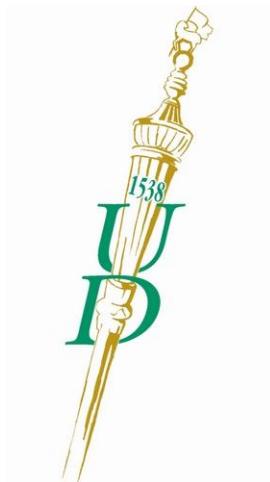


SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Investigations on the role of microRNAs and gene
polymorphisms in the pathogenesis of primary Sjögren's
syndrome**

by Ji-Qing Chen, MD

Supervisor: Margit Zeher, MD, PhD, DSc



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ALLERGOLOGY
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The Examination takes place at the Discussion Room of Building C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 11:00 a.m. on 8th of May, 2017

Members of the Defense Committee: Zsuzsanna Bata, MD, PhD, DSc
Gabriella Szűcs, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1:00 p.m. on 8th of May, 2017

1. Introduction

Autoimmune diseases are characterized by the dysregulation of immune system which results in the breakdown of tolerance to self-antigen. Even though the accurate etiology and pathogenesis of the majority of these diseases are still not clear in details, by now we know that complex elements, including genetic, environmental, hormonal factors may provoke the autoimmune processes leading to the development of the disease. These encourage us to investigate the role of certain genetic factors in details such as specific gene receptor polymorphisms and the expression profile of microRNAs (miRNAs) in primary Sjögren's syndrome (pSS).

2. Background

2.1 Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is a slowly progressive systemic autoimmune inflammatory disease that primarily affects middle-aged women (female to male ratio: 9:1), although it may be found in all ages including childhood. The target organs are primarily exocrine glands, such as salivary and lachrymal glands. Therefore, patients show typically symptoms of dry mouth and dry eyes. Beside the pathognomonic glandular symptoms (GS), other systemic symptoms, denoted as extraglandular manifestations (EGMs) can also develop during the disease course in approximately one third of the patients. These patients frequently complain about easy fatigability, Raynaud's phenomenon, muscle pain and joint pain. The most common EGMs are articular, cutaneous, pulmonary, renal involvement and neuropathy.

The typical histologic feature of minor salivary gland biopsies in pSS is the formation of ectopic germinal centers by infiltration of mononuclear cells, which are predominantly CD4+ T-cells and CD20+ B-cells, plasma cells, macrophages, natural killer and dendritic cells (DC).

The pathogenesis of the disease is still not fully understood, but there is no doubt that it is a multifactorial process in which autoimmune cascades damage the target tissue. Both intrinsic (genetical) and extrinsic (environmental) factors play the critical role in the disease development based on our current knowledge. In genetically susceptible individuals, certain external factors, such as hormonal changes, external environmental factors, viral infections, may trigger the autoreactive processes.

2.2 Vitamin D receptor gene polymorphisms

Vitamin D has lots of essential biological effects, including calcium homeostasis regulation, functional vascular events mainly vasodilation, modification of cell proliferation and differentiation, immunomodulatory and anti-inflammatory effects. The active form of vitamin D has various modulatory effects on monocytes and macrophages, dendritic cells, as well as T and B cells. It mainly decreases T helper (Th) cell response and enhances function of T regulator cells. Also it helps to increase proliferation of monocytes and macrophages but decrease proliferation of T and B cells. The physiological effects are achieved by binding to a member of the nuclear receptor superfamily which is called the vitamin D receptor (VDR). Only VDR isoform has been isolated in contrast to the other members of the subfamily which includes the retinoid receptors, the thyroid hormone receptors and the peroxisome proliferator activated receptors. The target DNA sequences binds to VDR as heterodimer with the retinoid X receptor, resulting in the induction of target gene expression by a series of co-activators that modify the basal transcriptional apparatus.

VDR gene is located on chromosome 12 (12q12-q14), and till now, more than 470 single nucleotide polymorphisms (SNPs) were reported. Some of SNPs play a key role in the modification of the uptake of 1,25-(OH)₂-vitamin D₃. Therefore, it has been considered that these SNPs could be a risk factor for the development of autoimmune diseases. Although VDR gene has more than 100 restriction endonuclease cutting sites, 4 of them are known polymorphisms, FokI and the other 3 are in linkage disequilibrium

like BsmI, ApaI and TaqI. The FokI and TaqI SNPs are located in exon 2 and exon 9. The other two BsmI and ApaI are located in intron 8 and intron 9, respectively. There is no significant difference in vitamin D levels between pSS patients and normal controls. On the contrary, many studies reported about the association between VDR gene polymorphisms and higher risks of certain autoimmune disease such as SLE, RA, MS, psoriasis, Crohn's disease, Behcet's disease and other autoimmune diseases. But so far, no study has evaluated the association between VDR gene polymorphisms and pSS. Therefore, we determined whether these VDR gene polymorphisms (BsmI, TaqI, ApaI and FokI SNPs) are genetic risk factors of pSS in Hungarian population or not. Since pSS patients have two main groups of symptoms which are denoted as GS and EGMs, we studied the genotype frequencies in both patients group.

2.3 MicroRNA

The discovery of microRNAs (miRNAs) and their critical role in genetic control opened new avenues in understanding of the intricate interplay of inherited and acquired factors leading to disease development. MiRNAs are single-stranded, endogenous non-coding RNAs, ranging from 18 to 25 nucleotides in length. After the first study revealing the posttranscriptional regulatory role of non-coding RNAs on gene expression in *Caenorhabditis elegans*, small RNAs with similar functions were identified in other animal models, such as *Drosophila* and zebrafish. Since then, several studies on miRNAs were reported in both animal models and humans, which shed light on their biology and mechanism of action. It is already known that mature miRNAs interact with specific messenger (m)RNAs to regulate gene expression. Target mRNA is recognized by the 2–7 nucleotides of the so called ‘seed’ region of miRNAs. When the complementary base pairing is perfect or near-perfect, endonucleotic cleavage is induced, which leads to the degradation of mRNAs. When the base pairing is incomplete, the formation of double-stranded RNAs resulting from the binding of miRNAs, leads to translational repression. MiRNAs regulate approximately 90% of

