMP-13 gene expression as compared to the "no drug" control (c) ($n = 6$ for each treatment group). The "no drug" group has been treated with TNF-α, but not celecoxib.

celecoxib, the direct addition of PGE2 negates the beneficial effect of celecoxib on MMP, CII, and aggrecan levels [30, 31]. Additionally, research by Attur et al. shows that exogenous PGE2 increases MMP-13 expression which in turn increases collagen breakdown and reduces proteoglycan synthesis [30]. In a separate study this group of researchers demonstrated that treatment with celecoxib results in decreased MMP-1 expression. In our study, PGE2, MMP-13, and MMP-1 expressions are all observed to decrease after celecoxib treatment. NSAIDs could potentially affect cartilage through inhibition of PGE2 production [14, 31]. Other studies show that NSAIDs function to prevent cartilage damage. Cartilage from patients treated before joint replacement surgery with the COX-2 inhibitor celecoxib showed a higher rate of proteoglycan synthesis and a better retention of the newly formed proteoglycans. In contrast, nonspecific COX inhibitors showed a tendency towards a decreased rate of proteoglycan synthesis [18]. The *in vitro* concentration of celecoxib used in these experiments was taken from Mastbergen et al. who demonstrated that proteoglycan turnover was greatest at 10 μM. *In vivo* dosing of celecoxib (10 mg/kg) was adapted from Cottrell and O'Connor's dosage for mice [23]. Our dose of piroxicam

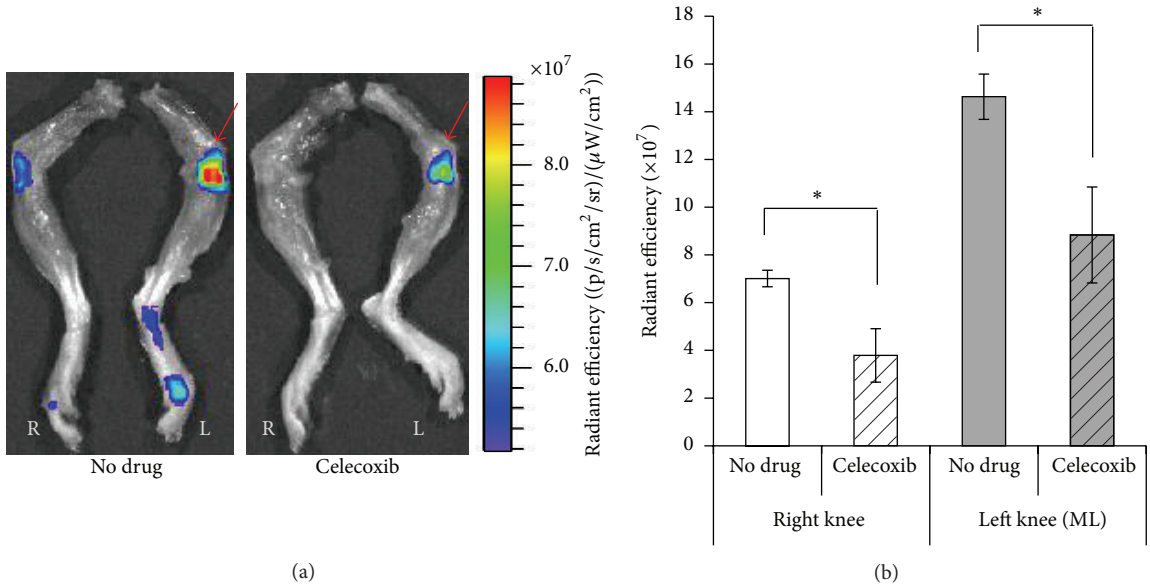


FIGURE 5: Optical imaging of fluorescently labelled MabCII antibody in PTOA model mice with and without treatment with celecoxib. IVIS scanning shows fluorescent CII targeted antibody binding to the damaged cartilage surface. (a) shows the antibody binding in greater quantity to the loaded left knee in both the drug treated and nondrug treated cases. Antibody binding to the loaded knee of the celecoxib treated group is lower than the loaded knee of the nondrug treated group. The red arrow indicates a mechanically loaded knee. (b) quantifies this binding by measuring fluoresce intensity and calculating radiant efficiency. Results are parallel (a) ($n = 6$ for each treatment group).

is the maximum dose possible without yielding significant effects on cell division and viability as demonstrated by Chang et al. [32]. The *in vitro* data obtained in this study shows that chondrocytes treated with celecoxib produce fewer arthritis-associated mediators such as PGE2 and MMP-1 than chondrocytes treated with piroxicam or prednisone and more anabolic indicators, namely, CII and aggrecan.

In our study and others, nonselective COX inhibitors, represented in our experiment by piroxicam, have been shown to have minimal beneficial effect on proteoglycan turnover and repair [33, 34]. This difference in NSAID effect supports COX-2 involvement in catabolic activity regulation in cartilage, whereas COX-1 activity may have a more constitutive role in chondrocytes [14, 35]. We chose to investigate the COX-2 inhibitor *in vivo* with the hypothesis that preserving the COX-1-mediated constitutive role, while inhibiting the induced COX-2 activity, would optimize OA treatment and prevention. Furthermore, few studies have described the effects of celecoxib on cartilage destruction *in vivo* [14, 36–39]. In *ex vivo* studies, 4 weeks of celecoxib treatment is shown to have beneficial effects on proteoglycan synthesis rates and proteoglycan retention, though no differences in the histopathological Mankin score were observed [38]. Studies of this kind are limited by sample size and duration and the lack of a reproducible animal model for OA and drug monitoring. Other animal models of PTOA require direct surgical manipulation and lead to aggressive and rapid joint destruction. Our studies utilize a PTOA animal model that requires no invasive intervention. The mechanical loading technique is easily reproducible and provides an alternate nonsurgical means of replicating the pathophysiology of PTOA.

Trauma which gives rise to osteoarthritic change is accompanied by injury of the adjacent soft tissues resulting in an increase of proinflammatory cytokines such as TNF-α in the joint space. As such, the combined action of trauma-induced and cytokine-induced processes in cartilage characterizes the early development of PTOA (Lewis et al.). Coupled with a noninvasive method of monitoring OA damage, this model could help researchers continuously monitor OA progression and the efficacy of drugs over the disease course. The noninvasive monitoring technique described in this experiment uses a fluorescently labeled monoclonal antibody targeted to CII (MabCII-NIF) and IVIS scanning. Previous studies by this lab have shown this method to be effective at identifying early stages of OA corresponding to histopathological Mankin scores less than 3 [19, 28]. Early changes of PTOA occur within 24 hours of joint injury. These changes include chondrocyte apoptosis and a significant surge in proinflammatory cytokines, nitric oxide, free radicals, and MMPs resulting in cartilage matrix damage [4, 40–44]. Therapies to target this phase need to be given early in disease when both clinical symptoms and histopathological Mankin scores are low [4].

As shown at our animal study the celecoxib reduced MMP expression and delayed the progress of arthritic damage in PTOA. However, for long-term therapy with this drug, the side effect of cardiac risk must be considered [45, 46]. The targeted antibody used in this study selectively binds to tissue demonstrating early OA changes and can be conjugated to a drug-encapsulating nanoscale liposome (nanosome) [19]. Delivery of therapeutic agents such as celecoxib with this nanosome may bring timely effective treatment to the site of

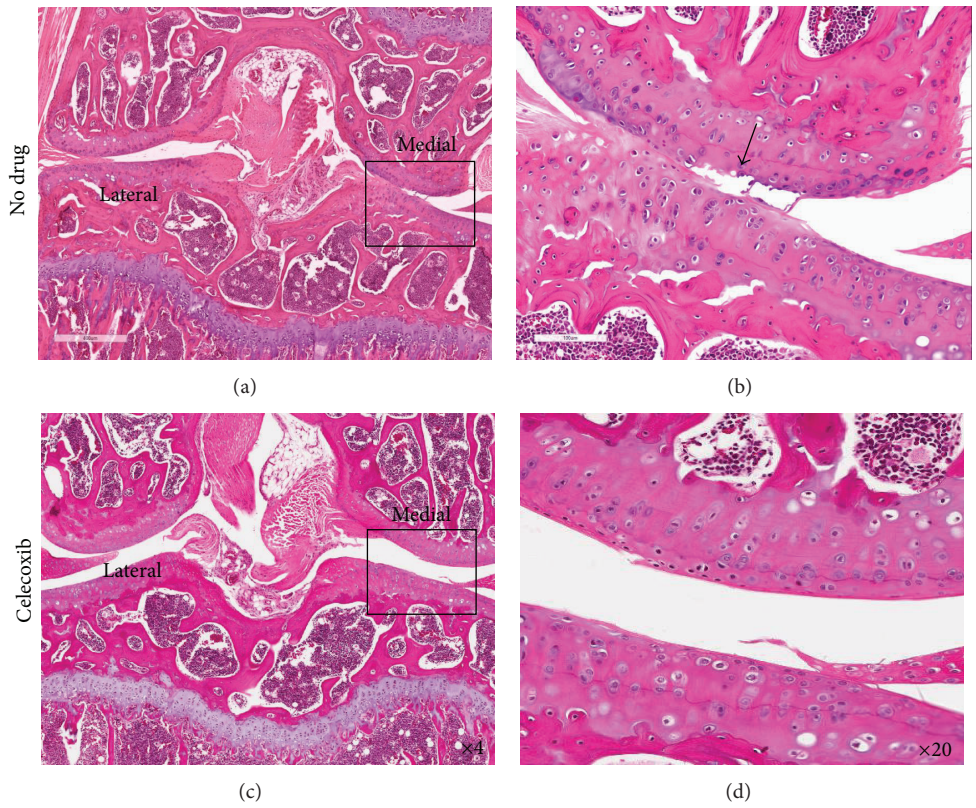


Figure 6: Histopathology of mechanically loaded knee joints with and without treatment with celecoxib. This figure shows H&E stained coronal sections of loaded mouse knees. Twenty histological sections, each taken 200 μm apart, were analyzed for arthritic joint damage across the entire joint. Sections at the same depth relative to the patella were compared. (a) and (b) are from the mechanically loaded knees of mice receiving no drug treatment (only saline). (c) and (d) are from the mechanically loaded knees of mice treated with celecoxib. In both cases, the lateral tibial and femoral plateaus have minimal damage. The medial plateaus sustained more damage, and thus higher magnification ((b) and (d)) compares treated (d) and untreated medial plateaus (b).

