Review

Primary Sjögren's syndrome: Clinical phenotypes, outcome and the development of biomarkers

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ABSTRACT

Primary Sjögren's syndrome (pSS) is a complex autoimmune disease with distinct clinical phenotypes and variable outcomes. The systemic form of the disease is characterized by immune complex mediated manifestations and is complicated by lymphoma as a result of a polyclonal B cell hyperactivity that is evolving into B cell malignancy. In the past decades, well-established clinical and serological markers have been described in the literature to identify high-risk patients and to predict lymphoma development. However, specific biologic treatments have proven ineffective to control the disease. Significant research effort has been made to reveal the major underlying biological events in this subgroup and identify biomarkers for early diagnosis, prognosis and response to treatment. In this review, we summarize the current data for the proposed histological, molecular and genetic biomarkers.

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Contents

1. Introduction

Sjögren's syndrome (SS) is a slowly progressive, systemic autoimmune disease characterized by chronic inflammatory infiltration of the salivary and lacrimal glands. Although pSS is mainly confined to the exocrine glands, almost every organ can be potentially affected, reflecting the systemic nature of the disease [1]. The relatively limited

TCR repertoire of the infiltrating T cells and the presence of hyperreactive B memory cells producing plethora of autoantibodies, in the typical periepithelial lesion of the affected tissues, suggest autoimmunity to play an important role in the pathogenesis of the disease [2–6]. The majority of pSS patients present with glandular symptoms while a subset may develop extraglandular and extraepithelial manifestations. Although sicca symptoms may be quite disabling, extraepithelial complications and especially lymphoma affect morbidity and survival among pSS population [1,7]. Sjögen's syndrome evolves slowly following a rather benign course with the majority of patients to have a stable clinical picture for a long time. However, when disease is usually diagnosed, patients already experience oral and eye dryness for many years, implying that the underlying pathological process has been

already established and therefore the disease is considered advanced [1]. The diverse clinical phenotypes and outcomes of pSS, the slowly progressive clinical course and the fact that the disease is already advanced when it becomes clinically apparent raise challenges regarding the therapeutic interventions in pSS.

Based on the experience from other autoimmune diseases, many biologic regimens have been tested to treat pSS patients [8]. However, biologic agents have proven ineffective to control the disease and improve the quality of life among pSS patients. Several reasons should be considered to interpret carefully this therapeutic failure. One important reason is the fact that the scientific community does not have reliable tools to assess the overall response to treatment. While ESSDAI is a useful research tool for patients with the systemic form of the disease, ESSPRI is based on the subjective perception of patients about dryness and is a measure of self-assessment and not an objective marker of hyposalivation [9]. In addition, the estimation of salivary flow is characterized by diurnal and personal variations and is determined by many factors. On the other hand, the unique clinical features of pSS mentioned previously have not been taken into account, and as a result, patients who participated in these studies represent different clinical subsets, masking the potential clinical benefit in some of them. The distinct clinical phenotypes and the diversity of the clinical course point out the necessity to develop biomarkers capable of stratifying pSS patients according to clinical, histological and molecular criteria. Ideally, biomarkers are expected not only to offer an early diagnosis of pSS but also to predict the disease outcome and the possible response to specific biologic treatments. In this review, we summarize the recent advances in the field of clinical and basic research to indentify and introduce useful biomarkers in the clinical practice.

2. The wide clinical spectrum of Sjögren's syndrome

The wide clinical spectrum of pSS extends from a well-tolerated and benign exocrinopathy restricted to the salivary and lacrimal glands to severe life-threatening conditions such as vasculitis and lymphoma [1]. This enriched clinical picture is considered to result from two distinct underlying immunopathologic phenomena: the lymphocytic infiltration around the epithelium of the affected tissues and the B cell hyperactivity. Over the past decades, it has been shown that the epithelium plays a critical role in the pathogenesis of the disease justifying the term "autoimmune epithelitis" for pSS [10]. Salivary gland epithelial cells form pSS patients have been found to express MHC class I and II molecules as well as adhesion and costimulatory molecules critical for activation and regulation of naïve T cells [11–18]. In addition, the epithelium secretes cytokines and chemokines implicated in the recruitment and differentiation of T and B lymphocytes [19–21]. The epithelial cells that orchestrate and attract lymphocytes in the site of lesion die by apoptosis releasing autoantigens, capable of fueling and perpetuating the local autoimmune response. The release of autoantigens may be also mediated by epithelial cells through exosomes [22]. Gradually, the epithelium is destroyed and replaced by fibrotic tissue producing clinical symptoms related to the dysfunction of the damaged tissue. The characteristic periepithelial lymphocytic infiltration of the salivary and lacrimal glands can be also observed in other organs such as the liver, the kidney and the bronchi/bronchiolars, producing the systemic extraglandular manifestations of the disease [10]. On the other hand, B cell hyperactivity, as reflected by the hypergammaglobulinemia and the production of autoantibodies, has been associated with extraepithelial immune complex mediated manifestations such as vasculitis, purpura, glomerulonephritis and peripheral nephropathy and the development of lymphoma [1,23]. The production of cryoglobulins and the deposition of immune complexes to the affected tissues represent the main mechanisms that drive the pathogenesis of the exraepithelial manifestations. The initial event that drives the activation of the epithelium is not known, although latent viral infections are implied to be involved in this process through ligation of PRRs of the innate immunity [24,25].