protein-coding genes, and play a central role in various biological processes including immune cell lineage commitment, differentiation, proliferation, apoptosis and maintenance of immune homeostasis. Alterations in miRNA regulation seem to be highly related to the development of immune dysfunctions and autoimmunity. In the last years, changes in miRNA expression have been identified in certain autoimmune diseases including RA, SLE and pSS, as well. Consequently, these molecules may be regarded as novel and attractive biomarkers specific for different autoimmune disorders; moreover, it has been also suggested that miRNA-targeting treatment might be more selective than the other therapeutic regiments in autoimmune diseases. However, it has to be taken into consideration that a given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNAs, thus, the intricate interplay between specific miRNAs and the functionally targeted genes is not elucidated yet. Additionally, genome-wide surveys identified many SNPs in the predicted miRNA target sites, as well as in miRNAs themselves. In some instances, SNPs have been shown to alter miRNA function, thus possibly contributing to disease development. The better understanding of the immune regulatory mechanisms of miRNAs by pathway-based exploratory analyses and the mapping and characterization of miRNA SNPs may help not only to elucidate the pathogenesis of autoimmune conditions but also can lead to the development of complex therapeutic approaches in patients with immunological disorders.

3. Aims of the study

To discover the pathogenesis of pSS on the field of gene polymorphisms and miRNAs expression profile, we have formulated the following study goals:

- To investigate the frequency of BsmI (*rs1544410*), FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) polymorphism genotypes in pSS patients and to determine whether VDR gene polymorphisms are associated with Sjögren's

syndrome;

- To detect the expression levels of miR-155 and its target gene SOCS1 in pSS patients;
- To examine miRNA expression profiles in pSS patients in order to remark the associations between the expression of dysregulated miRNAs and the development of disease.

4. Patients and methods

4.1 Study on vitamin D receptors gene polymorphisms

4.1.1 Patients

We have enrolled 105 pSS patients (53 with GS and 52 with EGMs; mean age 59.4 years, range from 26 to 82 years old, 100 females and 5 males) in the study, recruited from the Outpatient Clinic for systemic autoimmune diseases at the Division of Clinical Immunology, University of Debrecen. The diagnosis of pSS was established according to the European-American consensus criteria. We recruited 93 healthy individuals (mean age 41.2 years, range from 14 to 70 years; 52 females and 41 males) taking no immunosuppressive or immunomodulating medications to serve as controls. Informed written consent was obtained from the subjects, and the study has been approved by the ethics committee of the University of Debrecen. All experiments carried out were in compliance with the Declaration of Helsinki.

Among all 105 patients with pSS, 52 had EGMs, while 53 had only GS. The distributions of EGMs of patients were as following: polyarthritis n=44, Raynaud's phenomenon n=18, vasculitis n=5, polyneuropathy n=5, thyroiditis n=5, myositis n=4, pulmonary fibrosis n=3, primary biliary cirrhosis n=2, renal tubular acidosis n=1, pericarditis n=1, anti-phospholipid syndrome n=1, neck lymphadenopathy n=1, and marginal zone B cell lymphoma n=1.

4.1.2 Genomic DNA extraction

High molecular weight DNA for genotyping was extracted from peripheral blood, which was collected in EDTA Vacutainers. Genomic DNS was extracted according to the manufacturer's recommendation using a QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany). DNA was quantitated by UV absorption at 260 and 280 nm and stored at -20 °C until analyzed.

4.1.3 Genotyping of BsmI polymorphism

The identification of BsmI (*rs1544410*) polymorphism was performed through the allele-specific quantitative real-time polymerase chain reaction (qPCR) using Taqman® SNP Genotyping assays (Applied Biosystems, Foster City, CA, USA) with Corbett Rotor-Gene 3000 thermo cycler. The Taqman® SNP Genotyping assay, which contains the predesigned mixture of unlabeled PCR primers and the Taqman® Minor Groove Binding group probes (FAM™ and VIC® dye-labelled), using the same thermal conditions to work together with Taqman® Universal PCR Master Mix which consists of AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTPs and optimized buffer components. The BsmI G/A polymorphism genotype determination was achieved through allele-specific labelling which were FAM™ labelled probe for allele A detection and VIC® labelled probe for allele G detection. The multiplex reactions give 2 alleles' information at one time by shown different amplification pattern.

4.1.4 Genotyping of FokI , ApaI and TaqI polymorphisms

The genotype for FokI, ApaI and TaqI polymorphisms of VDR gene was determined by the digestion pattern of the amplified DNA fragment using polymerase chain

reaction followed by restriction fragment length polymorphisms. All the products of hydrolysis were separated on 3 % agarose gel and visualized with UV light.

FokI (rs10735810)

PCR amplified a selected fragment of 265bp by using specific primers, each primer as following: 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3' (F) and 5'-ATG GAA ACA CCT TGC TTC TCC TCC CTC-3' (R) (BioBasic Canada Inc.). The FokI restriction enzyme recognizes the sequence of 5'...GGATG(N)...3' and cut it if T nucleotide is present and reveals the C/T substitution in exon 2 of VDR gene.

ApaI (rs7975232)&TaqI (rs731236)

PCR amplified a selected fragment of 740bp by each of the following primer pairs: 5'-CGG TCA GCA GTC ATA GAGG-3' (F) and 5'-CAG TGT GTT GGA CAG GCG-3' (R) (BioBasic Canada Inc.).

The ApaI restriction enzyme recognizes the sequence of 5'...GGGCCC...3' and reveals the C/A substitution in intron 9 of VDR gene.

TaqI restriction endonucleases enzyme recognizes the sequence of 5'...TCGA...3' and reveals the T/C substitution in exon 9 of VDR gene.

