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

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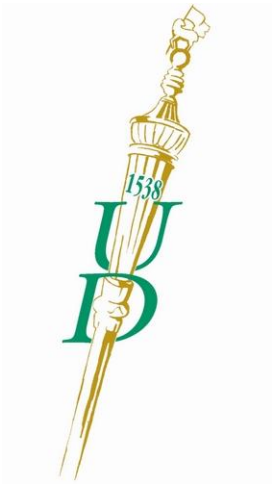

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Abbreviations

Ago	argonaute
AITD	autoimmune thyroid disease
BAFF	B-cell activating factors of the TNF family
CLRs	C-type lectin-like receptors
CXCR	chemokine receptor type
DC	dendritic cells
DGCR8	Drosha and DiGeorge syndrome critical region gene 8
DN	CD19+IgD-CD27- double negative B cells
dsRNAs	double-stranded RNAs
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EGMs	extraglandular manifestations
GC	germinal center
GS	glandular symptoms
GTP	guanosine triphosphate
HLA	human leukocyte antigen
IFN	interferon
Ig G	immunoglobulin G
IL	interleukin
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor

LD	linkage disequilibrium
LEF1	lymphoid enhancer-binding factor 1
LPS	lipopolysaccharide
miRNAs	microRNAs
mRNA	messenger RNA
MSI1	musashi RNA-binding protein 1
MS	multiple sclerosis
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	nucleotide-binding oligomerization domain-like receptors
PAMPs	pathogen-associated molecular patterns
PAX5	paired box 5
PBMCs	peripheral blood mononuclear cells
pre-miRNAs	precursor miRNA
pri-miRNAs	primary miRNA
PRRs	pattern recognition receptors
pSS	primary Sjögren's syndrome
PTEN	phosphatase and tensin homolog
RA	rheumatoid arthritis
RISC	RNA-induced silencing complex
RLRs	retinoic acid-inducible gene –I-like receptors
RNase	ribonuclease

RP	Raynaud’s phenomenon
RT	Reverse transcription
SGECs	salivary gland epithelial cells
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SNPs	single nucleotide polymorphisms
SOCS	Suppressor of cytokine signaling
STAT	signal transducer and activator transcription
stRNAs	small temporal RNAs
TCF7	Transcription Factor 7
TGF	transforming growth factor
Th	T helper cell
TLR	Toll-like receptors
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNF receptor associated factor
Treg	T regulatory cells
UTR	untranslated region
VDR	vitamin D receptor

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).

The Division of Clinical Immunology of the University of Debrecen is one of the largest immune centers for autoimmune disorders in Europe. Under the leadership of Prof. Dr. Margit Zeher, more than 1500 Sjögren's syndrome patients are regulary followed-up in our division. The large size of the patients' pool gives us a unique opportunity to carry out internationally recognized investigations on the pathogenesis of pSS.

I started my research at the division in 2011 as a medical student, and presented my results on consecutive conferences of the students' research society in 2012 and 2013. After graduating, I continued the genetic research in Sjögren's syndrome as part of my PhD program. I hereby summarize my research results in my doctoral dissertation.

2. Background

2.1 Sjögren's syndrome

Sjögren's syndrome 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 (1). The target organs of lymphocytic infiltration are primarily exocrine glands, such as salivary and lachrymal glands, which leads to immune-mediated secretory dysfunction. Therefore, patients show typical symptoms of dry mouth and dry eyes. Primary Sjögren's syndrome (pSS) is referred to the condition when patients do not have any additional systemic autoimmune diseases, while secondary Sjögren's syndrome coexists with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), scleroderma, or other autoimmune diseases (3).

2.1.1 Clinical features

Glandular symptoms (GS)

Glandular symptoms (GS) are the most common clinical features in pSS. Beside the typical xerostomia and xerophthalmia, other sicca symptoms can also develop in pSS patients. For instance, dryness of airway mucosa leads to hoarse voice, recurrent bronchitis and pneumonitis (3). Decreased vaginal secretion of female patients results in dyspareunia and impaired sexual function (4). Nasal, ear, and skin dryness cause local irritation (1).

Extraglandular manifestations (EGMs)

Beside the pathognomonic glandular symptoms, other systemic symptoms, denoted as extraglandular manifestations (EGMs) can also develop during the disease course in approximately one third of the patients (5). Patients with pSS complain about easy fatigability and muscle pain (1). Based on two large cohort studies in Spain and Italy in 2014, frequent severe manifestations of pSS patients include articular, cutaneous, pulmonary symptomes, renal involvement and neuropathy (6,7).

Articular and musculoskeletal involvements

Articular involvements including joint pain and nonerosive inflammatory arthritis have been reported in more than 50% of patients with pSS (7). Musculoskeletal manifestation can be a symmetric non-erosive polyarthritis which predominantly affects the small joints, or mild myositis without elevated serum muscle enzymes or alterations in the electromyogram. Mild myositis is characterized by focal lymphocytic infiltration in the muscle biopsy (1).

Raynaud's phenomenon (RP)

In pSS patients, the prevalence of RP is appr. 10-37%, which is milder than in scleroderma (3). RP is often presents in patients positive both for anti-Ro/SSA or anti-La/SSB autoantibodies.

Pulmonary involvements

The major manifestations of pulmonary involvement are dry cough in 50% of patients, and rhinosinusitis in 30% of patients. Subclinical abnormalities of pulmonary function, bronchoalveolar lavage, and computed tomography results are found in approximately

75% of patients (8). Abnormal high resolution computed tomography findings, such as interlobular septal thickening, micronodules, ground glass attenuation and parenchymal cysts, were seen in 65% of patients. Less predominant abnormal findings such as intralobular opacities, honey combing, bronchial wall thickening, bronchiectasis, and pleural irregularities were also found in the majority of pSS patients (9). Later stages may show histological evidence of pulmonary fibrosis (1).

Renal manifestations

Renal function can be affected by pSS either as epithelial disease causing tubulointerstitial nephritis or as an immune complex-mediated glomerulopathy (10). The early manifestations of renal involvement include interstitial nephritis associated with renal tubular dysfunction with or without acidosis (11). The clinical signs include mainly hypokalemic weakness (70%), and less commonly renal colic, nephrocalcinosis, osteomalacia and diabetes insipidus. In comparison with tubular involvement, glomerulonephritis is less common. Glomerulopathy associated with pSS is mostly linked to non-infective cryoglobulinemic vasculitis (10).

Cutaneous and vascular manifestations

A wide spectrum of cutaneous lesions is often observed in pSS patients. Approximately 10% of patients have annular erythema together with a polycyclic, photosensitive erythematosus rash (also known as subacute cutaneous lupus erythematosus), which could be also noticed in the neonatal cutaneous lupus erythematosus (12). Both are strongly associated with Ro/SSA autoantibodies.

Cutaneous vasculitis is reported in 10% of patients and develops in limited or systemic

necrotizing form (12). Small and medium-sized blood vessels are affected dominantly. The most common clinical features of small and medium sized vessels affected by vaculitis are purpura, skin ulcerations and recurrent urticaria. The pSS patients with vasculitis may often present progressive nervous system disease, mainly peripheral polyneuropathy (1).

Nervous system involvement

Sixty-four% of patients with pSS show manifestations of peripheral neuropathy, such as pure sensory neuropathy (or ganglionopathy), axonal polyneuropathy, mononeuritis multiplex or small-fiber neuropathy. The most characteristic neurologic complication of pSS is pure sensory neuropathy, caused by the damage of sensory neurons of dorsal root and Gasserian ganglia (13). Mononeuritis multiplex or polyneuropathy may manifest with the presence of anti-Ro/SSA, rheumatoid factor or cryoglobulinemia (1) .

Others symptoms and associated diseases

pSS patients have higher risk for developing lymphoma, which may occur later in the disease course. Frequent types of lymphomas are extranodal, low-grade marginal zone B cell lymphomas (14). One-half of pSS patients with the presence of anticardiolipin antibodies may develop sensorineural hearing loss. Primary biliary cirrhosis could also be one of the liver involvements (1). Other frequent clinical manifestations include splenomegaly, pericarditis and lymphadenopathy can be found in some pSS patients (15).

2.1.2 Histopathology

The typical histological feature of minor salivary gland biopsies in pSS is the formation of ectopic germinal center (GC) by infiltration of mononuclear cells, which are predominantly CD4+ T-cells and CD20+ B-cells, plasma cells, macrophages, natural killer and dendritic cells (DC) (1).

GC-like structures could be found in almost all sites of chronic inflammation. Since activities of GC-like structures are physiologically associated with secondary lymphoid organs, they are denoted as tertiary lymphoid microstructures. They generate somatically mutated high-affinity memory B cells and plasma cells. Moreover, GC-like structures are considered as strong predictive markers for lymphoma (16,17). The better understanding of the mechanisms of formation and persistence of GC-like structures helps to identify new potential therapeutic targets in the future.

2.1.3 Pathogenesis

The etiological name of the disease could be “autoimmune epithelitis” based on the critical role of clinical observations in salivary gland epithelial cells (SGECs) (18). The lymphocytic infiltrates is proximal to SGEC, resulting in secretion of numerous immunomodulatory molecules, such as cytokines, chemokines, adhesion molecules, major histocompatibility complex molecules, apoptosis-related molecules and functional innate immune receptors (3).

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.

Based on our current knowledge both intrinsic (genetical) and extrinsic (environmental) factors play critical role in the disease development. In genetically susceptible individuals, certain external factors, such as hormonal changes, external environmental factors, viral infections may trigger the autoreactive processes. The adhesion molecules (e.g. E-cadherin) and integrins of immune-competent cells recognize the autoantigens appear on the epithelial surface, triggering the "homing" process of activated immune cells and the lymphocytic infiltration of certain tissues (e.g. exocrine glands). Consequently leads to the destruction of the involved tissues and loss their function (19,20). Cell surface autoantigens appear during apoptosis leading to the breakdown of immune tolerance. The autoreactive immune processes that follow activation or apoptosis of glandular epithelial cells exposing autoantigens in genetically predisposed individuals may drive the immune-mediated tissue injury. Abnormalities related to the upregulation of type I interferon (IFN)-regulated genes (interferon signature), abnormal expression of B-cell-activating factor (BAFF) and activation of the IL-23/TH17 pathway are among the immune mediators implicated in the pathogenesis of autoimmune lesions within the salivary glands. Such abnormalities demonstrate the complex interplay between innate and adaptive immunity that contributes to autoimmune 'exocrinopathy'. Epithelial cell apoptosis may provide cellular proteins as autoantigens which subsequently drive the autoimmune response in pSS.