Almost all pSS patients experience oral and eye dryness at the time of diagnosis although in some rare cases other manifestations of the disease such as IN or even vasculitis may precede the main sicca symptoms [1]. Half of the patients present with unilateral or bilateral parotid swelling and a significant percentage of pSS patients complain of systemic dryness involving the nose, the trachea, the vagina and the skin, suggesting that other exocrine glands are also affected in the context of glandular involvement [1,26]. Briefly, the term glandular manifestations summarize the symptoms and signs originated from a generalized exocrinopathy, which usually involves the salivary and lacrimal glands but can be extended to other organs and tissues. Although grandular manifestations vary in severity and may affect the quality of life of pSS patients, they have not been associated with increased mortality and follow a rather stable clinical course for many years [1] Similarly, the systemic extraglandular manifestations are produced by the typical lymphocytic infiltration around the epithelium of target organs, such as the liver, the kidney and the bronchi/bronchiolars. Almost 5% of pSS patients are expected to have biochemical and histological features of primary biliary cirrhosis (PBC) as a result of the lymphocytic infiltration and destruction of the biliary epithelium [1]. Lymphocytic infiltration of the kidney interstitium and involvement of tubular epithelium may lead to distal renal tubular acidosis manifested by hypokalemic hyperchloremic metabolic acidosis with normal anion gap and nephrolithiasis/nephrocalcinosis [27]. Interstitial nephritis occurs in 30% of pSS patients in a subclincal form but overt disease ranges between 5% and 10% [1,27]. The most common type of pulmonary involvement is lymphocytic bronchitis/bronchiolitis affecting almost 20% of patients and is characterized by dry irritant cough and obstructive respiratory pattern [1]. Extraglandular manifestations evolve slowly with favorable outcome, although is some cases the ongoing pathologic process may lead to severe organ impairment and end-stage organ failure [1].

Sjögren's syndrome patients with extraepithelial manifestations constitute 10–15% of pSS population and represent the systemic form of the disease presenting with the clinical picture of vasculitis [1,7]. The detection of type II mixed monoclonal cryoglobulins, the low C4 complement serum levels and the deposition of immunoglobulins in the affected sites of these pSS patients support the notion that vasculitic manifestations are mediated by the deposition of immune complexes. The systemic form is clinically expressed by palpable purpura of the lower extremities and occasionally leg ulcers in 10% of pSS patients. Peripheral neuropathy due to inflammation of the vasa vasorum accounts for 1% among pSS patients and is usually manifested by sensorimotor axonal polyneuropathy and mononeuritis multiplex [1]. In the case of kidney, immune complexes are deposited within the glomerulus producing various clinical manifestations such as nephritic or nephrotic syndrome [28]. The extraepithelial manifestations appear late during the natural history of pSS and have been associated with increased morbidity and mortality. Patients of this group have increased risk for developing lymphoproliferative disorders since palpable purpura and low C4 serum levels have been found to be predictors of lymphoma [1,7]. It is obvious that the different biologic and immunologic aspects of the disease define the various clinical phenotypes and determine the outcomes. In this context, biomarkers should be able to differentiate the pSS subsets and focus on the systemic form of the disease that inevitably draws the clinical attention.

