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Gene Expression Profiles in Primary Sjögren's Syndrome With and Without Systemic Manifestations

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Objective. To investigate the gene expression profile in patients with Sjögren's syndrome that is characterized by different clinical phenotypes.

Methods. RNA from peripheral blood mononuclear cells was purified in 8 patients with glandular features (GFs) and widespread pain (WP) and 11 with extraglandular manifestations (EGMs) and then was analyzed by hybridization on a human gene chip exploring more than 40,000 human genes. Differentially expressed genes (DEGs) in the two subgroups (ie, those with false discovery rate–corrected P values ≤ 0.01) with respect to 20 healthy controls have been submitted to functional classification using a Gene Ontology database and were mapped to define the networks of protein to protein interactions (PPIs).

Results. The enriched pathway analyses of DEGs and of the highly interconnected modules identified in the PPI networks showed that the pathological processes characterizing the two subgroups were substantially different. The predominant pathways in patients with EGMs are related to T- and B-cell activation, Toll-like receptor, interferon signaling, and apoptosis. Conversely, pathological processes related to pain transmission and modulation are preferentially operative in patients with GFs and WP. These data suggest that a neuroinflammatory pathway driven by cytokines and chemokines may play a central role in triggering WP features in this phenotype of patients.

Conclusion. The present study supports the hypothesis that different biological pathways are operative in patients with primary Sjögren's syndrome with different clinical phenotypes. A better knowledge of these specific processes might help in tailoring more effective target therapies.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disorder whose typical manifestations are oral and ocular dryness. However, the clinical spectrum of pSS may vary from signs and symptoms consequent to the isolated involvement of the salivary and lachrymal glands, or sometimes of the other exocrine glands, up to manifestations related to the involvement of different organs or systems (1). Extraglandular manifestations (EGMs) are present in around 60% of patients and are usually distinguished in two types. The first one is characterized by lymphocytic infiltrates around the epithelial tissues of parenchymal organs, such as the lungs, kidneys, and liver. The second type is marked by autoantibodies or immune complex deposition in small vessels that may lead to the development of porpora, glomerulonephritis and peripheral neuropathy (1).

The fact that pSS can present such a wide variability of clinical features suggests that different pathogenetic mechanisms may be present in different patients. This hypothesis is supported by the finding that a) the number and organization of mononuclear cells as well as T- and B-cell ratio in the infiltrates of target tissues, b) gammaglobulins and antibody levels in the serum, and c) cytokine expression in both peripheral blood and glands can be different in patients with the disease limited to exocrine gland aggression or characterized by EGMs (2). Even the impact of fatigue, which represents one of the major contributors to the impaired quality of life in patients with pSS, is variable in different subsets of patients (3). Severe fatigue has been described in approximately one-

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third of patients and found to be closely associated with widespread pain (WP), anxiety, depression, and impaired sleep patterns (3). Unexpectedly, the levels of some proinflammatory cytokines that have been found to characterize this disease are inversely related to patient-reported levels of fatigue (4). In addition, some data suggest that WP, which is closely related to fatigue, is predominant in patients with a disease limited to glandular features (GFs) (5).

Because the presence of EGMs, hypergammaglobulinemia, cryoglobulinemia, hypocomplementemia, lymphopenia, and the germinal center–like organization of mononuclear infiltrates in the salivary glands are commonly ascribed to B-cell hyperactivity and are also predictors for the development of lymphoproliferation (6), targeting B cells has been postulated to be an effective therapeutic approach in patients with pSS. Therefore, treatment with rituximab, an anti-CD20 B-cell surface molecule inducing B-cell depletion in peripheral blood (7), has been tested in patients with pSS. Although preliminary open label and controlled pilot trials on rituximab therapy for pSS have shown improvement in selected subjective and objective parameters, two large randomized control trials (8,9) failed to reach the primary end points that were mainly represented by improvement of fatigue and sicca complaints. In contrast with these poor results, it has been clearly shown that rituximab therapy is effective in reducing the level of some serological markers of the disease, such as immunoglobulin M–rheumatoid factor, gammaglobulins, and B-cell number also in glandular infiltrates (10). Different hypotheses have been raised to explain these negative results. However, it is likely that rituximab ineffectiveness could be mainly ascribed to the differences in the biological pathways that are operative in different subsets of patients with pSS (10). Thus, a better knowledge of the genotypic patterns driving the phenotypic differentiation of patients may lead to identify the target therapy with the highest probability of success in improving the clinical condition and the quality of life of patients with pSS.

With the above considerations in mind, we planned the present study to specifically investigate the gene expression profile in patients with pSS with different phenotypical characteristics. To do so, we decided to select patients situated at the extremities of the clinical spectrum of the disease (ie, patients with disease limited to the involvement of salivary and lachrymal glands and only complaining of sicca symptoms and WP) and, on the opposite side, patients who, together with sicca manifestations, presented with systemic EGMs, such as small joint arthritis, vasculitis, lung or kidney involvement, peripheral neuropathy, and lymphoproliferative lesions.