The genetic background of pSS

The susceptibility genes reported previously, including human leukocyte antigens

(HLA-B8 - DW3, -DR3, -DQA1*0501), genes encoding chemokines, cytokines and transcription factors, showed positive correlation with the clinical manifestations and the serum immunological profile (21). Significant correlation with HLA-DRB1*0803 and Chinese pSS patients was revealed in 2015 (22). Formerly, an association between pSS and IL-10 promoter polymorphisms was demonstrated in a cohort of Finnish population (23). On the contrary, no correlation was found regarding IL-10 promoter polymorphism in Australian pSS patients (24). An increased frequency of the IL-1RN*2 allele has been reported in pSS (25); additionally, it was also demonstrated that a specific SNP in intron 3 of the Ro52 gene strongly correlated with the presence of anti-Ro52 autoantibodies in pSS (26).

In the last two decades, the application of modern genetic and genomic tools opened a new avenue for research on the genetic background of pSS. Consequently the comprehensive and unbiased nature of genome-wide screens removed the limitations of traditional hypothesis-driven candidate gene studies. Genome-wide association studies indicated that certain susceptibility genes, including interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4), and IL-12A, correlated with IFN signaling. Furthermore, B-lymphocyte kinase (a nonreceptor tyrosine kinase) and chemokine receptor type (CXCR) 5 are important for B-cell function and antibody production. Moreover, the TNFAIP3 interacts protein 1 gene which is involved in the negative regulation of the nuclear factor kappa B (NF-kB) pathway (3). Recently it is reported that the variation of functional serotonin transporter gene polymorphism significantly associated with the decreased platelet serotonin levels

in pSS patients (27).

Altered innate immunite reactions in pSS

Autoreactive processes can be triggered by infectious agents, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human T-cell leukemia lymphoma virus -1, human herpes virus -6, -8, via conserved molecular structures pathogen-associated molecular patterns (PAMPs) (1,28).

Toll-like receptors (TLRs)

TLRs are the most well-characterized pattern recognition receptors (PRRs), which are capable of potently activating different cell types, which could be highly expressed on most immune cells, as well as other cell types, including chondrocytes, endothelial cells and fibroblasts (29). Their downstream signaling pathways lead to the production of a wide range of immune-stimulatory cytokines and chemokines (30). Aberrant activation of TLRs may result in unrestricted inflammatory responses; therefore the family of TLRs may play a pivotal role in the development of autoimmune diseases.

The expression profile of various TLRs is quite different in pSS patients compared to that of healthy individuals (31). Cultured SGECs have been shown to express functional TLR2, TLR3 and TLR4 following treatment with their respective ligands. The TLR1, TLR2 and TLR4 mRNAs are significantly over-expressed in pSS SGECs compared to controls (31,32). The expression levels of TLR2, TLR3 TLR4 and myeloid differentiation primary response 88 (MyD88) are higher in the labial salivary glands of pSS patients compared to controls. Therefore, it is informative to assess them in

different salivary gland cell types, including acinar cells, infiltrating mononuclear cells and ductal epithelial cells. The TLR expression level showed similar pattern in all types of human salivary gland cells (33). As mentioned before, peptidoglycans could activate TLR2, which has been indicated to induce the production of interleukins (ILs) such as IL-17 and IL-23 in the peripheral blood mononuclear cells (PBMCs) of pSS patients via IL-6, STAT3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (34). A recent study revealed that increased number of naïve B cells and decreased number of pre-switched memory B cells were found in pSS patients. However, although TLR7 and TLR9 activity could be actually increased in pSS patients without an upregulation of the receptors, no significant differences were observed in TLR7 and TLR9 expression in B cells between pSS patients and healthy controls (35).

Type I interferon

Approximately 50% of pSS patients have an activated type I IFN in the blood and salivary glands tissue (3). Activation of type I IFN is usually triggered by viral infection. The innate immune response results in the production of IFN in pSS. However, there is also evidence of initiating IFN activation in pSS by RNA-containing immune complexes (e.g., Ro and La autoantibodies complexed to material from apoptotic or necrotic cells) (36). Patients with higher IFN activity are clinically similar: they exhibit hyper-gammaglobulinaemia and they are positive for anti-nuclear antibody (ANA) and anti-Ro/SSA. There is heterogeneity in the type I IFN response with high IFN correlated with a more severe disease phenotype in pSS patients (37).

Altered adaptive immune responses in pSS

The characteristic autoantibodies of SS are anti-Ro/SS-A and anti-La/SS-B, which are directed against the complex ribonucleoprotein antigens physiologically located in the cytoplasm. The presence of these autoantibodies enhances the production of apoptosis-inducing TNF-α. Antibodies against alpha-fodrin and M3 muscarinic receptors can also appear in the serum. The latter marker has parasympathetic effects and inhibits the transport of aquaporin channels to the apical cell surface which leads to the dysfunction of exocrine glands (1). The expression of costimulatory molecules (HLA-DR, -B7, CD40) leads to antigen presentation and T-cell activation, producing chemokines, cytokines and BAFF (38,39). Proinflammatory cytokines, including IL -1, -6, -7, -10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, are released during the activation of infiltrating CD4+ T cells and DCs. The activated T cells enhance the production of IL-1α, IL-6 and TNF-α. Stimulation of BAFF by IFN-α in DC and T cells results in the generation of autoreactive B cells and autoantibody producing plasma cells in pSS (1,20).

The alteration in the proportion of regulatory T cells [IL-10 producing T regulative type 1 (Tr1), CD4+ CD25+ Tregs, transforming growth factor (TGF)-β producing Th3] may also play an important role in the disease pathogenesis (40). The dysfunction of DCs, both myeloid and plasmacytoid cells, leads to the loss of peripheral tolerance by contributing to the persistence of autoreactive T cells, production of pro-inflammatory cytokines, enhanced antigen presentation and activation of inflammatory cells (39).

Abnormalities of glandular homeostasis, Tr1 deficiency and plasmacytoid DC

deregulation comprise an important network in the breakdown of peripheral tolerance and activation of autoreactive immune cells. Eventually, these lymphocytes promote autoantibody production and the further damage of epithelial cells by cytotoxic mechanisms (41).

The hyperactivity of B lymphocytes

The over-activation of B lymphocytes results in hypergammaglobulinemia, autoantibody production, disturbances of B cell subsets, GC formation in the salivary gland and the increased the risk for B-cell lymphoma in pSS (3).

The overexpression of B cell lymphoma apoptosis regulator gene (bcl)-2, BAFF and APRIL (a proliferation-inducing ligand) provides the apoptosis resistance of cells and prolongs their survival (42). The BAFF, one member of the TNF family, is regarded as a potent activator of B cells. BAFF is the natural ligand of three receptors: BAFF-R(BR3), a transmembrane activator and a calcium modulator. Higher serum level of BAFF and increased expression of BAFF in labial salivary glands were found in pSS, which is induced by type I and II IFNs (43,44). BAFF/BR3 pathway is a promising therapeutic target in pSS (45).

Sexual-related hormones and gender bias

The role of sexual-related hormones in the pathogenesis of pSS has been demonstrated in several experimental observations: the presence of estrogen receptors in the salivary glands, sialoadenitis of ovarectomized mice, B-cell lymphoma in estrogen-deficient mice, estrogen deficiency-dependent apoptosis,

autoimmune exocrinopathy, and prevention of glandular epithelial apoptosis and development of sialoadenitis by estrogen substitution (42–44)

. A recent work demonstarted that trisomy X (47, XXX) is associated with a higher prevalence in pSS compared to controls, which is in good agreement with the gender bias in the pathogenesis of pSS. (49)

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, DCs, as well as T and B cells (50). It mainly decreases T helper (Th) cell response and enhances Treg functions. It helps also to increasing the proliferation of monocytes and macrophages but decreases proliferation of T and B cells. According to the study of Cantorna and Mahon in 2004, vitamin D status could be regarded as an environmental factor which affects autoimmune disease prevalence (51,52).

The physiological effects are achieved by binding to a member of the nuclear receptor superfamily which is called the vitamin D receptor (VDR). Only one 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 (53–55).

The VDR gene is located on chromosome 12 (12q12-q14), and till now, more than 470 single nucleotide polymorphisms (SNPs) were reported. Some of the SNPs play a key role in the modification of the uptake of 1,25-$(OH)_2$-vitamin D_3. Therefore, it has been considered that these SNPs could be genetic susceptibility risk factor for the development of autoimmune diseases. Although VDR gene has more than 100 restriction endonuclease cutting sites, 4 of them are located within and/or associated with known polymorphisms: FokI (rs10735810/rs2228570) and the other 3 are in linkage disequilibrium (LD) like BsmI (rs1544410/rs7975128), ApaI (rs7962898/rs7975232) and TaqI (rs731236). The FokI and TaqI SNPs are located in exon 2 and exon 9. The other two, the BsmI and the ApaI are located in intron 8 and intron 9, respectively (54,56).

According to the paper of Szodoray et al. from 2010, there is no significant difference in vitamin D levels between pSS patients and normal controls (57). On the contrary, many studies reported about the association between VDR gene polymorphisms and higher risks of certain autoimmune disease such as SLE (54), RA (2), MS (58), psoriasis (59), Crohn's disease (60), Behcet's disease (61), autoimmune thyroid disease (AITD) (62) and autoimmune Addison's diseases (63). But so far, no study has been performed to evaluate the association between VDR gene polymorphisms and pSS. Therefore, we aimed to determine 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 distributions and allele frequencies in both patients group.