4.1.5 Statistical Analysis

Genotype frequencies were calculated by direct counting. Allele frequencies were calculated from genotype frequencies. For comparisons of mean values between patients and controls, statistical analysis was performed by the independent samples t test. Differences in genotypic and allelic distribution of VDR polymorphisms between patients and controls were determined by Pearson chi-square (χ^2) test using SPSS 20.0 statistical software. The P value less than 0.05 was regarded as statistically significant. Haplotype analysis was done by CHAPLIN 1.2 software. The possible haplotypes including genetic variants of three VDR polymorphisms (BsmI, ApaI, and TaqI) and four polymorphisms studied (FokI, BsmI, ApaI, and TaqI). Pairwise linkage disequilibrium (LD) between the VDR gene polymorphisms was computed, and LD

plots were constructed by Haplovview software version 4.2.

4.2 Study on miR-155 and its target gene SOCS1

4.2.1 Patients

Twenty-three patients with pSS (1 man, 22 women; mean age: 62.5 ± 12.4 years) were enrolled in the present study, recruited from Outpatient Clinic for systemic autoimmune diseases at the Division of Clinical Immunology, University of Debrecen. The diagnosis of pSS was established according to the European–American consensus criteria. Among patients, 17 showed EGMs, while 6 had only glandular symptoms. The distribution of EGMs of pSS patients were as follows: polyarthritis n=10, Raynaud's phenomenon n=10, thyroiditis: n=3, vasculitis n=2, lymphadenopathy n=1 and myositis n=1. Among patients, we found 16 patients were positive for anti-Ro/SSA autoantibody, while 5 of them were positive for both anti-Ro/SSA and anti-La/SSB autoantibodies. Labial salivary gland biopsy was taken only from the remaining 7 patients in order to meet diagnostic criteria; the biopsies showed focal lymphocytic sialadenitis. Ten age- and sex-matched healthy individuals (1 man, 9 women; mean age: 59.8 ± 9.7 years) served as controls. None of the patients or controls received any immunosuppressive or immunomodulating medications, or had ongoing infections during the study period. All patients and healthy individuals were positive for EBV specific serum immunoglobulin G (IgG). Informed written consent was obtained from the subjects, and the study has been approved by the ethics committee of the University of Debrecen. All experiments carried out were in compliance with the Declaration of Helsinki.

4.2.2 Sample handling

Peripheral blood samples obtained from each study subjects were collected and PBMCs

were separated by Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO, USA) density-gradient centrifugation.

4.2.3 miRNA and total RNA isolation, complementary DNA synthesis

MiRNA and total RNA were extracted from PBMCs using mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. RNA samples were quantitated by UV absorption at 260 nm and 280 nm. Reverse transcription (RT) reactions were carried out using TaqMan MicroRNA RT Kit (4366596, Applied Biosystems, Foster City, CA, USA) to convert miRNA to complementary DNA (cDNA). 50 ng of miRNAs were reverse transcribed in a total reaction volume of 15 µl containing 50 nmol/L specific stem-loop RT primer (RT 002287, Applied Biosystems), 0.25 mmol/L each of dNTPs, 50 U MultiScribe reverse transcriptase, 1x RT buffer, and 1U ribonuclease (RNase) inhibitor.

Total RNA sample of 1.0 µg was reverse transcribed using TaqMan Reverse Transcription Reagents (N808-0234, Applied Biosystems, USA) in a total reaction volume of 25 µl. The cDNA samples were stored at -20 °C until used.

4.2.4 RT-PCR quantification of miR-155

qRT-PCR analysis of miR-155 expression was carried out using TaqMan MicroRNA Assay kit according to the manufacturer's protocol (Applied Biosystems). The 20 µl PCR reaction included 1.33 µl RT product, 1× TaqMan Universal PCR master mix and 1 µl primers and probe mix of the TaqMan MicroRNA Assay kit (TM 002287). RNU48 miRNA was used as an internal control to normalize miRNA input. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method, presented as the fold-expression change in patient samples relative to their corresponding control

subject after normalization to the endogenous control. All experiments were carried out in triplicate. Relative quantification determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibration sample.

4.2.5 Relative quantification of gene expression for SOCS1

The target gene for real-time analyses was SOCS1. Pre-designed and labeled primer/probe sets (hs00705164) were selected from the Applied Biosystem's Assay-on-Demand product line (<http://appliedbiosystems.com>). Human β -actin, ACTB (4326315E), was used as endogenous control in the experiments.

Real-time PCR was performed in Corbett Rotor-Gene RG-3000 equipment. The PCR reaction was carried out in a 20 μ l reaction volume containing TaqMan Universal Master Mix (2x, 4331182, Applied Biosystems, USA). The PCR samples were placed in a Corbett Rotor-Gene 3000 equipment, which was set to detect FAM reporter dye simultaneously. Reactions were set up in duplicate. The C_t value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method, presented as the fold-expression change in patient samples relative to their corresponding control subject after normalization to the endogenous control.

4.2.6 Statistical analysis

The SPSS ver. 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. To assess the distribution of the data Kolgomorov-Smirnov test was used. In cases of normal distribution, we determined mean \pm standard deviation (SD) values and used independent samples *t*-test for statistical evaluation. The appropriate *p* values of < 0.05 were considered to be statistically significant.

4.3 miRNA expression profiling in pSS and SLE patients

4.3.1 Patients

Eight female patients with pSS (mean age: 55.12 ± 7.49 years, range from 42 to 64 years) and 8 female patients with SLE (mean age: 45.88 ± 10.58 years, range from 36 to 66 years) were enrolled in the study. All patients were recruited from the Outpatient Clinic for systemic autoimmune diseases at the Division of Clinical Immunology, University of Debrecen, where they received regular follow-up treatment. The recruitment of participants was between 14-12-2015 and 29-02-2016. The average disease duration was 13.40 ± 7.94 years in pSS, while 14.14 ± 9.14 years in case of SLE. The diagnosis of pSS was based on the European-American consensus criteria. Among pSS patients, 4 suffered from EGMs, while 4 had only glandular symptoms. The distribution of EGMs of pSS patients were as follows: polyarthritis n = 4, Raynaud's phenomenon n = 3, vasculitis n = 1. The exclusion criteria included therapy with immunosuppressive/immunomodulatory agents. Vasculitis or other EGMs needing immunosuppressive treatment were newly recognised. Patients with SLE fulfilled the corresponding diagnostic criteria for lupus. All of the SLE patients received per os methylprednisolone therapy with an average dose of 4 mg daily; the dose of the treatment did not exceed 8 mg methylprednisolone per day in any case of SLE patients. The blood samples were collected from SLE patients 24 hours after taking the regular methylprednisolone medication. We assessed the actual disease activity of SLE patients and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores were calculated. None of the lupus patients showed clinical activity or had SLEDAI score higher than 4. The control group consisted of seven age-matched (mean age: 49.78 ± 7.31 years, range from 39 to 61 years) healthy female volunteers. No patients or controls enrolled in this study had ongoing infections, either viral or bacterial. Table 1 summarizes the demographic and clinical data of the individuals included in the study.