PATIENTS AND METHODS

Patient selection. Patients classified as having pSS according with the American College of Rheumatology (ACR)-European League against Rheumatism (EULAR) criteria were enrolled in this study (11). We selected patients whose disease was limited to glandular involvement and therefore who only complain of GFs and WP as well as—on the other extreme—patients who were characterized by the presence of a wide spectrum of EGMs. An accurate workup was completed in all of the patients to accurately define their clinical and serological profile. Namely, each EGM was defined according to the European SS Disease Activity Index (ESSDAI) nomenclature (12), and the cumulative ESSDAI score for systemic activity was calculated. Furthermore, the presence of pain and fatigue was scored using the Likert scale adopted for these domains in the European SS Patient Reported Index (ESSPRI) (13). Furthermore, we classified patients as having WP according to ACR definition. Following these criteria, WP is defined as the presence of pain in at least three sites in the left and right sides of the body, above and below the waist, and in the axial skeleton, present for at least 3 months (14). For each enrolled patient, a clinical examination was performed to check the presence and number of fibromyalgia-associated tender points (14).

Gene expression analysis. An analysis of gene expression profiles was performed in pSS patients with only GFs as well as in pSS patients with EGMs and in 20 age- and sex-matched healthy donors.

Blood samples were collected in Vacutainer K2EDTA tubes, and peripheral blood mononuclear cells (PBMCs) were isolated upon stratification on Lympholyte, a miRNeasy mini kit, following the manufacturer's instructions (Qiagen).

cDNA preparation, sample hybridization, and scanning were entrusted to the Cogentech Affymetrix microarray unit (Campus IFOM IEO), and the whole procedure was performed as described in the Affymetrix protocols (Affymetrix).

Sample hybridization was performed on Human Clariom D gene chip (Thermo Fisher Scientific), which interrogates more than 40 000 human genes that are approximately represented by more than 120 000 probe sets.

Signal intensities of each probe set were analyzed using the Transcriptome Analysis Console (TAC) 4.0 software (Applied Biosystem). Background subtraction, normalization, and log-transformation of signal intensities were performed with The Signal Space Transformation–Robust Multi-Array Average algorithm.

The relative gene expression levels of each transcript were validated using the relative one-way analysis of variance (ANOVA) ($P \leq 0.01$) with multiple testing correction.

Transcripts that displayed an expression level at least 1.5-fold different in the test sample versus control sample ($P \leq 0.01$) were submitted to functional classification using the Gene Ontology (GO) annotations and pathways, and biological process enrichments in differentially expressed genes (DEGs) were analyzed by using Panther expression analysis tools (http://panth

Table 1. Main demographic, clinical, serological, and histologic features of patients with primary primary Sjögren's syndrome enrolled in the study. Patients 1, 2, 4, 8, 12, 13, 15, and 16 were those randomly selected from the two phenotypically different groups for the analysis of gene expression profile

Patient Number	Gender	Age (y)	Disease Duration (y)	Sicca Symptoms	WP[a]	ESSDAI Count	ESSPRI Fatigue Score	ESSPRI Pain Score	Specific EGMs	Serological Abnormalities	Focus Score[b]
1	M	75	3	no	no	55	8	2	VP, IP, PN, My, LE	ReC, Cryo, ANA, Ro, La	5
2	F	53	6	yes	no	19	6	3	AI, VP, PN	ReC, ANA, Ro	3
3	F	50	4	yes	no	11	5	2	AI, VP, LP, LE	ReC, ANA, Ro, La	1
4	F	59	7	yes	no	17	7	2	VP, My	ReC, ANA, Ro, La	3
5	F	52	1	yes	no	17	3	4	AI, VP, LP	ReC, Cryo, ANA, Ro, La	1
6	F	53	1	yes	no	29	10	2	AI, VP,	ReC, ANA, Ro	1
7	F	80	15	yes	no	7	7	3	AI, VP	ReC, ANA, Ro, La	4
8	F	49	3	yes	no	13	4	0	AI, VP	ReC, Cryo, ANA, Ro, La	1
9	F	31	1	yes	no	7	2	0	AI, LP	ANA	1
10	F	76	11	yes	no	47	5	2	AI, VP, IP, LE	ReC, Cryo, ANA, Ro, La	4
11	F	55	4	yes	no	13	6	4	AI, VP, LP	ReC, ANA, Ro, La	3
12	F	56	9	yes	yes	0	6	5	None	ANA, Ro, La	3
13	F	61	10	yes	yes	1	4	6	LP (transient)	Ro	2
14	F	70	11	yes	yes	0	3	4	None	ANA, Ro	3
15	F	76	13	yes	yes	0	6	8	None	ANA	2
16	F	71	12	yes	yes	0	6	8	None	ANA, Ro, La	4
17	F	58	2	yes	yes	0	5	7	None	ANA	1
18	F	45	1	yes	yes	0	3	8	None	ANA, Ro	1
19	F	46	8	yes	yes	0	10	10	None	Ana, Ro	2

Abbreviation: ANA, antinuclear antibodies; Cryo, cryoglobulins; EGM, extraglandular manifestation; IP, interstitial pneumonia; La, anti-La antibodies; LE, lymph node enlargement; LP, leukopenia; My, myositis; PN, peripheral neuropathy; ReC, reduced complement; Ro: anti-Ro antibodies; VP, vasculitic purpura.