2.3 MicroRNAs

2.3.1 The discovery miRNAs

In the early 1960s, the term of double-stranded RNAs (dsRNAs) was first described in correlation with viral infection. At that time, Montagnier et al. and Baltimore et al. found a small amount of RNA that sediment slightly faster than 16S in the poliovirus-infected HeLa cells. This lower density RNA compared to other viral RNAs were regarded as double-stranded form of poliovirus RNA (64,65).

Two decades later, during the study on the regulation of the heterochronic genes in Caenorhabditis elegans, it was found that mutations in the heterochronic genes lin-4, lin-14, lin-28 and lin-29 cause temporal transformations in diverse cell lines (66,67). In 1993, Wightman et al. identified several copies ranging from 14 to 19 base sequence motif in the lin-14 3'-untranslated region (UTR) that is complementary to a portion of the lin-4 RNAs. All seven sites in the lin-14 3'-UTR must be occupied by small lin-4 RNAs to induce posttranscriptional down-regulation of lin-14 which substantially reduces the LIN-14 protein expression. In other words, these small lin-4 RNAs showing a novel genomic role of regulatory pathway could be functionally considered as the first discovered miRNA (68,69).

Let-7 was reported in 2000 as another heterochronic switch gene in Caenorhabditis elegans. It was recognized to encode a 21-nucleotide regulatory RNA which is

complementary with 3'-UTR elements of the lin-14, lin-28, lin-41, lin-42 and daf-12 genes similarly to that reported previously on lin-4 (70,71). Pasquinelli et al. have detected let-7 RNA expression not only in Caenorhabditis elegans, but also in Drosophila, zebrafish, and in adult stages of annelids and mollusks while they did not detect lin-4 RNA in the above mentioned species. The lin-4 and let-7 RNA genes are not homologous to each other; the investigation of let-7 gave additional support to the function of non-coding RNA molecules (72). The investigation of lin-4, let-7 and other small temporal RNAs (stRNAs) presented similar features in different species in the following one year. More than 70 sequence-specific stRNAs have been reported. The class of stRNAs appear to be diverse and numerous. Consequently, the term miRNA was introduced for referring to stRNAs by a simultaneous publication of three related articles in Science in 2001 (73–75).

Ambros et al. set a standard nomenclature system with expression and biogenesis criteria in 2003. They established the “mir” prefix followed by a dash and a number to define each microRNA. The unique identifying number shows the order of discovery and naming. The capitalization and italics of “mir” prefix direct followed by the number resembles the genes that encode the same specific miRNA (76). As hundreds of miRNA were discovered, more detailed and precise ways were set to distinguish the mature forms and each stem-loop sequences in the nomenclature system. The format “mir-” indicates the primary miRNA transcripts (pri-miRNAs) and precursor miRNA (pre-miRNA) while “miR-” symbolizes the resultant mature miRNA. Suffixes were introduced to represent the homologous mature transcripts encoded by the same miR

number gene. The numerical suffixes followed by a dash demonstrate when the mature miRNAs are identical (eg miR1-1 and miR1-2). The letter suffixes are used while the mature miRNAs have nucleotides difference (eg miR10a and miR10b) (77).

2.3.2 The biology of miRNAs

The majority of miRNA genes are located in intergenic regions or they are oriented as antisense sequences to form independent transcription units. Most of the others reside in intronic regions of protein-coding genes (78). Human miRNAs are not always genomically isolated, sometimes several miRNAs are assembled as clusters for further transcription and expression (79).

MiRNA biogenesis and maturation occur first in the nucleus and then in the cytoplasm with the help of several proteins and enzymes (Fig. 1). The first step in miRNA biogenesis is the generation of pri-miRNAs from DNA molecules in the nucleus of the cell. Most miRNA genes are transcribed by RNA polymerase II to produce a few hundred to thousand nucleotide-long pri-miRNA (78). Pri-miRNAs are both capped and polyadenylated with a typical hairpin structure (80). These pri-miRNAs are recognized by an enzyme-protein complex and further cleaved into 70-100 nucleotide-long pre-miRNA. This complex is composed of Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) and denoted as microprocessor complex (81). Drosha is one of the two members of the ribonuclease (RNase) III family while DGCR8 is the dsRNA-binding protein which is deleted in DiGeorge syndrome (82). The pre-miRNA is then exported to cytoplasm through exportin 5, which is a member of the karyopherin

family of nucleocytoplasmic proteins. Exportin 5 recognizes a two-nucleotide overhang left by Drosha at the 3' end of the pre-miRNA hairpin, this process requires the guanosine triphosphate (GTP)-bound form of the Ran GTPase for providing energy (83).

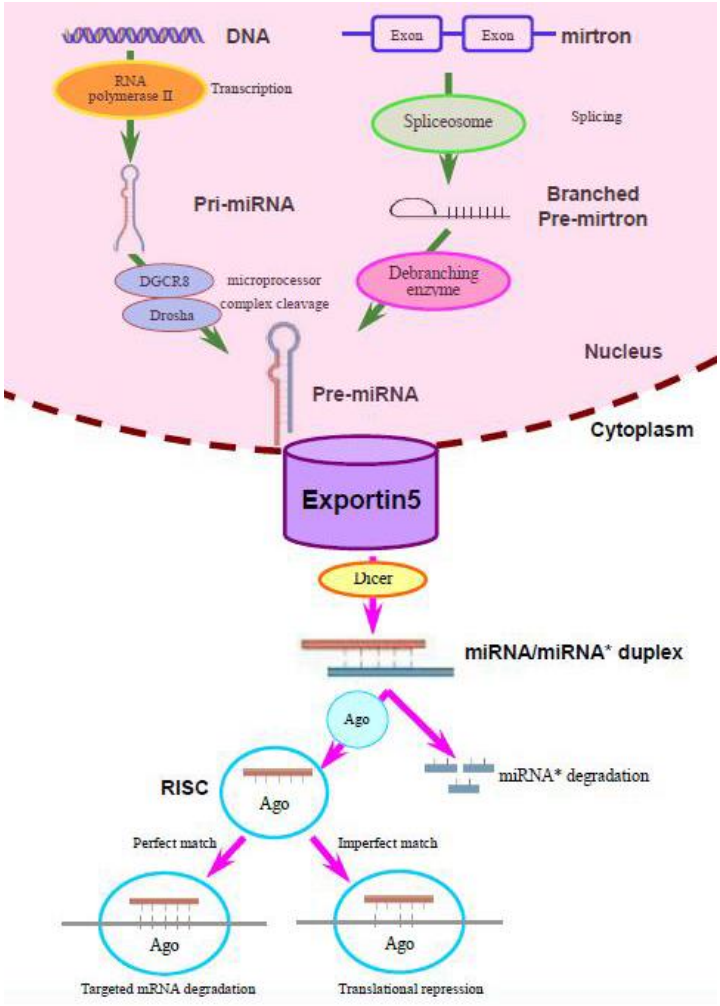

Figure 1. **microRNA biogenesis and mechanisms of action**

Most miRNAs are transcribed from genomic DNA by RNA polymerase II to generate typical hairpin structured primary miRNA transcripts (pri-miRNAs). This pri-miRNAs are recognized by the microprocessor complex (Drosha and DGCR8) and further cleaved into precursor miRNA (pre-miRNA).

The pre-miRNA then exported to cytoplasm through exportin 5. The pre-miRNA is

further cleaved by Dicer into the miRNA/miRNA* duplex. The guide strand loaded into the argonaute (Ago) proteins to generate the RNA-induced silencing complex (RISC), while the passenger strand (miRNA*) would eventually degrade. The perfect complementary base pairing match between miRNA and target messenger RNA (mRNA) induces target mRNA degradation, while the imperfect match results in the repression of the mRNA translation (84).

The noncanonical miRNA biogenesis pathway bypasses the microprocessor complex cleavage processing for another sort of pre-miRNAs, known as mirtrons, which are directly spliced out of introns by spliceosome. The branched pre-mirtrons then undergo lariat-mediated debranching to mimic the structural features of pre-miRNAs (85,86). Interestingly, mirtrons can not only be found in Caenorhabditis elegans and Drosophila, but also reported in mammals (87).

The pre-miRNAs have further processing to yield mature miRNA in the cytoplasm. The second member of the RNase III family named Dicer interacts with both 5' and 3' ends of the pre-miRNA and cleave the hairpin loop, processing to a 19-25 nucleotides miRNA/miRNA* duplex (88,89). The miRNA* regarded as passenger strand since it is less-stable, while the miRNA as guide strand. The miRNA/miRNA* duplex releases the helix structure after loaded into the Ago proteins. The guide strand remains in interaction with Ago to generate the RISC, which facilitate the binding of miRNAs to their targets (90). The passenger strand as complementary strand of the guide strand is degraded as a RISC complex substrate. However, a recent study demonstrated that

several miRNA* are stably expressed and may play an important role, as well (91).

2.3.3 miRNAs: mechanisms of action

The mature miRNA interacts with the 3'-UTR of specific mRNA to regulate gene expression. Target mRNA is recognized by 2-7 nucleotides of the 'seed' region of the miRNA (92). The complementary degree of the base pairing between the miRNA seed region and mRNA define the mechanism of gene regulation (93). When the complementary base pairing is perfect or near-perfect, Ago protein of the RISC complex induces the endonucleotic cleavage of the target mRNA resulting in deadenylation and degradation of the target mRNA fragment. When the base pairing is incomplete, the formation of dsRNA, resulting from the binding of miRNA, leads to translational repression (94–96). Repressed mRNAs aggregate in cytoplasmic foci called P-bodies, which are known sites of mRNA destabilization (97,98).

2.3.4 miRNAs in immune system

MiRNAs play critical roles not only in the development of immune system but also the regulation of both innate and adaptive immunity (99,100). MiRNAs function as translational repressors during stem cell fate and differentiation (101). MiR-181, miR-223 and miR-142s are strongly expressed in hematopoietic cells and play regulatory roles during hematopoietic lineage differentiation (102,103).