Informed written consent was obtained from all subjects enrolled in the investigation, and the study has been approved by the Ethics Committee of our University and the Policy Administration Services of Public Health of the Government Office (protocol number: IF-13052-9/2015). All experiments carried out were in compliance with the Declaration of Helsinki.

4.3.2 Sample handling

Peripheral blood samples obtained from each study subjects were collected and PBMCs were separated by Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO, USA) density-gradient centrifugation.

4.3.3 RNA isolation

RNA samples were isolated from PBMCs using Trizol reagent (MRC, Cincinnati, Ohio, USA) according to manufacturer's protocol. RNA quantity was measured by UV photometry using NanoDrop instrument (Themo Fisher Scientific, Waltham, MA, USA). RNA quality was checked by Agilent BioAnalyzer and RNA samples with RIN > 7 were used for the further applications.

4.3.4 RNA sequencing library preparation

Sequencing libraries for small RNA-Seq were generated from 1 µg total RNA using TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Fragment size distribution and molarity of libraries were checked on Agilent BioAnalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA, USA). Subsequently single read 50 bp sequencing run was performed on Illumina HiScan SQ instrument (Illumina, San Diego, CA, USA).

4.3.5 Determination of B cell subsets

For the identification of different lymphocyte subpopulations, PBMCs were stained then assessed using flow cytometer, as described previously. For identification of B cell subsets, we used the combination of IgD-fluorescein isothiocyanate (FITC)/CD27-phycoerythrin (PE)/CD19-phycoerythrin-Cyanine dye 5 (PE-Cy5) (Beckman Coulter Inc, Fullerton, CA, USA and Immunotech, Marseille, France) and CD38-FITC/CD27-PE/CD19-PE-Cy5/CD24-allophycocyanin (APC) (BD Biosciences, San Diego, CA, USA and Beckmann Coulter and BioLegend, San Diego, CA, USA). According to the expression of IgD, CD27, CD38 and CD24 cell surface markers, the following B cell subsets were identified: CD19⁺IgD⁺CD27⁻ naive B cells, CD19⁺IgD⁺CD27⁺ non-switched IgM memory B cells, CD19⁺IgD⁻CD27⁺ switched memory B cells, CD19⁺IgD⁻CD27⁻ double negative (DN) B cells, CD19⁺CD38⁻CD24^{hi}CD27⁺ primarily memory B cells, CD19⁺CD38^{hi}CD24^{hi}CD27⁻ transitional B cells, CD19⁺CD38⁺CD24⁺ mature-naive B cells and CD19⁺CD38^{hi}CD27^{hi} plasmablasts. Cells were quantified as their percentage in the CD19⁺ lymphocyte population. Fluorescence Minus One controls were used in all procedures. The stained cells were measured by FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data was analysed using FlowJo Software (Treestar, Ashland, OR, USA).

4.3.6 Data analysis

CASAVA software was used for pass filtering and demultiplexing process. Sequenced reads were aligned to Human Genome v19 and bam files were generated. Further statistical analyses were executed using StrandNGS software (Agilent Technologies, Santa Clara, CA, USA). Relative small RNA expression levels were calculated using DESeq algorithm. To find differentially expressed small RNAs between clinical conditions ANOVA analysis with Tukey post hoc test was performed.

To assess the distribution of the data Shapiro-Wilk normality test and Kolmogorov-

Smirnov test were used. The correlations between two variables were evaluated with Pearson's correlation coefficient, while in cases of non-normal distribution, Spearman's test was used. Differences were considered statistically significant at $p < 0.05$.

5. Results

5.1 Study on vitamin D receptor gene polymorphisms

5.1.1 Allele frequencies of VDR gene polymorphisms

After the calculation of the genotype distribution and allele frequency for BsmI (*rs1544410*), FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) polymorphisms, it is revealed that the alleles of the four polymorphisms were in Hardy-Weinberg Equilibrium both in controls and patients group.

The characteristics are shown separately for GS patients and EGMs patients, respectively. No significant difference was found in allele frequencies when data were compared between pSS cases and control individuals. No significant difference was observed when the pSS cases were grouped into GS cases and EGMs cases.

5.1.2 Genotype analyses

No significant difference was found in the genotype frequencies when the VDR gene polymorphisms genotypes of pSS patients and healthy individuals were compared. The same result has been confirmed between GS cases and EGMs cases. However, we recognized slightly increase prevalence of the Aa genotype in EGMs patients compared with GS patients, 57.69 and 39.62 %, respectively. Likewise, we also found a mild increased prevalence of the Tt genotype in EGMs patients compared with GS group and healthy group, 53.85, 41.51, and 39.79 %, respectively. These differences have not been proven significant. Genotype frequencies of VDR gene polymorphisms did not

differ between pSS cases and controls in any comparison performed.

5.1.3 Haplotype (BsmI- ApaI- TaqI) analyses

The haplotypes might provide valuable data where genotypes alone unable to do. The haplotype frequencies among the VDR-FokI, VDR-BsmI, VDR-ApaI, and VDR-TaqI polymorphisms in both the pSS patients and the healthy controls were evaluated by the CHAPLIN 1.2 software.

The estimated haplotype frequencies for VDR-BsmI, VDR-ApaI, and VDR-TaqI polymorphisms of the pSS patients and control individuals are shown in Table 4. The baT and BAt haplotypes were found the most frequent haplotypes in both the patient group (51 and 33 %) and the control group (48 and 31 %). These three-marker haplotype alleles were identified as the most frequent and corresponded haplotypes by Morrison et al. as well. The BaT haplotype was present neither in patients nor in the controls. Three haplotypes (BAT, Bat, and bAt) were relatively uncommon (frequency <10%) either in the patient group or in the control group.

