SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

STUDYING DIFFERENT ASPECTS OF THE BACKGROUND AND THE TREATMENT OF RHEUMATOID ARTHRITIS – OUR STORY "OF MICE AND MEN"

by Tímea Besenyei, MD

Supervisor: Prof. Zoltán Szekanecz, MD, PhD, DSc

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE
DEBRECEN, 2022

STUDYING DIFFERENT ASPECTS OF THE BACKGROUND AND THE TREATMENT OF RHEUMATOID ARTHRITIS – OUR STORY "OF MICE AND MEN"

by Tímea Besenyei, MD

Supervisor: Prof. Zoltán Szekanecz, PhD, DSc

Doctoral School of Clinical Medicine, University of Debrecen

Musculoskeletal Diseases Doctoral Program

Head of the **Examination Committee**: Prof. Árpád Illés, PhD, Dsc

Members of the Examination Committee: Prof. György Nagy, PhD, Dsc

Prof. István Balogh, PhD, DSc

The Examination takes place at the Lecture Hall of Bldg. B, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 6th December 2022, 11 am

Head of the **Defense Committee**: Prof. Árpád Illés, PhD, Dsc

Reviewers: Attila Balog, PhD

Zoltán Griger, PhD

Members of the Defense Committee: Prof. Árpád Illés, PhD, Dsc

Attila Balog, PhD

Zoltán Griger, PhD

Prof. György Nagy, PhD, Dsc

Prof. István Balogh, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. B, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 6th December 2022, 12:30 pm

1 INTRODUCTION

1.1 Overview of the etiopathogenesis of rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune-inflammatory disease that primarily targets the synovial joints. Its prevalence is approximately 0.5-1%. Genetic and environmental/lifestyle-related risk factors, along with autoimmunity trigger the etiopathogenesis of RA; epigenetic factors may also contribute to the etiology.

The role of genetic factors have been approximated to 60%. Both human leukocyte antigen (HLA-DR) and non-HLA genes have important role in the pathogenesis of RA. It has been known since the 1980s that the majority of RA patients share a short amino acid sequence motif in residues 70–74 of the 3rd hypervariable region of the major histocompatibility complex-II (MHC-II) molecule β1 chain. The encoding "shared-epitope" (SE) alleles (*HLA-DRB1*01*, *DRB1*04*, *DRB1*10*) have the strongest association with RA susceptibility. SE hypothesis was reconsiderated and a new classification system was validated by du Montcel et al, claiming that the susceptibility risk represented by the RAA motif is modulated by amino acids at positions 70 and 71. The new classification system categorizes SE alleles into S1, S2, S3P and S3D groups based on the amino acids at positions 70 and 71. S2 and S3P allele carriers have positive association with anticyclic citrullinated peptide antibody (ACPA) production, thus RA susceptibility. Among the non-HLA genes protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) and TNF receptor associated factor 1 (*TRAF1*) loci have the strongest association with RA. Both regions are primarily related to ACPA positive RA. More than 30 further single nucleotide polymorphisms (SNP) have been associated with RA, including *IL23R*, *FCGR*, *IRF5*, *CD40*, *STAT4*, *PADI4*, *CCR6*, *CCL21*. The role of *HLADRB1*, *PTPN22* and *IL23R* SNPs has also been confirmed in Hungarian RA patients.

Environmental and lifestyle-related factors (e.g. smoking, silica dust, caffeine, sugar-sweetened soda [fructose], contraceptives, etc.) also contribute to RA. They may induce protein citrullination, thus ACPA production. The significantly higher risk of ACPA positive RA among the smoker SE carriers has been confirmed in Hungarian RA patients; though this study -the first Central-Eastern European study of the associations between lifestyle-related factors and RA- has not found significant correlation between ACPA production and caffeine or oral contraceptive intake.

The genetic and environmental/lifestyle-related risk factors, along with autoimmunity provoke synovitis, the central inflammatory event in RA. It is a rather complicated process

involving numerous inflammatory cells and mediators. T cells have a key role in initiatiating and maintaining the synovitis. It is confirmed by the genetic significance of antigen-presentation, along with the recognition of citrullinated autoantigens (autoAgs) by T cells. The efficiency of B cell targeted therapy emphasizes the role of B-cells in the pathogenesis of RA. Their complex role includes antibody, cytokine and chemokine secretion, as well as antigen presentation. Innate immune system representative neutrophils are the dominant cell type of RA synovial fluid (SF), their role is though not quite clear: they have the ability to harm the joint structures via secretion of proteolytic enzymes and inflammatory mediators, however they may also impair joint T cells. Macrophages and endothelial cells are also present in high number in the RA synovium, the angiogenic mediators released by them provoke angiogenesis. Subsequently the expanded vascular endothelial surface may enable the extravasation of inflammatory leukocytes into the synovium, thus the progression of RA.

The fact that the monozygotic twin concordance rate for rheumatoid arthritis is only approximately 15% drew attention to the importance of epigenetic factors that regulate gene expression, thus imply connection between environmental and genetic factors. RA synovial fibroblasts were shown to contain a number of differentially (hypo- and hyper-) methylated genomic regions. Most of the affected genes were involved in matrix remodeling, leukocyte recruitment, immune responses and inflammation. A peripheral blood mononuclear cell (PBMC) DNA methylation profiling study described differentially methylated regions in the major histocompatibility complex loci that made important contribution to the genetic risk of RA.

1.2 The clinical picture – focusing on cardiovascular complications

The main motif in RA is chronic synovitis that may eventually lead to joint destruction. However, its extraarticular manifestations can damage a lot of other organs, e.g. the skin, eyes, lungs and even the central nervous system. Moreover RA is related to an increased mortality, with life expectancy reduced by 3-10 years. The main cause of mortality in RA is cardiovascular disease, principally due to RA-associated accelerated atherosclerosis that can be observed even in the early phase of RA. RA-associated atherosclerosis is related with both traditional, Framingham risk factors and inflammation-associated factors, the latter including ACPA, immunoglobulin (Ig) M rheumatoid factor (RF), circulating immune complexes, pro-inflammatory cytokines and Th0/Th1 T cells.

Manifest atherosclerosis is usually preceded by endothelial dysfunction, that can be detected with well-established imaging techniques, i.e. intima-media thickness (IMT), flow-mediated vasodilation (FMD) and nitroglycerine-mediated vasodilation (NMD). IMT is even included in the European Alliance of Associations for Rheumatology (EULAR) recommendations for clinical practice, based on the evidence that IMT is correlated with inflammatory activity and disease duration in RA, independently from traditional risk factors.

Although systemic inflammation plays critical role in the development of accelerated atherosclerosis in RA, traditional risk factors are also present, making the risk management quite complex. Yet, well-controlled disease activity has a beneficial effect on the vascular status of patients with accelerated atherosclerosis.

1.3 Briefly about the successes and the challenges in the therapy of rheumatoid arthritis

In RA, there is a very narrow "window of opportunity" to achieve remission or low disease activity, what is necessary to prevent joint damage, functional disability and unfavorable disease outcome. Thus, early diagnosis, followed by immediate treatment is essential. While the conventional synthetic disease-modifying antirheumatic drug (csDMARD) methotrexate (MTX) remains the anchor drug in RA, numerous biological therapies and targeted synthetic (ts) DMARDs have become available during the recent years. There is growing evidence that biologics not only slow down the arthritic process, but they have beneficial effect on the systemic manifestations of RA as well as comorbidities, such as cardiovascular disease.

In spite of the huge development of therapeutic possibilities there is still a number of patients who experience drug toxicity or ineffectiveness. Genetic signatures have been identified in the background of therapeutic response. For example, numerous SNPs in genes responsible for cellular drug transpont or folate metabolism have been associated with the efficacy or toxicity of MTX; various SNPs have also been described in relation to response to other csDMARDs. In addition, certain gene polymorphisms may predict the efficacy of biologics, primarily tumor necrosis factor-α (TNF-α) inhibitors. These therapeutical challenges make the development of new therapeutic modalities fundamental.