Innate immunity

The innate immune system is the first line of host defense and important in mechanisms against invading microorganisms; moreover, it forms the basis of the development of adaptive immunity. Host cells express diverse PRRs, including toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), retinoic acid-inducible gene -I-like-receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs). These can recognize a wide range of PAMPs. These mechanisms trigger the intracellular signaling pathways, which result in releasing of proinflammatory cytokines, chemokines and IFNs, as well as lead to the expression of co-stimulatory molecules (104). TLRs are the most characterized PRRs, which are capable of potently activating different cell types, which could be highly expressed on most immune cells (29). Their downstream signaling pathways lead to the production of a wide range of immune-stimulatory cytokines and chemokines. Aberrant activation of TLRs may result in unrestricted inflammatory responses therefore the family of TLRs may play a pivotal role in the development of autoimmune diseases (30). Among all ten TLR subtypes, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are generally regarded as extracellular receptors. However, the other members of TLR family (TLR3, TLR7, TLR8 and TLR9) locate in endosomal compartments and responsible for the recognition of nucleic acids derived from viruses, bacteria and the host, which comprise of intracellular receptors (105–108). TLR4 can recognize lipopolysaccharides (LPSs), which is the typical endotoxin for gram-negative bacteria. LPS-mediated inflammatory responses consequently induce overexpression of miR-146a/b, miR-132 and miR-155.

Upregulation of miR-146 leads to translational repression of its target genes IL-1 receptor-associated kinase (IRAK) 1 and tumor necrosis factors receptor associated factor (TRAF) 6 (109). miR-146 was recognized as negative regulator of RLRs in the *in vitro* model of mouse macrophages through targeting IRAK1, IRAK2 and TRAF6 (110). Exposure to LPS stimulates TNF-α secretion. Overexpression of miR-155 and the decreased expression of miR-125b may relate with elevated level of TNF-α. It was indicated that miR-155 targets the post-transcriptional regulation of genes coding for proteins enhancing TNF-α translation, including Fas-associated death domain protein, IkappaB kinase epsilon and tumor necrosis factor receptor (TNFR) superfamily-interacting serine-threonine kinase 1, while miR-125b targets the 3'-UTR of TNF-α transcripts (111). In miR-147 knockout mice, increased inflammatory cytokine expression was found in macrophages upon TLR stimulation such as ligands to TLR2, TLR3 and TLR4. Thus miR-147 was regarded as a negative regulator in TLR-activated inflammatory responses (112). A recent study revealed that miR-1303 production is also regulated by the NF-κB pathway (113).

MiR-146a and miR-155 influence IFN-type I synthesis in plasmacytoid DCs mediated by TLR-7 and TLR-9, while in T and B cells, a group of miRNAs including miR-21, miR-126, miR-146a, miR-155, miR-1246 and others might correlate with epigenetic modifications, support abnormal cytosine release, differentiation of cell subsets, B cell hyperactivity and autoantibody production (114).

Adaptive immunity

The adaptive immune system involves both T and B lymphocytes as major cellular components. One of the RNase III family enzymes, Dicer - as mentioned previously - is important in the biogenesis of miRNAs. In the early stage of T cell development, depletion of Dicer leads to reduction of T cell numbers both in the thymus and peripheral lymphoid organs (115). Dicer-deficient Th cells show aberrant cytokine secretion, such as increased expression of IFN-γ in the absence of exogenous cytokines and blocking antibodies (116). In early B cell progenitors, depletion of Dicer results in blocking at the pro- to pre-B cell transition since miR-17 mostly targets genes upregulated in Dicer-deficient pro-B cells (117).

IL-17 produced by Th17 cells are closely related to miR-326 and miR-155. It is shown that overexpression of miR-326 results in increased number of Th17 cells through targeting Ets-1 in MS patients and severe experimental autoimmune encephalomyelitis (EAE) mice (118). MiR-155 on the other hand is essential for DC production of cytokines which induce Th17 cell formation. MiR-155 knockout mice are recognized resistant to EAE (119). MiR-155 is down-regulated in human monocyte-derived DCs in response to LPS-induced inflammatory processes (120). MiR-155 expression is necessary for maintaining Treg cell proliferative activity under Foxp3 regulation in controlling the IL-2 signaling pathway by targeting the suppressor of cytokine signaling (SOCS) 1 (121). Like miR-155, miR-146a is not only relevant to the innate immune system but also critical in the adaptive immune system. Overexpression of miR-146a was found in Treg cells as a response to STAT1activation. The negative regulator of

STAT1 phosphorylation downstream of the IFN-γ receptor is SOCS1, which is additionally associated with Th1-mediated autoimmunity (122). In activated B cells, miR-181b results in the down-regulation of activation-induced cytidine deaminase mRNA and protein levels. By restricting activation-induced cytidine deaminase activity, miR-181b may prevent B cell malignant transformation (123).

The overexpression of miR-148a results in impaired B cell tolerance, which accelerates the development of autoimmune diseases. Moreover, miR-148a inhibits the expression of the autoimmune suppressor Gadd45α, the tumor suppressor phosphatase and tensin homolog (PTEN) and the pro-apoptotic protein Bim and protects immature B cells from apoptosis induced by engagement of B cell antigen receptor (124).

2.3.5 miRNAs in Primary Sjögren's syndrome

Alterations in miRNA regulation seem to be highly related to the development of immune dysfunctions and autoimmunity. Recently, several studies have focused on the role of miRNAs in autoimmune diseases and different expression profiles have been identified as biomarkers of certain autoimmune conditions.

The increase of Ro/SSA and La/SSB autoantigens is a common feature in pSS patients. miRNAs which are suspected to target Ro/SSA and La/SSB mRNAs in pSS are as follows: let-7b, miR-16, miR-181a, miR-200b-3p, miR-200b-5p, miR-223 and miR483-5p. The overexpression of miR-16 in minor salivary glands, miR-200b-3p in SGECs and miR-223 together with miR-483-5p in PBMCs of 29 pSS patients compared to 24 sicca-complaining controls have been shown previously. Significantly decreased

expression of miR200b-5p was reported in pSS patients with mucosa-associated lymphoid tissue lymphoma compared to pSS patients (125). Another study demonstrated the critical function of La/SSB as a global regulator of miRNA expression. The expression levels of La/SSB and the Dicer enzyme correlate positively with the risk of cancer development (126). Alevizos et al. generated microRNA microarray profiles from the minor salivary glands of patients with pSS who had low-grade or high-grade inflammation and impaired or normal saliva production, and compared the results with that observed in healthy control subjects. They found the over-expression of hsa-miR-768-3p, and down-regulation of hsa-miR-574 in patients' biopsies (127). Previously, our workgroup not only confirmed the over-expression of miR-146a/b in PBMCs of pSS but also demonstrated the unanticipated over-expression of its functionally targeted gene, TRAF6. Furthermore, we also reported decreased gene expression of IRAK1 (128). The over-expression of TRAF6 is surprising since miR-146a could inhibit the expression of TRAF6 (129). Recently, enhanced expression of miR-155 was reported in untreated Sjögren's syndorme (130). Of note, pSS patients treated with immunosuppressants also showed the over expression of miR-155. On the contrary, in Asian population the relative expression of miR-155 was lower in PBMCs of pSS patients not receiving any immunosuppressive treatment than the controls, which may emphasize the importance of the diverse genetic background of different ethnicities (131,132). A recent study demonstrated the over-expression of miR-181a in the PBMCs of pSS patients, which was associated with the up-regulation of several

virus-derived miRNAs, suggesting that viral infection may play a role in the disease [77].

Up-regulated expression of miR-34b-3p, miR-4701-5p, miR-609, miR-300, miR-3162-3p, and miR-877-3p in pSS monocytes compared to controls may relate with opposing of TGF-β signaling pathway and TLR/NF-κB pathways induced pro-inflammatory IL-12 secretion (134).

3. Aims of the studies

For the better understanding of the pathogenesis of pSS, with a special emphasis on the fields of gene polymorphisms and miRNAs expression profiles, we have formulated the following studies:

3.1 Study on vitamin D receptor gene polymorphisms

We determined the frequency of BsmI (*rs1544410*), FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) polymorphism of VDR gene in pSS patients together with healthy controls in order to analyze whether a relationship exists between polymorphisms/haplotypes in the VDR gene and susceptibility to Sjögren's syndrome. Based on the newly developed bioinformatics background we also established haplotype analysis.

3.2 Study on miR-155 and its target gene, SOCS1

We analyzed the expression rate of miR-155 and its functionally linked gene, the SOCS1 in the PBMCs of patients with pSS and healthy controls and to detect the association between miR-155 and SOCS1 in pSS patients.

3.3 miRNA expression profiling in pSS and SLE patients

We examined miRNA expression profiles in patients with pSS and SLE in order to remark the associations between the dysregulated miRNAs' expression and the

development of the diseases. Since the immunopathogenesis of pSS and SLE characterised by pronounced B cell hyperactivity along with specific autoantibody production, we paid a special attention on the association between miRNA expression levels and altered peripheral B cell distribution. Additionally, contrary to microarray analysis, the RNA sequencing technique gave us an opportunity to detect and recognize miRNAs with SNPs, as well.

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, Institute of Medicine, University of Debrecen. The diagnosis of pSS was established according to the European-American consensus criteria (135). 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 incompliance 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, RP 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

Peripheral blood was collected in EDTA vacutainers from all patients and controls. Blood samples were stored at 4 °C before extraction. High molecular weight DNA for genotyping was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Full blood samples were centrifuged for 10 min at 3000rpm. The amount of 200µl from buffy coat layer, which contains white blood cells and platelet concentrate, was transferred into Eppendorf tubes. After mixed well with the same amount of cell lysing buffer and 20µl protease, it was incubated at 56°C for 10 min. The lysate was resuspended by 200µl ethanol and centrifuged at 13000rpm for 15 sec. The mixture was applied into the spin column and spin (at 13000rpm) twice for 1 min each time. 500µl Buffer AW1 was added to wash. It was washed again with 500µl Buffer AW2 then 100µl Buffer AE was added and eluted in room temperature for 10 min. The concentration and purity of DNA were quantitated by measuring UV absorbance at 260/280 nm and stored at -20°C until analysed (136).