Pairwise LD was computed and LD plots were constructed using the Haplovview software version 4.2. LD analysis revealed a very strong LD ($r^2>0.8$) between ApaI and TaqI polymorphisms, a strong LD (r^2 between 0.67 and 0.7) between BsmI and ApaI or BsmI and TaqI polymorphisms, and a very weak LD ($r^2<0.3$) was observed between FokI and other polymorphisms in the control group. In patients, very strong ($r^2>0.8$) to moderate ($r^2=0.67 - 0.7$) LD was found between BsmI and ApaI, TaqI polymorphisms, ApaI and TaqI polymorphisms. No LD was observed between FokI and other polymorphisms in patients.

5.2 Study on miR-155 and its target gene SOCS1

5.2.1 Over-expression of miRNA-155 in patients with pSS

The relative expression of miRNA-155 was significantly higher in the PBMCs of patients with pSS than in those of 10 normal controls (miRNA-155: Sjögren patients: 2.65 ± 1.68 versus 1.0; $P = 0.003$).

5.2.2 Over-expression of SOCS1 in pSS patients

A significant and unanticipated over-expression of SOCS1 was also found in these patients compared to the controls (6.76 ± 6.4 versus 1.0; $P < 0.001$).

Of note, although both miRNA-155 and SOCS1 were significantly over-expressed in the PBMCs of patients with pSS compared to the data of healthy controls, there was no significant correlation between the expression values of the two genes in the pSS patients ($R = 0.023$, $P = 0.916$).

5.3 miRNA expression profiling in pSS and SLE patients

MiRNAs were isolated from the PBMCs of 8 SLE, 8 pSS and 7 healthy controls. The expression patterns were determined with Illumina next-generation sequencing technology. We carried out analysis of variance (ANOVA) together with Tukey's honest significant difference (HSD) test to evaluate the different expression patterns of miRNAs. We observed significant differences in the following cases: 25 different miRNAs were up-regulated and one miRNA was down-regulated in pSS patients compared to controls, while 135 miRNAs were over-expressed in SLE patients compared to healthy individuals.

Among all 135 significantly up-regulated miRNAs in SLE patients, 113 were over 2

fold change compared to controls. From those, let-7e-5p, miR-144-5p (11 fold change), miR-145-5p, miR-190a, miR-345-5p, miR-409-3p and miR-425-3p shown more than 5 fold change in patients group compared to healthy controls.

In pSS group, 25 miRNAs were significantly over-expressed in pSS compared to controls. Fourteen of them, namely, let-7e-5p, miR-16-1-3p, miR-20b-5p, miR-424-3p, miR-106a-5p, miR-15a-5p, miR-190a, miR-769-5p, miR-18a-5p, miR-21-3p, miR-145-5p, miR-425-3p, miR-34a-5p, miR-144-5p showed more than 2 fold change. Of note, all of these miRNAs were found to be elevated in the SLE group, as well. On the contrary, miR-150-5p was significantly down-regulated in pSS.

Interestingly, when we compared the miRNA profiles between the subset of pSS patients with glandular symptoms only and pSS patients with EGMs, we did not find any difference in miR-150-5p expression between the subgroups of pSS patients. On the contrary, we identified the over-expression of miR-148a in patients with EGMs, which miRNA did not show any change in the whole group of pSS patients, compared to the healthy individuals.

Finally, we also carried out the statistical comparison between the miRNA patterns of the SLE and pSS patients. We have found that 55 miRNAs significantly up-regulated in SLE compared to pSS. More than half of those have more than 2 fold change.

We also created the heat map representation of the individual expression levels of certain miRNAs determined in the PBMCs of SLE and pSS patients and control subjects.

As a next step, we analysed the associations between the expression levels of miRNAs and the measured B lymphocyte ratios within PBMCs. The miR-223-5p showed a positive association with naive B cell percentages ($R = 0.4859$, $p = 0.0298$), and a negative correlation with switched memory B cell ratios ($R = -0.5880$, $p = 0.0064$).

The miR-150-5p expression levels showed significant positive correlations with the percentages of CD19+IgD-CD27- double negative (DN) B cells ($R = 0.5523$, $p = 0.0215$) and plasmablasts ($R = 0.5409$, $p = 0.0250$). Furthermore, DN B cell ratios also

showed significant positive correlations with the expression levels of miR-155-5p ($R = 0.5410$, $p = 0.0204$). Additionally, we revealed positive correlation between miR-342-3p expression levels and percentages of plasmablasts, as well ($R = 0.5215$, $p = 0.0265$).

6. Discussion

6.1 Study on vitamin D receptor gene polymorphisms

Numerous cells involved in the operation of immune system express VDR and key vitamin D metabolizing enzymes which underlines the importance of vitamin D in immune regulation. Therefore we hypothesized that polymorphisms of the VDR gene may contribute to the pathogenesis of pSS as genetic susceptibility factors. Several data have been published on the involvement of VDR polymorphisms in the pathogenesis of autoimmune diseases. The FokI VDR polymorphisms have been recognized as the risk factor for several autoimmune diseases, such as SLE, RA, MS, psoriasis, AITD and autoimmune Addison's diseases. Even the recessive f allele gene of VDR regarded as a risk factor for the incidence of CMV Disease after kidney transplantation according to Zhao et al. study.

To our best knowledge, the possible relationship between pSS and VDR gene polymorphisms has not been investigated up to now. In the current study, our aim was to determine VDR gene BsmI (*rs1544410*), FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) genotypes in pSS patients and healthy controls. As the results of our investigations, there were no statistical differences of the FokI (FF, Ff, ff), BsmI (BB, Bb, bb), ApaI (AA, Aa, aa), and TaqI (TT, Tt, tt) genotypes and allelic frequencies between pSS patients and control individuals. Besides these findings, the haplotype frequencies among the VDR-FokI, VDR-BsmI, VDR-ApaI, and VDR-TaqI polymorphisms in both the patients with pSS and healthy controls were evaluated and showed nearly the same distribution. Analysis of FokI, BsmI, ApaI, and TaqI locus haplotype frequencies failed to show any association in the study groups, suggesting

that FokI, BsmI, ApaI, and TaqI polymorphisms of VDR gene are not associated with the development of pSS in the Hungarian population studied.