The fact that epigenetic changes may contribute to the development of RA and that - in contrast to genetic mutations – they are reversible, makes them good therapeutic candidates. The Aurora kinases are involved in the regulation of cell proliferation and their specific inhibitors are implicated as anticancer drugs. They belong to the serine/threonine kinase superfamily and - by regulating various steps in centrosome formation and chromosome segregation - Aurora kinases play a role in cell division.

1.4 The significance of murine models in studying rheumatoid arthritis

The completion of the human genome sequencing project led to enthusiasm in rheumatology research. However, after the early promising results of genome-wide association studies (GWAS), the progress has slowed down due to the extreme diversity of the human population, the polygenic nature of the disease and the fact that most of the significant genetic alterations are placed in non-protein coding regions of the genome. During the recent years only a few confirmatory studies, examinations of different ethnic groups and treatment-related meta-analysis studies have been published, and we are still very far from finding etiological correlations.

Studying murine models of RA may help to fill the gaps in human GWAS by allowing greater insights into the mechanisms of autoimmune T and B cell responses, as well as enabling functional studies, genome manipulation and the pretest of potential new therapies. Though animal studies are laborious and time-consuming, by using appropriate genetic combinations they can help finding disease-promoting genes, even, to investigate the role of a single gene. Moreover, by yielding better understanding of the mechanisms of the disease, they can be very useful in the development of more effective and appropriate treatments.

2 AIMS

This Ph.D. thesis includes 3 studies examining different aspects of the background and the treatment of rheumatoid arthritis. We evaluated RA risk alleles in humans and in correlating animal models (Study 1). We examined gene expression profiles in association with vascular pathophysiology in RA patients (Study 2). We assessed the role of epigenetic factors in the pathogenesis of RA (Study 3).

Our specific aims were as follows:

Study 1

Evaluation of RA risk alleles in humans and in correlating animal models

- To review the non-MHC risk alleles in RA and in the syntenic chromosome regions of animal models, paying particular attention to the results of our research group.

Study 2

Examination of gene expression profiles in association with vascular pathophysiology in RA patients

- To perform genomic analysis of RA patients with cardiovascular complications.
- To study gene expression profiles in association with clinical and vascular response to biological therapy.

Study 3

Studying the expression of epigenome modifiers, including Aurora kinase A and B, in immune cells of arthritic mice and RA patients

- To identify epigenetic factors that are associated with arthritis both in mice and RA patients.
- To find potential new therapeutic opportunities in the targeted inhibition of the abovementioned factors.

3 MATERIALS AND METHODS

3.1 Study 1

3.1.1 Mice

In many years our research group genotyped over 5000 inbred wild-type parents, approximately 500 F1 hybrids (all negative for cartilage proteoglycan (PG) aggrecan-induced arthritis (PGIA)) and 3200 F2 hybrids of six different genetic intercrosses using a total of 240 single sequence length polymorphism (SSLP) markers. The goal was to identify genetic alterations responsible for individual and overlapping qualitative (binary) quantitative trait loci (QTLs) that are linked to PGIA or collagen-induced arthritis (CIA) in the mouse genome. During this process a disease-suppressive chromosome region was inserted into a fully susceptible genetic background. F1 males were selected from the intercross of a PGIA susceptible BALB/c female and a resistant DBA/2 male (both MHC H2d) carrying the DBA/2 genomic region of interest. These F1 males were several times backcrossed with wild-type BALB/c females until the N1-NX generations had sufficient numbers of recombination. These Nx males were intercrossed with wild-type BALB/c females, and the resulting heterozygous Nx+1 males and females were intercrossed to establish a homozygous interval-specific congenic (IVSC) strain.

Then IVSC mice were tested for arthritis susceptibility, disease onset and severity and for over 15 biomarkers that might have some potential relevance for RA.

3.1.2 Literature review

Our main goal was to find human relevance of these animal studies. Therefore we performed a literature review and compared the results from our studies and from other rodent model experiments with the loci identified in human autoimmune diseases, preferably RA.

3.2 Study 2

3.2.1 Patients

Sixteen Caucasian, biologic-naive RA patients (15 females, 1 males); with a mean age of 53.7±5.7 (range: 42-60) years and a mean disease duration of 10.0±10.2 (range: 2-44) years

were included in the first part of Study 2. In the 2nd part of this study 19 biologic-naive RA patients (18 females, 1 males); with a mean age: 54.3±4.8 (range: 43-60) years and a mean disease duration: 12.1±10.9 (range: 2-44) years were recruited. Later 12 patients received etanercept (ETN), and 7 certolizumab pegol (CZP). All RA patients met the 2010 EULAR/ACR classification criteria for RA.

All blood samples were obtained after the subjects fasted overnight for 12 hours locally between 8:00 AM and 9:00 AM before the first admission of biologics. Medication remained unchanged during the study.

The inclusion criteria in both parts of the study included confirmed diagnosis of RA; age between 20 and 60, failure to respond to at least two DMARDs, active disease (DAS28 >3.2), anti-TNF therapy-naive patients. Corticosteroid therapy (prednisone ≤10mg per day) was allowed provided that the dosage had been stable for at least 2 months before entry. Non-steroidal anti-inflammatory drugs (NSAID) were also allowed in doses stable for at least one month before baseline. All patients received 10-25 mg/week oral MTX treatment, which had been stable for at least 4 weeks before baseline. Exclusion criteria included pregnancy or breastfeeding; current or recent malignancies; active infectious disease; patients with a history of arthritis or connective tissue disease other than RA; smoking.

Disease activity was assessed by determining the 28-joint Disease Activity Score (DAS28) at baseline and then 12 months after the initiation of anti-TNF treatment in Study 2/2.

Clinical responder (cR vs cNR) status was determined after 12 months of treatment with either ETN or CZP by the EULAR response criteria originally described by Van Gestel et al.

The Medical Research Council of Hungary gave ethical approval for this study (No. 9732-2/2012/EHR). In addition, the Institutional Review Board of the University of Debrecen Faculty of Medicine also approved the protocol. The study was in compliance with the Helsinki Declaration. Signed informed consent was obtained from all individuals providing blood sample.

3.2.2 Assessment of vascular physiology by ultrasound

Brachial artery FMD was assessed by ultrasound examination performed on the right arm. A 10 MHz linear array transducer (ultrasound system: HP Sonos 5500) was used by a single trained sonographer after 30 minutes resting in a temperature-controlled room (basal

value for FMD). A B-mode longitudinal section was obtained of the brachial artery above the antecubital fossa. In order to assess FMD, reactive hyperaemia was induced by the release of a pneumatic cuff around the forearm previously inflated to suprasystolic pressure for 4.5 minutes. Maximal flow velocity and the arterial diameter was continuously recorded for 90 seconds after deflation. Flow velocities, the baseline diameter, as well as FMD were ECG gated and detected offline. FMD values were expressed as % change from baseline (resting) value (FMD%). In a previous work of our team, we divided RA patients into "high (normal) FMD" and "low (impaired) FMD" subsets by defining a cut-off value of 5%. We used the same cut-off value in the present study.

The measurement of the IMT was carried out by a single observer using a duplex ultrasound system (HP Sonos 5500, 10 MHz linear array transducer). Longitudinal high-resolution B-mode ultrasound scan were employed over both right and left common carotid arteries, were R-synchronised and recorded. The offline measurements were performed 1 cm proximal to the carotid bulb in the far wall. IMT was defined as the distance between the first and second echogenic lines from the lumen taking the average of 10 measurements on both sides. IMT values were expressed in mm. In our previous work, we divided RA patients into "high (increased) IMT" and "low (normal) IMT" subsets by using a cut-off value of 0.65 mm. We used the same cut-off in the present study.