3. Mortality and risk factors in Sjögren's syndrome

Different groups have shown that pSS patients display increased mortality compared to the general population. In the largest studies, the standardized mortality ratio (SMR) varies from 1.02 (95% CI = 1.03–3.71) to 4.66 (95% CI = 3.85–5.60), suggesting that pSS has at least a minimal impact on patients' survival [1,7,29–34]. In most of

these studies, the leading cause of death was lymphoma. Theader et al., after studying a cohort of 265 pSS patients, found that the SMR was 1.17 (95% CI = 0.81–1.53), and this reduced survival rate was related only to lymphoproliferative malignancies with a specific SMR of 7.89 (95% CI = 2.89–17.18), directly correlating lymphoma with the risk of death in pSS [33]. Although the increased mortality in pSS is mainly attributed to lymphoma, other less common causes of death related to the syndrome have been reported in the literature. Severe forms of medium vessel vasculitis resembling polyarteritis nodosa may affect internal organs such as the gallbladder leading to death [35]. Similarly, progressive renal disease due to glomerulonephritis and less frequently interstitial nephritis may result in end-stage renal failure requiring hemodialysis and is associated with increased morbidity and mortality [28]. Finally, secondary causes of death due to infections after administration of immunosuppressive treatment in pSS patients with systemic complications may confer increased mortality risk. Interestingly, in a recent survival study with 1045 pSS consecutive patients, it was shown that apart from B cell lymphomas and severe organ impairment in the context of lung, renal and vasculitic involvement, infections and cardiovascular events were prominent causes of death implying that mortality is not correlated exclusively with autoimmunity itself [30].

The association between pSS and lymphoma is well documented. In one of the earliest studies conducted at the NIH, the estimated risk of developing lymphoma in pSS was 44-times higher compared to the general population [36]. In another meta-analysis, pSS had the highest SIR for lymphoma (18.9, 95% CI = 9.4–37.9) compared to SLE and RA [37]. The prevalence of lymphoma differs among different pSS populations and ranges from 2.7% to 9.8% [23,36,38–45]. Ioannidis et al. after studying a cohort of 723 pSS patients found that the 10-year risk of lymphoma was 4%, while the lifetime risk was estimated to be 5–10% [7]. Extranodal marginal zone B cell lymphomas of mucosa-associated tissue (MALT) are by far the most common histological type of pSS associated NHL followed by diffuse large B cell lymphomas (DLBC) with the salivary glands being the most common affected site [46]. MALT lymphomas are indolent with a favorable course, while DLBC lymphomas display a worse prognosis. In the past years, many predictors of lymphoma have been described [46,47]. In the Greek study mentioned above, a predictive classification model was proposed for pSS: patients with parotid gland enlargement or palpable purpura and low C4 serum levels or cryoglobulins are classified as type I with high risk for lymphoma, while patients without these adverse prognostic factors and therefore with negligible risk for future lymphoma development are classified as type II [7]. Taken together, patients with systemic complications and lymphoma development constitute the high-risk group of pSS population; the clinicians should be focused on regarding the diagnosis, prognosis and treatment. Although risk factors for lymphoma are of highly clinical importance, it is obvious that biomarkers should extend beyond the spectrum of clinical and serological predictors of lymphoma and reflect, if possible, more complex cellular, molecular and genetic aspects of the systemic form of disease and its complications including lymphomagenesis.

4. Biomarkers in Sjögren's syndrome

4.1. Biological significance and clinical utility

The systemic extraepithelial manifestations and the MALT lymphomas originated from the diseased salivary glands of pSS patients share B cell hyperactivity as a common underlying immunopathologic process. Extensive studies of the minor salivary glands (MSG) of pSS patients have shown that this marked B cell hyperactivity is mainly the result of an ongoing antigenic stimulation [48–50]. However, monoclonal products and cryoglobulins can be detected in the serum of pSS patients early during the disease course and especially type II cryoglobulins are considered one of the strongest predictors of lymphoma even at the time of disease diagnosis [1,7]. On the contrary, systemic immune

complex mediated manifestations and lymphomas are usually late complications of pSS [7,23,28]. Interestingly, in some cases, the monoclonal component has been associated with the presence of B cell monoclonal populations within the pathological lesions of the salivary glands [50,51]. The fact that B cell monoclonality may pre-exist at the time of pSS diagnosis in some patients and precede the appearance of extraepithelial manifestations and the development of lymphoma connotes that at the biological level there is a progressive and multistep process leading to malignant transformation within the salivary glands of pSS patients [1,7]. Indeed, all predictors of lymphoma and the adverse prognostic factors of pSS point out B cell monoclonality as the predominant biological event rather than the non-specific B cell hyperactivity. In accordance with this, pSS patients with anti-SSA, anti-SSB and hypergammaglobulinemia exhibit lower risk for lymphoma compared to those with mixed monoclonal cryoglobulins. A recent study concluded that serum free light chains and β2-microglibulin used as non-specific markers of B cell activation were correlated with diseases activity but not with lymphoma development [52]. During the transformation process, the B cell acquires various traits to overcome normal intrinsic and extrinsic barriers to malignant transformation that regulate cell proliferation and homeostasis, until it reaches the mature malignant phenotype clinically expressed as MALT lymphoma with invasion of the epithelium. Various factors contribute to lymphomagenesis including genetic factors, proto-oncogene activation, tumor suppression gene inactivation, dysregulation of immunoregulatory mechanisms and lymphoma promoting effects of the microenvironment and extracellular matrix [46]. This latent period between the premalignant phenotype of B monoclonal cells and the mature malignant phenotype of MALT lymphomas may last for a long time and offers the beneficial window for the use of the biomarkers.