[a]Widespread pain

[b]Focus score is the number of infiltrates of at least 50 mononuclear cells observed in 4 mm^2 of salivary glandular tissue from lip biopsy. The tender point count in patients with WP ranged from 8 to 14 out of the 18 classical points considered in the classification criteria for fibromyalgia (14).

erdb.org/) (1). Calculated by the binomial statistical test, *P* values ≤ 0.05 were considered as significant enrichment (15).

Validation of the microarray results by real-time polymerase chain reaction. The significantly different expression of genes observed in microarray assay was validated by testing some selected genes with real-time polymerase chain reaction (RT-PCR) in patients and healthy controls. The method adopted for this validation procedure is detailed in the Supplementary Methods section (16–18).

Protein-protein interaction, network construction, and network clustering. DEGs in test samples versus healthy controls that met the above-mentioned criteria were also mapped to the Search Tool for the Retrieval of Interacting Genes (STRING) database to analyze protein-protein interaction (PPI) pairs between the protein products of modulated genes, which were validated by experimental studies (19). A PPI network was designed, and a score of ≥0.7 was selected for each PPI pair.

The STRING database (version 10.5; http://string-db.org/) covers experimental interactions in more than 1000 fully sequenced organisms.

High-flow areas of the network (modules), characterized by the presence of highly connected proteins, were identified by a modular analysis employing the CFinder software tool, based on the Clique Percolation Method (20). The topological analysis of the built networks was performed with the Cytoscape software (21).

Ethical statement. The protocol for the present study was prepared in accordance with the guidelines of the 1975 Declaration of Helsinki and the recommendations of local ethical committee of University of Verona for this kind of study. Written, informed consent was obtained from all the enrolled patients and controls.

RESULTS

Patient population. Eight patients with disease expression limited to GFs and 11 patients characterized by EGMs constituted the two selected subgroups of patients.

The main demographic, clinical, and histologic findings of the patients are summarized in Table 1.

For the gene expression analysis, RNAs isolated from the PBMCs of four randomly selected patients from each subgroup (patients 1, 2, 4, and 8; and 12, 13, 15, and 16) were obtained. RNAs from the remaining patients of the two subgroups were used for the validation analysis using RT-PCR.

Gene expression analysis. In comparison with 20 healthy controls (volunteers), the DEGs were 2012 and 1679 in patients with GFs and EGMs, respectively. The complete lists of DEGs in the two subgroups, with the statistical difference compared with healthy controls and identification codes, are reported in Supplementary Tables 1 and 2.

The significantly different expression of some genes observed in microarray assay was confirmed using the RT-PCR method. The results of this validation test are reported in Supplementary Figure 1.

Selectively modulated genes in the two groups were 1021 and 573 in patients with GFs and EGMs, respectively. The lists of these genes are reported in Supplementary Table 3.

When the DEGs in the two subtypes underwent GO functional classification, it was evident that in both groups the large majority of DEGs belonged to the same biological processes. The modulated genes can be grouped into five main pathological processes: those related to apoptosis, immune and inflammatory response, Toll-like receptor (TLR) signaling and type-I interferon (IFN) signaling. Some less substantial differences between the subgroups were present in the relative proportions of genes referring to the main functional categories (Figure 1). Supplementary Tables 4 and 5 show a selection of genes included in the main biological processes in the two groups.

However, looking at the genes selectively modulated in the two subgroups and at their GO definition, we found that a limited series of genes involved in the sensory perception pathway were exclusively modulated in the group of patients with only GFs. Table 2 shows a selection of the above-mentioned genes.

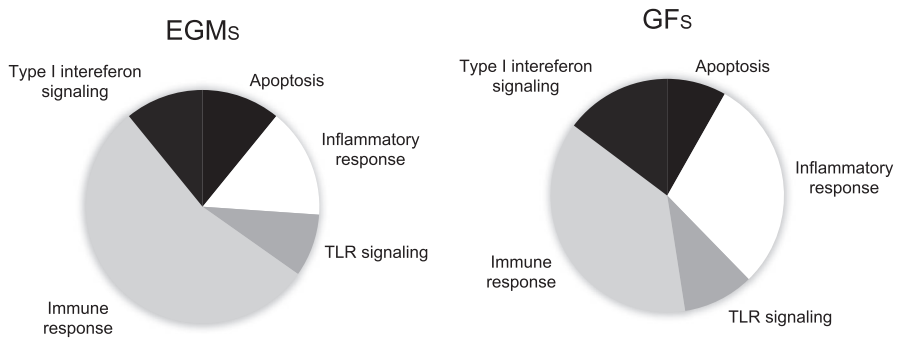

Figure 1. Biological processes activated in the two subgroups of patients with primary Sjögren's syndrome, according to Gene Ontology functional classifications of differentially expressed genes. The proportions of genes belonging to each biological process are represented in the pie charts and slightly differ in the two subgroups.