With respect to arterial stiffness, pulse wave velocity (PWV) was calculated automatically by a TensioClinic arteriograph system (Tensiomed Ltd, Budapest, Hungary) as the quotient of the distance between the jugular fossa and symphysis and the reflexion time. The arteriograph assesses this parameter from the oscillometric data obtained from the 35 mmHg suprasystolic pressure of the brachial artery. In order to obtain reproducible results, the patient had to rest in a supine position for at least 10 minutes before the assessment in a quiet room. PWV is expressed in m/s. If an artery is elastic, PWV is low. With decreased arterial elasticity, PWV rises. Based on our previous experience we used a cut-off value of 8 m/s, where PWV $\leq$ 8 m/s and PWV $>$ 8 m/s indicated "low (normal)" and "high (increased)" PWV, respectively.

In Study 2/2, we defined the arbitrary vascular responder status (vR vs vNR). This was determined by changes in FMD, IMT and PWV after one-year ETN or CZP therapy. Patients achieving at least 20% improvement in FMD, IMT or PWV were considered vascular responders (vR). Patients achieving $\geq$20% improvement in at least 2 out of the 3 vascular parameters (FMD, IMT, PWV) were considered Good Vascular Responders (GVRs).

3.2.3 PBMC and RNA isolation

Venous peripheral blood samples were collected (10 ml) in Venous Blood Vacuum Collection Tubes containing EDTA (BD Vacutainer K2EDTA). PBMCs were separated by Ficoll gradient centrifugation. Total RNA was extracted from PBMCs using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA quality was checked on Agilent Bioanalyzer 2100 (Agilent Technologies), all samples had a 28S/18S ratio between 1.5 and 2.0 and the RNA Integrity Number was between 9 and 10. Quantity was determined by NanoDrop (Thermo Scientific).

3.2.4 Microarray analysis and statistics

Affymetrix GeneChip Human Primeview array was used to analyze global expression pattern of 28869 well-annotated loci. 3'IVT Expression Kit (Affymetrix) and GeneChip WT Terminal Labeling and Control Kit (Affymetrix) were used for amplifying and labeling 250 ng of RNA samples. Samples were hybridized at 45°C for 16 hours and then standard washing protocol was performed using GeneChip Fluidics Station 450 and the arrays were scanned on GeneChip Scanner 7G (Affymetrix).

Microarray data were analyzed by Genespring GX12 software (Agilent Technologies). Affymetrix data files were imported using the RMA algorithm and median normalization was performed. To identify differentially expressed genes between clinical conditions, statistical analysis was performed using Mann-Whitney U test, p value <0.05 was considered to significant difference. We used hierarchical clustering and principal component analysis (PCA) to show the separation of clinical conditions by the differentially expressed genes. Microarray data were submitted to Gene Expression Omnibus (GEO), accession number: GSE126476.

Gene Ontology (GO) analysis was performed using Cytoscape 3.4.0 software (cytoscape.org) with ClueGO application. The settings were the following: GO biological process, GO immune system process, and KEGG human diseases pathways; statistical options: two-sided hypergeometric test and Benjamini-Hochberg FDR for multiple testing correction. Significantly enriched GO categories were considered to p value <0.05 and κ score <0.4.

The association between clinical and various vascular responses was analyzed by Pearson's correlation ($p<0.05$).

3.3 Study 3

3.3.1 Mice

Retired breeder female BALB/c mice were purchased from the National Cancer Institute (NCI, Frederick, MD) and were used for the generation of PGIA according to a standard protocol. Briefly, the mice were immunized with PG purified from knee joint cartilage of osteoarthritic patients who had undergone joint replacement surgery. PG was injected intraperitoneally (i.p.) three times (at three-week intervals, 100 μg PG core protein/injection) with 2 mg of dimethyldioctadecyl-ammonium bromide (DDA) adjuvant into BALB/c mice. Mice were assessed for arthritis in the limbs three times a week and were scored on a visual scale of 0 to 4 for each paw, thus yielding a maximum severity score of 16 for each mouse. To measure the therapeutic effect of VX-680 treatment on established arthritis, a microcaliper was used for measuring the thickness of the arthritic joints. CIA was generated by immunizing male DBA/1J mice (The Jackson Laboratory, Bar Harbor, ME) with 100 μg of human type II collagen (CII) in complete or incomplete Freund's adjuvant following the standard CIA protocol. DBA/2 mice (NCI) that are resistant to both PGIA and CIA were immunized with either cartilage PG or CII as described above. Serum samples were harvested prior to the treatment with VX-680 and at the end of experiment (on day 17–18 of treatments). IgG1 and IgG2a isotypes of autoantibodies (anti-mouse PG) were measured in serum samples of BALB/c mice. Animal experiments were approved by the Institutional Animal Care and Use Committee.

3.3.2 Human subjects

All patients (15 treatment-naive and 10 MTX-treated RA patients) and control individuals were recruited under Institutional Review Board – approved protocol, and gave informed consent. Human studies were approved by the Institutional Review Board at Rush University Medical Center, Chicago, IL, USA.

3.3.3 VX-680 treatment of BALB/c mice with PGIA

Prophylactic treatment with VX-680 was initiated six days prior to the expected onset of PGIA (in general arthritis develops 9–10 days after the third PG-immunization). Mice were i.p. injected with 40 mg/kg of VX-680 (Sellekchem, Houston, TX), which is an effective dose

described for cancer treatment, and which did not affected the viability of cells harvested by peritoneal lavage. VX-680 was dissolved in dimethyl sulfoxide (DMSO, 100 mg/ml) and diluted in 25% isopropyl alcohol. VX-680 was administered each day during the first week of the experiment and then every other day until the animals were sacrificed.

For therapeutic treatment, arthritic BALB/c mice were i.p. injected with 50 mg/kg of VX-680. Prior to the treatment, arthritic mice were selected to have approximately the same cumulative arthritis scores (~2.5±1.0, n=8–10). The mice received the first VX-680 treatment after the first symptoms (redness and swelling of the paws) of arthritis were observed and then received treatment every other day until the animals were sacrificed.

3.3.4 RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

Cells were collected from the spleens, bone marrow, and joint draining lymph nodes (LNs) of mice on day 17 after the third PG injection. Before the purification of lymphocytes, aliquots of the spleen, bone marrow and LN cells were used for RNA isolation. T and B cells were purified using antibody-coated magnetic beads (StemCell Technologies, Vancouver, BC, Canada). The RNA was isolated from aliquots of spleen cells and purified lymphocytes using a TRI reagent (Sigma-Aldrich, St. Louis, MO) immediately after the separation or purification. One μg of the total RNA was used for cDNA synthesis using iScript kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an iQ5 RT-PCR machine (Bio-Rad) and the SsoFast™ EvaGreen® Supermix (Bio-Rad). The primers were designed using PrimerQuest software and were manufactured by Integrated DNA Technologies (Coralville, IA). The specificity of the qRT-PCR product was monitored by a post-PCR melting curve analysis. Samples were tested in triplicate, and the iQ5 PCR software was used to calculate the normalized fold expression changes based on the ΔCt method. Measured Ct values were normalized to the β-actin or Gapdh internal control values.

The PBMCs from the blood samples from consenting, healthy individuals and DMARD treatment naive RA patients were separated on a Ficoll gradient within one hour after blood collection. The isolated mononuclear cells were stored in RNAlater® (Ambion/ LifeTechnologies, Grand Island, NY) until the RNA preparation was performed. The cDNA synthesis and qRT-PCR were performed as described for the mouse samples.

3.3.5 PCR arrays

Human (PHS-085) and mouse (PMM-085) "RT2Profiler™" PCR arrays (SABioscience, Fredrick, MD) were used for the study of arthritis-associated changes in the expression of 84 chromatin-modifying enzymes. We used the company's reagents for cDNA synthesis and followed the manufacturer's instructions for qRT-PCR.