Ideally, biomarkers should reflect the various stages and phenotypes of the disease, on the basis of the features through which the B cell evolves toward malignancy along with the principal mechanisms that drive this process. Given the explosive development of biotechnology and molecular biology, biomarkers should exceed the typical clinical and serological frame of lymphoma predictors and embrace additional measurable parameters reflecting the major cellular and molecular underlying events. In this way, biomarkers are expected to offer a number of advantages in clinical practice: (i) early diagnosis of the disease using as criterion whether the disease lies in the spectrum of the benign polyclonal, the premalignant/benign monoclonal or near the malignant stage of B cell evolution; (ii) correlation with the clinical phenotypes and subsets of pSS; (iii) prediction of severe systemic complications and lymphoma development; (iv) prediction of adverse outcome and risk of death; and (v) assessment of response to specific targeted treatments. For this purpose, different biological specimens could be used including serum, saliva and tissue from minor salivary glands. For a biomarker to be introduced in the clinical practice, some other requirements should be also fulfilled. Apart from its biological significance, the methodology to measure the variable must be applicable, reproducible and cost–benefit adequate. In addition, the biological specimen should be easily collected and processed. The usage of such biomarkers will allow a more sophisticated stratification of pSS patients according to the previously mentioned criteria and will offer the opportunity to design better clinical studies for assessing treatment efficacy. Considering the complexity of lymphomagenesis as a process, it would be useful to develop a set of biomarkers in order to achieve the maximum prognostic power. Although such a tool is not available, current advances in the field of cellular and molecular biology point out to this direction.

4.2. Clinical, serological and hematological markers

Many clinical, serological and hematological markers have been described in the literature as predictors of lymphoma, systemic involvement and death and are currently used in the clinical practice [46,47]. History of persistent parotid gland enlargement is by far the most

common predictor of lymphoma and is considered an independent risk factor in many series of pSS associated NHL patients [7,31,36,43,53,54]. Palpable purpura and skin ulcers in the context of small vessel vasculitis affecting mainly the lower extremities is another well recognized clinical entity that has been associated independently with the future development of lymphoma [1,7,33]. In addition, Skopouli et al. showed that palpable purpura is a significant predictor of glomerulonephritis, when present at the time of diagnosis (RR = 16.3, $p = 0.0024$) [1]. Voulgarelis et al. were the first who reported the occurrence of peripheral neuropathy in pSS patients with NHL, suggesting a predictive role for subsequent development of lymphoma [23]. Similarly, glomerulonephritis has been found to coincide with NHL in some pSS patients and has been associated with increased risk for lymphoma and death [1,28]. Lymphadenopathy, as a sign of lymphoid hyperactivity, was also found to be a valid predictor of lymphoma [7,36,43,47,55]. On the contrary, splenomegaly was proposed as an independent risk factor for lymphoma only in one study by Baimpa et al. (HR = 3.97, 95% CI = 1.49–10.60) [55]. Cohen et al. after using FDG PET/CT and a combined 6-point scale activity score derived from the lungs, parotid glands, submandibular glands and lymph nodes, found an association between the combined PET/CT activity score and pSS disease activity [56]. Furthermore, FDG uptake from the lymph nodes was marginally higher in pSS patients with lymphoma compared to those without (SUVmax = 5.4 vs. 3.2, $p = 0.05$), implying that since lymphadenopathy is a common finding among pSS patients, PET/CT could be a useful diagnostic tool for lymphoma. At this point, we would like to emphasize that all these clinical signs and manifestations, irrespectively of their prognostic value, may accompany cases of well-established lymphomas, and therefore the clinician should always rule out the possibility of an active lymphoproliferative disorder.