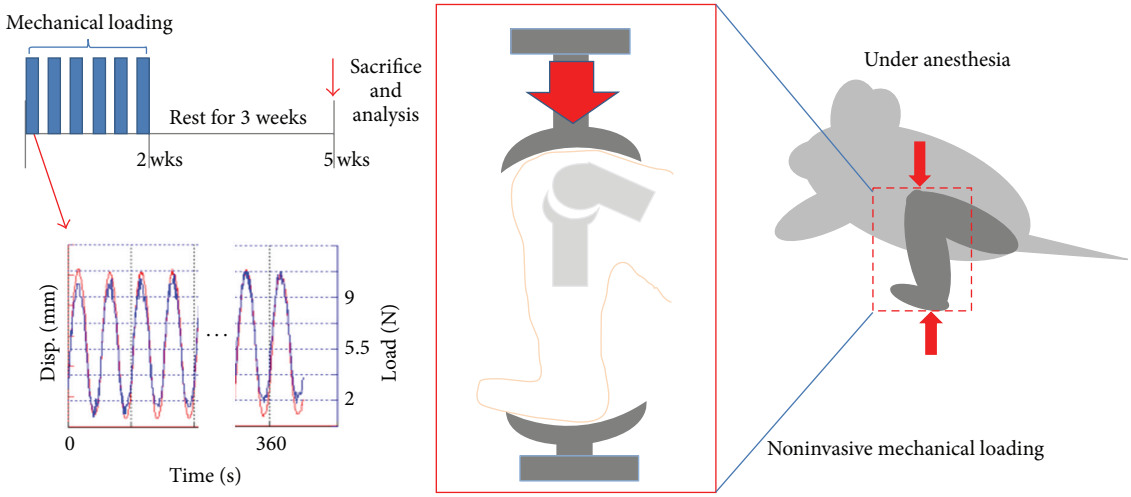


Figure 7: Mechanically loaded PTOA mouse model schematic diagram. This figure graphically represents the cycle of loading (6 cycles) over the course of 2 weeks followed by 3 weeks of rest. The figure also demonstrates the position of the mouse knee in the loading apparatus and the force versus time diagram.

joint damage, maximizing local drug delivery while minimizing systemic side effects. The mechanically loaded PTOA mouse model combined with IVIS scanning provides a means to monitor drug effects using this treatment method longitudinally without animal sacrifice [28]. The combination of early detection with aggressive, targeted pharmacologic treatment could have an enormous impact on the treatment and prevention of OA while reducing the side effect burden of common effective treatments.

Conflict of Interests

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

Acknowledgments

This work was supported with resources and the use of facilities at the Veterans Affairs Medical Center (VAMC) at Memphis, TN, USA, and a VA Merit Review Award and a VA Career Scientist Award from Department of Veterans Affairs (Karen A. Hasty), an R21 from National Institutes of Health (AR060408 to Karen A. Hasty) and William & Ella Owens Research Foundations (to Hongsik Cho). The authors would like to thank Christy Patterson who is the technical director at the University of Tennessee Health Science Center for her assistance with this study and invaluable comments.

References

[1] W. Zhang, R. W. Moskowitz, G. Nuki et al., "OARSI recommendations for the management of hip and knee osteoarthritis, part I: critical appraisal of existing treatment guidelines and systematic review of current research evidence," *Osteoarthritis and Cartilage*, vol. 15, no. 9, pp. 981–1000, 2007.

[2] F. Guilak, B. Fermor, F. J. Keefe et al., "The role of biomechanics and inflammation in cartilage injury and repair," *Clinical Orthopaedics and Related Research*, no. 423, pp. 17–26, 2004.

[3] F. C. Ko, C. Dragomir, D. A. Plumb et al., "In vivo cyclic compression causes cartilage degeneration and subchondral bone changes in mouse tibiae," *Arthritis & Rheumatism*, vol. 65, no. 6, pp. 1569–1578, 2013.

[4] S. A. Olson, P. Horne, B. Furman et al., "The role of cytokines in posttraumatic arthritis," *Journal of the American Academy of Orthopaedic Surgeons*, vol. 22, no. 1, pp. 29–37, 2014.

[5] P. G. Mitchell, H. A. Magna, L. M. Reeves et al., "Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage," *Journal of Clinical Investigation*, vol. 97, no. 3, pp. 761–768, 1996.

[6] G. N. Smith Jr., "The role of collagenolytic matrix metalloproteinases in the loss of articular cartilage in osteoarthritis," *Frontiers in Bioscience*, vol. 11, no. 3, pp. 3081–3095, 2006.

[7] J. Martel-Pelletier, C. Boileau, J.-P. Pelletier, and P. J. Roughley, "Cartilage in normal and osteoarthritis conditions," *Best Practice & Research: Clinical Rheumatology*, vol. 22, no. 2, pp. 351–384, 2008.

[8] M. B. Goldring, "Osteoarthritis and cartilage: the role of cytokines," *Current Rheumatology Reports*, vol. 2, no. 6, pp. 459–465, 2000.

[9] J. R. Vane, "Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs," *Nature: New Biology*, vol. 231, no. 25, pp. 232–235, 1971.

[10] L. J. Crofford, P. E. Lipsky, P. Brooks, S. B. Abramson, L. S. Simon, and L. B. A. van de Putte, "Basic biology and clinical application of specific cyclooxygenase-2 inhibitors," *Arthritis & Rheumatology*, vol. 43, no. 1, pp. 4–13, 2000.

[11] J. Martel-Pelletier, J. P. Pelletier, and H. Fahmi, "Cyclooxygenase-2 and prostaglandins in articular tissues," *Seminars in Arthritis and Rheumatism*, vol. 33, no. 3, pp. 155–167, 2003.

[12] C. J. Hawkey, "COX-2 inhibitors," *The Lancet*, vol. 353, no. 9149, pp. 307–314, 1999.

[13] C. O. Bingham III, "Development and clinical application of COX-2-selective inhibitors for the treatment of osteoarthritis and rheumatoid arthritis," *Cleveland Clinic Journal of Medicine*, vol. 69, supplement 1, pp. SI5–SI12, 2002.

[14] M. C. Zweers, T. N. de Boer, J. van Roon, J. W. J. Bijlsma, F. P. J. G. Lafeber, and S. C. Mastbergen, "Celecoxib: considerations regarding its potential disease-modifying properties in osteoarthritis," *Arthritis Research & Therapy*, vol. 13, article 239, 2011.

[15] E. V. Tchetina, J. A. di Battista, D. J. Zukor, J. Antoniou, and A. R. Poole, "Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy," *Arthritis Research & Therapy*, vol. 9. article R75, 2007.

[16] M. Attur, H. E. Al-Mussawir, J. Patel et al., "Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor," *The Journal of Immunology*, vol. 181, no. 7, pp. 5082–5088, 2008.

[17] S. C. Mastbergen, J. W. J. Bijlsma, and F. P. J. G. Lafeber, "Synthesis and release of human cartilage matrix proteoglycans are differently regulated by nitric oxide and prostaglandin-E2," *Annals of the Rheumatic Diseases*, vol. 67, no. 1, pp. 52–58, 2008.

[18] S. C. Mastbergen, F. P. J. G. Lafeber, and J. W. J. Bijlsma, "Selective COX-2 inhibition prevents proinflammatory cytokine-induced cartilage damage," *Rheumatology*, vol. 41, no. 7, pp. 801–808, 2002.

[19] H. Cho, J. M. Stuart, R. Magid et al., "Theranostic immunoliposomes for osteoarthritis," *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol. 10, no. 3, pp. 619–627, 2014.

[20] R. Tsutsumi, H. Ito, T. Hiramitsu et al., "Celecoxib inhibits production of MMP and NO via downregulation of NF-kappaB and JNK in a PGE2 independent manner in human articular chondrocytes," *Rheumatology International*, vol. 28, no. 8, pp. 727–736, 2008.

[21] J. Deschner, C. R. Hofman, N. P. Piesco, and S. Agarwal, "Signal transduction by mechanical strain in chondrocytes," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 6, no. 3, pp. 289–293, 2003.

[22] B. Poulet, R. W. Hamilton, S. Shefelbine, and A. A. Pitsillides, "Characterizing a novel and adjustable noninvasive murine joint loading model," *Arthritis & Rheumatism*, vol. 63, no. 1, pp. 137–147, 2011.

[23] J. Cottrell and J. P. O'Connor, "Effect of non-steroidal anti-inflammatory drugs on bone healing," *Pharmaceuticals*, vol. 3, no. 5, pp. 1668–1693, 2010.

[24] L. Overbergh, D. Valckx, M. Waer, and C. Mathieu, "Quantification of murine cytokine mRNAs using real time quantitative

reverse transcriptase PCR," *Cytokine*, vol. 11, no. 4, pp. 305–312, 1999.

[25] A. Giulietti, L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon, and C. Mathieu, "An overview of real-time quantitative PCR: applications to quantify cytokine gene expression," *Methods*, vol. 25, no. 4, pp. 386–401, 2001.

[26] J. Huang, L. R. Ballou, and K. A. Hasty, "Cyclic equibiaxial tensile strain induces both anabolic and catabolic responses in articular chondrocytes," *Gene*, vol. 404, no. 1-2, pp. 101–109, 2007.

[27] H. Cho, S. Lee, S.-H. Park, J. Huang, K. A. Hasty, and S.-J. Kim, "Synergistic effect of combined growth factors in porcine intervertebral disc degeneration," *Connective Tissue Research*, vol. 54, no. 3, pp. 181–186, 2013.

[28] H. Cho, E. Pinkhassik, V. David, J. M. Stuart, and K. A. Hasty, "Detection of early cartilage damage using targeted nanosomes in a post-traumatic osteoarthritis mouse model," *Nanomedicine: Nanotechnology, Biology and Medicine*, 2015.

[29] B. White, M. Schmidt, C. Murphy et al., "Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor κB (NF-κB) and tumour necrosis factor α (TNF-α) production in the THP-1 monocytic cell line," *British Journal of Haematology*, vol. 110, no. 1, pp. 130–134, 2000.

[30] M. Attur, H. E. Al-Mussawir, J. Patel et al., "Prostaglandin E_2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor," *The Journal of Immunology*, vol. 182, no. 10, pp. 5082–5088, 2009.

[31] M. M. Hardy, K. Seibert, P. T. Manning et al., "Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants," *Arthritis and Rheumatism*, vol. 46, no. 7, pp. 1789–1803, 2002.

[32] J.-K. Chang, G.-J. Wang, S.-T. Tsai, and M.-L. Ho, "Nonsteroidal anti-inflammatory drug effects on osteoblastic cell cycle, cytotoxicity, and cell death," *Connective Tissue Research*, vol. 46, no. 4-5, pp. 200–210, 2005.

[33] H. El Hajjaji, A. Marcelis, J.-P. Devogelaer, and D.-H. Manicourt, "Celecoxib has a positive effect on the overall metabolism of hyaluronan and proteoglycans in human osteoarthritic cartilage," *The Journal of Rheumatology*, vol. 30, no. 11, pp. 2444–2451, 2003.

[34] S. C. Mastbergen, N. W. D. Jansen, J. W. J. Bijlsma, and F. P. J. G. Lafeber, "Differential direct effects of cyclo-oxygenase-1/2 inhibition on proteoglycan turnover of human osteoarthritic cartilage: an in vitro study," *Arthritis Research & Therapy*, vol. 8, article R2, 2006.

[35] H. Nakamura, K. Masuko, K. Yudoh, T. Kato, and K. Nishioka, "Effects of celecoxib on human chondrocytes—enhanced production of chemokines," *Clinical & Experimental Rheumatology*, vol. 25, no. 1, pp. 11–16, 2007.

[36] M. A. Álvarez-Soria, G. Herrero-Beaumont, J. Moreno-Rubio et al., "Long-term NSAID treatment directly decreases COX-2 and mPGES-1 production in the articular cartilage of patients with osteoarthritis," *Osteoarthritis and Cartilage*, vol. 16, no. 12, pp. 1484–1493, 2008.

[37] M. A. Álvarez-Soria, R. Largo, J. Santillana et al., "Long term NSAID treatment inhibits COX-2 synthesis in the knee synovial membrane of patients with osteoarthritis: differential proinflammatory cytokine profile between celecoxib and aceclofenac," *Annals of the Rheumatic Diseases*, vol. 65, no. 8, pp. 998–1005, 2006.