3.3.6 Western blot analysis

For Western blot analysis, we used the same cells as those isolated for the total RNA preparation. Cell extracts were prepared in RIPA buffer with Protease Inhibitor Cocktail® (Roche, Indianapolis, IN). Protein concentrations were determined using the BCA Protein Assay kit™ (Pierce, Rockford, IL). Proteins (30 μg/lane) were resolved using 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (PBS, pH 7.5) for 1 hour and then incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: rabbit polyclonal anti-Aurora A (ab61114, Abcam, Cambridge, UK), rabbit polyclonal anti-Aurora B (#3094, Cell Signaling Technology, Beverly, MA) and mouse monoclonal (mAb) anti-Gapdh (clone 6C5, Abcam). Peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were developed using Plus Western Blotting Substrate (Pierce). The histone fractions were prepared from purified B cells and were resolved on a12% SDS-PAGE gel. Immunodetection of the phosphorylation-modified or un-modified histone H3 was performed as described above. The primary antibodies were rabbit polyclonal antibodies against phosphorylated Histone H3 Ser10 (H.H3-P, ab5176) or histone H3 (H.H3, ab1791) both from Abcam.

3.3.7 Tissue culture

For the apoptosis-related gene expression studies, spleen cells from mice with PGIA were cultured in 24-well tissue culture plates for 3 days in Dulbecco's Modifed of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were treated with 60, 300 or 1500 nM VX-680 or mock-treated with DMSO. RNA was isolated daily from

the treated spleen cells on the three subsequent days. The expression levels of the p53 and Puma genes were measured using qRT-PCR.

3.3.8 Flow cytometry

T and B cells isolated from the spleens, lymph nodes or bone marrow of mice were analyzed using multicolor flow cytometry. Briefly, 106 cells/well were seeded into 96-well U-bottom plates (BD Falcon), and the Fc receptors were blocked with anti-CD16/CD32 mAb (BioLegend, San Diego, CA) for 15 minutes at 4°C. The samples were incubated with fluorochrome-conjugated mAbs against mouse CD3, CD19, active caspase 3, IgM, IgD, or CD138 (mAbs from BioLegend or BD Biosciences, San Diego, CA) in 100 µl of flow cytometry staining/washing buffer (0.1% bovine serum albumin in PBS with 0.1% NaN3) for 30 min in the dark at 4°C. The cells were washed twice and analyzed on the same day. Data acquisition and analysis were performed using a FACS Canto II flow cytometer with an HTS module and FACS DIVA software (BD Flow Cytometry Systems, San Jose, CA). Initial gating was performed on lymphoid cells (based on the forward/side scatter (FSC/ SSC) parameters). The CD19+ cells were defined as total B cells and the CD3+ cells were defined as total T cells. Intracellular caspase 3 was detected after permeabilization with Cytofix/Cytoperm (BD Biosciences). B cell subsets were analyzed after co-staining with anti-CD19, anti-IgM, and anti-IgD mAbs (B1 and B2 cells), or with anti-CD19 and antiCD138 mAbs (plasmablasts/plasma cells).

3.3.9 Statistical analysis

Descriptive statistics were used to determine the group means and the standard error of the mean (mean ± SEM), unless otherwise stated. The difference between two groups was tested for statistical significance using a Student's t-test. When non-parametric distribution occurred (e.g., human samples), we used Mann-Whitney U test to compare results. $P \leq 0.05$ was considered to be statistically significant.

4 RESULTS

4.1 Study 1

4.1.1 Corresponding genomic loci of RA and rodent models of RA

Our and other research groups have identified 29 PGIA and 40 CIA loci in mice and a few corresponding QTLs in rats. We overviewed QTLs from all mouse genomic studies that correlate with one of the major RA risk loci confirmed in meta-analyses and choose two of them for more detailed examination: *Pgia26/Cia5/mCia21/Eae3* on mChr3, corresponding to the *PTPN22/CD2* allele on human Chr1; *Pgia2/Cia2/Cia3* on mChr2, corresponding to the *TRAF1/C5* allele on human Chr9. From the chosen strains IVSC were generated, the other congenic and subcongenic strains were cryopreserved.

A few mutated genes were identified by parallel sequencing of homogeneous genomic regions of disease-susceptible and disease-resistant IVSC mice. Not all of these genes had been previously associated with arthritis, but they had localised without exception in close proximity to a gene used to denote human RA risk alleles.

The aftermath of this study was a high-throughput sequence analysis of these genes and appropriate human genomic DNA samples.

4.2 Study 2

4.2.1 Gene expression profiles may differ in patients with normal vs impaired vascular pathophysiology

Eleven of the 16 RA patients had low (<5%) and 5 had high (≥5%) FMD values. Also 11 patients had low (≤0.65 mm) and 5 had high (>0.65 mm) IMT values. Low PWV (≤8 m/s) was observed in 9 and high (>8 m/s) in 7 patients.

Two up- and 12 down-regulated genes were associated with impaired versus normal FMD. The up-regulated genes were *CD74* and *ZNF718*, the down-regulated were *FOLR3*, *ADM*, *HP*, *DSC2*, *ANXA3*, *LILRA5*, *PLSCR1*, *AKAP12*, *VNN2*, *TCN1*, *HDC* and *NFIL3*.

Altogether 62 genes were up- and 129 were down-regulated in patients with increased IMT compared to those with normal values. The up-regulated genes were *G0S2*, *NRGN*, *ITGA2B*, *C3*, *FLNA*, *IRF5*, *ABCC3*, *CAPNS1*, *IL2RG*, *CCL4L1*, *ACTN1*, *HLAB*, *HLAC*,

TNFAIP3 and *MYO1G* among others. Down-regulated genes were including *PPP1CB*, *HLADRB4*, *IFNGR1*, *LRRN3*, *CCR2*, *CD46*, *IFI44L*, *IFIT1*, *TLR10*, *CD164*, *IFIT2*, *SMAD4* and *SGPP1*.

Altogether 32 genes showed differential expression between patients with increased PWV in comparison with patients with normal values. Most of these changes were modest, only two genes showed ≥ 2 fold difference: *HLAB/HLAC* was up- and *LRRN3* was down-regulated.

4.2.2 Association between clinical and vascular response following anti-TNF therapy

In Study 2/2, 13 patients proved to be cR and 6 to be cNR. Ten patients were FMD-20% responders, 9 were IMT-20% responders and 8 were PWV-20% responders. Eight patients achieved ≥20% improvement in at least 2 out of the 3 vascular parameters, among them 5 had response in all three.

We also compared the clinical and vascular responder status of the treated patients: IMT response had a consequent tendency of correlation with clinical response (R=0.418, p=0.075). Eight FMD, 8 IMT and 6 PWV responders, as well as 7 patients achieving GVR status were also cRs. Contrarily, 4 FMD, 5 IMT, 4 PWV and 5 global vascular NRs proved to be cNRs, respectively.

4.2.3 Baseline gene expression differs between clinical and vascular responders versus non-responders

The baseline gene expression study revealed that only 5 genes (*HLADRB4*, *TMEM176A*, *TMEM176B*, *IFI44* and *PLSCR1*) were significantly (≥2-fold) up-regulated in cR versus cNR patients ($p<0.05$). There were no significantly down-regulated genes.

FMD-20% responder status was associated with significant (≥2-fold) up-regulation of a single gene (*NEFL*) and down-regulation of two genes (*JUN* and *GYPB*) ($p<0.05$). IMT-20% responder status showed association with the up-regulation of 18 (e.g. various immunoglobulin and HLA genes, *TNFRSF17*, *CD74*, *FCRL5*, *CD79A*, *IFITM3*) and down-regulation of 12 genes (e.g. *CXCL5*, *ITGB3*, *NEFL*). PWV-20% responder status was associated with up-regulation of 3 genes (*IFNG*, *JUN* and *CCL4L1/L2*) and down-regulation of 5 genes (*HLAC*,

GNB4, *NRG1*, *NEFL* and *FKBP5*). GVR-20% responder status showed association with the up-regulation of 11 genes (e.g. various immunoglobulin genes, *SCN3A*, *CD79A* and *FCRL5*) and down-regulation of two genes (*NEFL* and *CES1/CES1P1*).