Regarding the serological markers, Tzioufas et al. demonstrated that IgMk type II cryoglobulins are detected in the serum of almost 20% of pSS [50] patients and 10 years later the same group proposed mixed monoclonal cryoglobulins as a significant risk factor for lymphoma [51]. Since then, many studies have documented the predictive value of mixed monoclonal cryoglobulins as an independent risk factor for lymphoma development and lower survival in pSS [1,30,53,55]. Hypocomplementemia has been also associated with lymphoma and increased mortality. More specifically, low C4 serum levels and cryoglobulinemia were reported as strong predictors of lymphoma by Skopouli et al. (RR = 7.5, $p = 0.0016$ and RR = 7.9, $p = 0.0012$, respectively) [1], while Iaonnidis et al. correlated low C4 serum levels and purpura with increased risk of death by lymphoma (HR = 4.39, $p = 0.001$ and HR = 3.0, $p = 0.019$, respectively) [7]. Notably, the detection of mixed monoclonal cryoglobulins and low C4 serum levels at disease diagnosis could also predict the development of glomerulonephritis in pSS patients (RR = 6.5, $p = 0.03$ and RR = 0.015 $p = 8.6$, respectively) [1]. Apart from lymphoma, in the study by Ramos-Casals et al., low C4 serum levels were independently correlated with RF, cryoglobulins, peripheral neuropathy and skin vasculitis implying that type II cryoglobulins with RF activity and low C4 serum levels could be also used as biomarkers of systemic involvement and vasculitis in pSS [57]. Similarly, Brito-Zeron et al. (2007) showed that the low C4 serum levels were independent predictor of vasculitis, while low C3 levels were proven strong predictor of both lymphoma and death [31]. Finally, the levels of beta microglobulin and serum free light chains have been found elevated in pSS patients with increased disease activity, although no association with lymphoma development was found [52]. The fact though that these serological markers reflect a non-specific B cell activation supports their use as indicators of the B polyclonal phase of the disease. The role of common hematological markers has been also studied but less extensively. A low CD4/CD8 ≤ 8 ratio and CD4 lymphopenia have been found to correlate with the appearance of DLBCL [58]. In this line, Baimpa et al. correlated the presence of neutropenia at diagnosis with the subsequent development of marginal zone B cell lymphomas while lymphocytopenia was associated with non-MZBCL and

mostly diffuse large B cell lymphomas [55]. The ESSDAI index is another useful tool that could be introduced in the clinical practice as a measure of systemic disease activity combining many categories of the markers mentioned previously [9]. Of interest, pSS patients with high activity in at least one ESSDAI domain and ESSDAI score >14 at the time of diagnosis have increased overall risk of death [30], while as mentioned previously for pSS-related NHL patients, high disease activity and IPI were the main risk factors for lymphoma outcome [59].

A variety of autoantibodies are also expected to define pSS subsets based on the described clinical associations. Previous studies have shown that pSS patients with anti-Ro/SSA and anti-La/SSB are expected to have more severe inflammatory infiltrations of the salivary glands and present more frequently with parotid gland enlargement and systemic extraglandular manifestations, reflecting a higher disease activity [60]. Interestingly, anti-Ro/anti-La-negative patients were found with lower prevalence of lymphoproliferative disorders and lower risk to develop lymphoma [53]. During the past years, a lot of effort has been focused on the role of ACA in pSS syndrome. In a study from Greece, the presence of ACA was associated with higher prevalence of dysphagia and lower prevalence of dry eyes, hypergammaglobulinemia and anti/SSA and anti/SSB antibodies [61]. In addition, Baer et al. after analyzing 1381 pSS patients found that ACA + patients had higher focus score suggesting a more severe glandular inflammation [62], while Baldini et al. supported that the patients with the overlap entity ACA + limited scleroderma/SS may experience a higher risk for NHL [63]. Regarding the anti-CCP antibodies in pSS, Ryu et al. after assessing the clinical features of 405 pSS patients supported that the detection of anti-CCP allows the prediction of progression to RA with an odds ratio 2.5 (95% CI = 1.7–3.7) [64]. However, in a Dutch study, pSS patients with positive anti-CCP antibodies were found to present with less severe symmetric polyarthritis compared to RA patients and less pronounced B cell activation [65]. Rheumatoid factor, especially in the presence of low C4 serum levels, should be interpreted as an indication of type II mixed cryoglobulinemia, and in this context, it has been associated with predictors of lymphoma and systemic disease [51]. Finally, the presence of AMA and ASMA points out to the diagnosis of primary biliary cirrhosis and autoimmune hepatitis, respectively, and constitutes either overlap entities with pSS or distinct subsets that need to be further defined.