[38] T. N. de Boer, A. M. Huisman, A. A. Polak et al., "The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: *ex vivo* evaluation of human cartilage tissue after *in vivo* treatment," *Osteoarthritis and Cartilage*, vol. 17, no. 4, pp. 482–488, 2009.

[39] J.-P. Raynauld, J. Martel-Pelletier, A. Beaulieu et al., "An open-label pilot study evaluating by magnetic resonance imaging the potential for a disease-modifying effect of celecoxib compared to a modelized historical control cohort in the treatment of knee osteoarthritis," *Seminars in Arthritis and Rheumatism*, vol. 40, no. 3, pp. 185–192, 2010.

[40] J. S. Lewis, W. C. Hembree, B. D. Furman et al., "Acute joint pathology and synovial inflammation is associated with increased intra-articular fracture severity in the mouse knee," *Osteoarthritis and Cartilage*, vol. 19, no. 7, pp. 864–873, 2011.

[41] P. Swärd, R. Frobell, M. Englund, H. Roos, and A. Struglics, "Cartilage and bone markers and inflammatory cytokines are increased in synovial fluid in the acute phase of knee injury (hemarthrosis)—a cross-sectional analysis," *Osteoarthritis and Cartilage*, vol. 20, no. 11, pp. 1302–1308, 2012.

[42] M. Bigoni, P. Sacerdote, M. Turati et al., "Acute and late changes in intraarticular cytokine levels following anterior cruciate ligament injury," *Journal of Orthopaedic Research*, vol. 31, no. 2, pp. 315–321, 2013.

[43] B. D. Furman, D. S. Mangiapani, E. Zeitler et al., "Targeting pro-inflammatory cytokines following joint injury: acute intra-articular inhibition of interleukin-1 following knee injury prevents post-traumatic arthritis," *Arthritis Research & Therapy*, vol. 16, no. 3, article R134, 2014.

[44] J. S. Lewis Jr., B. D. Furman, E. Zeitler et al., "Genetic and cellular evidence of decreased inflammation associated with reduced incidence of posttraumatic arthritis in MRL/MpJ mice," *Arthritis and Rheumatism*, vol. 65, no. 3, pp. 660–670, 2013.

[45] T. A. Holland and A. G. Mikos, "Advances in drug delivery for articular cartilage," *Journal of Controlled Release*, vol. 86, no. 1, pp. 1–14, 2003.

[46] M. Nasr, "In vitro and in vivo evaluation of proniosomes containing celecoxib for oral administration," *AAPS PharmSciTech*, vol. 11, no. 1, pp. 85–89, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 279890, 13 pages
http://dx.doi.org/10.1155/2015/279890

Research Article

Comparative Effectiveness of Tocilizumab and TNF Inhibitors in Rheumatoid Arthritis Patients: Data from the Rheumatic Diseases Portuguese Register, Reuma.pt

Vasco C. Romão,[1,2] Maria José Santos,[2,3] Joaquim Polido-Pereira,[1,2] Cátia Duarte,[4] Patrícia Nero,[5] Cláudia Miguel,[6] José António Costa,[7] Miguel Bernardes,[8] Fernando M. Pimentel-Santos,[5,9] Filipe Barcelos,[6] Lúcia Costa,[8] José António Melo Gomes,[6] José Alberto Pereira da Silva,[1] Jaime Cunha Branco,[5,9] José Canas da Silva,[3] José António Pereira da Silva,[4] João Eurico Fonseca,[1,2] and Helena Canhão[1,2]

[1] *Rheumatology Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon Academic Medical Centre, Edifício Egas Moniz, Avenida Prof. Egas Moniz, 1649-028 Lisboa, Portugal*
[2] *Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Avenida Prof. Egas Moniz, 1649-028 Lisboa, Portugal*
[3] *Rheumatology Department, Hospital Garcia de Orta, Avenida Prof. Torrado da Silva, 2801-951 Almada, Portugal*
[4] *Rheumatology Department, Centro Hospitalar Universitário de Coimbra, Praceta Mota Pinto, 3000-076 Coimbra, Portugal*
[5] *Rheumatology Department, Hospital Egas Moniz, Centro Hospitalar Lisboa Ocidental, Rua da Junqueira 126, 1349-019 Lisboa, Portugal*
[6] *Rheumatology Department, Instituto Português de Reumatologia, R. Beneficência 7, 1050-034 Lisboa, Portugal*
[7] *Rheumatology Department, Unidade Local de Saúde do Alto Minho, Hospital Conde de Bertiandos, Largo Conde de Bertiandos, 4990-041 Ponte de Lima, Portugal*
[8] *Rheumatology Department, Centro Hospitalar São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal*
[9] *CEDOC, NOVA Medical School, Nova University, Campo dos Mártires da Pátria 130, 1169-056 Lisboa, Portugal*

Correspondence should be addressed to Helena Canhão; helenacanhao@gmail.com

Received 5 December 2014; Accepted 11 February 2015

Academic Editor: Guixiu Shi

Objectives. To compare the effectiveness of TNF inhibitors (TNFi) and tocilizumab in rheumatoid arthritis (RA) treatment, according to different response criteria. *Methods*. We included RA patients registered in the Rheumatic Diseases Portuguese Register treated with TNFi or tocilizumab for at least 6 months, between January 2008 and July 2013. We assessed remission/low disease activity (LDA) at 6 months according to DAS28, CDAI, and SDAI, as well as Boolean ACR/EULAR remission and EULAR response rate, adjusting for measured confounders. *Results*. Tocilizumab-treated patients ($n = 95$) presented higher baseline disease activity and were less frequently naïve to biologics compared to TNFi users ($n = 429$). Multivariate logistic regression analysis including the propensity score for receiving tocilizumab showed that patients treated with tocilizumab were more likely to achieve remission or LDA according to DAS28 (OR = 11.0/6.2, 95% CI 5.6–21.6/3.2–12.0), CDAI (OR = 2.8/2.6, 95% CI 1.2–6.5/1.3–5.5), or SDAI (OR = 3.6/2.5, 95% CI 1.5–8.7/1.1–5.5), as well as a good EULAR response (OR = 6.4, 95% CI 3.4–12.0). However, both groups did not differ in Boolean remission (OR = 1.9, 95% CI 0.8–4.8) or good/moderate EULAR response (OR = 1.8, 95% CI 0.8–4.5). *Conclusions*. Compared with TNFi, tocilizumab was associated with greater likelihood of achieving DAS28, CDAI, and SDAI remission/LDA and EULAR good response. Boolean remission and EULAR good/moderate response did not differ significantly between groups.

1. Introduction

Tumour necrosis factor inhibitors (TNFi) were the first biological agents introduced in the treatment of rheumatoid arthritis (RA). They have been widely used for over 15 years now and extensive evidence of their efficacy and effectiveness has accumulated, arising from numerous randomised clinical trials (RCTs) and large observational studies [1–6]. Tocilizumab, a monoclonal antibody targeting the interleukin-6 receptor, has become available one decade later and has progressively gained its place into RA treatment algorithms [7]. It has now been included in the last 2013 European League Against Rheumatism (EULAR) recommendations as one of the potential first line biologic drugs, alongside TNFi [7], after methotrexate (MTX) and/or other synthetic diseases modifying antirheumatic drugs (DMARDs) failure, a guidance followed by several national rheumatology societies [8].

TNFi are highly efficacious, both in monotherapy and in combination with synthetic DMARDs, such as MTX. Several indirect comparisons of RCTs and register-based observational studies failed to show significant differences in effectiveness between TNFi, although there are no available RCTs specifically addressing this issue [3–6, 9–12]. Likewise, tocilizumab presents good response rates, not only with concomitant MTX, but also in monotherapy [13, 14]. The only available head-to-head study, comparing tocilizumab and adalimumab in monotherapy, revealed higher clinical response with the former [15]. While RCTs directly assessing both classes of drugs in combination with synthetic DMARDs are missing, indirect comparisons through systematic reviews of RCTs have reported similar American College of Rheumatology (ACR) 50 responses [11, 12, 16–19] between tocilizumab and TNFi, with only one of these studies suggesting a higher ACR70 response rate with tocilizumab [16].

Real life observational data have confirmed the effectiveness of tocilizumab, with studies reporting 39–54.9% of the patients achieving remission according to disease activity score-28 joints (DAS28) [20–22] and 50.7% reaching ACR50 response at 24 weeks [22]. However, there are few observational register-based studies comparing the effectiveness of different biologic drug classes in real life circumstances. In one of such studies, Yoshida et al. compared the remission rates after 6 months of treatment with tocilizumab or TNFi and found that although the percentage of patients reaching DAS28-erythrocyte sedimentation rate (ESR) remission was higher with tocilizumab, the rates of stringent Boolean remission were similar in both groups [23]. This finding reflects the profound effect of tocilizumab upon inflammatory markers, due to the direct inhibition of IL-6, a major activator of the acute phase response [24]. Thus, response rates to tocilizumab might be overestimated when considering composite scores that include inflammatory markers, such as the DAS28, where ESR or C-reactive protein (CRP) has a high weight on the overall score [24].

With this in mind, we aimed to compare the effectiveness of TNFi and tocilizumab in RA treatment, according to different response criteria, in patients followed for at least 6 months in a multicentre nationwide cohort. We further looked at assessing the impact of previous biologic therapies on treatment response.

2. Methods

2.1. Patients. The Rheumatic Diseases Portuguese Register, Reuma.pt, is a nationwide clinical register established in 2008 and used in daily practice by nearly all rheumatology centers in Portugal [25]. Biologic therapy for RA has been available in Portugal since 2000, with the introduction of etanercept and infliximab. Adalimumab was approved in 2003 and the three drugs currently account for the majority of treatments. Tocilizumab and golimumab have become available in 2009 and 2010, respectively, and have progressively been incorporated into the daily clinical practice. The decision to initiate, switch, or maintain biologic treatment is guided by the SPR recommendations [8], which make no formal statement about which agent(s) should be considered as first line option(s).

We included patients fulfilling ACR 1987 revised RA criteria, starting tocilizumab or TNFi (adalimumab, etanercept, golimumab, or infliximab; certolizumab was not available in Portugal during the time frame of the study) between January 2008 and July 2013, who were treated for at least 6 months and had available DAS28 scores at baseline and follow-up.

All patients provided written informed consent as part of their enrolment in Reuma.pt, which is approved by competent authorities in Portugal, including the Health National Directorate and the National Board of Data Protection. The study was conducted according to the Declaration of Helsinki, as revised in Fortaleza (October 2013) and was approved by the Santa Maria Hospital Ethics Committee.