Table 2. Overexpressed genes in patients with glandular features and widespread pain, which are members of the "sensory perception" gene set, according to the Gene Ontology definition (GO: 0007600)

Gene Symbol	Gene Descriptors	Function[a]	Fold Change	*P* value
IFNG	Interferon, gamma	Proinflammatory cytokine	10.35	0.0009
TRIM26	Tripartite motif containing 26	Transcription factor	2.38	0.0021
TRIM25	Tripartite motif containing 25	Transcription factor	2.18	0.0057
EDN1	Endothelin 1	Inducer of pain perception	3.41	0.009
PML	Promyelocytic leukemia protein	Regulator of synapsis strength	2.58	0.003
ANPEP	Alanyl (membrane) aminopeptidase	Inducer of nociceptive pain	9.02	0.0026
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Receptor of proinflammatory cytokine	2.09	0.0088
P2RY1	Purinergic receptor P2Y, G-protein coupled, 1	Receptor involved in synaptic transmission	2.1	0.0079
P2RX4	Purinergic receptor P2X, 4	Receptor involved in synaptic transmission	3.52	<0.0001
ADRB1	Adrenoceptor beta 1	Receptor involved in perception of pain	1.86	0.009
GPR75	G protein–coupled receptor 75	Chemokine receptor component	2.13	0.0044
GPR132	G protein–coupled receptor 132	Nociceptive receptor component	2.87	0.0032
GPR137B	G protein–coupled receptor 137B	Sensory receptor component	3.08	0.0006
GPR37L1	G protein–coupled receptor 37 like 1	Endothelin receptor component	1.98	0.0025
ADCY5	Adenylate cyclase 5	Nociception inducer (via cAMP)	1.89	0.0027
GNG5	Guanine nucleotide binding protein (G protein),	Chemokine receptor component γ5	2.2	0.0018
RGS16	Regulator of G-protein signaling 16	Modulator of chemokine receptor	2.78	0.0018
GNG2	Guanine nucleotide binding protein (G protein),	Chemokine receptor component γ2	2.03	0.005
LIF	Leukemia inhibitory factor	Interleukin-6 family cytokine	2.24	0.0046
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Transcription factor	4.84	0.0046
FOSL1	FOS-like antigen 1	Postsynaptic stimulation product	3.78	0.0004
CSNK1E	casein kinase 1, epsilon	Inducer of neuropathic pain	2.24	0.0062
GRINA	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1	Postsynaptic receptor sensible to glutamate release	2.21	0.0044

[a]Function that the listed genes may have in the context of sensory perception.

Notably, the most strongly overexpressed gene in this list was that which codifies for IFN-γ.

Pathway enrichment analyses showed that almost completely different signaling networks were preferentially enriched in patients with GFs and WP in comparison with patients with EGMs. In this latter subgroup of patients, the B-cell activation and TLR signaling pathways were strongly enriched together with the pathways related to 5 hydroxytryptamine (5HT)-4 receptor signaling and angiotensin II (Ang II) receptor signaling (Table 2). Conversely, the most enriched pathways in the subgroup of patients with GFs and WP were those related to beta-adrenergic and Notch signaling, 5HT-1 receptor, and corticotropin releasing factor (CRF) receptor signaling (Table 3).

PPI analysis of DEGs allowed the identification of two networks that represented all the functional interactions among modulated genes in the two subgroups of patients. Also, with this kind of analysis, relevant differences could be observed in the number of genes, composition, density of edges (Figure 2A and B) in the PPI network of DEGs in patients with GFs and WP in comparison with that derived in patients with EGMs.

The network obtained from PPI analysis of DEGs in patients with GFs and WP included 585 codifying genes and 2166 pairs of interactions (edges) (Figure 2A). This PPI network was then submitted to cluster analysis to identify the more highly interconnected nodes or modules (M) that are more probably involved in determining the typical feature characteristics of different subsets of patients. This modular analysis showed the presence of 13 modules that were submitted to pathway enrichment analysis. In particular, we observed that several genes involved in pain perception and in nociceptive signal transduction were present in M0 and M2 (Figure 3A). M0 was characterized by the presence of genes codifying for endothelin 1 precursor (EDN1), G-protein subunit γ 2 and 5 (GNG2; GNG5), lysophosphatidic acid receptor 2 and 5 (LPAR2; LPAR5), which were also present in M2. In addition, genes codifying for C-C chemokine receptor 5 (CCR5), taste

Table 3. Enriched pathways in the two subgroups of patients obtained by Panther analysis

Pathways enriched in patients with EGMs	*P* Value
B-cell activation (P00010)	1.49E-02
Toll receptor signaling pathway (P00054)	2.67E-02
5HT-4 type receptor mediated signaling pathway (P04376)	3.40E-02
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	4.54E-02
Pathways enriched in patients with GFs and WP	***P* Value**
Beta adrenergic receptor signaling pathway (P04377)	1.42E-02
Notch signaling pathway (P00045)	3.70E-02
5HT1 type receptor mediated signaling pathway (P04373)	4.67E-02
Corticotropin releasing factor receptor signaling pathway (P04380)	4.89E-02

Abbreviation: EGM, extraglandular manifestation; GF, glandular feature; WP, widespread pain.