4.3. Histological markers

The histology of the minor salivary glands is one of the most fundamental approaches to understand the pathogenesis of the disease and to establish the diagnosis of pSS. In one of the first studies, it was demonstrated that the extension of the inflammatory infiltration of salivary glands was greater in patients with anti-Ro/SSA and anti-La/SSB antibodies, and a significant correlation was found with vasculitis, splenomegaly, lymphadenopathy and the total number of extraglandular manifestations, implying it is a measure of systemic and severe involvement [60]. In a recent study, Carrubi et al. after evaluating the clinical utility of the degree of inflammation in the minor salivary gland biopsy concluded that the focus score was independently associated with lymphoma [66], while Risselada et al. defined that pSS patients with FS ≥ 3 are at increased risk for subsequent lymphoma development [67]. The ectopic germinal center (EGC)-like structures that may appear in the minor salivary glands of pSS patients have also drawn attention as possible sites of B cell hyperactivity and MALT lymphomas. Theader et al. reported that 25% of pSS patients may have EGC-like lesions in minor salivary gland biopsy at diagnosis and these patients display increased risk for developing NHL compared to those without ($p < 0.001$) [67]. However, in another study, after multivariate analysis, EGC-like structures were associated only with extraglandular manifestations [68]. Finally, in a small series of 21 pSS patients with lymphoma, the investigators could not document an association among ectopic germinal centers, B cell monoclonality and lymphoma [69]. Given that the ectopic germinal center formation is mediated by lymphotoxins and specific

chemokines such as CXCL12, CXCL13, CCL11, CCL19 and CCL21, Nocturne et al. reported increased CXCL13 and CCL11 serum levels in pSS associated NHL patients, while CXCL13 serum levels were also correlated with disease activity [70]. Surprisingly, Barone et al. found increased expression of CXCL12 in the infiltrated ductal epithelium and malignant B cells of pSS patients with MALT lymphoma, suggesting a regulatory role for this chemokine to promote survival of B cells [71]. Investigating the role of ectopic germinal centers to mediate class switch recombination, Bombardieri et al. detected expression of activation-induced cytidine deaminase (AID) by follicular dendritic cells within EGC and by interfollicular large B cells in T rich areas around ECG in salivary glands of pSS patients [72]. On the contrary, in pSS patients with MALT, AID was expressed only by numerous residual EGC and not by neoplastic marginal-like B cells. Taken together, the distribution of AID within the histological structures and the cell populations of the minor salivary glands could reflect at some extent the various stages of the disease toward malignancy.

Furthermore, the composition of the inflammatory infiltrate of the minor salivary glands seems to reflect the distinct underlying pathogenetic mechanisms and the severity of the lesion. Christodoulou et al., after classifying the inflammatory lesions into mild, intermediate and severe according to Tarpley score, showed that T cells predominate in mild lesions (60% of total infiltrating cells), whereas B cells is the major population in the intermediate (45% of total infiltrating cells) and advanced lesions (50% of infiltrating cells) [73]. The reduction of T cells with severity was attributed to a decline of CD4 cells since the number of CD8 cells remained stable (15% of total infiltrating cells). The numbers of macrophages and B cells were directly correlated with the degree of inflammation in contrast with the numbers of T cells. In another study, the presence of macrophages in the lesions of minor salivary glands was correlated with salivary gland enlargement ($p = 0.01$), while dendritic cell infiltration was associated with salivary gland enlargement ($p = 0.03$) and C4 hypocomplementemia ($p = 0.05$) [74]. In addition, strong expression of IL-18 that was observed mainly in B cell rich areas and was positively associated with focus score ($p = 0.001$) and negatively with C4 serum complement levels ($p = 0.02$). On the contrary, infiltration by IL-12 expressing cells was negatively associated with focus score ($p = 0.003$) and positively with C4 serum levels ($p = 0.05$) [74]. Although the histological pattern of the lesion regarding the B, T, macrophages and dendritic cells remains unchanged from the time of diagnosis, patients with adverse prognostic factors should perform another biopsy to monitor disease progression toward lymphomagenesis [75]. Given that different cell types either T or B cells drive the major immune responses in mild and advanced lesions, respectively, the histological pattern could be used as an overall biomarker to tailor treatment options. The role of Foxp3 Tregs has been also studied in pSS by Christidoulou et al. who demonstrated that the higher incidence of Foxp3 Tregs cells was associated with the inflammation severity as reflected by focus score ($p = 0.05$), while lower frequencies were correlated with low C4 serum levels ($p = 0.012$), a well-established predictor of lymphoma [76].