2.2. Statistical Methods. The coprimary outcomes were the proportion of patients in remission according to DAS28 (<2.6), CDAI (≤2.8), SDAI (≤3.3), and ACR/EULAR Boolean criteria (tender joint count-28 joints (TJC28) ≤ 1, swollen joint count-28 joints (SJC28) ≤ 1, CRP ≤ 1 mg/dL, and patient global health (PGH) ≤ 1/10). Secondary outcomes included proportion of patients reaching at least low disease activity (LDA) according to DAS28 (<3.2), CDAI (CDAI ≤ 10), and SDAI (≤11); frequencies of EULAR good (change in DAS28 > 1.2 and DAS28-6 months ≤ 3.2) and good/moderate response (change in DAS28 > 0.6 and DAS28-6 months ≤ 5.1 or change in DAS28 > 1.2 and DAS28 > 5.1); and, finally, change in DAS28, ESR, TJC28, SJC28, visual analogue scale (VAS), and health assessment questionnaire (HAQ). Covariates of interest included age at biologic start, gender, race (Caucasian versus non-Caucasian), disease duration, years of education, seropositivity (if anti-citrullinated protein antibody (ACPA) and/or rheumatoid factor (RF) positive), erosive disease (if erosions identified at X-rays of hands/feet at any time in disease), previous biologic therapy status (biologic-naïve versus previously exposed to ≥1 biologic), number of previous biologics, smoking status (current smokers versus noncurrent smokers), cardiovascular comorbidity (hypertension, dyslipidaemia, heart disease, or diabetes mellitus), and concomitant MTX and corticosteroid therapy. At 6 months,

data were collected on TJC/SJC28, ESR, CRP, VAS (PGH and physician global assessment (PhGA)), and HAQ.

Baseline and follow-up data were compared according to biologic treatment using ANOVA, Student's t-test or chi-square tests, where applicable, both for each biologic separately and for biologic class (TNFi/tocilizumab). Bonferroni tests were applied, when significant differences were detected in ANOVA. We further performed stratification on previous biologic therapy status, to account for the potential relationship between previous biologic therapies and current therapy effectiveness.

To try to accommodate for patient- and disease-related confounders, we used multiple logistic regression and propensity score-based methods to explore the relationship between biologic class and treatment response. We built logistic regression models predicting binary response outcomes using stepwise backward elimination, including covariates with P value < 0.1 in the univariate analysis and those thought to be clinically meaningful (age, sex, seropositivity, number of previous biologics, disease duration, and baseline disease activity). In order to avoid overadjusting, individual components of the disease activity score were not considered. Variables conferring a greater than 10% change on the main regression coefficient (biologic class) were included in the final model.

A propensity score estimating the likelihood of receiving tocilizumab was generated, using a *logit* function and including baseline variables potentially related to biologic class that did not contain significant numbers of missing values: age, age-squared, sex, number of previous biologics, disease duration, baseline DAS28, TJC, SJC and concomitant treatment with MTX, corticosteroids, and other DMARDs. We then included this propensity score as a covariate in the univariate and multivariate logistic regressions in order to account for potential residual confounding. Finally, we conducted caliper 1 : 5 matching with replacement on the propensity score using the *psmatch2* command of Stata for each of the outcomes separately. Matching strategies significantly reduced the overall mean bias (e.g., 5.4% for the DAS28 matching), while decreasing the number of patients subject to the analysis, as expected.

All statistical analyses were performed using Stata version 12.1 (StataCorp, College Station, TX, USA) and P value was considered significant at <0.05.

3. Results

Five hundred and twenty-four patients fulfilled the inclusion criteria, 95 treated with tocilizumab and 429 with TNFi (106 adalimumab, 202 etanercept, 43 golimumab, and 78 infliximab). The baseline characteristics of the population are represented in Table 1. Patients from different groups had similar demographic characteristics, with expected distributions of variables such as age, gender, disease duration, smoking, or cardiovascular comorbidities, compatible with an established RA population. Frequencies of seropositivity (RF and/or ACPA), erosive disease and concomitant treatment with MTX, or low-dose corticosteroids were similar between groups considering either each biologic separately or biologic class. However, tocilizumab-treated patients were less frequently naïve to biologic therapy, had received a higher number of previous biologic agents, and had more active disease, as translated by significantly higher SJC28, PhGA, DAS28, CDAI, and SDAI. Furthermore, comparing patients by biologic class revealed higher mean ESR/CRP and increased proportions of patients with high disease activity according to all indexes in the tocilizumab group.

At follow-up (Table 2), only DAS28 and ESR were lower in the tocilizumab group compared to all TNFi ($P < 0.001$). Bonferroni tests after ANOVA regarding CRP at 6 months revealed that there were no significant differences between tocilizumab and each TNFi separately ($P > 0.05$ for all two-group comparisons). All other disease activity measures were similar between the groups. However, considering changes from baseline values, tocilizumab users presented a significantly greater decrease in DAS28, CDAI, SDAI, and inflammatory markers (ESR and CRP), as well as in the SJC28 and PhGA than patients treated with TNFi (Table 2).

3.1. Remission and EULAR Response. More than half of tocilizumab-treated patients were in DAS28 remission at 6 months, a significantly higher proportion than observed for TNFi users (OR = 4.4, 95% confidence interval (CI) 2.8–7.0; Figure 1(a)). However, no significant differences were seen for remission rates according to CDAI (OR = 1.6, 95% CI 0.8–3.2), SDAI (OR = 1.9, 95% CI 0.97–3.9), or Boolean definition (OR = 1.1, 95% CI 0.6–2.3) criteria. Similarly to DAS28 change and remission, nearly two-thirds of the tocilizumab group had a good EULAR response, compared to one-third of TNFi users (OR = 3.6, 95% CI 2.3–5.7; Figure 1(b)). When considering the achievement of good/moderate EULAR response, the differences between groups were smaller, with 89.5% and 79.9% for tocilizumab and TNFi groups, respectively (OR = 2.1, 95% CI 1.07–4.2, $P = 0.03$).

3.2. Response according to Previous Exposure to Biologics. Stratification according to previous biologic therapy exposure revealed that biologic-naïve patients treated with tocilizumab had higher odds of achieving remission, not only according to DAS28 (OR = 7.6, 95% CI 4.0–14.5), but also according to CDAI (OR = 2.6, 95% CI 1.2–5.8) and SDAI (OR = 3.0, 95% CI 1.3–6.8; Figure 2(a)). No significant differences were seen with the more stringent Boolean definition (OR = 1.6, 95% CI 0.7–3.7; Figure 2(a)). Regarding EULAR response, 76.9% of biologic-naïve patients in the tocilizumab group presented a good response, compared to 35.1% of TNFi users (OR = 6.2, 95% CI 3.2–12.1; Figure 2(b)). Considering patients previously exposed to biologic therapy and although the numbers are considerably smaller (TNFi = 64 and tocilizumab = 43), the results were similar to those observed in the overall population analysis, with significant differences being seen only for DAS28 remission (OR = 2.8, 95% CI 1.2–6.6; Figure 2(a)). Likewise, rates of EULAR response in this subgroup of patients were lower in comparison to those that were biologic-naïve, namely, 48.8% and 23.4% of EULAR good response in the tocilizumab and TNFi groups, respectively

Table 1: Baseline characteristics of included rheumatoid arthritis patients.

	Adalimumab ($n = 106$)	Etanercept ($n = 202$)	Golimumab ($n = 43$)	Infliximab ($n = 78$)	Tocilizumab ($n = 95$)	P value[$]	TNFi ($n = 429$)	P value[¥]
Age (years)	52 ± 11.0	53.1 ± 13.3	55.2 ± 11.4	54.9 ± 11.9	53.8 ± 10.9	0.558	53.5 ± 12.3	0.791
Female	98 (92.5)	172 (85.2)	38 (88.4)	67 (85.9)	82 (86.3)	0.459	375 (87.4)	0.772
Caucasian ($n = 456$)	85 (92.4)	166 (95.4)	27 (96.4)	67 (89.3)	80 (92.0)	0.424	345 (93.5)	0.607
Disease duration (years, $n = 489$)	12.3 ± 10.0	11.1 ± 9.0	10.2 ± 8.5	13.1 ± 10.6	10.7 ± 9.0	0.339	11.7 ± 9.5	0.372
Education (years, $n = 387$)	7.2 ± 4.7	7.4 ± 4.7	7.5 ± 3.6	6.2 ± 4.1	7.4 ± 4.6	0.464	7.1 ± 4.5	0.611
Current smokers ($n = 450$)	11 (11.6)	23 (13.0)	2 (8.0)	7 (10.1)	12 (14.3)	0.884	43 (11.8)	0.522
CV comorbidity ($n = 467$)	50 (52.1)	68 (39.5)	14 (36.8)	28 (38.9)	40 (44.9)	0.258	160 (42.3)	0.654
Seropositive ($n = 463$)	80 (87.0)	142 (80.2)	29 (76.3)	61 (92.4)	73 (81.1)	0.107	312 (83.7)	0.564
Erosive ($n = 380$)	18 (25.4)	37 (23.7)	7 (25.9)	13 (23.6)	16 (22.5)	0.994	75 (24.3)	0.757
Previous biologics	0.24 ± 0.61	0.16 ± 0.38	0.09 ± 0.29	0.14 ± 0.39	0.81 ± 1.13	**<0.001**	0.17 ± 0.44	**<0.001**
Biologic-naïve	88 (83.0)	170 (84.2)	39 (90.7)	68 (87.2)	52 (54.7)	**<0.001**	365 (85.1)	**<0.001**
MTX	86 (81.1)	164 (81.2)	36 (83.7)	67 (85.9)	75 (79.0)	0.813	353 (82.3)	0.447
MTX dose (mg/week)	19.6 ± 4.4	18.9 ± 4.5	19.4 ± 5.2	19.6 ± 3.8	18.2 ± 4.2	0.279	19.3 ± 4.4	0.069
Corticosteroids	81 (76.4)	153 (75.7)	35 (81.4)	65 (83.3)	77 (81.1)	0.586	334 (77.9)	0.493
Corticosteroids dose (mg/day)	7.4 ± 3.3	7.3 ± 2.9	7.2 ± 2.8	7.1 ± 2.7	6.7 ± 2.4	0.530	7.3 ± 3.0	0.097
TJC28	11.1 ± 8.2	10.1 ± 7.3	9.2 ± 6.8	11.3 ± 8.2	12.4 ± 7.5	0.092	10.5 ± 7.6	**0.028**
SJC28	7.0 ± 5.5	6.5 ± 4.7	6.9 ± 4.6	7.2 ± 5.7	10.4 ± 6.4	**<0.001**	6.8 ± 5.1	**<0.001**
ESR (mm/h, $n = 522$)	36.2 ± 22.9	36.9 ± 27.2	38.9 ± 27.1	37.7 ± 24.4	45.6 ± 27.1	0.073	37.1 ± 25.6	**0.004**
CRP (mg/dL, $n = 491$)	2.2 ± 2.6	2.0 ± 3.1	2.2 ± 2.7	1.9 ± 1.9	2.8 ± 3.2	0.266	2.1 ± 2.7	**0.035**
PGH (mm, $n = 496$)	58.7 ± 24.5	56.4 ± 22.9	59.5 ± 20.2	60.5 ± 23.6	59.8 ± 24.3	0.648	58.0 ± 23.2	0.496
PhGA (mm, $n = 376$)	47.3 ± 20.1	51.5 ± 20.0	51.0 ± 19.1	54.4 ± 19.2	60.0 ± 17.9	**0.002**	51.0 ± 19.8	**0.001**
DAS28	5.5 ± 1.4	5.4 ± 1.3	5.4 ± 1.2	5.6 ± 1.4	6.1 ± 1.1	**0.001**	5.4 ± 1.3	**<0.001**
CDAI ($n = 376$)	27.7 ± 14.8	28.0 ± 12.8	26.0 ± 11.5	29.8 ± 14.9	33.3 ± 13.2	**0.037**	28.1 ± 13.6	**0.003**
SDAI ($n = 361$)	29.9 ± 15.4	30.6 ± 13.8	27.6 ± 12.0	31.7 ± 15.7	35.6 ± 13.1	0.056	30.4 ± 14.4	**0.006**
HAQ ($n = 415$)	1.6 ± 0.7	1.4 ± 0.6	1.5 ± 0.7	1.5 ± 0.6	1.6 ± 0.6	0.158	1.5 ± 0.6	0.150
High disease activity								
DAS28 (>5.1)	68 (64.2)	120 (59.4)	28 (65.1)	51 (65.4)	74 (77.9)	**0.044**	267 (62.2)	**0.004**
CDAI (>22, $n = 376$)	46 (60.5)	93 (65.0)	14 (51.9)	38 (64.4)	56 (78.9)	0.068	191 (62.6)	**0.009**
SDAI (>26, $n = 361$)	39 (54.2)	78 (58.7)	13 (48.2)	33 (55.9)	53 (75.7)	**0.036**	163 (56.0)	**0.003**

Continuous variables presented as mean ± standard deviation; categorical variables are expressed as number (percentage). Final number of patients is indicated where data was missing. P value significant at <0.05; significant differences highlighted in bold. [$]Comparison of groups according to biologic using ANOVA or chi-square test, as appropriate; [¥]comparison of TNFi versus tocilizumab groups using Student's t-test or chi-square test, as appropriate. CDAI: clinical disease activity index; CRP: C-reactive protein; CV: cardiovascular; DAS28: disease activity score-28 joints; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; MTX: methotrexate; PGH: patient global health; PhGA: physician global assessment; SDAI: simplified disease activity index; SJC28: swollen joint count-28 joints; TJC28: tender joint count-28 joints; TNFi: tumour necrosis factor inhibitors.

