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Expression profile of genes regulating cellular response to cytokine stimulus in Sjögren's syndrome

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Abstract

The existence of abnormal cytokine profile in Sjögren's syndrome (SS) may contribute to find potential SS biomarkers and accompanying comorbidities. We aimed to investigate and compare gene expressions in the labial salivary glands of SS and of healthy subjects (HS) by means of microarray analysis. The study group comprised 8 SS patients and 8 HS in microarray analysis. The relative gene expression changes were validated with real time quantitative polymerase chain reaction in 25 SS and 20 HS. Among the differently expressed genes belonging to the "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response" ontology groups with a fold change >1.5 and with a p value < 0.05, the adiponectin, C1Q and collagen domain containing (ADIPOQ), S100 calcium binding protein A9 (S100A9), leptin receptor (LEPR), and fatty acid binding protein 4 (FABP4) gene expressions in SS group were higher than in HS. Increased expression of the assessed genes are determined by the presence of SS and the accompanying inflammation and may be a valuable diagnostic biomarker of SS. Their selected systemic effects may suggest their role as potential prognostic biomarkers of the risk of accelerated atherosclerosis and cardiovascular complications in SS.

Running title: Cytokines in Sjögren's syndrome

Keywords: Sjögren's syndrome, adiponectin, fatty acid binding protein, cytokines, labial salivary glands, leptin receptor

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Introduction

Sjogren's syndrome (SS) is a chronic inflammatory, autoimmune disease of the exocrinopathy type that mainly involves the salivary and lacrimal glands. It is characterized by the presence of focal lymphocytic inflammatory infiltrations in target tissues termed in salivary glands as focal lymphocytic sialadenitis (FLS), which lead to its main clinical manifestation that is dry mouth [1,2]. Xerostomia decreases general oral health and predisposes to tooth caries.

Although multiple organ systems may be affected in SS, causing a broad spectrum of extraglandular manifestations, its characteristic feature is a predominance of the local symptoms limited to the exocrine glands over those related to other organs and systems. Moreover, some SS patients do not progress to the more severe disease state and to the extraglandular disease manifestations. However, activation of the immune system and a perpetuated inflammatory process in SS may play a role as the predisposing factor for the development of other associated comorbidities. They act as disease-dependent hidden subclinical pathologies, which under favorable conditions can develop into full-blown disease entities. SS and the pathological processes induced by it may favor the development of other diseases that could not only be explained by the possible existence of concurrent traditional risk factors and genetic determinants. It is believed that a complex interaction between traditional and genetic but also disease-specific features leads to a premature and accelerated pathological processes in autoimmune diseases such as SS. These observations necessitate the implementation of a new strategy in predicting, preventing and treating selected pathologies in SS patients. A key player in this strategy is to look for biomarkers and genes related to these pathological processes. Identification of new biological markers in SS patients that reflect the changes characteristic to it may help in the early diagnosis of SS and accompanying comorbidities, the selection of SS patients who are most at risk, as well as increase their monitoring and implement appropriate preventing therapy.

Inflammatory cytokines that maintain chronic inflammation and reflect activation of inflammatory cells are potential markers helpful in the diagnosis and monitoring of SS and associated comorbidities. Previous studies revealed changes in the inflammatory cytokine profile due to inflammation of the salivary glands in patients with eating disorders such as anorexia [3]. Apart from their traditional pro-inflammatory or anti-inflammatory function, cytokines play a multifactorial and complex role in other processes including the initiation of vascular endothelial damage, lipoprotein metabolism changes, inducing a proatherosclerotic profile and insulin resistance as well as the thrombosis promotion by the secretion of procoagulant factors [4]. Moreover, cytokines may play autocrine and paracrine action regulating function of the exocrine glands in SS. The potential source of biomarkers involved in autoimmune diseases are adipocytes, which are widely distributed within loose connective tissue. Adipocytokines secreted by these adipocytes exert an influence on the immune system by activating macrophages, secreting proinflammatory cytokines and stimulating neutrophil chemotaxis [5]. The participation of adipocytes and their secreted adipokines is particularly strongly expressed in SS, where the destruction of the secretory epithelium by inflammatory infiltrates in the salivary glands is correlated with the subsequent proliferation of adipose tissue and increased fibrosis. Labial salivary glands (LSG) through their participation in the SS diagnosis, based on the occurrence of FLS in microscopic examination of salivary gland specimens and in its pathogenesis by presenting the activation of immune cells and the presence of adipose tissue, make them a valuable source of biomarkers involved in the pathology of SS and the accompanying subclinical disorders.

Therefore, we performed gene expression profiling to better understand and identify potential biomarkers for the prognosis, prediction or diagnosis of this condition. In this study, we aimed to investigate and compare gene expression locally in LSG obtained from SS patients and from healthy subjects (HS) by means of microarray analysis. We performed gene ontology and pathway analysis to define cellular and molecular pathways differentially expressed in SS that may be causally related to the development of the diseases. In the first stage, our goal is to find genes whose expression is changed in SS patients and that they could be potential SS biomarkers and the concurrent comorbidities.