4.1.3 Genotyping of the BsmI polymorphism

Identification of the BsmI (*rs1544410*) polymorphism was performed with 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 (137). The PCR reaction was carried out in a 20µl reaction volume containing 1.0µl genomic DNA (50ng/µl), 1.0µl Taqman® SNP Genotyping assay (Taqman® probes) (20x), 10.0µl Taqman® Universal PCR Master Mix (2x, 4331182, Applied Biosystems) and 8.0µl DNase-free water. Reactions were set up in duplicate. The program was running as follows: initial denaturing at 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase; 40 cycles of 92 °C for 15 s as denaturation, 60 °C for 1 min as primer annealing and extension.

4.1.4 Genotyping of FokI , ApaI and TaqI polymorphisms

The genotype for FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) 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 restriction digestion were separated on 3 % agarose gel and visualized with UV light (138).

FokI (rs10735810)

PCR amplified a selected fragment of 265bp by using specific primers, which was carried out in the mixture of 4µl genomic DNA (100ng/µl), 5µl 10xDreamTaq buffer, 1mM dNTP, 2µl of each primer as following: 5'-AGC TGG CCC TGG CAC TGA CTC

TGC TCT-3’ (F) and 5’-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3’ (R) (BioBasic Canada Inc.) and 1µl of DreamTaqI polymerase. The program was running as follows: initial denaturing at 95 °C for 10 min; 45 cycles of 95 °C for 30 s as denaturation, 70 °C for 30 s as primer annealing and 72 °C for 30 s as elongation; final incubation at 72 °C for 5 min.

The 20µl PCR product was digested by adding 2.5µl FastDigest Green Buffer, 0.5µl FastDigest FokI restriction endonucleases (Fermentas) and 2.0µl distilled water. The digestion took 1 hour at 37°C. The FokI restriction enzyme recognizes the sequence of 5’…GGATG(N)…3’ and cuts it if T nucleotide is present and reveals the C/T substitution in exon 2 of VDR gene. Then gel electrophoresis was performed in agarose gel as described before. Since the buffer already contains the loading dye, only 15µl digested product was needed for running the gel (139) (Fig. 2).

ApaI (rs7975232)&TaqI (rs731236)

PCR amplified a selected fragment of 740bp by mixture of 2µl genomic DNA (100ng/µl), 15µl Immumix (Bionline) and 2µl 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 program was running as follows: initial denaturation at 94 °C for 4 min; 40 cycles of 94 °C for 30 sec as denaturation, 70 °C for 1 min as primer annealing and 72 °C for 1 min as elongation; final incubation at 72 °C for 4 min. Ten µl PCR product was digested by adding 2.0µl 10xBuffer B with BSA, 1.0µl ApaI restriction endonucleases (Fermentas) and 7.0µl distilled water. The digestion took 4 hours at 37°C. The ApaI restriction enzyme recognizes the sequence of

5'…GGGCCC…3' and reveals the C/A substitution in intron 9 of VDR gene. Then we performed the 3% agarose gel electrophoresis. 10µl digested product together with 2µl loading dye were needed for running the gel. The program was carried out at 150V for 50 min. After electrophoresis, gel could be taken photo under UV light using Multiimage Light Cabinet by Alpha Imager 1220 analyzer (Alpha Innotech Co.),

The other 10µl PCR product was digested by adding 2.0µl 10xBuffer TaqI with BSA, 0.5µl TaqI restriction endonucleases (Fermentas) and 7.5µl distilled water. Incubated in 65°C for 1 hour is needed for digestion. TaqI restriction endonuclease enzyme recognizes the sequence of 5'…TCGA…3' and reveals the T/C substitution in exon 9 of VDR gene (53). (Fig. 2)

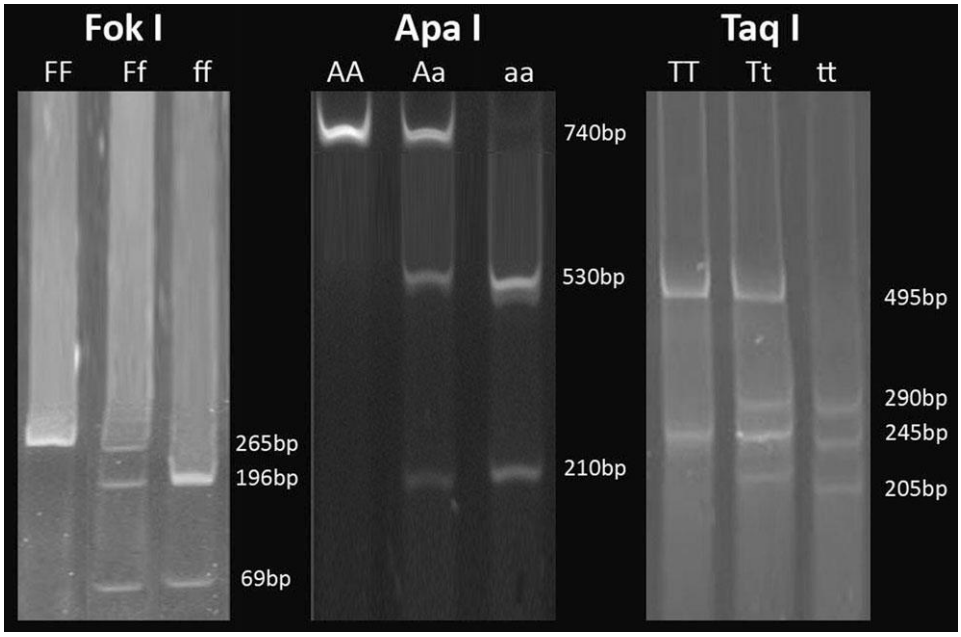

Figure 2. **Genotyping of the FokI, ApaI, and TaqI VDR polymorphisms**

The PCR products were digested with FokI, ApaI, and TaqI digestion of the amplified region of the VDR gene producing different fragments indicative for the specific genotypes. The absence and/or presence of the enzyme recognition site were identified

by SYBR Green I staining of fragments separated in a 3 % agarose gel. Genotypes were assigned as FF, Ff, and ff for the VDR-FokI polymorphisms; AA, Aa, and aa for the ApaI polymorphisms; TT, Tt, and tt for the TaqI polymorphisms

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 ($\chi 2$) test using SPSS 20.0 statistical software. The P value less than 0.05 was regarded as statistically significant. Haplotype analysis was done by the 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 LD between the VDR gene polymorphisms was computed, and LD plots were constructed by Haploview software version 4.2 (140).

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, Institute of Medicine, University of Debrecen. The diagnosis of pSS was established according to the European–American consensus criteria (135). Among patients, 17 showed EGMs, while 6 had only glandular symptoms. The distribution of EGMs of pSS patients were as follows: polyarthritis n=10, RP n=10, thyreoiditis: n=3, vasculitis n=2, lymphadenopathia 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 sialoadenitis. 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 (Ig G). 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, cDNA 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. cDNA samples were stored at -20 ºC 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). Twenty 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). Reactions were placed in a Corbett Rotor-Gene 3000 Sequence Detection System at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15s and 60 °C for 1 min. 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 $2^{-\Delta\Delta Ct}$ 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 µl 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. Thermal cycling was initiated by incubation at 50 °C for 2 min and 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase, respectively. After this initial step, 50 cycles of PCR were performed. Each PCR cycle consisted of heating to 95 ºC for 15 s for melting and 60 ºC for 1 min for annealing and extension. 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 $2^{-\Delta\Delta Ct}$ 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, UDA) software 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 ± 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, Institute of Medicine, 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 (135). 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, RP 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 (141,142). 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. 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 accordance with the Declaration of Helsinki.

	pSS patients	SLE patients	Healthy controls
Number	8	8	7
Age in years, mean ± SD [range]	55.12 ± 7.49 [42 - 64]	45.88 ± 10.58 [36 - 66]	49.78 ± 7.31 [39 - 61]
Gender, male/female	0/8	0/8	0/7
Disease duration in years mean ± SD [range]	13.40 ± 7.94 [3-22]	14.14 ± 9.14 [2-32]	n.a.
Extraglandular involvement, n (%)	4 (50)	n.a.	n.a.
SLEDAI score	n.a	1.75 ± 1.67 [0 - 4]	n.a.

Table 1. Demographic and clinical data of study individuals.

pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; SD, standard deviation; SLEDAI, systemic lupus erythematosus disease activity index

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 (143). For identification of B cell subsets, we used the combination of IgD-fluorescein isothiocyanate (FITC)/CD27-phycoerythrin (PE)/CD19-phycoerythrin-Cyanine dye 5 (Beckman Coulter Inc, Fullerton, CA, USA and Immunotech, Marseille, France) and CD38-FITC/CD27-PE/CD19-PE-Cy5/CD24-allophycocyanin (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.

Genotype distributions distribution for the four polymorphisms studied are summarized in Table 3. 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 stratified into GS cases and EGMs cases.

5.1.2 Genotype analyses

No significant difference was found in the genotype distribution when the VDR gene polymorphisms of pSS patients and healthy individuals were compared (Table 2). The same result has been confirmed between GS cases and EGMs cases. However, we recognized a slightly increased 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 the GS group and the group of healthy controls, 53.85, 41.51, and 39.79 %, respectively.

These differences have not been proven significant. Genotype and allele frequencies of VDR gene polymorphisms did not differ between pSS cases and controls in any comparison performed.