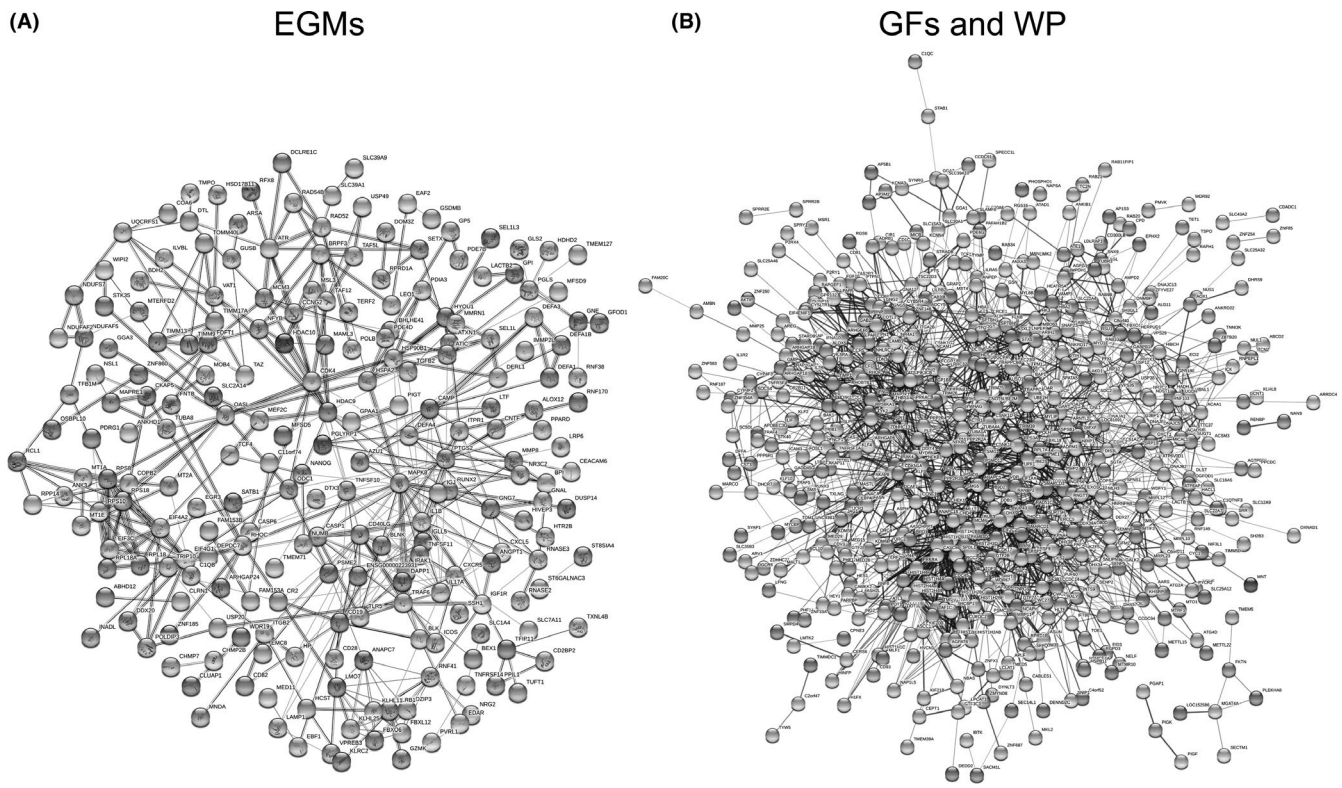

Figure 2. Protein-to-protein interaction networks of differentially expressed genes in patients with primary Sjögren's syndrome characterized by the presence of extraglandular manifestations (EGMs) (**A**) and with disease expression limited to glandular features (GFs) and widespread pain (WP) (**B**).

type 2 receptor 3 (TAS2R3), and adenylate cyclase 5 (ADCY5) entered in the composition of this module. Besides the genes shared with M0, M2 also included cysteinyl leukotriene receptor 1 (CYSLTR1), P2Y purinoreceptor 1 (P2RY1), and G-coupled receptor 132 (GPR132).

The enrichment analysis applied to M0 and M2 showed that, among the pathways represented in these modules, there are those related to G-proteins, beta adrenergic receptors, 5HT receptors, muscarinic receptors, gamma-aminobutyric acid (GABA) receptor, CRF receptor signaling, and to proinflammatory cytokine-chemokine signaling (Figure 3A).

In contrast with M0 and M2, M11 contained genes encoding for anaphase-promoting complex subunit 5 and 16 (ANAPC5; ANAPC16) and for histone H2B type 1-B proteins (HIST1H2-BB, -BJ, and -BL), CCAT/enhancer-binding protein beta (CEBPB), and nuclear factor NF-kappa-B subunit 1 (NFKB1). The analysis for enriched pathways demonstrates that these genes are involved in apoptosis and in immune response, namely in B- and T-cell activation and TLR signaling (Figure 3A).

From the PPI analysis of genes modulated only in the subgroup of patients with EGMs, we obtained a PPI network connecting 440 genes (Figure 2B), and from this network, four hyperconnected modules were extracted. The pathway enrichment analysis of the four modules showed that no pathways were significantly enriched in M0 and M2, whereas in M1 and M3 we observed the enrichment of signaling pathways related to apoptosis, T- and B-cell activation, TLR and IFN-γ signaling, as well as co-stimulatory and proinflammatory cytokine signaling (Figure B). The complete list of the genes in the modules derived from PPI network analysis in the two subgroups of patients and the related enriched pathways are reported in Supplementary Tables 6 and 7.