Materials and methods

The study group comprised 8 patients diagnosed with primary Sjögren's syndrome (pSS). Eight HS were included as healthy controls (HS). All samples were analyzed by microarray gene expression profiling. The study group used for validation of the relative changes in gene expression comprised 25 SS patients and 20 HS. The SS group met the American-European Consensus Group (AECG) diagnostic criteria of 2016 [6]. HS group did not meet these criteria. Exclusion criteria included the presence of IgG4 syndrome, lymphoma, sarcoidosis, graft-versus-host disease, head and neck radiotherapy, essential mixed cryoglobulinemia and infection by hepatitis B or C virus or human immunodeficiency virus. The thorough patient's examination included laboratory tests, histopathological examinations of LSG and tests for the assessment of salivation and lacrimation. The microscopic confirmation of SS was based on the presence of FLS with a focus

score (FS) ≥1 per 4 mm^2 of glandular tissue [7]. Laboratory assessments included anti-Ro/SSA and anti-La/SSB, ANA profile, Erythrocyte Sedimentation Rate (ESR), rheumatoid factor (RF). Ocular and oral dryness were assessed using Schirmer's test and unstimulated whole salivary flow (USWSF), respectively. Volumes of ≤1.5 mL/15 min in USWSF were marked as a diagnostic criterium of xerostomia. USWSF was performed using the standardized collection procedure as described [8]. Lacrimation in Schirmer's test ≤5 mm in one eye was marked as a positive result [9].

Labial salivary gland samples were harvested from the lower lip as described [10]. LSG samples from HS were obtained during surgical removal of the mucocele. Following surgery, some LSG samples were immediately stored in RNA later at −20 °C for molecular studies. Other samples used for histopathological examination were fixed in 10% buffered formalin.

Tissue samples were homogenized using a homogenizer. Total RNA was extracted from the LSG samples using a Universal RNA Purification Kit (EURx, Gdańsk, Poland, cat.: E3598). The concentration of total RNA was measured from the optical density (OP) at 260 nm, and the RNA purity was determined based on the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Each sample of RNA was diluted to 100 ng/μL for a subsequent microarray assay, 100 ng of RNA from each sample was taken. For the real-time quantitative polymerase chain reaction (RT-qPCR) validation approach, RNA samples were resuspended in 40 μl of RNase-free water and stored at −80°C. Then, 350 ng of an RNA sample was used for reverse transcription and reverse-transcribed into cDNA using smART RT-qPCR Kit (EURx, Gdańsk, Poland, cat.: E0806), according to the manufacturer's protocol.

The Affymetrix procedure and methods of analysis were described previously [11]. Total RNA (100ng) was converted to double-stranded cDNA. In the next step, labeled complementary RNA (cRNA) was synthesized and amplified by in vitro transcription of the double stranded cDNA template (GeneChipTM 3'IVT PLUS Reagent Kit, Applied BiosystemsTM, Foster City, CA, USA). Obtained cRNA was fragmentated by divalent cations and elevated temperature. Fragmentated and labeled cRNA (7.5 μg) were hybridized to Human Genome U219 Array Strip (45°C/16 h, Applied Biosys-temsTM, Foster City, CA, USA). Then, microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station. Subsequently the array strips were scanned by Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis software. All analyses were performed by Bioconductor and R programming language. Each CEL file was merged with a description file. In order to correct background, normalize and summarize results, we used Robust Multiarray Averaging (RMA) algorithm. Statistical significance of analyzed genes was performed by moderated t-statistics from the empirical Bayes method. Obtained p value was corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on p value below 0.05 and expression fold higher than 1.5.

Differentially expressed genes (DEGs) were subjected to the selection of genes involved in an immune response. The gene lists were uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery), where "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response" GO BP terms were obtained. Expression data of these genes were subjected to hierarchical clustering procedure and presented as a heatmap graph. Detailed analysis of genes belonging to selected GO BP terms were presented as plots using "GOplot" library [12].

The calculations for clinical, serological and demographic parameters were carried out with Microsoft Excel 2016 and STATISTICA software (v.13 TIBCO Software Inc., USA). Continuous data are presented as medians and or mean and standard deviation (±SD). Distributions of continuous variables were evaluated for normality using the Shapiro-Wilk test. Differences between the two groups were tested using the Mann-Whitney U test. Categorical variables are presented in contingency tables and their associations were tested, depending on the number of cases, with chi ^{2} test or Fisher's exact test. $p < 0.05$ was considered statistically significant.

The relative changes in gene expression results obtained from the microarray analysis were validated for four gene of interest: adiponectin, C1Q and collagen domain containing (ADIPOQ), S100 calcium binding protein A9 (S100A9), leptin receptor (LEPR) and fatty acid binding protein 4, adipocyte (FABP4) with the use of RT-qPCR. qPCR was performed using a Light Cycler® 96 Real-Time PCR System, Roche Diagnostics GmbH (Mannheim, Germany) with SYBR Green I as a detection dye (SG qPCR Master Mix (EURx, Gdańsk, Poland, cat.: E0401)). The primers used for qPCR (**Tab. 1**) were designed using Primer 3 software, using the exon–exon design approach, as an additional method to avoid the possible amplification of genomic DNA fragments. The primers were also designed using the sequence of several transcript variants of genes of interest available in the Ensembl database.