BsmI Genotypes	**EGMs**	**GS**	**Patients**	**Controls**
BB	9.60%	13.46%	12.60%	15.9%
Bb	55.70%	40.38%	47.41%	46.2%
bb	34.60%	46.15%	40.51%	37.8%
P-value	0.3966	0.5868	0.6791	
BsmI Alleles	**EGMs**	**GS**	**Patients**	**Controls**
b	0.63	0.66	0.64	0.61
B	0.37	0.34	0.36	0.39
ApaI genotypes	**EGMs**	**GS**	**Patients**	**Controls**
AA	17.31%	22.64%	20%	24.73%
Aa	57.69%	39.62%	48.57%	50.54%
aa	25%	37.74%	31.43%	24.73%
P-value	0.5045	0.2804	0.5331	
ApaI Alleles	**EGMs**	**GS**	**Patients**	**Controls**

A	0.46	0.42	0.44	0.5
a	0.54	0.58	0.56	0.5
TaqI genotypes	**EGMs**	**GS**	**Patients**	**Controls**
TT	32.69%	41.51%	37.14%	40.86%
Tt	53.85%	41.51%	47.62%	39.79%
tt	13.46%	16.98%	15.24%	19.35%
P-value	0.2959	0.9887	0.5905	
TaqI Alleles	**EGMs**	**GS**	**Patients**	**Controls**
T	0.6	0.62	0.61	0.61
t	0.4	0.38	0.39	0.39
FokI genotypes	**EGMs**	**GS**	**Patients**	**Controls**
FF	34.62%	33.96%	34.28%	42.25%
Ff	50%	45.28%	47.62%	43.66%
ff	15.38%	20.76%	18.10%	14.08%
P-value	0.7033	0.6061	0.6133	
FokI Alleles	**EGMs**	**GS**	**Patients**	**Controls**
F	0.6	0.57	0.58	0.64

f	0.4	0.43	0.42	0.36

Table 2. Genotype frequencies and allele distribution in Hungarian pSS patients and controls.

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 3. The baT and BAt haplotypes were found to be the most frequent haplotypes in both the patient group (51 and 33 %) and the control group (48 and 31 %). These three-marker haplotypes were identified as the most frequent ones and corresponded well with the haplotypes reported by Morrison et al. (144). 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.

Three-marker haplotype	**Frequency (%)**	
	Patients	**Controls**
BaT	0	0

Bat	0	3.16
bAt	1.55	1.69
bat	1.87	4.16
BAT	2.31	1.02
bAT	9.16	10.16
BAt	33.59	31.46
baT	51.52	48.35

Table 3. Calculated haplotype frequencies among the VDR-BsmI, VDR-ApaI, and VDR-TaqI polymorphisms in patients and controls

Pairwise LD was computed and LD plots were constructed using the Haploview software version 4.2. LD analysis revealed a very strong LD (r^2>0.8) between the ApaI and TaqI polymorphisms, a strong LD (r^2 between 0.67 and 0.7) between the BsmI and ApaI or BsmI and TaqI polymorphisms, and a very weak LD (r^2<0.3) was observed between the 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 the BsmI and ApaI, TaqI polymorphisms, the ApaI and TaqI polymorphisms (Fig. 3). No LD was observed between the FokI and other polymorphisms in patients.

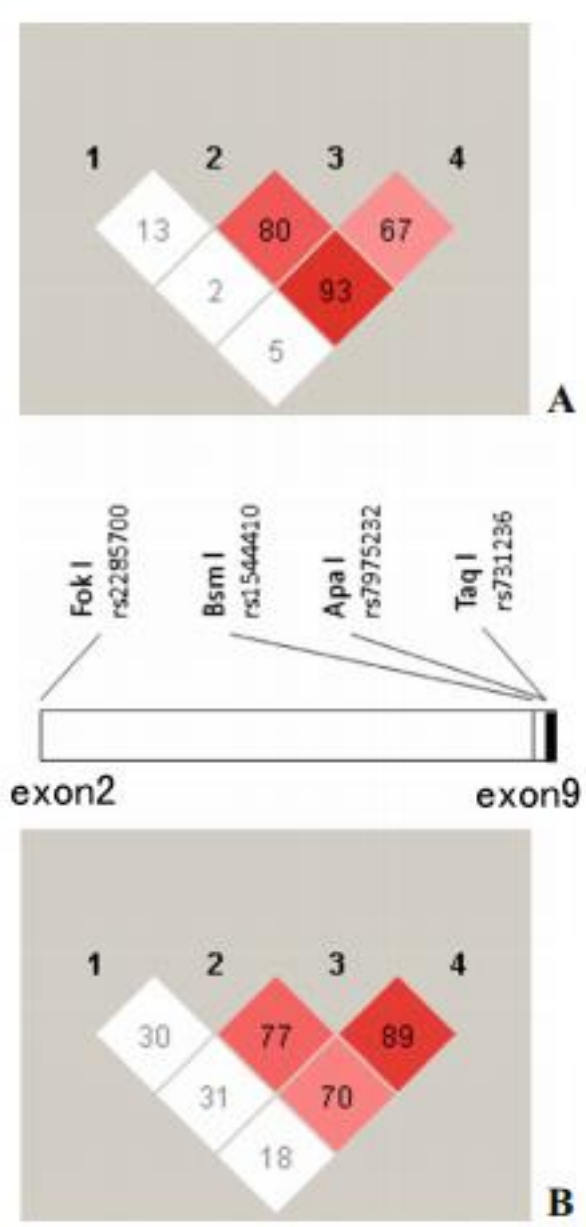

Figure 3. **Four common gene polymorphisms and pattern of linkage disequilibrium (LD) of the VDR gene in Sjögren's syndrome patients (A) and in healthy controls (B)**

Graphical presentation of the VDR gene with the location of polymorphisms studied. Numbers in the boxes represent the correlation coefficient value of LD (r^2) value multiplied by 100. The intensity of the dark color of the boxes represents strength of

linkage disequilibrium (r^2) with dark boxes having high LD and white boxes having low LD.

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 patents: 2.65 ±1.68 *versus* 1.0; *P*= 0.003). These data are shown in Fig. 4.

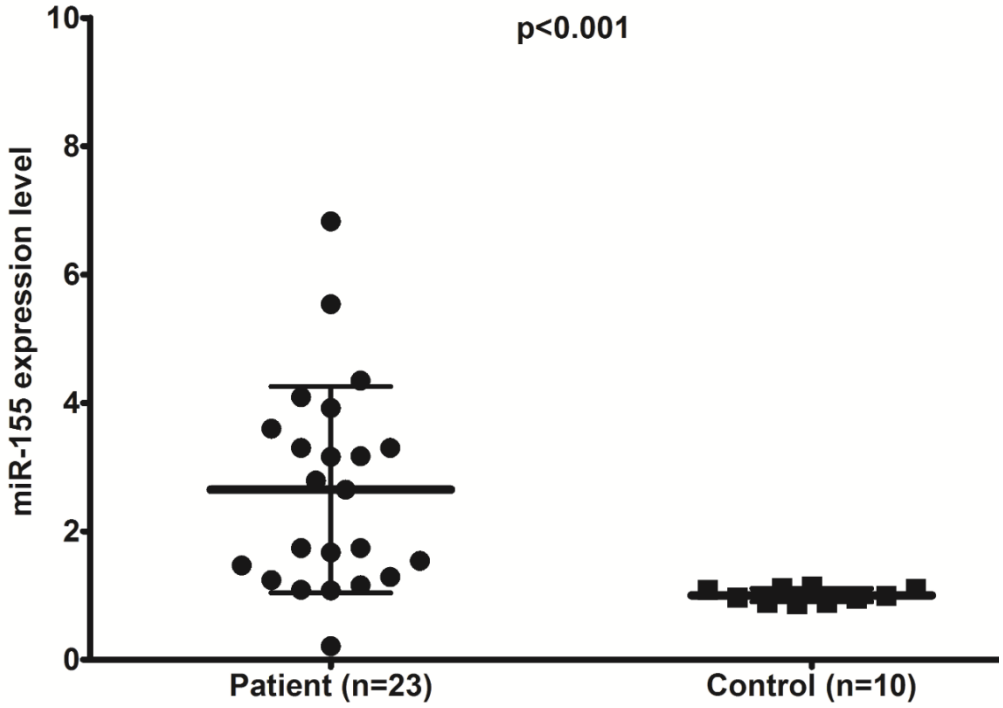

Figure 4. **Over-expression of miR-155 in pSS patients compared with normal controls**

Real-time polymerase chain reaction quantification of miR-155 template in relative

values is presented in a dot-plot diagram. Bars show the mean and the standard deviation (SD). The numbers of patients and healthy individuals are given (n). Significant over-expression of miR-155 was found in pSS compared to healthy controls (*P < 0.001).

5.2.2 Over-expression of SOCS1 in pSS patients

Furthermore, 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; $P < 0.001$). These data are shown in Fig. 5.

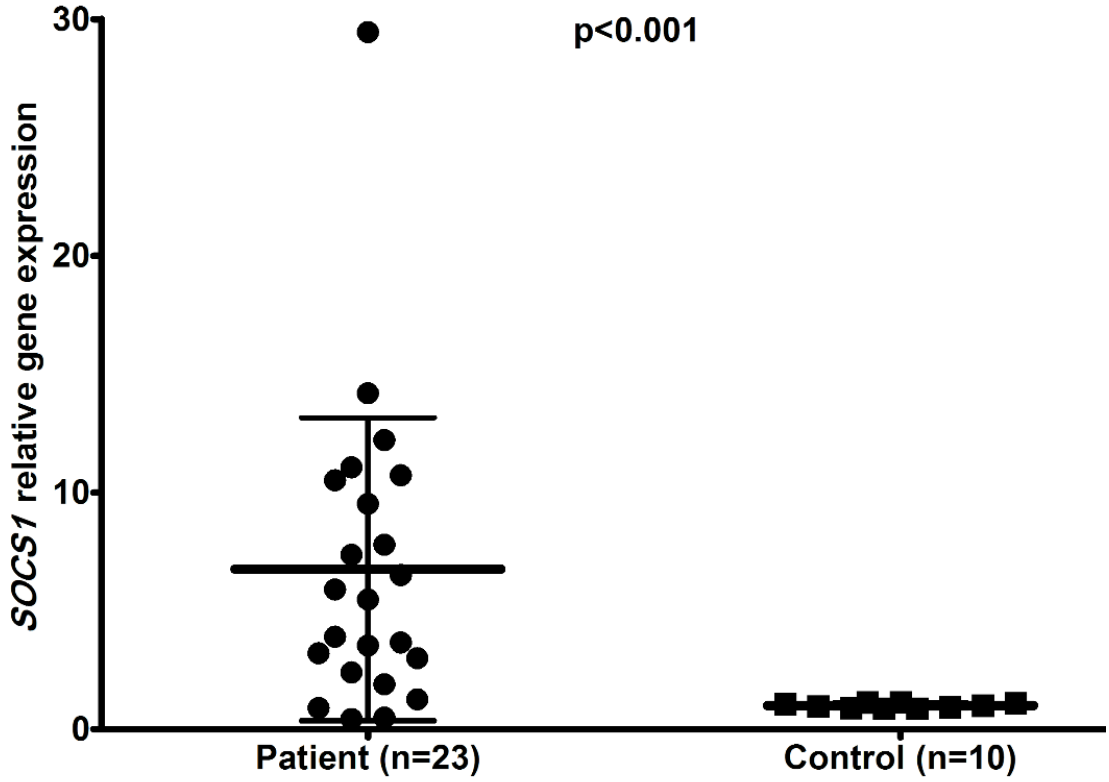

Figure 5. **Over-expression of SOCS1 in pSS patients compared with normal controls**

Real-time polymerase chain reaction quantification of SOCS1 in relative values is presented in a dot-plot diagram. Results are calculated as relative gene expression values. Bars show the mean and the standard deviation (SD). The numbers of patients and healthy controls are given (n). Significant over-expression of SOCS1 was found in pSS patients compared to healthy controls (*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 showed 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 the patients' group compared to healthy controls (Fig.s 6).