DISCUSSION

We report here the results of gene expression analysis carried out in two strongly differentiated subsets of patients with pSS: those with disease limited to GFs, who also complained of WP, and those who presented with a variety of EGMs.

DEGs were functionally classified and submitted to a pathway enrichment analysis to find the most crucial signaling network that could be operative in the two subgroups.

The number and type of DEGs were different in the two subtypes of patients. Furthermore, patients with disease limited to GFs had a higher number of DEGs. This may indicate that this kind of patient could have a wider spectrum of pathological pathways.

In both subgroups, most of the modulated genes belonged to the same few biological processes that are known to be peculiarly activated in pSS (22). However, only in the GFs group were genes involved in sensory and pain perception modulated.

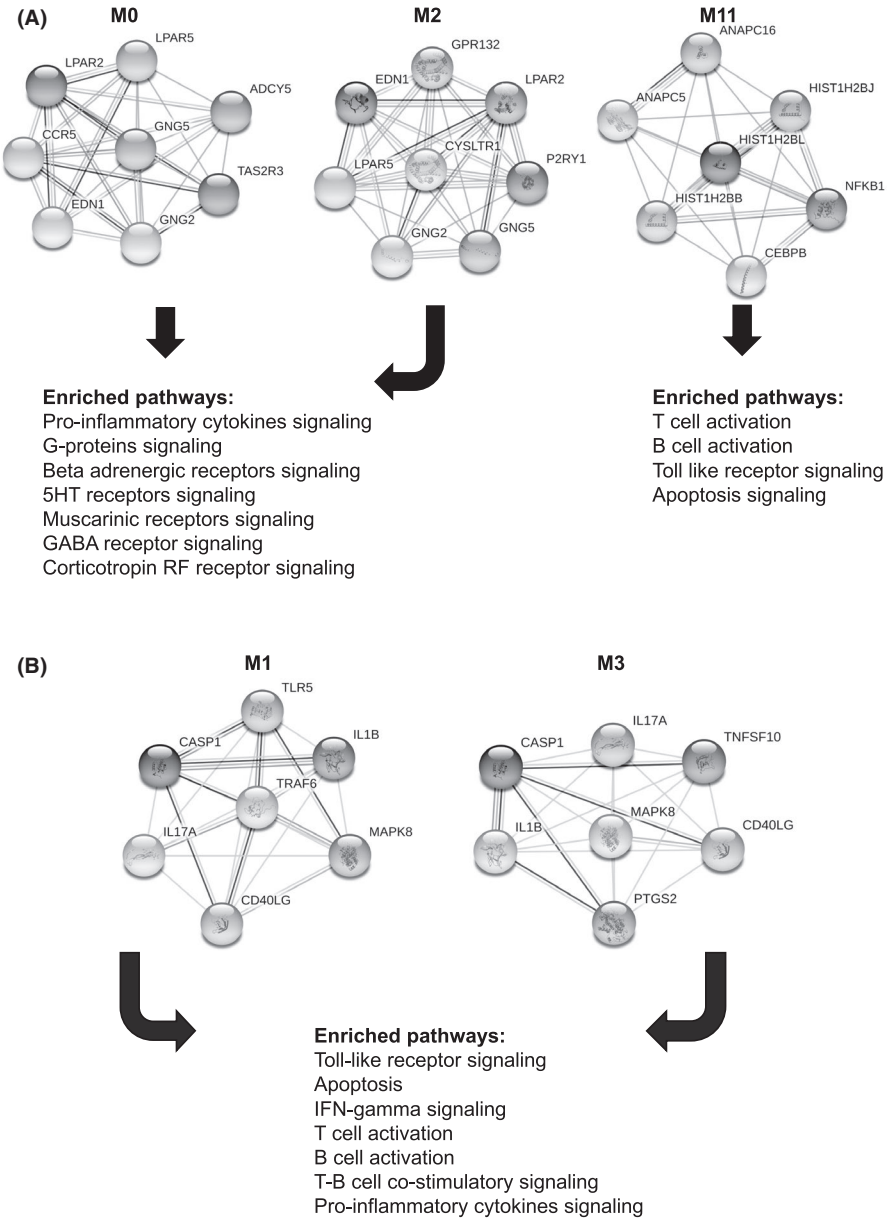

Figure 3. Highly interconnected modules obtained by cluster analysis of the protein-to-protein interaction networks in patients with glandular features and widespread pain (**A**), and in patients with extraglandular manifestations (**B**). The enrichment analysis of these modules allows recognition of the pathways represented in each module (listed in the lower part of the figure). For the definition and function of the genes present in the modules, see the Results section of the text.

We then performed a cluster analysis of the PPI networks to detect areas with the highest levels of interconnections among modulated genes (modules) and their associated pathways, which could be the drivers of the most important pathological mechanisms that are operative in the two subsets of patients.