TABLE 1 Sequences of primers used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis

GENE NAMES	FORWARD PRIMER (5′–3′)	REVERSE PRIMER (5′–3′)	PRODUCT SIZE (BP)
ADIPOQ	TGGAGTACAGTGACACGACC	CATGCCTGTAATCCCAGCAC	235
S100A9	GGACCTGGACACAAATGCAG	CCTGGCCTCCTGATTAGTGG	239
LEPR	TGCCTCCATCCAGTGTGAAA	CGCACCTGAACAGCATAGAC	223
FABP4	GGGCCAGGAATTTGACGAAG	ACTTCAGTCCAGGTCAACGT	227

TABLE 2 Demographic, laboratory, serological and histological characteristics of the SS and HS study groups used in microarray analysis and in RT-qPCR approach

PARAMETERS	MICROARRAY ANALYSIS			RT-QPCR		
	HEALTHY SUBJECTS (HS)	SS PATIENTS (SS)	*P* VALUE	HEALTHY SUBJECTS (HS)	SS PATIENTS (SS)	*P* VALUE
Number of individuals	8	8		20	25	
Gender, Female/ Male, *n*	7/1	7/1		17/3	24/1	
Age, mean ± SD years	42.87 ± 18,5	56.1250 ± 9,29	0.206[a]	46.7 ± 17.32	52.8 ± 12.29	0.372[a]
Age, median (range), years	34.5 (24-66)	57 (38-67)	0.206[a]	49 (15-68)	54 (25-79)	0.372[a]
Focus score [&], *n* 1 2 3 4 median	 0 0 0 0 0	 2 3 2 1 2	 **<0.001[a]**	 0 0 0 0 0	 5 13 4 3 2	 **<0.001[a]**
Ro/SSA or La/SSB antibodies positive, *n,* (%)	*0* (0)	*7* (87.5)	**0.001[b]**	*0* (0)	10 (40)	**0.001[b]**
Schirmer's test (mm/5 min): Left eye, median Right eye, median	 7.5 11	 5 9	 0.329[a] 0,559[a]	 11 12	 6 7	 **0.024[a]** **0.037[a]**
Antinuclear antibodies positive (ANA), *n,* (%)	*0* (0)	*7 (*87.5*)*	**0.001[b]**	*0* (0)	*15* (60)	**0.00002[c]**
Rheumatoid factor, positive *n,* (%)				*0* (0)	*5* (20)	0.056[b]
ESR (mm), median	9.5	12.5	0.342[a]	9.5	18.00	**0.000356[a]**
USWSF, mean ± SD ml/15 min	4.81 ± 3.35	2.18 ± 1.36	0.055[a]	4.12 ± 2.67	1.62 ± 0.86	**0.000353[a]**

n, number; SD, standard deviation; a, Mann-Whitney U test; b , Fisher's exact test; c , chi 2 test; & the number of foci/4 mm2 of the tissue; RF, rheumatoid factor; %, percentage; ESR, erythrocyte sedimentation rate; USWSF, unstimulated whole salivary flow

The RT--PCR program included a 15-min denaturation step, followed by a three-step amplification program: denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The specificity of reaction products was checked by determining the melting pointsR (0.1°C/s transition rate).

Levels of analyzed gene expression was normalized in reference to hypoxanthine phosphoribosyltransferase 1 (HPRT1) and β-actin (ACTB) genes as an internal control. For target cDNA quantification, we have performed relative normalized quantification with the $2^{-\Delta\Delta CT}$ method.

The normality of the observed data distribution was assessed by the Shapiro–Wilk test, followed by the unpaired two probes t-test (ADIPOQ and LEPR) or nonparametric the Mann–Whitney U test (S100A9 and FABP4) to identify statistically significant differences between the compared mean values.

Results

The summarized demographic, laboratory, serological and histological characteristics of SS patients and HS used in microarray and RT-qPCR analyzes are presented in **table 2**.

We used Human Genome U219 Array Strip for the analysis of transcriptomes of LSGs of SS patients and HS. The microarray gene expression analysis allowed us to study the gene expression of 49,387 transcripts. We selected genes with more than 1.5- fold changes and p-values less than 0.05 for downstream analysis. The obtained gene list was uploaded to the Database for Annotation, Visualization and Integrated Dis-covery (DAVID) software and subjected to gene ontology analysis. This paper focused on the genes encoding cytokines involved in the immune response, so we have chosen to further our analysis by using Gene Ontology Biological Process (GO BP) terms that cover the above processes: "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response". The 31 genes involved in those processes were clustered using hierarchical clustering and presented as heatmaps (**Fig. 1**).

It is worth mentioning that 24 genes were upregulated, which is the greater part of the list of genes used for hierarchical clustering and only 7 genes were downregulated. The 10 most significantly upregulated and all of the downregulated genes, their symbols, fold changes and p- values are shown in the supplemental table (**Tab. 3**).

In the next part of analysis, we focused on the z-scores, which indicate whether the molecular function is more likely to be decreased (negative value) or increased (positive value). The z-scores were presented as segments of inner circles in **figure 2**.

As can be seen from the figure, expression of most genes was increased (green dots) in all ontological groups. The z-scores of above-mentioned GO BP terms had positive values, so the processes described by these GO BP terms were upregulated.