Table 2: Disease activity at 6-month follow-up and respective change from baseline.

	Adalimumab ($n = 106$)	Etanercept ($n = 202$)	Golimumab ($n = 43$)	Infliximab ($n = 78$)	Tocilizumab ($n = 95$)	P value[§]	TNFi ($n = 429$)	P value[¥]
DAS28	3.8 ± 1.4	3.5 ± 1.4	3.5 ± 1.2	3.8 ± 1.4	2.8 ± 1.6	**<0.001**	3.6 ± 1.4	**<0.001**
ΔDAS28	1.6 ± 1.4	1.9 ± 1.4	1.9 ± 1.4	1.8 ± 1.4	3.3 ± 1.6	**<0.001**	1.8 ± 1.4	**<0.001**
CDAI ($n = 327$)	12.4 ± 9.3	11.7 ± 10.0	10.6 ± 6.5	12.7 ± 10.2	10.5 ± 10.3	0.690	12.0 ± 9.6	0.254
ΔCDAI	15.1 ± 12.5	16.1 ± 14.8	16.0 ± 8.5	17.0 ± 14.3	22.7 ± 15.7	**0.017**	16.0 ± 13.6	**0.001**
SDAI ($n = 299$)	13.7 ± 11.1	12.4 ± 10.5	13.1 ± 13.0	14.0 ± 10.7	11.6 ± 11.5	0.778	13.1 ± 10.9	0.343
ΔSDAI	16.1 ± 13.3	17.6 ± 16.1	15.2 ± 11.6	18.2 ± 14.9	25.2 ± 16.5	**0.007**	17.1 ± 14.7	**0.0003**
TJC28	4.8 ± 5.8	3.7 ± 5.4	2.7 ± 3.2	4.6 ± 5.9	3.6 (4.8)	0.109	4.0 ± 5.5	0.508
ΔTJC28	6.3 ± 6.9	6.5 ± 7.5	6.5 ± 6.3	6.6 ± 7.5	8.7 ± 7.7	0.100	6.4 ± 7.2	**0.006**
SJC28	2.3 ± 3.0	1.7 ± 2.6	2.3 ± 2.9	2.2 ± 3.2	2.6 ± 3.9	0.179	2.0 ± 2.9	0.111
ΔSJC28	4.6 ± 4.8	4.7 ± 4.9	4.6 ± 4.4	5.0 ± 4.9	7.8 ± 6.6	**<0.001**	4.7 ± 4.8	**<0.001**
ESR (mm/h, $n = 516$)	26.2 ± 21.6	23.7 ± 19.4	22.1 ± 15.1	25.9 ± 21.0	10.3 ± 14.5	**<0.001**	24.6 ± 19.9	**<0.001**
ΔESR	10.1 ± 21.2	13.5 ± 22.5	15.5 ± 21.9	12.0 ± 23.0	35.2 ± 25.1	**<0.001**	12.6 ± 22.2	**<0.001**
CRP (mg/dL, $n = 465$)	1.4 ± 2.7	0.81 ± 1.4	1.9 ± 7.3	1.0 ± 1.7	0.5 ± 1.3	**0.023**	1.1 ± 2.9	**0.045**
ΔCRP	0.7 ± 3.0	1.2 ± 3.1	0.1 ± 7.5	1.0 ± 1.7	2.3 ± 3.2	**0.006**	0.9 ± 3.5	**0.0009**
PGH (mm, $n = 481$)	39.8 ± 25.8	34.9 ± 23.8	41.7 ± 22.0	38.9 ± 24.9	35.0 ± 24.0	0.295	37.4 ± 24.4	0.385
ΔPGH	18.7 ± 31.6	21.5 ± 26.4	18.3 ± 19.9	21.5 ± 28.9	25.5 ± 30.0	0.525	20.5 ± 27.7	0.132
PhGA (mm, $n = 327$)	25.8 ± 17.3	24.0 ± 17.9	22.7 ± 14.7	28.3 ± 20.4	20.0 ± 19.4	0.160	25.1 ± 18.0	**0.041**
ΔPhGA	20.5 ± 21.4	26.7 ± 23.2	29.1 ± 19.1	27.4 ± 21.6	39.8 ± 25.6	**0.0001**	25.5 ± 22.2	**<0.001**
HAQ ($n = 323$)	1.1 ± 0.7	1.0 ± 0.7	1.1 ± 0.7	1.3 ± 0.7	1.0 ± 0.7	0.201	1.1 ± 0.7	0.522
ΔHAQ	0.5 ± 0.7	0.4 ± 0.6	0.3 ± 0.5	0.3 ± 0.5	0.6 ± 0.6	0.053	0.4 ± 0.6	**0.016**

Absolute values and relative change in disease activity composite scores and markers at 6 months of therapy in rheumatoid arthritis patients, according to biologic therapy. Continuous variables presented as mean ± standard deviation; categorical variables are expressed as number (percentage). Final number of patients is indicated where there was missing data. P value significant at <0.05; significant differences highlighted in bold. [§]Comparison of groups according to biologic using ANOVA or chi-square test, as appropriate; [¥]comparison of TNFi versus tocilizumab groups using Student's t-test or chi-square test, as appropriate.

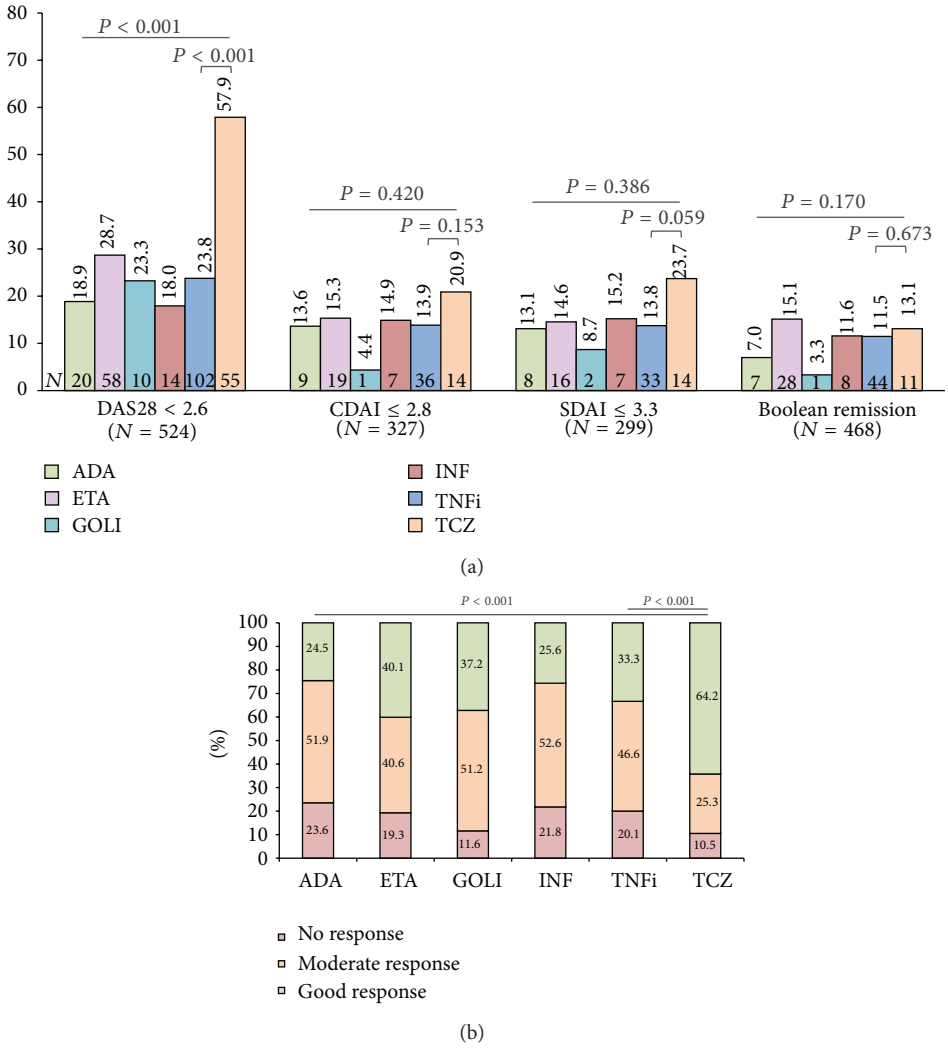


Figure 1: Frequencies of remission (a) and EULAR response (b) at 6 months according to biologic treatment. Tocilizumab- (TCZ-) treated patients had higher rates of DAS28 remission and EULAR response. Similar proportions of TCZ and TNFi users were in remission according to CDAI, SDAI, and Boolean remission criteria. P value significant at <0.05. ADA: adalimumab; ETA: etanercept; GOLI: golimumab; INF: infliximab.

(OR = 3.1, 95% CI 1.4–7.1). Considering the achievement of at least a moderate EULAR response, differences between groups were less striking, being significant only in the biologic-naïve subgroup (OR = 5.3, 95% CI 1.3–22.4, P = 0.011) and not in patients previously exposed to biologics (OR = 2.3, 95% CI 0.9–5.7, P = 0.075).

3.3. Low Disease Activity at 6 Months. The proportions of patients achieving, at 6 months, at least a LDA state according to each of the indexes are represented in Figure 3 ((a) overall population analysis; (b) stratifying on previous biologic status). Notably, more than half of the patients reached LDA according to CDAI (≤10) or SDAI (≤11) regardless of type of treatment. As seen for remission, in the overall population analysis there were significant differences between groups favoring tocilizumab only for the DAS28 definition of LDA (OR = 2.6, 95% CI 1.6–4.1; Figure 3(a)). Interestingly, there was a better concordance between indexes than for remission, particularly in the tocilizumab group, with 64% of patients in LDA/remission according to every definition. Biologic-naïve patients also had higher odds of achieving at least a LDA state when treated with tocilizumab, compared to those in the TNFi group: defined by DAS28 (OR = 4.6, 95% CI 2.4–9.0), CDAI (OR = 2.8, 95% CI 1.3–6.1), or SDAI (OR = 2.5, 95% CI 1.1–5.7; Figure 3(b)). As for patients previously exposed to biologics, there were no statistically significant differences between both drug classes in terms of achieving at least LDA according to any of the criteria (Figure 3(b)).