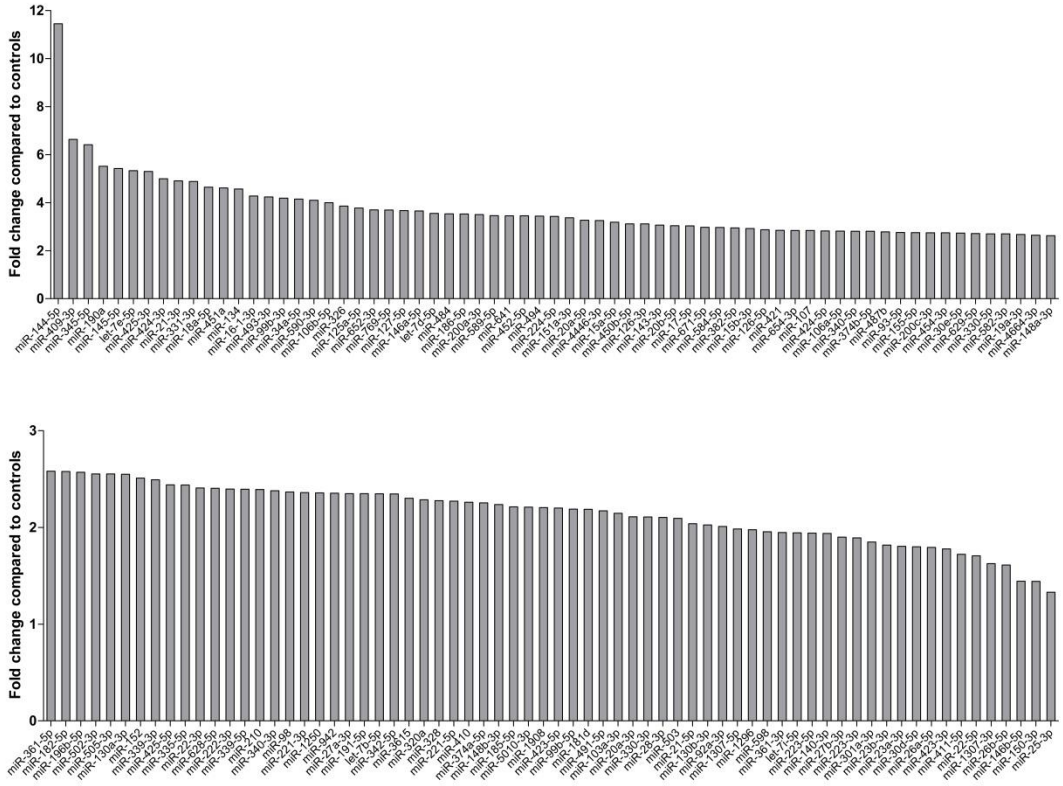

Figure 6. **Fold changes of microRNAs in SLE patients compared to controls.** MicroRNAs (miRNAs) were analysed with Illumina next-generation sequencing technology in SLE patients compared to controls. A total of 135 miRNAs were over-expressed and 113 miRNAs of them showed more than 2-fold difference. Particularly, miR-144-5p, let-7e-5p, miR-145-5p, miR-190a, miR-345-5p, miR-409-3p and miR-425-3p show more than 5-fold change in the patients' group compared to healthy controls.

In the pSS group, 25 miRNAs were significantly over-expressed 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 (Fig.s 7 A and B).

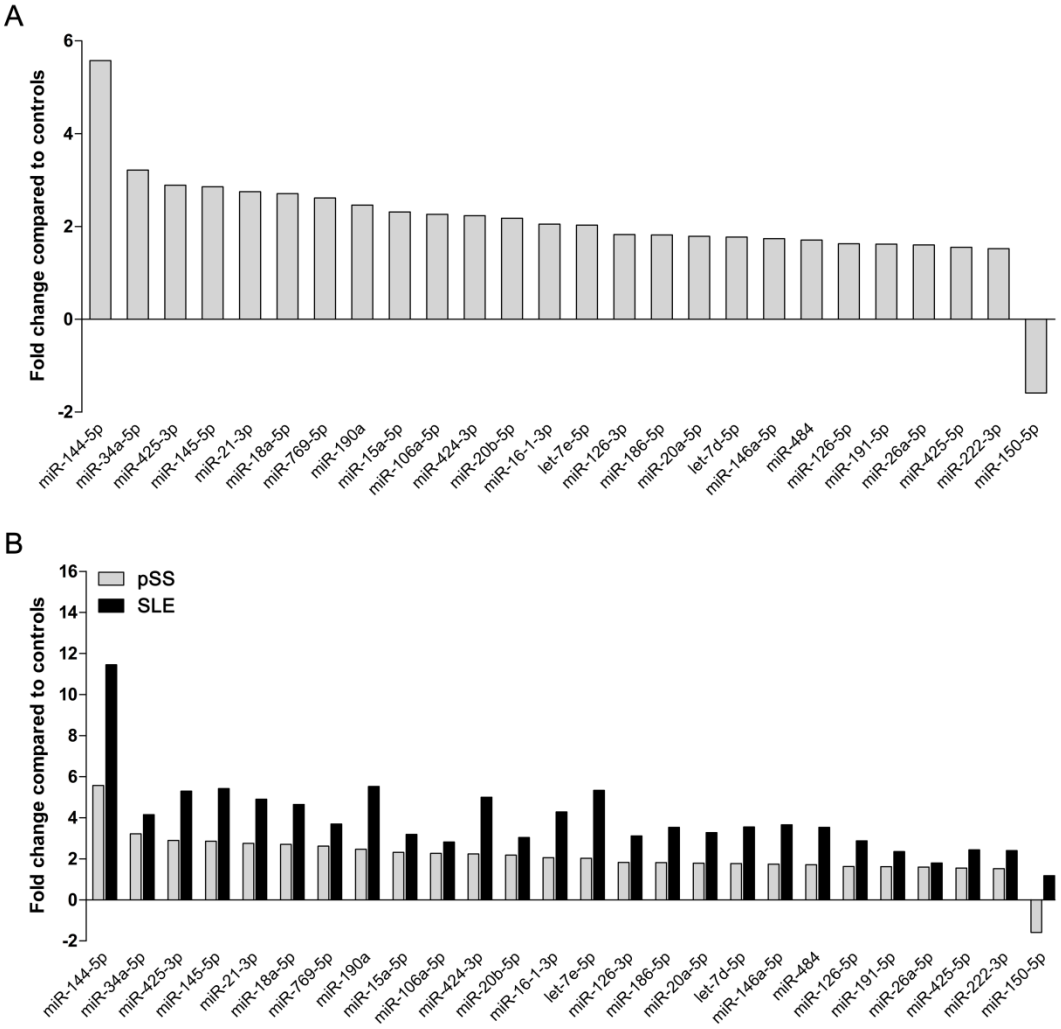

Figure 7. **Fold changes of microRNAs in pSS and SLE patients compared to controls.** (A) A total of 26 microRNAs (miRNAs) showed altered expression levels in patients with pSS compared to controls; among them, 14 miRNAs displayed more than

2-fold change. Expression of miR-150-5p was down-regulated in pSS. (B) All of these miRNAs were also analysed in patients with SLE and found to be elevated as well.

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 total group of pSS patients, compared to the group of 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 were significantly up-regulated in SLE compared to pSS. More than half of those have more than 2-fold change (Fig. 8).

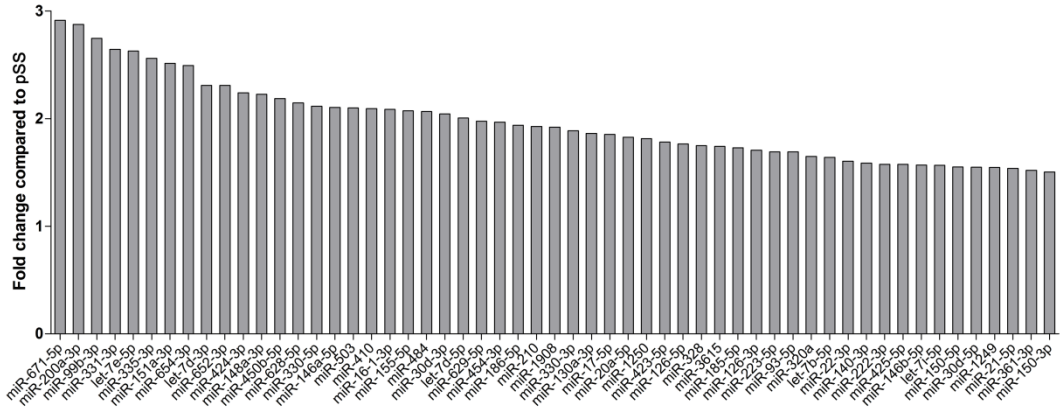

Figure 8. **Fold changes of microRNAs in SLE patients compared to pSS patients.** Fifty-five microRNAs (miRNAs) were significantly up-regulated in SLE patients compared to pSS patients. At least half of them have more than 2-fold change.