In the gene ontology database, single genes may belong to many ontological terms. For this reason, we used plots with visualization of logFC values

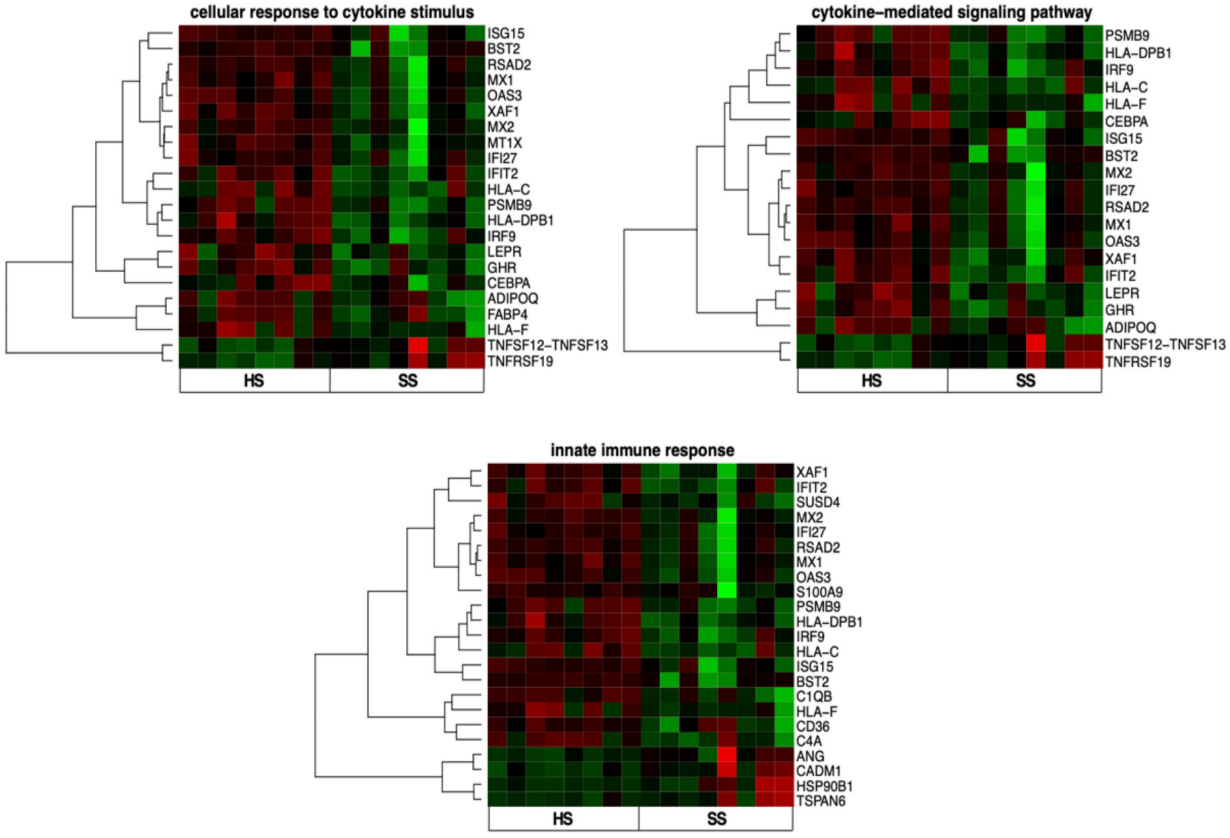

FIGURE 1 Heatmaps presenting differentially expressed genes involved in "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response" based on GO BP terms. Each row on the Y axis represents a single transcript. The red color indicates downregulated genes while the green color is for upregulated genes

TABLE 3 The 10 most significantly upregulated and 7 significantly downregulated genes involved in "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response" GO BP terms

GENE SYMBOL	GENE NAME	FOLD CHANGE	P.VAL
ADIPOQ	adiponectin, C1Q and collagen domain containing	4.88	<0.05
S100A9	S100 calcium binding protein A9	4.42	<0.05
LEPR	leptin receptor	3.39	<0.05
IF127	interferon, alpha-inducible protein 27	3.18	<0.05
C4A	complement component 4A (Rodgers blood group)	3.15	<0.05
FABP4	fatty acid binding protein 4, adipocyte	3.06	<0.05
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	3.04	<0.05
BST2	bone marrow stromal cell antigen 2	2.83	<0.05
GHR	growth hormone receptor	2.68	<0.05
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	2.60	<0.05
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	-1.86	<0.05
TSPAN6	tetraspanin 6	-1.97	<0.05
TNFRSF19	tumor necrosis factor receptor superfamily, member 19	-2.31	<0.05
TNFSF12-TNFSF13	TNFSF12-TNFSF13 readthrough	-2.39	<0.05
CADM1	cell adhesion molecule 1	-2.39	<0.05
ANG	angiogenin, ribonuclease, RNase A family, 5	-2.50	<0.05
HLA-DPB1	major histocompatibility complex, class II, DP beta 1	-2.61	<0.05