The baT haplotype was found as the highest frequent haplotype in the patient group and the control group. Uitterlinden et al. has reported the frequencies of the BsmI-ApaI-taqI haplotypes of VDR gene in different ethnic groups. The distribution of the most frequent haplotype (baT) was different among Caucasians, Asians, and Africans, 43, 75 and 26 %, respectively. We calculated slightly higher frequency for the baT haplotype in the patient group and the control group, 51 and 48 %, respectively. Despite of Caucasian origin of our study subjects, a slightly elevated distribution of the baT haplotype might be explained by the population genetics. Haplotype frequencies of VDR gene may vary within Caucasian population as well as the strength of the LD between different VDR polymorphisms.

Alterations in VDR gene expression and VDR protein activity could lead to deregulation of vitamin D uptake, metabolism, and serum levels of the biologically active vitamin D. Furthermore, certain polymorphisms of the VDR gene may have regulatory effects on vitamin D function and metabolism. Szodoray et al. recently reported that the vitamin D levels were found similar in both the overall pSS patients and controls in the Hungarian population they studied. Similarly, no significant differences were found in vitamin D levels of the blood in patients with and without EGMs. Allele frequencies we have found for VDR-FokI, VDR-BsmI, VDR-ApaI, and VDR-TaqI in pSS patients might help to explain the correlation between the genotypes of VDR gene of pSS patients and normal blood level of vitamin D in patients examined previously.

6.2 Study on miR-155 and its target gene SOCS1

The enhanced expression of SOCS1 in parallel with the elevated miR-155 expression has been an unanticipated and surprising finding because several earlier observations described the reciprocal character of their occurrence and coexistence. The high level

expression of miR-155 was found to suppress the expression of SOCS1 in the PBMCs of patients with RA recently. pSS patients treated with immunosuppressant also showed over expression of miR-155 while in Asian population the relative expression of miR-155 was lower in PBMCs of pSS patients (not receiving any immunosuppressant) compared to controls. Our results are in line with the observations of Pauley et al., since we measured enhanced miR-155 expression in the PBMCs of our patients. Although a large part of Pauley's patients received secretagogues or immunosuppressants, they also enrolled patients without any therapy and they examined the possible associations between increased miR-155 expression and medications, however they found no correlations.

MiR-155 is encoded within the B cell integration gene cluster located on chromosome 21. This region also carries an IL-6 dependency gene regulating cell growth. The interplay between EBV and B lymphocytes in the context of infection, immunity and diseases is widely known. The involvement of EBV infection in the insurgence and persistence of Sjögren's syndrome is also an accepted aspect in the pathogenesis of disease. Pri-miR-155 and miR-155-5p were over-expressed in EBV infected B cells displaying type III latency but not in EBV infected cells displaying type I latency in cancer cells. Recently, Croia et al. reported that EBV infection is selectively associates with ectopic lymphoid structures in the salivary glands of patients with Sjögren's syndrome. Based on their observations, EBV infection appears to contribute to local growth and differentiation of Sjögren-specific autoreactive B cells. In addition, EBV virus latent membrane protein 1 (LMP1) could up-regulate miR-155 in nasopharyngeal carcinoma cell line.

LMP1 is essential for EBV-mediated B cell transformation. LMP1 can functionally mimic the TNF receptor superfamily member CD40, which is an activating receptor constitutively expressed on B cells, macrophages together with DCs. However, LMP1 and CD40 use TRAF proteins to mediate signaling. Moreover, TLR 4 pathway involves not only the SOCS1 protein but TRAF6. TRAF6 is a convergence point for both

upstream and downstream signaling cascades. Formerly, we reported elevated expression level of TRAF6 in pSS patients which suggests the role of EBV infection in the pathogenesis of pSS. In the literature, there are several cell types in which various EBV related alterations were described concerning miR-155 and SOCS1. We assume that the over-expression of miR-155 in the PBMCs of our pSS patients is linked anyhow to EBV infections passed off earlier as each of their sera were positive for anti-EBV IgG. In addition, EBV infection itself could induce the expression of both SOCS3 and SOCS1 resulting in a survival pathway for the virus by the blockage of IFN production in primary monocytes. However, it was a crucial fact that the expression levels of neither miR-155 nor SOCS1 were increased in the peripheral PBMCs of healthy controls, although all of them were also positive for anti-EBV. Therefore, we do think that noteworthy differences may exist in the persistence of EBV in the peripheral PBMCs of healthy subjects and patients with pSS,

It is important to take into consideration that pSS is a clinically heterogeneous disease and we worked with a relatively small sample size; therefore our results should be confirmed in an independent set of pSS cases and healthy controls in future studies.

We assume that the simultaneous and unanticipated over-expression of miR-155 and SOCS1 can serve as a marker of Sjögren's syndrome, similarly to our earlier observation on the concurrent over-expression of miR-146a and TRAF6. In both phenomena the difference in the EBV virus induced immune responses between the healthy subjects and patients with pSS can be one of the causative factors, but to prove this hypothesis further studies are needed.