As in the present study, the few previous studies in this field of gene expression analysis in pSS patients was performed using PBMCs (23,24). Although these studies were not completely comparable because of differences in adopted gene platforms, oligonucleotide probe sets, applied analytical software and statistical analysis, they unanimously demonstrated the central role of IFN type I and its induced genes in the pathogenesis of pSS (23,24) as well as the association of this gene signature with the presence of anti-Ro/La antibodies (23). In PBMC, pathways also related to T- and B-cell activation via their respective receptor signaling appeared to be overexpressed (23,24). However, in previous studies, in contrast with the present one, no preliminary phenotypical characterization and distinction was made in the studied cohorts to analyze possible correlations between genotypic and phenotypic patterns.

Although some locally activated pathways are certainly important to explain the biological processes specifically operative

in target tissues, it has been largely demonstrated that the analysis of gene signature in PBMCs can be considered the mirror of the main mechanisms that may drive the pathogenesis of some systemic autoimmune diseases, such as rheumatoid arthritis and systemic lupus (25,26), but also of other organ-specific immune-mediated disorders, such as multiple sclerosis (27). The fact that a transcription profile similar to that found in PBMCs has been observed in salivary tissue of patients with pSS (24) and, more recently, in cells isolated from synovial tissue of patients with psoriatic arthritis (28), seems to further support this assumption.

When the analysis of the most enriched pathways was performed in the present study, the results we obtained indicate that the predominant signaling networks in the two subsets of patients were different. Because the methods to define biological processes and enriched pathways in gene expression analysis are different, to obtain not completely concordant results from the application of these procedures is only apparently contradictory. Two sets of genes can be part of the same biological process, according to the GO classification system, but can be assigned to different signaling pathways when enrichment analysis is applied.

The most enriched pathways that characterized patients with GFs and WP are linked to pain perception and modulation processes (beta-adrenergic receptor, CRF, and 5HT receptors signaling) (29–31). The activation in PBMCs of some signaling pathways that are commonly believed to be peculiar of neuroendocrine system, but not of cells belonging to the immune system, is not surprising. Some studies have shown that a specific gene signature is present in PBMCs in non immunoe-related conditions (32) and in neuropsychiatric disorders (33). This is considered the direct consequence of the continuous biological cross talk between the neuroendocrine system and innate immune cell population that induces a reciprocal regulation (34).

Notch signaling is another pathway we found to be enriched in patients with GFs and WP. The evolutionary conserved Notch signaling is a ubiquitous pathway that is involved in many biological processes in adulthood. It has been shown that Notch signaling is involved in many inflammatory conditions (35). Probably via the TLR signal, Notch signaling is activated in macrophages and dendritic cells and thus induces these cells to proliferate and produce inflammatory cytokines, such as TNF-α and IL-1β. Thus, Notch signaling plays multiple roles in both innate immune response and inflammation. Furthermore, Notch signaling has an important role in synaptic plasticity and inflammation in the central nervous system (CNS). It seems to have an important role in the development of neuropathic pain as demonstrated by the fact that the intrathecal administration of a Notch signaling inhibitor may prevent the development of neuropathic pain (36).

The most enriched pathways in pSS patients with EGMs are those related to B-cell activation and TLR signaling. This confirms the previous data indicating that both innate and adaptive immune response are enhanced in this subset of patients.

Another pathway that appeared to be enriched in the patients with EGMs is that related to angiotensin II. Angiotensin II exerts proinflammatory and immune-mediated actions through the interaction with angiotensin II receptor type 1 expressed on the surface of macrophages and T cells by inducing the activation of different kinases, such as mitogen-activated protein kinase (MAPK) and extracellular signal–activated kinase (ERK), which are involved in many intracellular transduction signals (37). In this pathway, the arrestin system is able to influence various signaling systems and modulate chemokine receptor trafficking and signaling as well as apoptosis mechanisms (38). Conversely, the fact that the 5HT receptor signaling pathway is also enriched in this subgroup suggests that the activation of pain perception pathways is an important pathogenetic moment, even in this distinct phenotype of patients.

The differences in gene expression in the two subgroups of pSS patients, with opposite phenotypic features, appear more evident when looking at the PPI network cluster analysis and the related enriched pathway analysis. The most interconnected genes modulated in patients with EGMs belong to pathways involved in innate (apoptosis, TLR and IFN signaling, proinflammatory cytokines) and adaptive (T- and B-cell activation and cooperation) immune responses, which are largely documented in many biological studies as playing fundamental roles in pSS (22).

Conversely, by analyzing the PPI network derived from patients with GFs and WP, only one module (M11) was composed of genes that can be referred to enriched pathways involved in innate and adaptive immune responses, whereas M0 and M2 are dominated by components that belong to pathways that are involved in sensory perception and pain modulation mechanisms (39). In details, LPAR2, P2RY, beta-adrenergic receptor, and CRF receptor are involved throughout different biological mechanisms in central and peripheral pain sensitivity, whereas 5HT receptor systems have a double function of descending facilitation or inhibition of pain, and GABA receptor plays an important role in the inhibition of ascending painful stimuli (40).

Of particular interest are M0 and M2, in which the enriched pathway called “inflammation mediated by chemokines and cytokines” is interfaced with genes and their related pathways, which are involved in the process of pain perception and modulation. According to the enriched pathway description, CCR5, ADCY5 (present in M0), GNG2, and GNG5 (present in both M0 and M2) are the modulated genes that are components of this pathway. Notably, most of the genes present in M0 and M2 are also included among those that, according to the GO definition, are members of “sensory perception” gene set (Table 2).