3.4. Multivariate Analyses. Table 3 presents the results of logistic regression and propensity scores-based analyses to determine the effect size of tocilizumab versus TNFi in predicting each of the discussed outcomes. Multivariate logistic regression, adjusting for age, disease duration, baseline

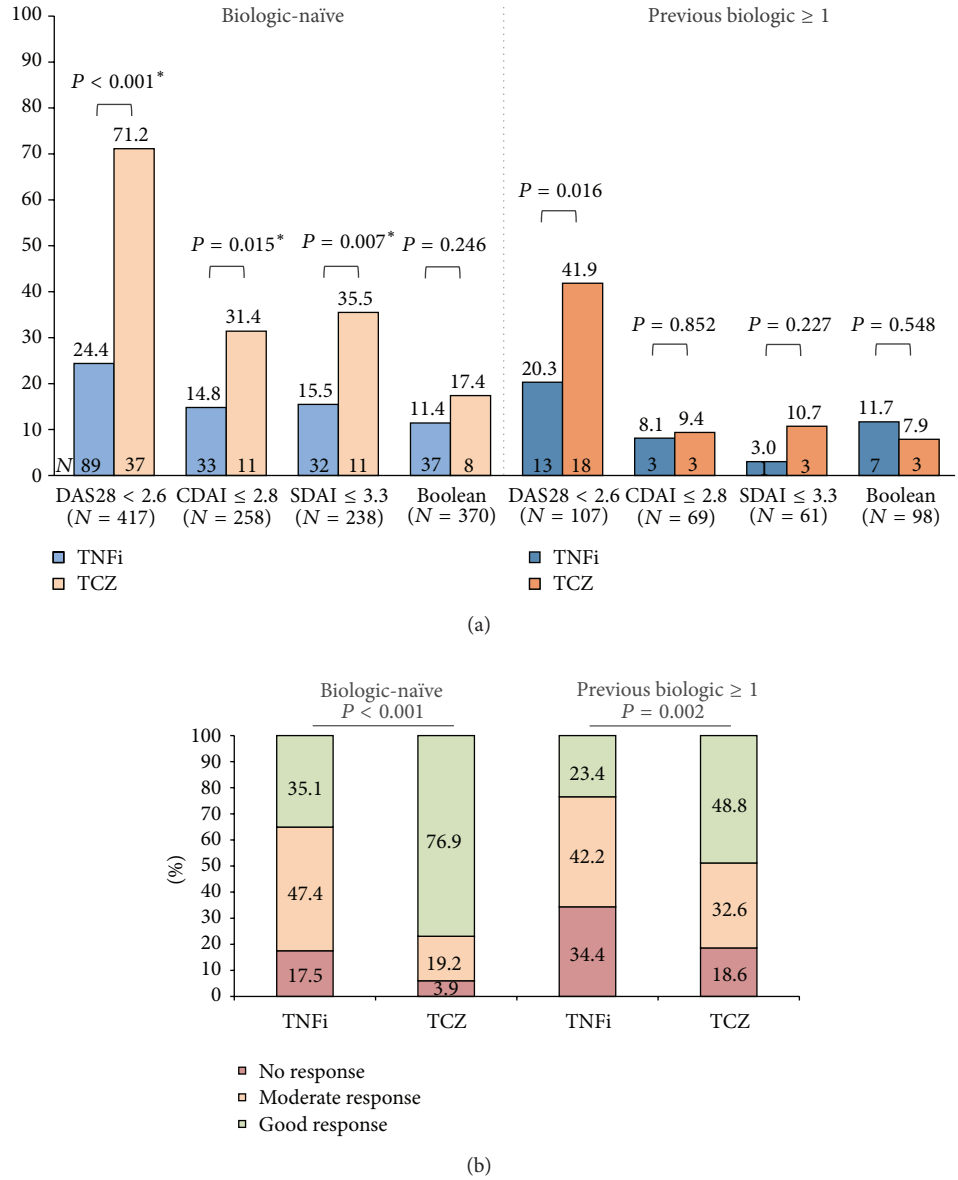


Figure 2: Remission (a) and EULAR response rate (b) at 6 months stratified by previous biologic therapy. (a) Biologic-naïve patients treated with TCZ had significantly higher rates of DAS28, CDAI, and SDAI remission, with nonsignificant differences in the more stringent Boolean remission. TCZ-treated patients previously exposed to at least 1 biologic had greater frequencies of DAS28 remission than those treated with TNFi, with similar rates of CDAI, SDAI, and Boolean remission. (b) EULAR response rates were significantly higher in the TCZ group for both previous biologic statuses, although the differences were greater in biologic-naïve patients. P value significant at <0.05.

disease activity (DAS, CDAI, or SDAI, as appropriate), and number of previous biologics, revealed that tocilizumab-treated patients had higher odds of achieving remission and LDA according to DAS28, CDAI, and SDAI. There were no significant effects of biologic class on reaching Boolean remission. Good and good/moderate EULAR responses were also more likely to occur in tocilizumab-treated patients using this approach. The inclusion of the propensity scores predicting biologic class into the logistic regression model decreased the effect size of treatment group on the outcomes, although not changing the inference made for DAS28, CDAI or SDAI remission/LDA, Boolean remission, or EULAR good response. However, the odds of achieving a good/moderate EULAR response were no longer different between biologic therapy classes. Propensity score matching alone or in combination with multivariate logistic regression confirmed this finding and further revealed no significant effect of biologic class on reaching LDA according to SDAI. Achieving CDAI remission or LDA was not different between groups in the propensity score-matched analysis, although the regression analysis performed in the matched population did reveal significant differences for reaching remission/LDA, favoring TCZ. All other outcomes remained unchanged using this approach.

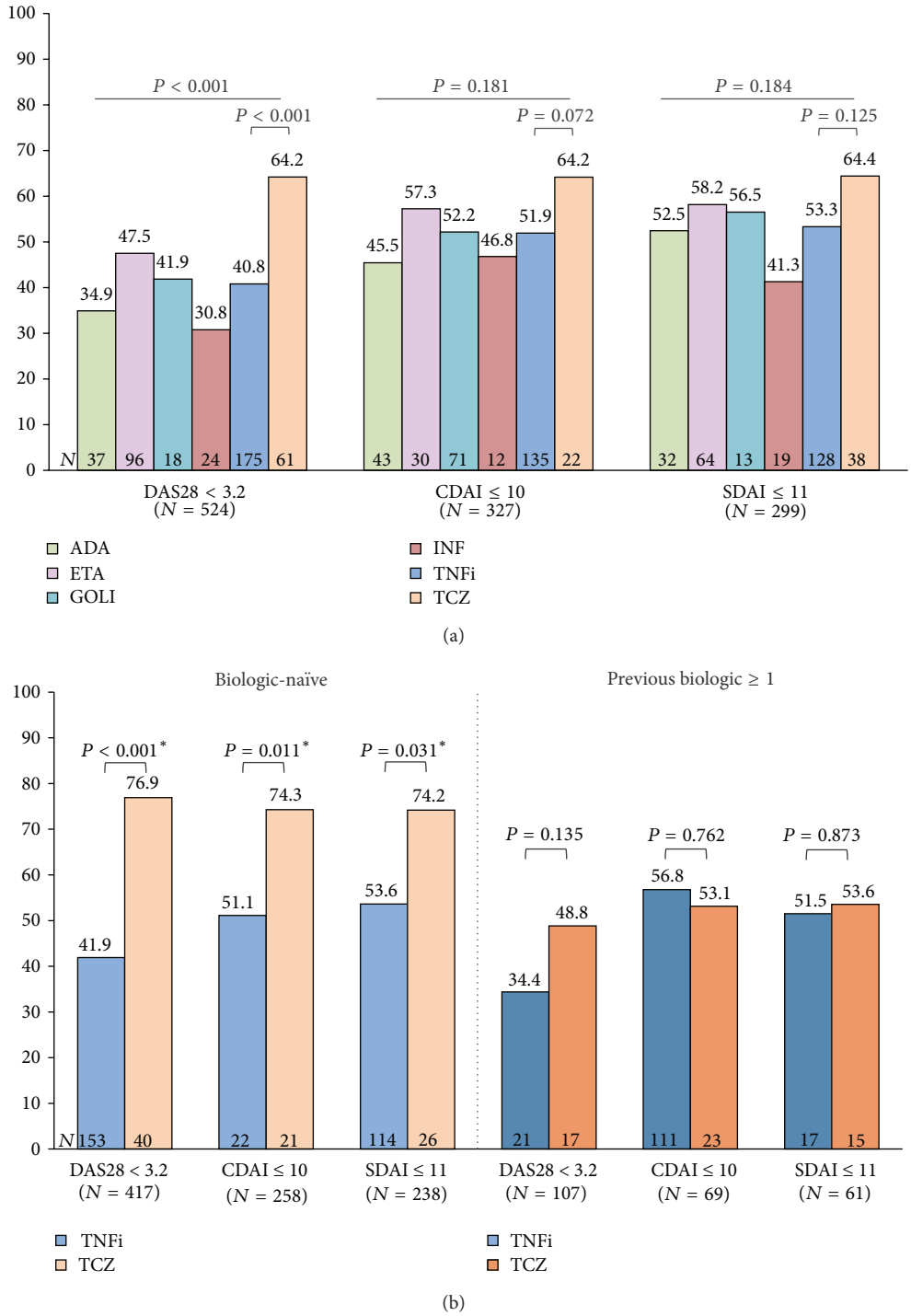


Figure 3: Low disease activity at 6 months according to treatment (a) and previous biologic therapy (b). (a) Significantly more patients treated with TCZ reached a state of at least DAS28 low disease activity (LDA), with no significant differences for CDAI and SDAI cutoffs. (b) Biologic-naïve patients in the TCZ group had significantly higher proportions of DAS28, CDAI, and SDAI LDA compared to TNFi users. On the contrary, in patients previously exposed to at least 1 biologic the frequencies of LDA according to all indexes were similar between drug class groups. *P* value significant at <0.05.

4. Discussion

In this study we compared the effectiveness of two classes of biologic therapies in RA patients registered in our national register, Reuma.pt. We found that patients treated with tocilizumab were more likely to achieve DAS28, CDAI, and SDAI remission/LDA, as well as good EULAR response at 6 months, when adjusting for confounding factors. On the

Table 3: Logistic regression and propensity score-based analyses to predict treatment response with tocilizumab versus TNFi.