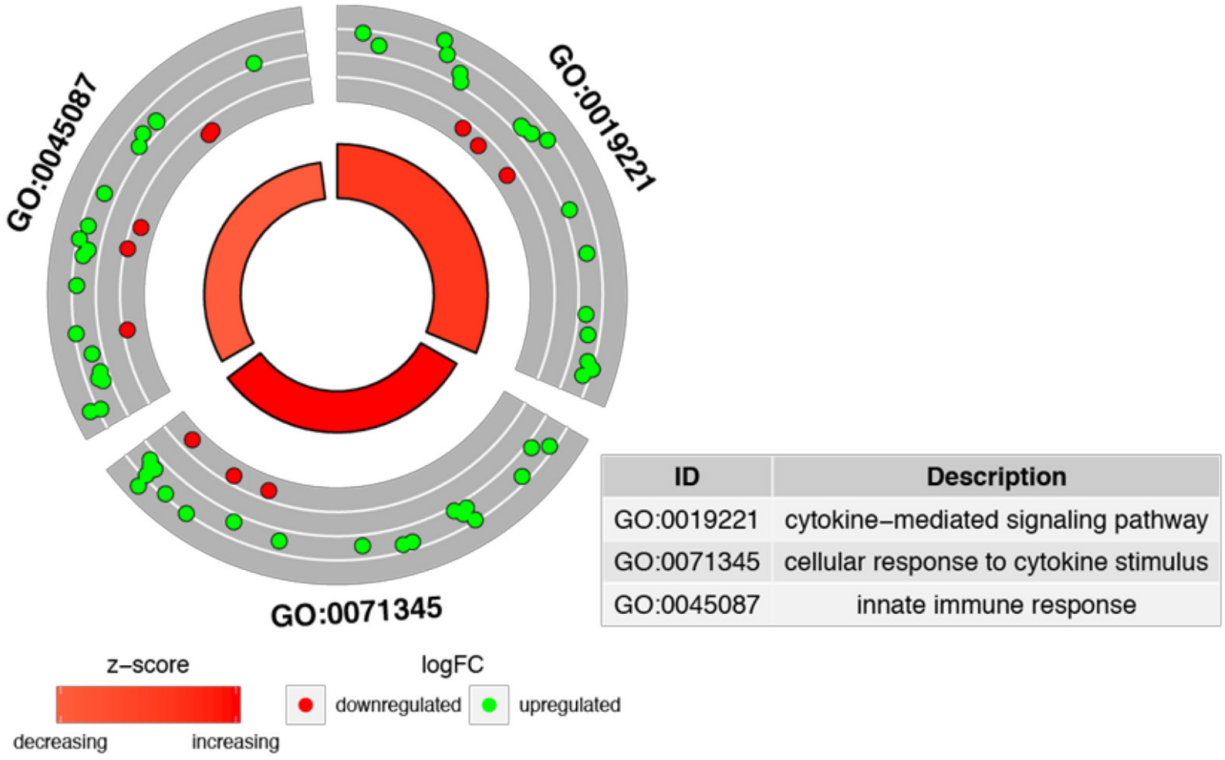

FIGURE 2 The circular scatter plots of differentially expressed genes involved in "cellular response to cytokine stimulus", "cytokine-mediated signaling pathway" and "innate immune response" GO BP terms. Each dot represents a single gene. The z-scores were presented as segments of inner circles

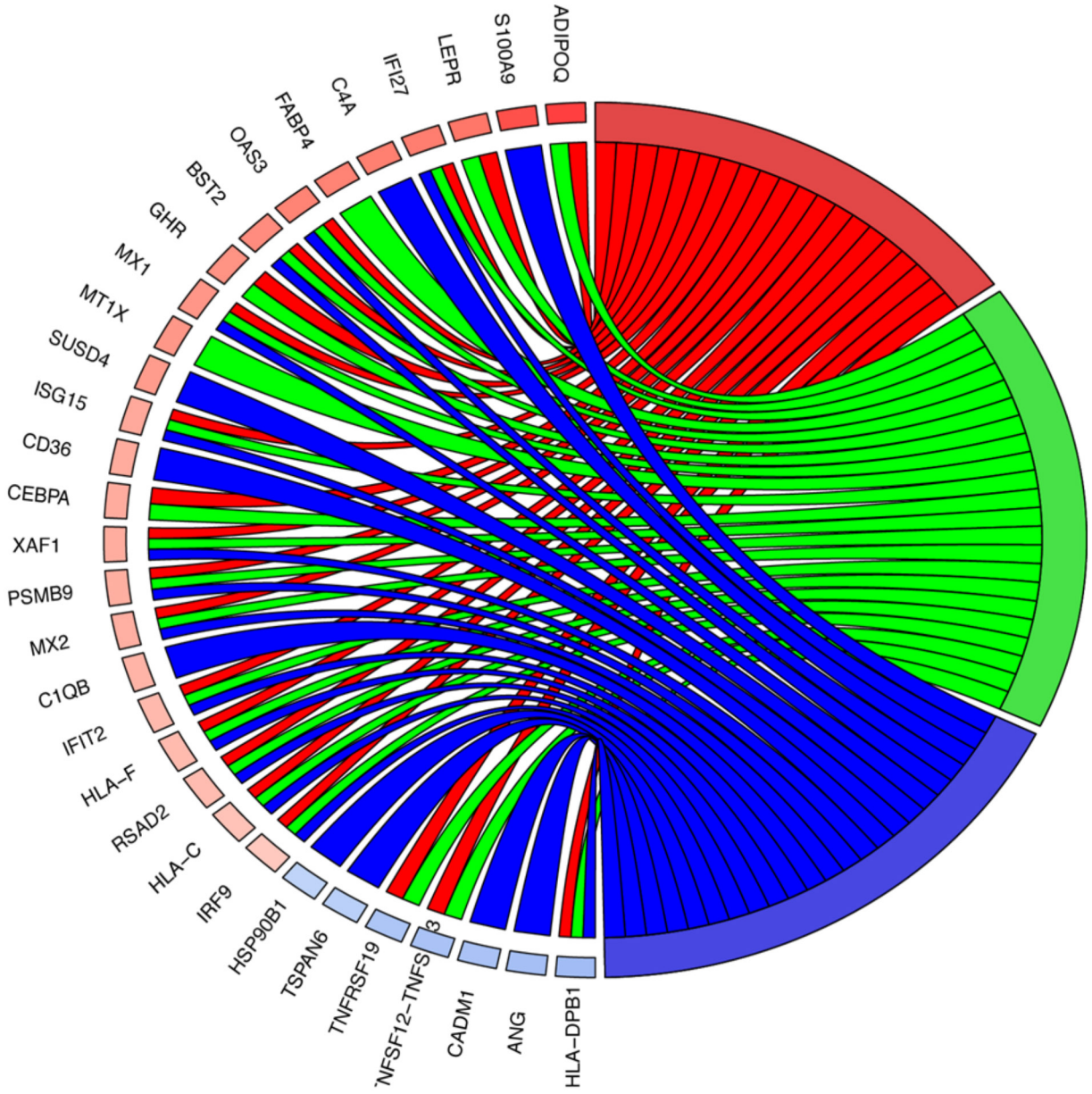

FIGURE 3 Analysis of enriched gene ontological groups involved in "cellular response to cytokine stimulus", "cytokine--mediated signaling pathway" and "innate immune response" GO BP terms. The network plot presenting the linkages of genes and GO BP terms