GNG2 and GNG5 are the γ2 and γ5 subunits of specific heterodimeric G proteins and thus are essential constituents of some subtypes of G protein–coupled receptors (GPCRs). The GPCRs are ubiquitously distributed in practically all cells and systems, although the large majority of them have been estimated to be located in the peripheral nervous system (PNS) and CNS, and

drive here important functions in nociceptive signal transmission from the periphery and modulation of the upcoming neuropathic signals (41,42). The γ subunit, together with the β subunit, are constituents of particular GPCR subtypes that are specifically linked and activated by all types of chemokines, ie, α (C-X-C), β (C-C), γ (CX3C), and δ (XC) chemokines (43,44). The activation of these receptors is followed by the formation of the second messenger, the cyclic adenosine monophosphate (cAMP), which is catalyzed by ADCY. cAMP production is only the first step of a cascade of events, which includes activation of multiple kinases and transcription factors that exert differentiated effects on PBMCs, like T-cell proliferation, migration, aggregation, and production of proinflammatory cytokines as well as B cell antigen–stimulated proliferation (45).

Different chemokine receptors have been identified as promising therapeutic targets in autoimmune diseases. This approach, however, appears to be extremely complicated because of the redundancy of chemokines as well as the remarkable complexity and variety of chemokine receptors involved in the recruitment of multiple types of immune and inflammatory cells (46). Some interesting results regarding the role of chemokines in activating the autoimmunity process, and the possibility to consider this mechanism as a new potentially effective therapeutic challenge, come from animal studies, namely, by the demonstration that the inhibition of G protein βγ subunit signaling may abrogate nephritis in lupus-prone mice (47).

On the other hand, there is a large amount of data demonstrating that the biological sequence represented by chemokine linkage to their specific GPCRs, ADCY catalytic action, cAMP intracellular release, and specific kinase activation is an essential moment in inducing neuropathic pain and its central processing (48,49). A confirmation of this aspect comes from the observation that ADCY5 knockout mice have a markedly reduced pain response in acute and chronic conditions (50). The main actors in the development of neuropathic pain and pain sensitization mechanisms are the glia cells, namely, the microglia cells resident in PNS and spinal cord (51). Spinal microglial cells, but also peripheral nociceptors, and afferent postsynaptic neurons express chemokine receptors, and the activation of these receptors induces the ADCY-catalyzed cAMP production, the subsequent p38 MAP kinase phosphorylation leading to the production of proinflammatory cytokines and growth factors (52–54). It has also been shown in many experimental studies that T cell–released IFNγ is one of the main activators of microglia cells; this step is considered crucial for the induction of neuropathic pain (39). The fact that IFNγ is markedly overexpressed in patients with GFs and WP may suggest that this mechanism could be important even in inducing pain sensitization in this subset of patients.

This complex system can be maintained and even enhanced by the ability of activated microglia cells to induce a similar activation of astrocytes, which are also important actors for the induction of presynaptic/postsynaptic starting of excitatory ascending stimuli. Because astrocytes are predominantly located in the brain, it has been postulated that these biological processes may also induce central pain sensitization (39).

The data obtained in the present study using PBMCs suggest the possibility that specific mediators of inflammatory processes may play a role in activating different pathways involved in the development of neuropathic pain and central and peripheral sensitization. We can hypothesize that the same machinery of soluble mediators could become operative in glia and neuronal cells of both the PNS and CNS and thus may trigger the development of WP in pSS as well in other autoimmune disease where similar clinical features have been described (55).

In summary, our data suggest that different pathways driven by DEGs are operative in patients with pSS characterized by a disease limited to GFs and WP as compared with patients phenotypically marked by the presence of EGMs. In the former subtype, the phenotypical expression of the disorder could be predominantly driven by mechanisms of innate immunity and by a cascade of inflammatory cytokines and chemokines. The chronic production of these proinflammatory mediators could induce pain sensitization and WP.

Conversely, adaptive immune response pathways appear to dominate the scene in patients belonging to the other subtype of disease that is phenotypically characterized by EGMs. Whether these differences may be conditioned by differences in genetic susceptibility, as genoma-wide association studies may suggest (56), or modulated by post-transcriptional epigenetic phenomena like DNA methylation defects, histone modifications or microRNA-related activity (57) remains to be clarified. Further studies are needed to confirm the present preliminary data and to better understand these phenomena. More advanced results could both open the possibility to a more precise stratification of patients with pSS according to the pathological processes activated in different phenotypic subsets and offer the opportunity to adopt more precisely tailored therapeutic strategies.

AUTHOR CONTRIBUTIONS

Drs. Vitali and Dolcino wrote the article. All authors approved the final version of the article to be published.

Study conception and design. Vitali, Puccetti, Del Papa, Lunardi.

Acquisition of data. Dolcino (gene expression analysis), Minniti, Pignataro, Maglione, Del Papa (clinical and serological characterization of patients).

Analysis and interpretation of data. Vitali, Dolcino, Del Papa, Puccetti, Lunardi.

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