4.2.4 Network analysis of the differentially expressed genes

Because IMT responder status was associated with the differential expression of reasonably high number of genes, we decided to perform GO analysis on IMT-, but not on FMD- or PWV-associated genes. The aim of GO analysis was to determine functional categories of those differentially expressed genes, which showed minimum 2-fold change up- or down-regulation. The overrepresented functional categories of the differentially expressed IMT-associated genes included the positive regulation of immune effector process, Golgi vesicle budding and the regulation of glucose transport among others.

4.3 Study 3

4.3.1 Disease-associated expression changes of a subset of chromatin-modifying enzymes in arthritic mice and RA patients

To identify those chromatin-modifying factors that are involved in the pathogenesis of arthritis we analysed the expression of 84 genes that encode key enzymes known to be specific chromatin modifiers. PCR arrays were used to detect changes in gene expression. RNA samples originated from spleen cells of PGIA and control mice. An analogous PCR array was used to investigate the expression of similar human genes, using RNA samples purified from PBMCs of DMARD-naive RA patients.

In this study, 2.58- to 4.44-fold increases were detected in the expression of 6 genes and 9 showed 2.15- to 4.60-fold downregulation in mice with PGIA. Among the aforementioned genes Aurora kinase A and B were chosen for further analyses because these encode the best characterized histone-modifying enzymes out of the genes identified in our study. Both of them exhibited ≥2-fold upregulation in all of the tested subjects. Upregulation of 9 genes were detected in RA patients and 4 of these (*AURKA*, *AURKB*, *ESCO2* and *NEK6*) also showed increased expression in mice with acute arthritis.

Data were verified by qRT-PCR that showed 2.8- to 4.2-fold upregulation of both Aurora kinases ($P<0.01$) and 1.5- to 3.5-fold upregulation of the other genes. Five further genes showed significant suppression in RA patients compared to healthy individuals and 2 of them (*HDAC1* and *SETDB2*) were also found to be regulated similarly in mice with PGIA.

When studying the expression of the two Aurora kinases in patients treated with low-dose MTX we found that both were significantly downregulated following treatment.

To explore which cell type(s) displays modified Aurora kinase expression in arthritis, we separated T and B lymphocytes from the spleen cells of PGIA mice and found lower expression levels in T cells. Hence we concluded that B lymphocytes might be the main mononuclear cell type that expresses high levels of the Aurora kinases in arthritis. Additional experiments showed significant upregulation of both enzymes in the bone marrow but not in the LNs of PGIA mice.

4.3.2 Phosphorylation of histone H3 by the Aurora kinases in arthritis

The phosphorylation of histone H3 causes chromatin condensation and subsequent mitosis which may lead to the elevated B cell activity in arthritic mice. We performed a Western blot analysis using purified cell lysates from mouse B lymphocytes and kinase-specific antibodies in order to investigate whether the increased expression of the Aurora kinases resulted in elevated protein levels; and found that kinase-specific signals were strong in arthritic mice, however almost undetectable in the control group. The fact that Aurora kinase A and B selectively interact with and phosphorylate histone H3 on serine 10 benefited us in assessing enzymatic activity. We found that the phosphorylation level of serine 10 was at least 7 times higher in arthritic mice compared to control mice.

4.3.3 Elevated expression of Aurora kinases is characteristic of the onset of arthritis and is associated with the acute phase of polyarthritis in animal models of RA

After our previous experiments it was still unknown whether these changes in the expression of Aurora kinases were specific for arthritis-associated lymphocyte activation. The BALB/c mouse strain is highly susceptible to PGIA and resistant to CIA (however immunization with CII can provoke an immune reaction in these mice); while the DBA/1J mouse strain is susceptible to CIA and resistant to PGIA. After immunisation of BALB/c mice

with human PG or CII, expression levels of Aurora kinase A and B were measured using qRT-PCR. Although we found moderately increased expression rate of both Aurora kinases following the first injection of CII, subsequent immunizations did not change the expression levels, which successively returned to baseline levels by week seven. The expression pattern was notably different in response to PG immunization which provoked a gradual increase of Aurora kinase expression. The expression levels reached their peak at the onset of arthritis.

The CIA model in DBA/1J mice confirmed this pattern of the Aurora kinase expression. As the mice had increased symptoms of CIA the levels of both Aurora kinase transcripts elevated gradually.

At last, we investigated if PG immunization could stimulate the expression of the Aurora kinases in PGIA- and CIA-resistant DBA/2 mice. Even though a mild increase was detectable, it was markedly lower than in the PG or CII immunized BALB/c mice.

Altogether, a continuous increase was observed in the expression of the Aurora kinases until they reached a peak just before or at the time of the onset of arthritis. However, in the chronic phase of the disease the expression levels declined to baseline.

4.3.4 Preventive and therapeutic treatment of PGIA mice with Aurora kinase inhibitor VX-680

VX-680 is a specific inhibitor of both Aurora kinases, that -by causing polyploidity- finally leads to apoptotic cell death.

We began studying the effects of VX-680 on cultured splenocytes isolated from arthritic BALB/c mice. 300 μM VX-680 induced elevated expression of the apoptosis-promoting transcription factor p53 and the Puma genes, that was associated by decreased splenocyte viability.

We continued with assessing if VX-680 is able to prevent the development of arthritis in PGIA mice. We began to administrate VX-680 6-7 days prior to the expected onset of arthritis and continued the treatment until the end of the experiment. qRT-PCR and histone phosphorylation assays showed that VX-680 treatment reduced both the expression and activity

of the two Aurora kinases. The total B cell count (CD19+) significantly decreased in the treated animals (49.2±1.3% to 38.4±2.8%; P=0.0044).

To evaluate its treatment potential in PGIA, we started to administrate VX-680 (or vehicle) following the first signs of arthritis and continued every other day until the end of the experiment. Swelling of the peripheral joints was significantly reduced in the VX-680-treated animals compared to the control group. The assays demonstrated the downregulation of the Aurora kinase genes in association with reduced phosphorylation of the histone substrates. An elevated number of active caspase 3+ B lymphocytes was detected at the end of the therapeutic treatment (day 11), implying that VX-680 induced apoptotic events in B cells. Overall, the number of apoptotic CD19+ cells increased from 4.66±1.88% to 28.57±3.5% (P=0.00048), and the total B cell number was 30% less in the VX-680-treated animals (47±5.4%) compared to the control group (76.9±2.2%) (P=0.0068). The proportion of intracellular active caspase 3+ B lymphocytes (but not T cells) was elevated in the spleens of VX-680- treated mice. We detected the decrease of B cell count (involving both B1 [IgM high/IgD low] and B2 [IgD high/IgM low] subsets) in the spleens and bone marrow, but not in the LNs. However the proportion of CD19+CD138+ B cells (antibody-secreting plasmablasts and plasma cells) was not reduced.

5 DISCUSSION

5.1 Study 1: Evaluation of RA risk alleles in humans and in correlating animal models

Given the huge importance of animal models in studying the etiopathogenesis and treatment of RA it is easy to understand the existence of so many types of them. Though one has to be aware of the differences. From genetic and clinical aspect PGIA and CIA displays the greatest similarity to RA. PGIA is characterized with RF and ACPA positivity for which is considered to be a model of seropositive RA whereas CIA is a seronegative RA model. For establishing the congenic and subcongenic strains for our experiments we used MHC-matched arthritis-susceptible and arthitis-resistant mice to exclude the dominant effect of MHC.

Detailed examination of *Pgia26/Cia5/mCia21/Eae3* on mChr3 (corresponding to the *PTPN22/CD2* allele on human Chr1) and *Pgia2/Cia2/Cia3* on mChr2 (corresponding to the *TRAF1/C5* allele on human Chr9) identified a few mutated genes, some of these had not been previously associated with arthritis. However not only the genomic identity but also the corresponding functional defects need to be identified to confirm the role of these genes in RA.