We have constucted a heat map (Fig. 9) to represent the detected miRNA expression differences (145) in the PBMCs of SLE and pSS patients and control subjects.

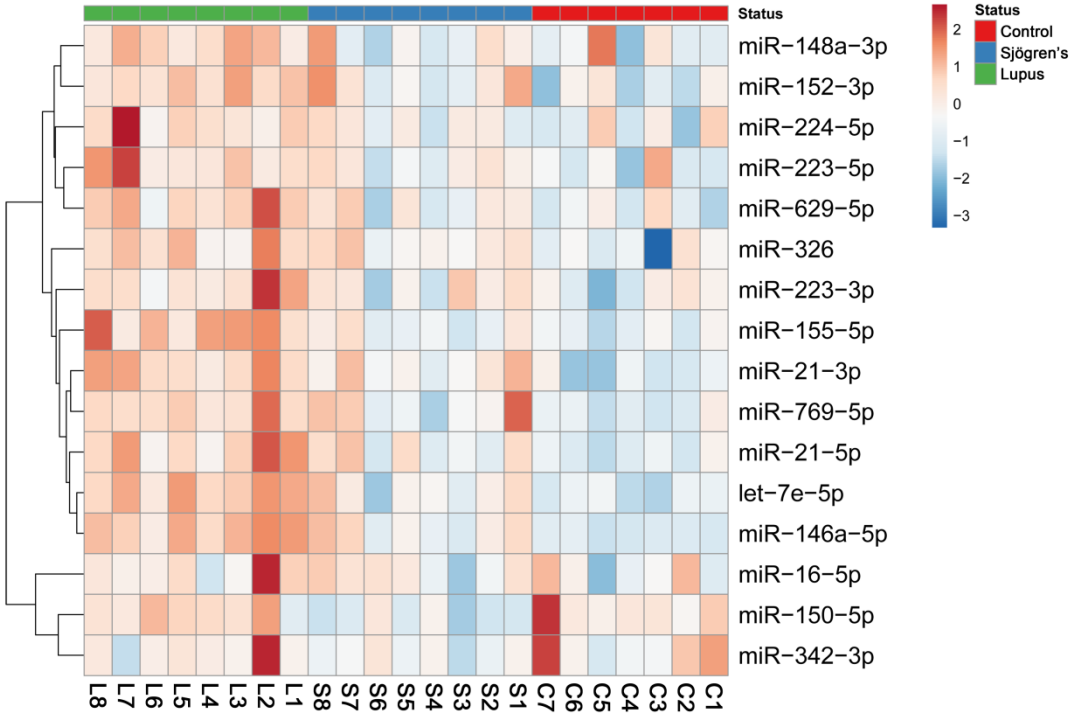

Figure 9. **Expression profile of certain microRNAs in study individuals.** Heatmap was generated using the expression data of 16 miRNAs potentially contribute to autoimmune disorders. The rows represent the individual miRNAs and columns represent our study individuals. Elevated expression level was signed by red colour.

As a next step, we analysed the associations between the expression levels of miRNAs and the measured B lymphocyte ratios within PBMCs. 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) (Fig.s 10 A and B).

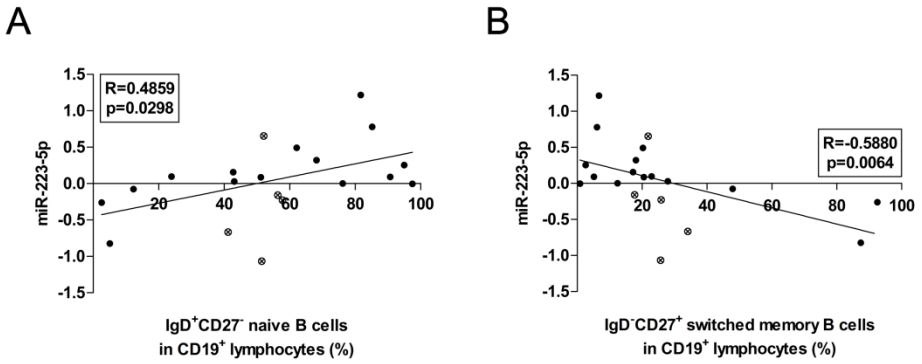

Figure 10. **Associations between the expression levels of miR-223 and the percentages of peripheral lymphocyte subsets.** The expression levels of miR-223-5p correlated positively with the proportions of naive B cells (A) and showed a negative correlation with switched memory B cell percentages (B). Each data point represents an individual subject. Black dots represents patients, while clear dots with an x show control subjects.

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) (Fig.s 11 A and B). Furthermore, DN B cell ratios also showed significant positive correlations with the expression levels of miR-155-5p (R = 0.5410, p = 0.0204) (Fig. 11C). Additionally, we revealed a positive correlation between miR-342-3p expression levels and percentages of plasmablasts (R = 0.5215, p = 0.0265) (Fig. 11D).

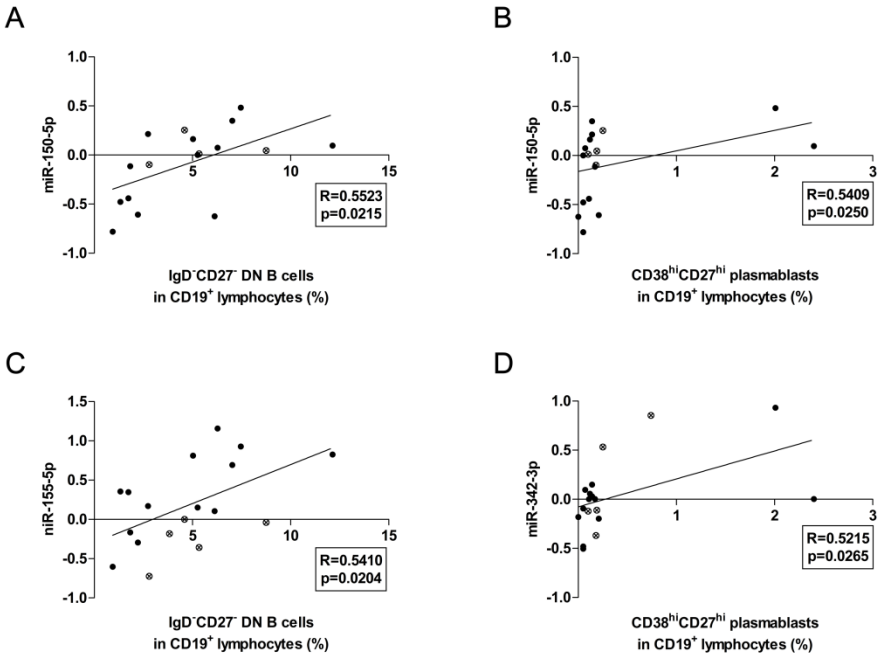

Figure 11. **Associations between the expression levels of certain microRNAs and the percentages of peripheral lymphocyte subsets.** The expression of miR-150-5p correlated positively with the ratios of both DN B cells (A) and plasmablasts (B), while other microRNAs (miRNAs), namely miR-155-5p (C) and miR-342-3p (D) showed positive associations with DN B cell and plasmablast ratios, respectively. Each data point represents an individual subject. Black dots represents patients, while clear dots with an x show control subjects

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 (146). 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 (54), RA (2), MS (58), psoriasis (59), AITD (62) and autoimmune Addison's diseases (63). 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 (147).

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 (148). 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 (149). 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 (57). 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 (57).

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 (121,150,151). The high level expression of miR-155 was found to suppress the expression of SOCS1 in the PBMCs of patients with RA recently (152). pSS patients treated with immunosuppressant also showed over expression of miR-155(131) while in Asian population the relative expression of miR-155 was lower in PBMCs of pSS patients (not receiving any immunosuppressant) compared to controls (132). 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(153). 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 (154). The involvement of EBV infection in the insurgence and persistence of Sjögren's syndrome is also an accepted aspect in the pathogenesis of disease(155). 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 (156). 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 (157). In addition, EBV virus latent membrane protein 1 (LMP1) could up-regulate miR-155 in nasopharyngeal carcinoma cell line (158).

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 (159). 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(160). 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 (128). 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 moderate 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 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 IRAK1 and TRAF6, thus it serves as a negative feed-back for immune activation (109). In T and B cells, a 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 (114). Our workgroup previously reported the over-expression of miR-146 in pSS patients compared to controls (128). Regarding SLE, a former study reported the decreased expression of miR-146a in the PBMCs of Chinese SLE patients (161). 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 (162,163). miR-16 is one of the critical regulators of TLR-mediated immune responses, and it can promote NF-kB regulated transactivation of the IL-8 gene by the suppression of silencing mediator for retinoid and thyroid hormone receptor (164). MiR-16 was found to be up-regulated in patients with RA (165,166), 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 (167). 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 Gadd45α, the tumor suppressor PTEN and the pro-apoptotic protein Bim (124). 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 (168). 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 (169). In autoimmune disorders such as rheumatoid arthritis, systemic sclerosis or psoriasis, miR-155 showed higher expression in patients' PBMCs (84). 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 (170,171). 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 (172); while on the contrary, up-regulation was shown in SLE patients in Asian population (166). 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 (173). 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 (174). 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 (175), 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 (176). 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 (177). MiR-150-5p was reported to be over-expressed in autoimmune pancreatitis compared to chronic pancreatitis, pancreatic cancer and healthy controls (178). 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 (143). 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. References

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8.2 List of publications

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Registry number: DEENK/72/2017.PL
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., Póliska, 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 Sjogren'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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5. **Chen, J. Q.**, Szodoray, P., Zeher, M.: Toll-Like Receptor Pathways in Autoimmune Diseases.
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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.
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   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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9. Keywords

Primary Sjögren's syndrome (pSS); vitamin D receptor (VDR) gene polymorphism [BsmI (*rs1544410*), FokI (*rs10735810*), TaqI (*rs731236*) and ApaI (*rs7975232*) polymorphisms]; microRNA (miRNA); Suppressor of cytokine signaling (SOCS)

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11. Appendix

Publications related to the dissertation