6.3 miRNA expression profiling in pSS and SLE patients

In our study, we revealed moderated alterations in the expression patterns of miRNAs in patients with pSS. On the contrary, lupus patients showed more pronounced changes in miRNA expression profile, compared to the results of healthy subjects. Furthermore, the over-expressed miRNAs in pSS showed elevated expression levels in SLE, as well;

although the decreased expression of miR-150 was observed in pSS patients only. We compared the expression profiles of pSS and SLE patients in order to get a better insight into the disease-specific changes in the expression of different miRNAs. In total, 25 miRNAs were over-expressed in both SLE and pSS, including miR-146a, miR-16 and miR-21. Consequently, the observed alterations of these miRNAs could be part of the immunological processes developing in both investigated diseases. The over-expression of miR-146a/b is induced by LPS-mediated inflammatory responses and leads to translational repression of its target genes interleukin-1 receptor-associated kinase (IRAK) 1 and tumor necrosis factors receptor associated factor (TRAF) 6, thus serving as a negative feed-back for immune activity. In T and B cells, group of miRNAs including miR-21, miR-146a, miR-155 and others might correlate with epigenetic modifications, support abnormal cytosine release, differentiation of cell subsets, B cell hyperactivity and autoantibody production. Our workgroup previously reported the over-expression of miR-146 in pSS patients compared to controls. Regarding SLE, a former study reported the under-expression of miR-146a in the PBMCs of Chinese SLE patients. Nevertheless, our present study revealed the over-expression of miR-146a in PBMCs of our SLE patients. This variance between European and Chinese SLE patients could be explained by the difference in genetic background as well as various external factors, such as dietary habits, exposure to different infectious agents and other environmental elements, which may have effects on miRNAs expression. The miR-16 is one of the critical regulators of TLR-mediated immune responses, and it can promote NF- κ B regulated transactivation of the interleukin (IL)-8 gene by the suppression of silencing mediator for retinoid and thyroid hormone receptor (SMRT). MiR-16 was found to be up-regulated in patients with RA, and based on our observations and considering the role of IL-8 in SLE, the miR-16 could play an important role in lupus as well. MiR-21 is regarded to be one of the DNA methylation associated miRNAs. It shows higher expression level in PBMCs of SLE patients compared to healthy controls. MiR-21 has a pluripotent role in serving to link distinct lymphocyte signaling pathways.

and promote B and T cell activation in lupus. Overall, the elevated miR-21 expression observed in our SLE and pSS patients could be a part of autoimmune machinery, but presumably the consequence of an initial trigger, and not the reason.

In the present study, we found several over-expressed miRNAs in SLE, which levels were not changed in pSS. Hereby, we discuss the significance of miR-148a-3p, miR-152, miR-155, miR-223, miR-224, miR-326 and miR-342 in details. Over-expression of miR-148a impaired B cell tolerance by promoting the survival of immature B cells after the engagement of the B cell antigen receptor by suppressing the expression of the autoimmune suppressor Gadd45 α , the tumor suppressor PTEN and the pro-apoptotic protein Bim. In our present study, we reported the over-expression of miR-148a in SLE; however, we also observed the same higher level expression in pSS patients suffering from EGMs. Taken together, the enhanced expression of this miRNA seems to be associated with systemic autoimmune processes, rather than a specific autoimmune disorder. It was also found that three members of the miR-148 family, including miR-148a, miR-148b and miR-152, are negative regulators of the innate response and Ag-presenting capacity of DCs. In the present study, the expression levels of both miR-148a-3p and miR-152 were elevated in the PBMCs of SLE patients. Beside the aforementioned miRNAs, miR-155 was also elevated in lupus, which is regarded as a central modulator of immune responses. Activated B and T cells show increased miR-155 expression, the same goes for macrophages and DCs as well. MiR-155 is crucial for proper lymphocyte development and maturation and has been shown to be required for antibody production. Vigorito et al. reported that B cells lacking miR-155 generate reduced extrafollicular and GC responses and fail to produce high-affinity IgG1 antibodies, and that the transcription factor Pu.1 is a direct target of miR-155-mediated inhibition. In autoimmune disorders such as rheumatoid arthritis, systemic sclerosis or psoriasis, miR-155 showed higher expression in patients' PBMCs. In the present study, we observed enhanced expression of miR-155 in the PBMCs of our SLE patients, which was associated with the increased peripheral DN B cell percentages in lupus. A previous

report proposed that the presence of these cells could be the result of an extrafollicular differentiation process in secondary lymphoid organs. Their activation does not require T cell interaction. Furthermore, due to the expression of CXCR3, after receiving activation signals, DN B cells could migrate to inflamed tissues. Taken together, our observations underline the importance of miR-155 in not only GC but also in extrafollicular responses in SLE. We observed elevated miR-223 expression levels in SLE. The present data on this miRNA is controversial, since it was reported that miR-223 is down-regulated in SLE patients with active nephritis in Danish population; while on the contrary, up-regulation was shown in SLE patients in Asian population. In our study, we revealed the over-expression of both miR-223-3p and miR-223-5p in our SLE group; additionally, we demonstrated positive association with naive B cells, and negative association with switched memory B cells. These associations are supported by the recent findings showing that the expression of miR-223 in naive B cells blocks the differentiation of naive B cells into GC B cells by repressing LIM domain only 2 and MYB Proto-Oncogene Like 1. Conversely, a few highly up-regulated miRNAs in naive B cells, such as miR-223, miR-150 and miR-342-5p, were also down-regulated in activated peripheral blood B cells, suggesting that they regulate B cell activation. Regarding miR-224, its over-expression facilitates activation-induced cell death in Jurkat cells. Enhanced expression of miR-224 accelerates T cell activation-induced cell death by suppressing apoptosis inhibitor (API) 5 expression and associated with LN by enhancing STAT-1 expression in patients with SLE. Our observation on over-expressed miR-224 in PBMCs underlines its significance in SLE pathogenesis. Additionally, we found elevated expression levels of miR-326, which was reported to be over-expressed in PBMCs from type 1 diabetic patients with ongoing islet autoimmunity, however, its significance in lupus development needs further clarification. miR-342-5p, similar to miR-491-5p, has predicted binding sites within the 3'UTR of three genes involved in Wnt signaling [Transcription Factor 7 (TCF7), musashi RNA-binding protein 1 (MSI1), and paired box 5 (PAX5)]. A recent study indicated that these miRNAs also participate

in the regulation of TCF7, MSI1, and PAX5 genes. PAX5 upregulates lymphoid enhancer-binding factor 1 (LEF1) (TCF7-related protein, regulator of Wnt signaling) and interacts directly with LEF1 in B cells. In our study, we observed the significant elevation in both miR-342-5p and miR-491-5p expression; moreover, miR-342-5p showed a strong positive correlation with plasmablast ratios in SLE. This is an important result, since PAX5 is one of the key transcription factors for plasma cell differentiation.