We have continued this work with high-throughput sequence analysis keeping in mind that our is only one of the many possible ways for the identification of the RA-related defects of the genome.

5.2 Study 2: Examination of gene expression profiles in association with vascular pathophysiology in RA patients

RA has been associated with accelerated atherosclerosis, increased cardiovascular morbidity and mortality. Both HLA and non-HLA genes have been implicated in susceptibility to RA and atherosclerosis. The role of common genetic factors determining both RA and atherosclerosis has occurred. Although single SNPs - both *HLA-DRB1* and non-HLA alleles - were revealed in the background of RA-driven atherosclerosis in previous studies, no complex genomic studies have been performed in association with cardiovascular pathophysiology in RA. The effect of anti-TNF therapy on vascular function in RA was neither analysed. That's why we conducted a pilot study in which clinical data, vascular pathophysiology and patterns of differentially expressed genes were compared.

We found differentially regulated genes in association with vascular pathophysiology as well as connected with clinical and vascular responses to anti-TNF treatment. Numerous RA-driven atherosclerosis associated SNPs had been described before, identifying genes encoding HLA, pro-inflammatory cytokines and several other molecules related to autoimmunity and inflammation. Our study gave similar results regarding SNPs. However there was no previous complex genomic study or genetic study on biologic-treated patients to compare our results with.

A few genes showed differential expression in patients with abnormal (low) FMD. We found more differentially expressed genes associated with abnormal (high) IMT. A $\geq$ 2-fold change in gene expression was considered significant. 1 gene was significantly up- and 1 was significantly downregulated in patients with abnormal (high) PWV. The differentially expressed genes primarily included MHC-related ones, but there were also cytokine-, adhesion molecule-, integrin- and interferon-related genes among them.

In the second part of study 2 19 RA patients received TNF-inhibitor therapy (either ETN or CZP) for 1 year. Thirteen patients showed clinical response in the end of the year, but only 10 of them had accordance between the clinical and any form of vascular response. Clinical response showed association with the upregulation of only 5 genes. Some genes showed differential expression in IMT responder RA patients, but only a very few genes correlated with FMD, PWV or GVR response to anti-TNF therapy. Here, differential expression was observed principally in immunoglobulin- and HLA-related genes, other affected genes included cytokine-, chemokine- and interferon-related ones.

Two vanin genes (*VNN1* and *VNN2*) displayed differential expression in study 1/2. These molecules have been implicated in vascular pathology and fibrosis underlying certain autoimmune conditions and atherosclerosis, however their role in the pathogenesis of RA is unknown.

Several interferon-related genes (e.g., *IFI44*, *IFIT1*, *IFITM3*, *IRF5*) showed differential expression in both part of the study. Among them *IRF5* genetic variants are known to be associated with cardiovascular complications in RA.

Neurofilament light polypeptide (*NEFL*) gene correlated significantly with FMD, IMT, PWV and GVR responses. Neurofilament proteins are known to be associated with nervous system involvement in SLE, however they are also observable in the synovial cytoskeleton in RA.

The expression of leucine-rich repeat neuronal 3 (*LRRN3*) gene was different in RA patients with abnormal IMT and PWV. Leucine-rich repeat kinases (*LRRK*) have a role in aging, RA and other arthritides, along with neuroinflammation.

We observed the differential expression of the proto-oncogene encoding *JUN* gene in association with FMD-20% and PWV-20% responses. The association between Jun, c-Jun N-terminal kinase (*JNK*) and the pathogenesis of RA and atherosclerosis has been known for a long time.

The abovementioned genes are just a few examples to demonstrate the role of the differentially expressed genes in the pathogenesis of RA and other autoimmune diseases.

5.3 Study 3: Studying the expression of epigenome modifiers, including Aurora kinase A and B, in immune cells of arthritic mice and RA patients

Most of the epigenetic studies in RA were performed on synovial fibroblasts finding that DNA hypomethylation and elevated expression of certain histone modifiers characterizes these activated cells. Pro-inflammatory cytokine production is increased by these histone modifiers, stimulating the immune cell ingress into the synovium. However we studied arthritis-specific expression changes in mononuclear cells isolated from arthritic animals and RA patients, finding 14 genes that were significantly up- or downregulated in all of the investigated RA patients.

We - for the first time - identified all of the major chromatin-modifying enzyme families that can be involved in autoimmune arthritis. These include histone kinases (*AURKA, AURKB* and *NEK6*), acetyltransferases (*ESCO2* and *KAT2B*), deacetylases (*HDAC1* and *HDAC11*), methyltransferases (*SETD6, SETDB2* and *PRMT6*), demethylases (*KDM5C* and *KDM6B*) and protein degradation-promoting ubiquitin ligases (*DZIP3* and *UBE2B*). A lot of these genes have enzyme products with such activating or repressing function that can modulate the activity of the NF-κB transcription factor. As RA is known to be controlled by several NF-κB-regulated pro-inflammatory cytokines these findings suggest an obvious link to arthritis. For example H.H3-P is known to be an important epigenetic signal for the recruitment of NF-κB to the promoters of a number of cytokine genes. *SETD6*, which is a known negative regulator of NF-κB signaling is downregulated in arthritis. The relevance of SETD6 in the pathogenesis of arthritis is supported by the fact that it can interact with NEK6 and the Aurora kinases, enzymes

identified by us as arthritis-promoting genes. Other genes, like *HDAC11* and *KDM6B* can affect inflammatory pathways differently. For example the overexpression of *HDAC11* suppresses the anti-inflammatory cytokine IL-10 enhancing antigen presentation, therefore has considerable contibution to autoimmunity. *KDM6B* eliminates strong repressive epigenetic transcription signals (i.e. histone H3 K27 tri-methylation), its overexpression has been implicated in macrophage activation.

Though all of the aforementioned enzymes are principally considered to be histone modifiers, they may target other intracellular proteins according to a number of studies. For example, NEK6 can phosphorylate the STAT3 transcription factor that is known to have a role in the induction of the IL-6 cytokine family. Therefore, the identification of target genes and the exploration of the complex regulatory network among these enzymes is absolutely necessary for the complete understanding of the epigenetic components of arthritis and our work took the first steps in this direction. We demonstrated – by using different mouse models of RA - that the increased expression of the Aurora kinases is not part of the physiological lymphocyte activation and differentiation, instead it is specific to arthritis. Although Aurora kinase expression was increased in both mouse T cells and B cells, we detected approximately double level in B cells, suggesting that pathologically high Aurora kinase expression is most prominent in the B cells. The Aurora kinases cause increased B cell activity in arthritic mice by the phosphorylation of histone H3. Aurora kinase A and B expression levels progressively increased prior to and reached a plateau at the onset of arthritis. Because of their involvement in the regulation of certain NF-κB controlled genes, it is reasonable to hypothesize that Aurora kinases may play important role in the pathogenesis of arthritis.

Preventive treatment with specific Aurora kinase inhibitor VX-680 delayed the onset, the therapeutic application significantly reduced the severity of arthritis. We could prove that VX-680 treatment promotes B cell elimination by inducing B cell apoptosis. Significant decrease in the autoantibody levels was not observed in association with B cell apoptosis, probably because autoantibodies have long half-life in the circulation. However, in order to prove this hypothesis a much longer treatment period or the adaptive transfer of VX-680-treated B cells to antibody secretion-deficient mice would be necessary, which is way beyond the scope of our current study. The effectivity of anti-CD20 treatment in anti-TNF therapy resistant RA emphasises the significance of our results.

Although a 2-fold upregulation of the Aurora kinases was detected in mouse T cells, VX-680 treatment did not lead to significant T cell apoptosis. There may be additional anti-apoptotic factors that reverse the effect of VX-680 in activated T cells.