and relationship between genes and selected GO BP terms (**Fig. 3**).

The strongest upregulated genes from examined GO BP terms included, among others: ADIPOQ, S100A9, and LEPR.

Statistically significant correlations were observed between ADPIOQ, S100A9, LEPR, and FABP4 gene expression in SS patients compared to HS. These genes were subjected to validation with qPCR. The **table 4** is showing microarray results and RT-qPCR validation for gene of interest.

In RT-qPCR analysis significantly higher FABP4 (p=0.038), LEPR (p=0.049) and ADIPOQ (p=0.043) transcript levels in SS patients were observed compared to HS. SS patients and HS showed similar mRNA levels of S100A9 (p=0.656) in RT-qPCR analysis. The results were presented as a bar graph as a comparison between the fold changes (FC) values obtained for each analyzed gene in the microarray analysis and in RT-qPCR approach. As can be seen, the direction of changes in expression (higher mRNA levels in SS patients compared to HS) was

TABLE 4 Comparison of ADPIOQ, S100A9, LEPR, and FABP4 gene expression results for SS patients in relation to HS obtained in microarray approach and in RT-qPCR validation

GENE SYMBOL	GENE NAME	MICROARRAY		RT-QPCR	
		FOLD CHANGE	P-VALUE	FOLD CHANGE	P-VALUE
ADIPOQ	Adiponectin, C1Q and collagen domain containing	4.88	**<0.05**	5.71	**<0.05**
S100A9	S100 calcium binding protein A9	4.42	**<0.05**	1.32	>0.05
LEPR	Leptin receptor	3.39	**<0.05**	3.06	**<0.05**
FABP4	Fatty acid binding protein 4, adipocyte	3.06	**<0.05**	2.95	<0.05

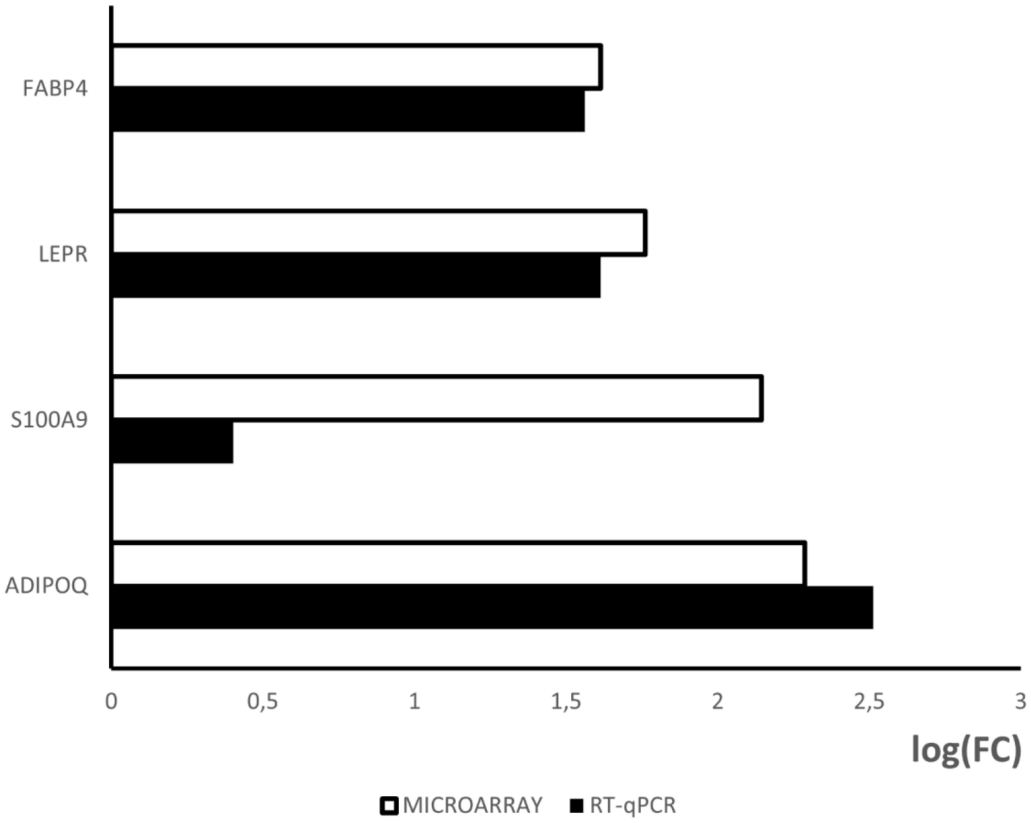

FIGURE 4 RT-qPCR quantitative validation of microarray results presented in the form of a bar graph. The graph shows the relative changes in gene expression results for SS patients (n=25) in relation to the transcript levels obtained from HS (n=20). FC was presented in its logarithmic form to provide a clear comparability of the results

confirmed in all examples. However, the scale of differences in transcription levels varied between both methods analyzed (**Fig. 4**).