We observed the down-regulation of miR-150-5p, which is a novel and unique finding in pSS. Former studies showed that miR-150-5p might emerge as a master regulator of gene expression during immune cells differentiation and immune response processes. It plays an important role in the inhibition of B cell activation and differentiation, and its regulation ability in immune cellular process might contribute to the host defence against invading pathogens. Dysregulated expression of miR-150-5p in immune cells might result in autoimmunity. MiR-150-5p was reported to be over-expressed in autoimmune pancreatitis compared to chronic pancreatitis, pancreatic cancer and healthy controls. Based on our observations miR-150-5p is down-regulated in pSS, on the contrary, in SLE patients, miR-150-5p expression levels were not decreased but elevated, albeit not significantly. We also revealed that miR-150-5p expression levels associated positively with the percentages of DN B cells and plasmablasts. Our results are in accordance with the recently reported observations, namely, DN B cell percentages are lower in pSS, but higher in SLE, while plasmablast ratios are increased in SLE but not in pSS. Based on these important differences, we assume that the under-expression of miR-150-5p potentially contributes to the differentiation and activation of B cells leading to the development of specific autoimmune processes in pSS.

In the present study, we not only depict the alterations in miRNA expression profiles in SLE and primary Sjögren's syndrome, but we were the first to compare the changes in miRNA expression profiles between the two autoimmune diseases at the same time. However, we also have to mention some limitations of this study. SLE and pSS are

clinically heterogeneous diseases and we worked with a relatively small sample size; therefore, our results should be confirmed in an independent set of patients. Some of the aforementioned miRNAs may be regarded as novel biomarkers for the investigated autoimmune disorders; however, functional experimental studies are also required to verify and establish the causal associations between the aberrantly expressed miRNAs and the development of SLE and pSS.

In conclusion, the observed differences in miRNA expression profiles in Sjögren's syndrome and systemic lupus erythematosus, and the better understanding of the immune regulatory mechanisms of the relevant miRNAs may help to elucidate the pathogenesis of the diseases. Certain miRNAs, as potential biomarkers, may not only help in the early diagnosis and prediction of prognosis, but also could assist in identifying potential targets for therapeutic interventions in the future.

7. Summary

Our study was the first in the literature which focused on the VDR gene polymorphisms in pSS; however, there were no differences in the distribution of BsmI, TaqI, ApaI, and FokI genotypes and the common haplotypes between pSS patients and healthy controls. We hypothesize that these polymorphisms of the VDR gene are not associated with the development of pSS in the Hungarian population.

We observed the over-expression of miR-155 in the PBMCs of pSS patients. SOCS1 gene was also over-expressed in patients. This unanticipated phenomenon might be a laboratory characteristic of Sjögren's syndrome, and presumably a consequence of the noteworthy difference in the pSS immune system reacting with EBV.

Our results on miRNAs expression profiles indicated that in SLE patients 135, while in pSS patients 26 miRNAs showed altered expression. Interestingly, the 25 miRNAs including miR-146a, miR-16 and miR-21, which were over-expressed in pSS patients, were found to be elevated in SLE group, as well. On the contrary, we observed the down-regulation of miR-150-5p, which is a novel and unique finding in pSS. Levels of

several miRNAs over-expressed in SLE, were not changed in pSS, such as miR-148a-3p, miR-152, miR-155, miR-223, miR-224, miR-326 and miR-342. Expression levels of miR-223-5p, miR-150-5p, miR-155-5p and miR-342-3p, which miRNAs are potentially linked to B cell functions, showed associations with the B cell proportions within peripheral blood mononuclear cells. The observed differences in miRNA expression profiles and the better understanding of immune regulatory mechanisms of miRNAs may help to elucidate the pathogenesis of SLE and pSS.

8. Appendix



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Subject: PhD Publikációs Lista

Candidate: Ji-Qing Chen
Neptun ID: XIKZ6P
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Chen, J. Q.**, Papp, G., Pólska, S., Szabó, K., Tarr, T., Bálint, B. L., Szodoray, P., Zeher, M.: MicroRNA expression profiles identify disease-specific alterations in systemic lupus erythematosus and primary Sjögren's syndrome. *PLoS One. [Epub ahead of print]*, 2017.
IF: 3.057 (2015)
2. **Chen, J. Q.**, Zilahi, E., Papp, G., Sipka, S., Zeher, M.: Simultaneously increased expression of microRNA-155 and suppressor of cytokine signaling 1 (SOCS1) gene in the peripheral blood mononuclear cells of patients with primary Sjögren's syndrome. *Int. J. Rheum. Dis. [Epub ahead of print]*, 2015.
DOI: <http://dx.doi.org/10.1111/1756-185X.12804>
IF: 1.914
3. Zilahi, E., **Chen, J. Q.**, Papp, G., Szántó, A., Zeher, M.: Lack of association of vitamin D receptor gene polymorphisms/haplotypes in Sjögren's syndrome. *Clin. Rheumatol. 34* (2), 247-253, 2015.
IF: 2.042



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List of other publications

4. **Chen, J. Q.**, Papp, G., Szodoray, P., Zeher, M.: The role of microRNAs in the pathogenesis of autoimmune diseases.
Autoimmun. Rev. 15 (12), 1171-1180, 2016.
DOI: <http://dx.doi.org/10.1016/j.autrev.2016.09.003>
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Clin. Rev. Allergy Immunol. 50 (1), 1-17, 2016.
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6. Bodoki, L., **Chen, J. Q.**, Zeher, M., Nagy-Vincze, M., Griger, Z., Zilahi, E., Dankó, K.: Vitamin D Receptor Gene Polymorphisms and Haplotypes in Hungarian Patients with Idiopathic Inflammatory Myopathy.
Biomed Res. Int. 2015, 1-8, 2015.
DOI: <http://dx.doi.org/10.1155/2015/809895>
IF: 2.134

Total IF of journals (all publications): 22,95

Total IF of journals (publications related to the dissertation): 7,013

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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