	N	Remission			Low disease activity		
		Odds ratio	95% CI	P value	Odds ratio	95% CI	P value
		DAS28 < 2.6			DAS28 < 3.2		
Multivariate LR	489	13.3	6.9–25.4	**<0.001**	7.3	3.9–13.6	**<0.001**
Univariate LR + PS	489	8.3	4.4–15.4	**<0.001**	5.5	2.9–10.5	**<0.001**
Multivariate LR + PS	489	11.0	5.6–21.6	**<0.001**	6.2	3.2–12.0	**<0.001**
PS matching	259	7.9	4.3–14.6	**<0.001**	5.0	2.7–9.2	**<0.001**
PS matching + multivariate LR	259	12.3	6.0–25.4	**<0.001**	7.5	3.7–15.1	**<0.001**
		CDAI ≤ 2.8			CDAI ≤ 10		
Multivariate LR	308	2.9	1.3–6.6	**0.009**	3.0	1.5–6.1	**0.002**
Univariate LR + PS	308	2.5	1.1–5.6	**0.022**	2.3	1.1–4.6	**0.019**
Multivariate LR + PS	308	2.8	1.2–6.5	**0.016**	2.6	1.3–5.5	**0.010**
PS matching	179	2.1	0.92–5.0	0.078	2.0	1.00–4.1	**0.048**
PS matching + multivariate LR	179	3.3	1.3–8.4	**0.015**	2.6	1.2–5.6	**0.017**
		SDAI ≤ 3.3			SDAI ≤ 11		
Multivariate LR	282	4.1	1.7–9.5	**0.001**	2.9	1.4–6.3	**0.005**
Univariate LR + PS	282	3.1	1.3–7.0	**0.008**	2.2	1.04–4.8	**0.038**
Multivariate LR + PS	282	3.6	1.5–8.7	**0.005**	2.5	1.1–5.5	**0.024**
PS matching	158	2.6	1.1–6.4	**0.029**	1.6	0.8–3.5	0.209
PS matching + multivariate LR	158	4.0	1.5–10.8	**0.007**	2.2	0.95–5.0	0.065
		Boolean					
Multivariate LR	442	2.1	0.91–4.8	0.083			
Univariate LR + PS	442	1.8	0.76–4.0	0.184			
Multivariate LR + PS	442	1.9	0.77–4.8	0.159			
PS matching	216	1.2	0.54–2.9	0.629			
PS matching + multivariate LR	216	1.4	0.54–3.9	0.463			
		Good EULAR			Good/moderate EULAR		
Multivariate LR	489	6.8	3.8–12.3	**<0.001**	2.5	1.1–5.9	**0.035**
Univariate LR + PS	489	6.3	3.4–11.8	**<0.001**	1.8	0.8–4.1	0.143
Multivariate LR + PS	489	6.4	3.4–12.0	**<0.001**	1.8	0.8–4.5	0.182
PS matching	259	6.2	3.3–11.6	**<0.001**	2.2	0.93–5.2	0.074
PS matching + multivariate LR	259	7.8	4.0–15.4	**<0.001**	2.4	0.98–6.1	0.056

The odds ratio and 95% confidence interval (95% CI) for the effect of tocilizumab versus TNF inhibitors (TNFi) in the considered outcomes are represented according to the statistical methodology used. Multivariate logistic regression (LR) adjusted for other significant confounders as described in Section 2, namely, age, disease duration, number of previous biologics, and baseline disease activity (DAS28 for DAS28/Boolean remission, DAS28 low disease activity (LDA), and EULAR response; CDAI for CDAI remission/LDA; SDAI for SDAI remission/LDA). Propensity scores (PS) predicting biologic class were calculated and incorporated in the analysis, via LR and/or matching (caliper 1 : 5 with replacement). P value significant at <0.05; significant differences highlighted in bold.

other hand, the modelled probability of Boolean remission did not differ between groups and neither did the likelihood of achieving a good/moderate EULAR response when taking into account the propensity score for receiving tocilizumab.

Similar results were obtained by Yoshida et al., with fewer patients per group, in the single centre CABUKI register, where DAS28-ESR remission was more frequent in tocilizumab-treated patients (47.8% versus 25%, $P = 0.006$), Boolean remission was similar, and previous biologic therapy had a significant impact on DAS28 remission frequencies [23]. In another study and unlike us, Takahashi et al. found no significant differences in proportions of EULAR good response, DAS28-CRP remission, and LDA between patients treated with tocilizumab or adalimumab, while confirming higher frequencies of EULAR good/moderate response in the tocilizumab group [26]. No adjusted results were reported for these comparisons, an aspect that might help explain these discrepancies [26].

We have found an impressive frequency of 57.9% DAS28 remission at 6 months. These rates are higher than those observed in published RCTs (27% OPTION [27], 30.1% RADIATE [28], 39.9% ADACTA [15], and 40.4% ACT-RAY [13]) but similar to other observational register-based studies (39–58% DANBIO [20], 53.3% Michinoku Tocilizumab Study Group [29], 47.8% CABUKI [23], and 54.9% FRAB [21]). The EULAR response rates, based on DAS28, were also similar to those found in RCTs [13, 15, 27] and observational studies [20, 26] that reported them. While it is understandable that the remission rates seen in register-based studies are higher than those observed in RCTs, due to factors such as selection and attrition bias associated with observational studies in a real life setting, it is intriguing that such discrepancies are also seen among different registers. Although the magnitude of the difference in the proportion of patients achieving remission at 6 months is not very large (39% minimum in DANBIO [20], 57.9% maximum in Reuma.pt), several aspects can explain these findings. In observational studies, treatment is selected based on objective criteria and also on subjective physician attitudes and expectations, which greatly vary according to country or region. Western European practices certainly differ from Asian ones and even between Portugal (Reuma.pt) and Denmark (DANBIO) factors associated with treatment selection are quite different. Furthermore, visual analogue scale scores are highly subjective, influenced by local cultural factors, and can have a profound impact on the assessment of disease activity. Other potential explanations for these results include genetic factors accounting for variable responses between populations with distinct backgrounds and different frequencies of concomitant treatment with MTX, other DMARDs, and corticosteroids, which might be crucial for suppressing minimal disease activity and attaining remission.

The results of our study confirm that the inclusion of inflammatory markers in the assessment of response to therapy is of extreme importance when analyzing the effectiveness of drugs such as tocilizumab that, through a profound inhibition of IL-6-driven inflammation, markedly suppress ESR and CRP, even within the normal range limits, and might overestimate remission rates, as shown by Smolen and Aletaha [24]. These authors suggest the use of CDAI and SDAI remission/LDA might be more appropriate to compare treatment responses and, in fact, we have found no differences between biologic classes in the overall population analysis [24]. However, in biologic-naïve patients CDAI and SDAI remission were more frequent in the tocilizumab group, with OR of 2.6 ($P = 0.015$) and 3.0 ($P = 0.007$), respectively. Similar observations were made after multivariate analyses adjusting for several confounders. It should be noted, though, that the CDAI/SDAI-based analyses forced the exclusion of a significant number of patients due to missing values, and this might somewhat weaken the conclusions. However, sensitivity analysis revealed that simply excluding patients with missing values and performing a univariate analysis for the effect of biologic class on response rates did not yield the same results as the multivariate approaches (data not shown). This suggests that our results are not merely explained by the exclusion of patients in regression models.

We have also found that previous biologic therapy had an important effect on response to treatment. In fact, analysis of the biologic-naïve subgroup of patients revealed higher remission rates, especially for tocilizumab, not only according to DAS28, a finding already reported by others [21, 23], but also with CDAI and SDAI, which differed significantly between biologic class. While it seems reasonable that tocilizumab response is better in biologic-naïve patients compared to those having failed a biologic previously, the differences at 6 months between biologic class in terms of CDAI/SDAI remission/LDA might be at least partly explained by the fact that at baseline these subpopulations were more similar between groups, which was mainly due to less active disease in tocilizumab users and slightly more active disease in the TNFi group (data not shown; the only significant differences were SJC28 and DAS28, still both higher with tocilizumab). On the other hand, for TNFi users, response rates did not differ greatly between the biologic-naïve patients and both the overall population and those previously exposed to at least one biologic. This was also seen in the study by Yoshida et al. [23] and, in our opinion, might be related to two findings: first, most of the TNFi group (85.1%) was naïve to biologic therapy and, thus, the overall group mostly represented the characteristics of the biologic-naïve subpopulation; secondly, biologic-naïve TNFi users had higher baseline DAS28 (5.5 versus 5.1, $P = 0.041$) and SJC28 (7.1 versus 4.8, $P < 0.001$) compared to those that were previously exposed to biologic therapy, thus counteracting the potentially beneficial effect of being a first line user.

Our study has several limitations. Given its observational nature it is prone to different types of confounding, for which we have tried to account for by using propensity scores and multivariate logistic regression. However, residual and unmeasured confounding cannot be avoided and this may limit some of the conclusions. Furthermore, extrapolation of these results to drug efficacy is not possible. More specifically, the fact that there were several baseline differences between biologic class groups suggests that treatment was chosen, at least partially, based on the characteristics of the patients, as would be expected in a clinical practice setting. We used propensity scores to try to adjust for this, although we could not include every baseline variable of interest into the score due to missing data. However, given that RCTs addressing comparative effectiveness of biologics are unlikely to be conducted, observational studies are one of the ways to address this issue.

Other potential limitations should be also taken into account. In this study we provide data at 6 months, which might not be extendable to more prolonged follow-up times. We have also not analysed discontinuation rates or reasons, mainly due to missing values and short follow-up time, and this might limit the translation of conclusions into clinical practice. On the other hand, the fact that we have only included patients with available DAS28 at 0 and 6 months may also imply some degree of bias. Still, considering that Reuma.pt is a clinical practice register, data will be missing due to random reasons such as different likelihood of different clinicians to fill in all fields, rather than due to more or less severe disease activity. Another point to be

focused is that we only included data on baseline concomitant MTX/corticosteroid treatment and did not assess whether these treatments were discontinued during follow-up. Nevertheless, given the relatively short follow-up and considering the longstanding nature of the disease in most cases, it is reasonable to assume that the combination therapy status did not alter significantly for the majority of the population.

5. Conclusions

In conclusion, using data from Reuma.pt, we found that treatment with tocilizumab was associated with higher rates of DAS28 remission/LDA and EULAR good response at 6 months. Similar observations were made also for CDAI and SDAI remission in biologic-naïve patients. Adjusting for other potential confounders confirmed higher response rates with tocilizumab according to DAS28, CDAI, and SDAI criteria. Boolean remission was similar between groups, suggesting that the use of more stringent remission criteria blurs the differences between drug classes. Similar, larger and longer observational studies from other registers are needed to confirm these results and give further insight into therapeutic decisions in the managements of RA patients.

Abbreviations

ACPA: Anti-citrullinated protein antibodies
ACR: American College of Rheumatology
CDAI: Clinical disease activity index
CI: Confidence interval
CRP: C-reactive protein
DAS28: Disease activity score-28 joints
DMARDs: Disease modifying antirheumatic drugs
ESR: Erythrocyte sedimentation rate
EULAR: European League Against Rheumatism
HAQ: Health assessment questionnaire
LDA: Low disease activity
MTX: Methotrexate
OR: Odds ratio
PGH: Patient global health
PhGA: Physician global assessment
RA: Rheumatoid arthritis
RCTs: Randomised clinical trials
Reuma.pt: Rheumatic Diseases Portuguese Register
RF: Rheumatoid factor
SDAI: Simplified disease activity index
SJC28: Swollen joint count-28 joints
TNFi: Tumour necrosis factor inhibitors
TJC28: Tender joint count-28 joints
VAS: Visual analogue scale.