High expression of Aurora kinases was observed in the mononuclear cells of untreated RA patients, which decreased following MTX treatment. The fact that both low-dose MTX and VX-680 reduces Aurora kinase expression suggests that these kinases can be promising target of pharmacological intervention.

6 SUMMARY

The main characteristic of RA is chronic synovitis that may lead to joint destruction, thus pain, disability and deterioration of life quality. Moreover RA is associated with accelerated atherosclerosis, the primary cause of increased mortality in RA. Genetics, environment and autoimmunity is involved in the pathogenesis of RA; there is also growing evidence about the contribution of epigenetic factors. Although this is a thoroughly studied and very important field of science, many aspects of RA etiopathogenesis are yet to be clarified. The prevalence and the burden of the disease make a highly effective treatment necessary and there is still a huge demand of new therapeutic possibilities.

This thesis consists of 3 studies investigating the background and the treatment of rheumatoid arthritis. We evaluated RA risk alleles in humans and in corresponding animal models (Study 1). We performed gene expression analysis of vascular pathophysiology in RA patients (Study 2). We investigated arthritis-associated epigenetic factors and the therapeutic opportunities in their targeted inhibition (Study 3).

Our observations are summarized below:

Study 1

Detailed examination of *Pgia26* locus on mChr3 (corresponding to the *PTPN22/CD2* allele on human Chr1) and *Pgia2* locus on mChr2 (corresponding to the *TRAF1/C5* allele on human Chr9) revealed a few RA risk alleles, some of these had not been formerly associated with arthritis.

Study 2

We performed the first complex genomic study regarding RA-associated cardiovascular pathophysiology, along with clinical and vascular response to anti-TNF treatment. The differentially expressed genes we found in association with vascular pathophysiology primarily included MHC-related ones, but there were also cytokine-, adhesion molecule-, integrin- and interferon-related genes among them. With regard to the clinical and vascular response to anti-

TNF therapy, the differential expression was observed principally in immunoglobulin- and HLA-related genes, among the other affected genes there were cytokine-, chemokine- and interferon-related ones.

Study 3

We were the first to identify all major chromatin-modifying enzyme families that can be involved in autoimmune arthritis. Aurora kinase A and B were chosen for further analyses because these encode the best characterized histone-modifying enzymes out of them. The increased expression of the Aurora kinases proved to be arthritis specific. Preventive treatment with VX-680 delayed the onset, while therapeutic application significantly diminished the severity of arthritis, suggesting that it can be a promising new direction of antirheumatic drug development.

7 NEW FINDINGS, RESULTS AND CLINICAL RELEVANCE

This thesis consists of 3 studies investigating the etiopathogenesis and a promising treatment possibility of RA.

The clinical relevance and our new findings are listed below:

Study 1

We established congenic and subcongenic strains that allowed the detailed examination of *Pgia26* locus on mChr3 (corresponding to the *PTPN22/CD2* allele on human Chr1) and *Pgia2* locus on mChr2 (corresponding to the *TRAF1/C5* allele on human Chr9). This has led to the identification of a few gene mutations whose association with arthritis was previously unknown. Further investigation of animal models may provide an opportunity to functionally investigate these newly identified gene mutations, which may lead to a better understanding of the human disease process.

Study 2

We performed the first complex genomic study regarding cardiovascular pathophysiology, along with clinical and vascular response to anti-TNF treatment in RA. Our study confirmed a different expression pattern associated with vascular pathology in the case of MHC, cytokine, adhesion molecule, integrin and interferon genes. Altered expression of immunoglobulin, HLA, cytokine, chemokine, and interferon genes was observed in association with the response to TNF inhibitors. Continued studies may provide a better understanding of the background of RA-associated atherosclerosis and the mechanism of effect of TNF inhibitors

Study 3

- We - for the first time - identified all the major chromatin-modifying enzyme families that can be involved in autoimmune arthritis. Most of these genes had been studied in connection with carcinogenesis, but their significance in the pathogenesis of arthritis is novel and had not been investigated before.

- We proved that Aurora kinase inhibitor VX-680 led to more favorable outcome in PGIA, therefore it can be a promising new candidate in the rheumatological drug development.

8 LIST OF ABBREVIATIONS

ACPA	anticyclic citrullinated peptide antibody
ACR	American College of Rheumatology
Aurka	Aurora kinase A
Aurkb	Aurora kinase B
CIA	collagen-induced arthritis
CII	human type II collagen
cNR	clinical non-response/responder
cR	clinical response/ responder
csDMARD	conventional synthetic disease-modifying antirheumatic drug
CZP	certolizumab pegol
DAS28	Disease Activity Score 28
DDA	dimethyldioctadecyl-ammonium bromide
DMEM	Dulbecco's Modifed of Eagle's Medium
DMSO	dimethyl sulfoxide
ETN	etanercept
EULAR	European Alliance of Associations for Rheumatology
FBS	fetal bovine serum
FMD	flow-mediated vasodilation
FSC	forward scatter
GEO	Gene Expression Omnibus
GO	Gene Ontology
GVR	good vascular response/responder
GWAS	genome-wide association study
H.H3	histone H3
HLA	human leukocyte antigen
Ig	immunoglobulin
IMT	intima-media thickness
IVSC	interval-specific congenic
mAb	monoclonal antibody
MHC-II	major histocompatibility complex-II
MTX	methotrexate
NCI	National Cancer Institute

NMD	nitroglycerine-mediated vasodilation
NSAID	non-steroidal anti-inflammatory drug
PAGE	SDS-polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PG	proteoglycan
PGIA	cartilage proteoglycan aggrecan-induced arthritis
PTPN22	protein tyrosine phosphatase non-receptor type 22
PWV	pulse wave velocity
RA	rheumatoid arthritis
RF	rheumatoid factor
qRT-PCR	quantitative real-time polymerase chain reaction
QTL	quantitative trait locus
SE	shared epitope
SEM	standard error of the mean
SF	synovial fluid
SNP	single nucleotide polymorphism
SSC	side scatter
SSLP	single sequence length polymorphism
TNF-α	tumor necrosis factor-α
TRAF1	TNF receptor associated factor 1
tsDMARD	targeted synthetic disease-modifying antirheumatic drug
vNR	vascular nonresponse/responder
vR	vascular response/responder

9 ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, Professor Zoltán Szekanecz for his continuous support, guidance, encouragement and patience throughout my PhD. His knowlegde and mentality inspired me greatly to become a rheumatologist.

I am honestly grateful to my mentor, Professor Tibor T. Glant for giving me the chance to work in his research team and to learn so much from him.

I would also like to thank Professor Katalin Mikecz for being so supportive during the time I spent in Chicago and ever since then.

I would like to express my warm gratitude to Tibor A. Rauch PhD for his support and friendship.

The work presented in this thesis would not have been possible without my co-authors and my former colleagues from University of Debrecen and Rush University Medical Center, Chicago, IL; I would like to express my sincere thankfulness to all of them.

I owe a debt of gratitude to all the patients who participated in the studies.

I really appreciate the sacrifice of the mice of our experiments.

I am forever thankful to my family: my parents for protecting and encouraging me at the same time and for setting me good example; my sister for always being there for me; my husband who was also my fellow researcher; my little son for all his love which helps me through everything.