These variations usually are small (FABP4, LEPR and ADIPOQ), while for S100A9 exhibit major discrepancies between the two analyses. This fact may be explained by differences in precision of those two methods because the microarray approach used to analyze the full transcriptome is mainly qualitative. However, validation of the results with quantitative RT-qPCR often gives variable results.

Discussion

Natural potential candidate biomarkers in SS are inflammatory cytokines and their encoding genes, the profile of which is disrupted in SS, and which may function not only as local inflammatory mediators but also as systemic determinants of SS and the accompanying pathologies. In our study, we identified and confirmed the higher expression of genes potentially involved in SS. Moreover, the genes identified in this study and their increased expression in SS patients compared to healthy subjects creates per-

spectives for further research on their potential role in other pathological processes in SS based on their already known neuroendocrine and systemic effects.

The first group of the potential biomarkers identified in our study are leptin and adiponectin, named adipokines, which influence the immune system as well as the neuroendocrine system. Adipokines show significant homology with the structure of interleukins (IL) IL-6, IL-11, IL-12 and granulocyte colony-stimulating factor involved in SS pathology. Their structural characteristic is implicative in regulating immune responses and in acting as proinflammatory cytokines [13]. In addition, adipokines receptors are found on T and B lymphocytes, monocytes / macrophages, natural killer cells, neutrophils and dendritic cells. The increased LEPR expression detected in our study reflects the activation of these immune cells observed in SS and the increased secretion of proinflammatory cytokines. Moreover, leptin enhances the ability of macrophages to phagocytose pathogens that may trigger epithelial inflammation in the initial stage of SS leading to the increased secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6 and IL-12 [13]. Leptin reflects the shift of the immune response towards Th1 lymphocytes by stimulating the secretion of TNF-α, In-terferon gamma (IFN-γ) and IL-2, and suppressing the response from lymphocytes Th2 by reducing IL-4 secretion, which is typical for SS. In the salivary glands of SS patients, the secretion of IL-1β, IL-6 and TNF-α and the activation and migration of lymphocytes with the formation of lymphocytic infiltrates lead to apoptosis of epithelial cells and the destruction of glandular tissue, which is then replaced by fibrotic and adipose tissue. One of the postulated causes of salivary gland destruction in SS is leptin/OB-R pathway promoting IL-4 secretion from B lymphocytes and inducing salivary gland epithelial cell apoptosis [14]. Leptin secretion in B lymphocytes and the expression of leptin receptor is increased in early and advanced stages of SS. Moreover, a proapoptotic effect of leptin/OB-R signaling is dependent on elevated IL-4 secretion [14]. Divergent results were obtained by Erbasan et al who investigated the expression of leptin and its receptor (LEPR) in minor salivary glands in SS patients and in healthy subjects. The distribution and staining intensity of leptin and LEPR were similar in both groups and there was no correlation between focal lymphocytic infiltration and leptin and leptin receptor [5]. In contrast to leptin and its receptor, it is postulated that adiponectin plays a protective role on submandibular cells. Decreased local adiponectin might be important factors resulting in the submandibular epithelial cell dysregulation in SS-specific sialadenitis [15]. Moreover, Katsiougiannis et al. reported higher expression of adiponectin and its two receptors AdipoR1 and AdipoR2 in the salivary glands of SS patients compared to healthy people [16]. These findings suggest that adiponectin and leptin may play opposite function in salivary gland destruction in SS. The same authors suggested that adiponectin presents the divergent function of pro or antiinflammatory and antiapoptotic effects dependently on the energy stress condition, which indicates a link between the metabolic and cellular function of adiponectin [17]. Apart from the distribution of adipokines and their receptors in the salivary glands, their levels in body fluids is higher in SS patients compared to healthy controls. Although Zinkevičiene et. al reported no differences in serum adiponectin levels between SS patients and non-autoimmune sicca syndrome patients other studies confirmed that serum or salivary adiponectin levels were higher in SS patients than in controls [4,18,19]. Moreover, serum levels of leptin and adiponectin correlated with selected parameters of metabolic syndrome and other cardiovascular risk factors such as higher diastolic blood pressure, glucose, triglycerides and LDL level [4]. These results may suggest that increased levels of adipokines could be a valuable predictor of cardiovascular disease development in SS, and this correlation is modulated by the inflammation severity in SS.

Another candidate biomarker for SS is S100A8/A9, known as calprotectin, which has been studied so far as a sensitive marker of the activity of autoimmune diseases [20]. In recent studies its levels correlated with Sjögren's Syndrome Disease Activity Index (ESSDAI) [21]. Nicaise et al detected higher S100A8/A9 serum levels in SS patients compared to healthy controls. Moreover, the same authors detected that IL-1β, TNF-α, IL-6, and IL-17A stimulated the release of S100A8/A9 in peripheral blood mononuclear cells (PBMCs) in SS patients. Additionally, S100A8/A9 induced the production of IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-17A and IL-22 by PBMCs, which would indicate a mutual relationship between pro-inflammatory cytokines and S100A8/A9 [22]. The elevated expression of S100A9 detected in our study is a result of the activation of macrophages and neutrophils during the inflammatory process in epithelial tissue in salivary glands. Recent studies reported that serum calprotectin levels may be an alternative parameter to ESR and C-reactive protein (CRP), determining the severity of the inflammatory process and allowing for treatment, monitoring and response to the applied treatment [23]. Its highest levels are observed in the initial stage of inflammation and show significant sensitivity to the duration of the disease. This evolution of serum calprotectin levels in inflammatory autoimmune diseases that are characterized by subsequent periods of exacerbation and remission may explain the fact that we were not able to confirm the statistical significance of higher S100A9 expression in SS patients differing in activity and disease duration compared to HS during the validation of