Conflict of Interests

Vasco C. Romão has received speaker's fees from Merck Sharp and Dohme. Joaquim Polido-Pereira has received speaker's and/or consulting fees from Roche, Merck Sharp and Dohme, Pfizer, and Tecnimede. Miguel Bernardes has received speaker's and/or consulting fees from Abbvie, Roche, Merck Sharp and Dohme, Pfizer, and Bristol-Myers Squibb. Filipe Barcelos has received speaker's fees from Merck Sharp and Dohme and travel grants for participation at meetings. José António Pereira da Silva has benefited from speaker's fees and research support from Roche, MSD, Pfizer, and Abbvie. João Eurico Fonseca has received research grants from Abbott, Merck Sharp and Dohme, Pfizer, Roche, and UCB Pharma and speaker's fees and/or consulting fees from Abbvie, Merck Sharp and Dohme, Pfizer, Roche, and UCB Pharma. Helena Canhão has received research grants from Abbott, Merck Sharp and Dohme, Pfizer, Roche, and UCB Pharma. All other authors did not report any relevant financial disclosures.

Authors' Contribution

Vasco C. Romão has done study conception and design, data collection, data and statistical analysis, paper drafting, critical revision, and final approval of paper. Maria José Santos has done data collection, critical revision, and final approval of the paper. Joaquim Polido-Pereira has done data collection, critical revision, and final approval of the paper. Cátia Duarte has done data collection, critical revision, and final approval of the paper. Patrícia Nero has done data collection, critical revision, and final approval of the paper. Cláudia Miguel has done data collection, critical revision, and final approval of the paper. José António Costa has done data collection, critical revision, and final approval of the paper. Miguel Bernardes has done data collection, critical revision, and final approval of the paper. Fernando M. Pimentel-Santos has done data collection, critical revision, and final approval of the paper. Filipe Barcelos has done data collection, critical revision, and final approval of the paper. Lúcia Costa has done data collection, critical revision, and final approval of the paper. José António Melo Gomes has done data collection, critical revision, and final approval of the paper. José Alberto Pereira da Silva has done data collection, critical revision, and final approval of the paper. Jaime Cunha Branco has done data collection, critical revision, and final approval of the paper. José Canas da Silva has done data collection, critical revision, and final approval of the paper. José António Pereira da Silva has done data collection, critical revision, and final approval of the paper. João Eurico Fonseca has done study conception and design, data collection, data analysis, paper drafting, critical revision, and final approval of paper. Helena Canhão has done study conception and design, data collection, data and statistical analysis, paper drafting, critical revision, and final approval of paper. All authors read and approved the final paper.

Acknowledgments

Reuma.pt has been supported by unrestricted grants from Abbvie, Merck Sharp and Dohme, Pfizer, UCB Pharma, and Roche. The authors would like to acknowledge Fernando Martins, IT, from the Portuguese Society of Rheumatology for his technical support and management of Reuma.pt data.

References

[1] P. C. Taylor and M. Feldmann, "Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis," *Nature Reviews Rheumatology*, vol. 5, no. 10, pp. 578–582, 2009.

[2] M. C. Hochberg, J. K. Tracy, M. Hawkins-Holt, and R. H. Flores, "Comparison of the efficacy of the tumour necrosis factor α blocking agents adalimumab, etanercept, and infliximab when added to methotrexate in patients with active rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 62, Supplement 2, pp. 13–16, 2003.

[3] K. J. Aaltonen, L. M. Virkki, A. Malmivaara, Y. T. Konttinen, D. C. Nordström, and M. Blom, "Systematic review and meta-analysis of the efficacy and safety of existing TNF blocking agents in treatment of rheumatoid arthritis," *PLoS ONE*, vol. 7, no. 1, Article ID e30275, 2012.

[4] H. Canhão, A. M. Rodrigues, A. F. Mourão et al., "Comparative effectiveness and predictors of response to tumour necrosis factor inhibitor therapies in rheumatoid arthritis," *Rheumatology*, vol. 51, no. 11, Article ID kes184, pp. 2020–2026, 2012.

[5] J. D. Greenberg, G. Reed, D. Decktor et al., "A comparative effectiveness study of adalimumab, etanercept and infliximab in biologically naive and switched rheumatoid arthritis patients: results from the US CORRONA registry," *Annals of the Rheumatic Diseases*, vol. 71, no. 7, pp. 1134–1142, 2012.

[6] K. L. Hyrich, K. D. Watson, A. J. Silman, and D. P. M. Symmons, "Predictors of response to anti-TNF-α therapy among patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register," *Rheumatology*, vol. 45, no. 12, pp. 1558–1565, 2006.

[7] J. S. Smolen, R. Landewé, F. C. Breedveld et al., "EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update," *Annals of the Rheumatic Diseases*, vol. 73, no. 3, pp. 492–509, 2014.

[8] J. E. Fonseca, M. Bernardes, H. Canhão et al., "Portuguese guide lines for the use of biological agents in rheumatoid arthritis—october 2011 update," *Acta Reumatologica Portuguesa*, vol. 36, no. 4, pp. 385–388, 2011.

[9] I. Flouri, T. E. Markatseli, P. V. Voulgari et al., "Comparative effectiveness and survival of infliximab, adalimumab, and etanercept for rheumatoid arthritis patients in the Hellenic Registry of Biologics: low rates of remission and 5-year drug survival," *Seminars in Arthritis and Rheumatism*, vol. 43, no. 4, pp. 447–457, 2014.

[10] J. A. Markenson, A. Gibofsky, W. R. Palmer et al., "Persistence with anti-tumor necrosis factor therapies in patients with rheumatoid arthritis: observations from the RADIUS registry," *The Journal of Rheumatology*, vol. 38, no. 7, pp. 1273–1281, 2011.

[11] C. Salliot, A. Finckh, W. Katchamart et al., "Indirect comparisons of the efficacy of biological antirheumatic agents in rheumatoid arthritis in patients with an inadequate response to conventional disease-modifying antirheumatic drugs or to an anti-tumour necrosis factor agent: a meta-analysis," *Annals of the Rheumatic Diseases*, vol. 70, no. 2, pp. 266–271, 2011.

[12] M. Schoels, D. Aletaha, J. S. Smolen, and J. B. Wong, "Comparative effectiveness and safety of biological treatment options after tumour necrosis factor α inhibitor failure in rheumatoid arthritis: systematic review and indirect pairwise meta-analysis," *Annals of the Rheumatic Diseases*, vol. 71, no. 8, pp. 1303–1308, 2012.

[13] M. Dougados, K. Kissel, T. Sheeran et al., "Adding tocilizumab or switching to tocilizumab monotherapy in methotrexate inadequate responders: 24-week symptomatic and structural results of a 2-year randomised controlled strategy trial in rheumatoid arthritis (ACT-RAY)," *Annals of the Rheumatic Diseases*, vol. 72, no. 1, pp. 43–50, 2013.

[14] G. Jones, A. Sebba, J. Gu et al., "Comparison of tocilizumab monotherapy versus methotrexate monotherapy in patients with moderate to severe rheumatoid arthritis: the AMBITION study," *Annals of the Rheumatic Diseases*, vol. 69, no. 1, pp. 88–96, 2010.

[15] C. Gabay, P. Emery, R. van Vollenhoven et al., "Tocilizumab monotherapy versus adalimumab monotherapy for treatment of rheumatoid arthritis (ADACTA): a randomised, double-blind, controlled phase 4 trial," *The Lancet*, vol. 381, no. 9877, pp. 1541–1550, 2013.

[16] G. J. D. Bergman, M. C. Hochberg, M. Boers, N. Wintfeld, A. Kielhorn, and J. P. Jansen, "Indirect comparison of tocilizumab and other biologic agents in patients with rheumatoid arthritis and inadequate response to disease-modifying antirheumatic drugs," *Seminars in Arthritis and Rheumatism*, vol. 39, no. 6, pp. 425–441, 2010.

[17] E. Turkstra, S.-K. Ng, and P. A. Scuffham, "A mixed treatment comparison of the short-term efficacy of biologic disease modifying anti-rheumatic drugs in established rheumatoid arthritis," *Current Medical Research and Opinion*, vol. 27, no. 10, pp. 1885–1897, 2011.

[18] A. Pierreisnard, N. Issa, T. Barnetche, C. Richez, and T. Schaeverbeke, "Meta-analysis of clinical and radiological efficacy of biologics in rheumatoid arthritis patients naive or inadequately responsive to methotrexate," *Joint Bone Spine*, vol. 80, no. 4, pp. 386–392, 2013.

[19] M. Gallego-Galisteo, A. Villa-Rubio, E. Alegre-Del Rey, E. Márquez-Fernández, and J. J. Ramos-Báez, "Indirect comparison of biological treatments in refractory rheumatoid arthritis," *Journal of Clinical Pharmacy and Therapeutics*, vol. 37, no. 3, pp. 301–307, 2012.

[20] H. C. Leffers, M. Østergaard, B. Glintborg et al., "Efficacy of abatacept and tocilizumab in patients with rheumatoid arthritis treated in clinical practice: results from the nationwide Danish DANBIO registry," *Annals of the Rheumatic Diseases*, vol. 70, no. 7, pp. 1216–1222, 2011.

[21] Y. Nakashima, M. Kondo, T. Fukuda et al., "Remission in patients with active rheumatoid arthritis by tocilizumab treatment in routine clinical practice: results from 3 years of prospectively registered data," *Modern Rheumatology*, vol. 24, pp. 258–264, 2014.

[22] G. R. Burmester, E. Feist, H. Kellner, J. Braun, C. Iking-Konert, and A. Rubbert-Roth, "Effectiveness and safety of the interleukin 6-receptor antagonist tocilizumab after 4 and 24 weeks in patients with active rheumatoid arthritis: the first phase IIIb real-life study (TAMARA)," *Annals of the Rheumatic Diseases*, vol. 70, no. 5, pp. 755–759, 2011.

[23] K. Yoshida, Y. Tokuda, H. Oshikawa et al., "An observational study of tocilizumab and TNF-α inhibitor use in a Japanese community hospital: different remission rates, similar drug survival and safety," *Rheumatology*, vol. 50, no. 11, pp. 2093–2099, 2011.

[24] J. S. Smolen and D. Aletaha, "Interleukin-6 receptor inhibition with tocilizumab and attainment of disease remission in rheumatoid arthritis: the role of acute-phase reactants," *Arthritis and Rheumatism*, vol. 63, no. 1, pp. 43–52, 2011.

[25] H. Canhão, A. Faustino, F. Martins, and J. E. Fonseca, "Reuma. PT—the rheumatic diseases portuguese register," *Acta Reumatologica Portuguesa*, vol. 36, no. 1, pp. 45–56, 2011.

[26] N. Takahashi, T. Kojima, A. Kaneko et al., "Clinical efficacy of abatacept compared to adalimumab and tocilizumab in rheumatoid arthritis patients with high disease activity," *Clinical Rheumatology*, vol. 33, no. 1, pp. 39–47, 2014.

[27] J. S. Smolen, A. Beaulieu, A. Rubbert-Roth et al., "Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial," *The Lancet*, vol. 371, no. 9617, pp. 987–997, 2008.

[28] P. Emery, E. Keystone, H. P. Tony et al., "IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial," *Annals of the Rheumatic Diseases*, vol. 67, no. 11, pp. 1516–1523, 2008.

[29] Y. Hirabayashi and T. Ishii, "The DAS28-ESR cutoff value necessary to achieve remission under the new Boolean-based remission criteria in patients receiving tocilizumab," *Clinical Rheumatology*, vol. 32, no. 1, pp. 123–127, 2013.