4.4. Molecular markers

In the past decade, saliva has become an attractive biological specimen for research not only because it is easily accessible but also because of the recent advances in the field of biotechnology. Proteomic analysis has been applied to whole or parotid saliva of pSS patients providing some new insights about the diagnosis and classification of pSS [77]. Saliva contains a variety of proteins although in lower concentrations compared to serum and is suitable for proteomics. In this line, Ambatipudi et al. after employing multidimensional protein identification technology in a pooled sample of parotid saliva from pSS patients found that 240 proteins were either upregulated or downregulated compared to healthy controls and after further analysis 100 related pathways were identified, highlighting the dynamics of this approach

[78]. However, regarding the type of sample, one should keep in mind that the proteome of whole saliva may originate not only for the local diseased glandular tissue but also from the serum and the oral microenvironment. In a systemic review by Baldini et al. [77], the research effort of different groups to identify potential biomarkers in the saliva of pSS patients using proteomic analysis was explored. Eight studies were found, in which the unstimulated whole saliva was analyzed, although in 2 out of 8 studies, parotid saliva was collected. Overall, the identified proteins could be classified into three main categories: acinar-related secretory proteins such as amylase, inflammatory proteins and immune-related proteins [77]. As expected, acinar proteins were found decreased in pSS patients compared to controls reflecting the destruction of salivary glands, while inflammatory proteins were increased as a result of a persistent and chronic inflammatory state. Interestingly, b2 microglobulin, immunoglobulin k light chain and immunoglobulin gamma light chain were elevated indicating an underlying B cell hyperactivation. However, in all these studies, no protein or group of proteins were correlated with specific clinical phenotype of pSS or typical predictors of systemic disease and lymphoma [77]. In a recent study by Delaleu et al. [79], after developing an antibody assay with 187 proteomic biomarkers to identify the salivary proteomic profile of pSS patients, it was found that 61 proteins were significantly changed compared to asymptomatic individuals, with 60 of them to be upregulated and 1 downregulated. Most of these proteins were associated with B cell mediated responses. Notably, a four- and six-component biomarker signature based on IL-4, IL-5 and clusterin managed to successfully classify pSS patients and differentiate them from the healthy subjects. Although, saliva proteomics is a promising research and diagnostic tool, it does not have the capacity yet to define pSS subsets and to mirror the underlying pathological and immunological events at the level of inflammatory glandular lesions [80]. The suggestion to replace minor salivary gland biopsy by saliva proteomics raises questions. Although minor salivary gland biopsy is an invasive method, it is not accompanied by major complications and provides important information not only to safely diagnose pSS but also to guide treatment choices. On the other hand, the introduction of novel biotechnologies into the tissue level may guide saliva proteomics and improve the clinical utility of biomarkers. To this direction, Hu et al. performed gene expression and proteomic analysis in parotid gland tissues from non-pSS, pSS and pSS associated MALT lymphoma patients and identified 8 co expressed genes and 70 upregulated proteins that could distinguish lymphoma from non-lymphoma pSS patients, underling the important role of glandular tissue specimen and new biotechnologies [81].

Micro-RNAs are well-conserved, small non-coding RNAs of 19–25 nucleotides that are involved in RNA silencing and post-transcriptional regulation of gene expression by either mRNA degradation or blocking of translation [82–84]. A single miRNA molecule is usually complementary to the 3UTR of more than one mRNAs, thus regulating the expression of many different genes. Indeed, the expression pattern of miRNAs seems to reflect the physiological state of a cell or a tissue and specific patterns have been associated with disease states. Lately, the role of miRNAs in pSS and more specifically in the saliva has been revealed [84–87]. Michael et al. after collecting whole saliva from healthy volunteers, managed to isolate exosomes and to document the presence of miRNAs with quantitative PCR and microarray profiling [87]. The presence of miRNAs in salivary exosomes points out glandular cells as the possible source and offers another research tool for identifying potential biomarkers. Alevizos et al., recruited 16 pSS patients and 8 healthy individual, collected MSG biopsy specimens and classified patients according to the severity of the inflammatory lesion and the level of salivary flow [88]. Micro-RNAs were isolated from homogenized tissues and analysis was performed to identify miRNA expression patterns that could distinguish pSS patients from controls. In addition, miRNAs expression was compared between patients with mild and severe inflammation as well as between patients with low and preserved salivary function to find possible expression alterations. Two selected miRNAs following

opposite expression patterns were found, with miR-768-3p to increase and miR-574 to decrease with high focus scores. In addition, 9 miRNAs (miR-765, miR-181a, miR-766, miR-335, miR-16, miR-671, miR-663, miR-340, hsa-miR-155, miR-5100) were associated with decreased salivary flow. The authors concluded that distinct mRNA profiles are correlated with the degree of inflammation in the MSG and with the functional state of the salivary glands, suggesting miRNAs as a set of biomarkers for pSS [88]. Accordingly, our group explored the role of miR200b – known to regulate oncogene expression and play an important role in tumorigenesis and metastasis – in the minor salivary glands of pSS patients without lymphoma, with pre-lymphoma and lymphoma. Preliminary results revealed that the expression levels of miR200b were inversely correlated with the progression toward lymphoma and thus it could be used as a biomarker for lymphoma development.