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/241/2021.PL
Subject: PhD Publication List

Candidate: Tímea Besenyei
Doctoral School: Doctoral School of Clinical Medicine
MTMT ID: 10047984

List of publications related to the dissertation

1. Póliska, S., **Besenyei, T.**, Végh, E., Hamar, A. B., Pusztai, A., Váncsa, A., Bodnár, N., Szamosi, S., Csumita, M., Kerekes, G., Szabó, Z., Nagy, Z., Szűcs, G., Szántó, S., Zahuczky, G., Nagy, L., Szekanecz, Z.: Gene expression analysis of vascular pathophysiology related to anti-TNF treatment in rheumatoid arthritis.
   Arthritis Res. Ther. 21 (1), 94, 2019.
   IF: 4.103

2. Glant, T. T., **Besenyei, T.**, Kádár, A., Kurkó, J. E., Tryniszewska, B., Gál, J., Soós, G., Szekanecz, Z., Hoffmann, G., Block, J. A., Katz, R. S., Mikecz, K., Rauch, T. A.: Differentially expressed epigenome modifiers, including aurora kinases A and B, in immune cells in rheumatoid arthritis in humans and mouse models.
   Arthritis Rheum. 65 (7), 1725-1735, 2013.
   DOI: http://dx.doi.org/10.1002/art.37986
   IF: 7.871

3. **Besenyei, T.**, Kádár, A., Tryniszewska, B., Kurkó, J. E., Rauch, T. A., Glant, T. T., Mikecz, K., Szekanecz, Z.: Non-MHC Risk Alleles in Rheumatoid Arthritis and in the Syntenic Chromosome Regions of Corresponding Animal Models.
   Clin. Dev. Immunol. 2012, 1-14, 2012.
   DOI: http://dx.doi.org/10.1155/2012/284751
   IF: 3.064

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

Szilárd Póliska and Tímea Besenyei are shared first authors of publication nr. 1.

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

4. Soós, B., Kurkó, J. E., **Besenyei, T.**, Szabó, Z., Szántó, S., Meskó, B., Póliska, S., Nagy, L., Laki, J., Glant, T., Mikecz, K., Szekanecz, Z.: A rheumatoid arthritis genetikája és genomikája: I. patogenetikai vonatkozások.
Magyar Reumatol. 54 (1), 7-17, 2013.

5. Kurkó, J. E., **Besenyei, T.**, Laki, J., Glant, T. T., Mikecz, K., Szekanecz, Z.: Genetics of Rheumatoid Arthritis: a Comprehensive Review.
Clin. Rev. Allergy Immunol. 45 (2), 170-179, 2013.
DOI: http://dx.doi.org/10.1007/s12016-012-8346-7
IF: 4.728

6. Szekanecz, Z., Meskó, B., Póliska, S., Váncsa, A., Szamosi, S., Végh, E., Simkovics, E., Laki, J., Kurkó, J. E., **Besenyei, T.**, Mikecz, K., Glant, T. T., Nagy, L.: Pharmacogenetics and pharmacogenomics in rheumatology.
Immunol. Res. 56 (2-3), 325-333, 2013.
DOI: http://dx.doi.org/10.1007/s12026-013-8405-z
IF: 3.525

7. Glant, T. T., Adarichev, V. A., Boldizsár, F., **Besenyei, T.**, László, A., Mikecz, K., Rauch, T. A.: Disease-promoting and -protective genomic loci on mouse chromosomes 3 and 19 control the incidence and severity of autoimmune arthritis.
Genes Immun. 13 (4), 336-345, 2012.
IF: 3.675

8. Egelston, C., Kurkó, J. E., **Besenyei, T.**, Tryniszewska, B., Rauch, T. A., Glant, T. T., Mikecz, K.: Suppression of dendritic cell maturation and T cell proliferation by synovial fluid myeloid cells from mice with autoimmune arthritis.
Arthritis Rheum. 64 (10), 3179-3188, 2012.
DOI: http://dx.doi.org/10.1002/art.34494
IF: 7.477

9. Szekanecz, Z., **Besenyei, T.**, Paragh, G., Koch, A. E.: Actualités sur l'angiogenése synoviale.
Revue du Rhumatisme. 77 (1), 14-20, 2010.
DOI: http://dx.doi.org/10.1016/j.rhum.2009.11.014

10. Szekanecz, Z., **Besenyei, T.**, Szentpétery, Á., Koch, A. E.: Angiogenesis and vasculogenesis in rheumatoid arthritis.
Curr. Opin. Rheumatol. 22 (3), 299-306, 2010.
DOI: http://dx.doi.org/10.1097/BOR.0b013e328337c95a
IF: 4.497

11. Szekanecz, Z., **Besenyei, T.**, Paragh, G., Koch, A. E.: New insights in synovial angiogenesis.
Joint Bone Spine. 77 (1), 13-19, 2010.
DOI: http://dx.doi.org/10.1016/j.jbspin.2009.05.011
IF: 2.46

12. Szekanecz, Z., **Besenyei, T.**, Paragh, G., Koch, A. E.: Angiogenesis in rheumatoid arthritis.
Autoimmunity. 42 (7), 563-573, 2009.
DOI: http://dx.doi.org/10.1080/08916930903143083
IF: 2.813

13. Szekanecz, Z., Pákozdi, A., Szentpétery, Á., **Besenyei, T.**, Koch, A. E.: Chemokines and angiogenesis in rheumatoid arthritis.
Front. Biosci. (Elite Ed). 1, 44-51, 2009.

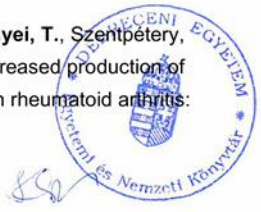
14. Pákozdi, A., **Besenyei, T.**, Paragh, G., Koch, A. E., Szekanecz, Z.: Endothelial progenitor cells in arthritis-associated vasculogenesis and atherosclerosis.
Joint Bone Spine. 76 (6), 581-583, 2009.
DOI: http://dx.doi.org/10.1016/j.jbspin.2009.04.007
IF: 2.25

15. **Besenyei, T.**, Pákozdi, A., Végvári, A., Szabó, Z., Szekanecz, Z.: Az endothelsejtek, a leukocytamigráció, a kemokinek és az angiogenezis jelentősége a gyulladásos reumatológiai kórképekben.
Magyar Immunol. 7 (1-2), 4-21, 2008.

16. Szekanecz, Z., Kerekes, G., Dér, H., Sándor, Z., Szabó, Z., Végvári, A., Simkovics, E., Soós, L., Szentpétery, Á., **Besenyei, T.**, Szűcs, G., Szántó, S., Tamási, L., Szegedi, G., Shoenfeld, Y., Soltész, P.: Accelerated atherosclerosis in rheumatoid arthritis.
Ann. N. Y. Acad. Sci. 1108, 349-358, 2007.
DOI: http://dx.doi.org/10.1196/annals.1422.036
IF: 1.731

17. Simkovics, E., **Besenyei, T.**, Szabó, Z., Szentpétery, Á., Szodoray, P., Szűcs, G., Szántó, S., Szekanecz, Z.: Első hazai tapasztalat a rheumatoid arthritis rituximabkezelésével: egy polirezisztens beteg esete.
Magyar Immun. 6 (3), 42-47, 2007.

18. Szekanecz, É., Sándor, Z., Antal-Szalmás, P., Soós, L., Lakos, G., **Besenyei, T.**, Szentpétery, Á., Simkovics, E., Szántó, J., Kiss, E., Koch, A. E., Szekanecz, Z.: Increased production of the soluble tumor-associated antigens CA19-9, CA125, and CA15-3 in rheumatoid arthritis: potential adhesion molecules in synovial inflammation?
Ann. N. Y. Acad. Sci. 1108, 359-371, 2007.
DOI: http://dx.doi.org/10.1196/annals.1422.037
IF: 1.731



UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

19. Szántó, S., Szűcs, G., Szamosi, S., **Besenyei, T.**, Szentpétery, Á., Szekanecz, Z.: Rheumatoid arthritises betegek adalimumab kezelésével szerzett kezdeti tapasztalataink. *Magyar Reumatol. 48*, 98-104, 2007.
20. **Besenyei, T.**: A reumatológiában alkalmazott biológiai terápia nemkívánatos következményei és a megelőzés lehetőségei. *Magyar Reumatol. 45* (1), 23-28, 2004.

Total IF of journals (all publications): 49,925
Total IF of journals (publications related to the dissertation): 15,038

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

04 May, 2021

DEBRECENI EGYETEM
Egyetemi és Nemzeti Könyvtár