microarray results using RT-qPCR. Furthermore, calprotectin is deemed as a damage-associated molecular marker of injury and its increased expression can reflect the severity of inflammation locally in the epithelium of salivary glands. In salivary glands, higher expression of S100A8/A9 correlated with the focus score [22]. Moreover, Jazzar et all reported that salivary levels of S100A8/A9 may be a helpful biomarker in differentiating between SS patients with lymphoma and at higher risk of lymphoma [24]. These results confirm a strong proinflammatory effect of S100A8/A9 locally in salivary glands as well as systematically in circulation. One of the postulated systemic effect of calprotectin is its potential involvement in atherosclerotic process. It is worth noting that a higher serum calprotectin levels in SS correlated with a higher prevalence of carotid atherosclerosis independently of traditional risk factors [25]. These results may suggest that the mere presence of SS and the accompanying chronic inflammation increases the risk of accelerated development of atherosclerosis and cardiovascular diseases, and indicates the need to monitor this development in SS patients with calprotectin as a valuable prognostic biomarker.

The last candidate biomarker presented in our study is FABP4. It has been postulated that epidermal FABP is a valuable marker of the lacrimal glands destruction in SS, and its reduced concentration in tears in SS patients compared to healthy subjects is the result of ocular surface epithelial barrier damage [26]. Moreover, this decrease correlated with the severity of dry eye. The differences in FABP expression were also observed in saliva between SS patients and healthy volunteers, and these noted changes reflected disease activity and SS severity [27]. Although, FABP4 expression obtained in our study is increased in SS compared to healthy subjects contrary to previously presented findings, these observations confirm that FABP may be a valuable diagnostic marker of SS, its activity and the severity of accompanying exocrinopathies. Its higher expression in SS patients detected in labial salivary glands specimens may be explained at the local level by the greater share of adipose tissue that replaces glandular tissue in SS. FABP4 is one of the most abundant proteins in adipose tissue and acts as a marker of adipocyte differentiation from preadipocytes into adipocytes. However, its expression is detected in macrophages, particularly upon inflammatory activation. Thus, the increased expression may reflect the activation of macrophages in a similar mechanism as presented for other detected cytokines. The main promoter of FABP expression in macrophages is peroxisome proliferator-activated receptor γ (PPARγ). PPARγ plays an antiinflammatory function contrary to the proinflammatory function of FABP4. PPARγ agonists inhibit the IL-1β pathway in lacrimal glands in SS as well as decrease serum levels of IL-6 and TNF-α, and increase serum levels of IL-4 in SS patients. Moreover, its reduced activity promotes the activation of the IL-1β and NF-κB pathways in ductal epithelial cells in SS patients. Except its local antiinflammatory effects revealed in salivary and lacrimal glands, PPARγ may have a systemic metabolic effect via the PPARγ–FABP4 pathway. PPARγ regulates glucose homeostasis and lipid metabolism [28]. The other factors which increase FABP expression in macrophages are rapamycin, immunosuppressants responsible for hyperlipidemia induction and liver X receptor agonists involved in cholesterol transport and triglyceride synthesis [28]. Low FABP4 levels play a protective function against proatherosclerotic factors and thus, the higher levels could predispose to the development of atherosclerosis. The involvement of FABP4 in lipolysis and lipogenesis, macrophage activity, inflammation of adipose tissue and in the process of atherosclerosis can postulate the use of its serum levels as a marker of obesity, metabolic syndrome and insulin resistance.

The limitations of this study arise mainly from the selection of both SS and HS groups, the disease duration and the heterogeneity of accompanying symptoms. The disease activity could also determine and modify the results obtained in our study. Another limitation of this study is the difference between the cellularity and gene profile of different cell types within LSG, including periductal, acinar and infiltrated mononuclear cells.

Conclusions

Our study is an attempt to identify genes whose expression is increased in SS patients and whose participation in signaling pathways and metabolic processes may predispose to other comorbidities. Most of the observed changes in the expression of these genes are a consequence of the inflammatory process and activation of inflammatory cells, which locally leads to the increased proliferation of adipose tissue that replaces the glandular tissue in the salivary glands. Additionally, prolonged inflammation in SS will intensify the systemic effect of the genes we determined and the cytokines encoded by them. In our opinion, an important systemic effect of all genes detected in our study is their potential proatherosclerotic activity. This may suggest that there is an increased risk of the development of other diseases such cardiovascular complications, metabolic syndrome and endocrinopathies in patients with SS. Apart from the activity of the inflammatory process, an important factor that may increase this risk is the duration of the disease, which contributes to the accumulation of all negative effects of the disease. Antiinflammatory therapy can help to reduce the risk of accelerated development of potential atherosclerosis and cardiovascular complications by eliminating nontraditional and SS-associated risk factors.

Ethical approval
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Bioethics Committee of Poznan University of Medical Sciences, Poland (number 403/17).

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Conflicts of interest
The authors declare they have no conflict of interest.

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