B cell activating factor (BAFF) is a well-known trophic agent involved in B cell homeostasis, differentiation and proliferation through activation of the NF-kB pathway and/or upregulation of anti-apoptotic molecules [89–91]. In previous studies, BAFF has been proposed to be implicated in autoimmune diseases and NHL [90,92]. More specifically, increased serum levels and specific SNPs of BAFF have been found in NHL patients of B cell origin [93,94]. In pSS, increased serum levels of BAFF were linked to anti-Ro antibodies and EGC and were correlated with high ESSDAI score as well as the presence of B cell clonal populations in the salivary glands of pSS patients [95–97]. Finally, Flt3 ligand was proposed as a biomarker for lymphoma development in pSS. The Flt3/Flt3L pathway is expressed by lymphoid progenitors in the bone marrow and is capable of delivering survival, differentiation and apoptotic signals [98]. Tobon et al. measured the Flt3L serum levels of 369 pSS patients and found a positive correlation with disease activity, purpura and predictors of lymphoma such as lymphopenia and hypocomplementemia. Interestingly, ROC curve analysis revealed a significant predictive value of elevated Ftl3 serum levels for the development of lymphoma [99].

4.5. Genetic markers

Genetic factors are important contributors in the pathogenesis of diseases. Alterations in gene expression may have an impact on cell phenotype, function and homeostasis, especially in multistep biological processes such as tumorigenesis. Most of diseases have a complex genetic background with more than one gene to be implicated and drive the cellular and molecular events. In the past decades, the different regulatory levels of gene expression were revealed offering new insights to better understand cellular function and to connect molecular biology with clinical manifestations and phenotypes. Single nucleotide polymorphisms (SNP) are common genetic variations of a single nucleotide at specific positions in the genome that may affect gene expression. There are SNPs that have been associated with certain diseases or traits, while others seem to offer a protection against specific diseases. Regarding pSS, Khuder et al. studied the gene expression from saliva and salivary gland biopsy specimens of 52 patients and 51 healthy controls [100]. The authors identified 55 genes as possible genomic biomarkers to distinguish pSS patients from healthy individual and after further analysis they proposed 19 genes with 95% classification accuracy. Similarly, Hu et al., as mentioned previously, proposed 8 co expressed genes (GRB2, ARHGDIB, CD40, PSMB9, ALDOA, PRDX5, PARC, PPIA) as genomic markers to identify pSS associated NHL patients. After gene ontology functional analysis, these genes were found to be involved in protease and ribosome degradation, translation, signal peptides MHC class I, cell communication and integrin mediated adhesion [81]. In the past years, many gene polymorphisms have been reported in the literature to be associated with pSS either positively or negatively. An SNP polymorphism rs11575837 in the promoter of the NCR3/NKp30 activating receptor of NK cells was found to downregulate gene expression and protect from pSS [101], while Miceli-Richard et al. reported that the CGGGG insertion/deletion polymorphism of the IRF5 promoter was associated with decreased levels of mRNA in PBMCs and higher risk to develop pSS [102]. Furthermore, 2 STAT4 SNPs, rs7574865 and rs7582694 were found more frequently among pSS population compared to controls [103]. Lately, a polymorphism of TNFAIP3 was described to play a role in pSS lymphoma patients [104]. TNFAIP3 is upregulated by TNFa or other cytokines and inhibits NF-kB activation. Apart from its role in immunity and inflammation, TNFAIP3 has been found to be inactivated in B cell lymphomas [105], while recently the rs2230926 polymorphism was found to confer high risk for pSS associated NHL [106]. Finally, specific SNPs in CCL11, IL17A, ICA1, PKN1 and NF-kB-related genes (TANK, IKBKE, CARD8) were reported by Reksten et al. in pSS patients with EGC compared to those without [107].

Lately, cumulative data highlighted the role of genetic variants of the BAFF/BAFF-R pathway in the development of lymphoma in pSS. Hildrebrand et al. first described a mutation in the cytoplasmic tail of the receptor in patients with NHL [108]. This mutation encodes a His159Tyr substitution and leads to enhanced recruitment of TRAF 2,3,6 and subsequent activation of both the NF-kB1 and NF-kB2 pathways along with increased production of immunoglobulins after CD40 ligation. In a study by Nezos et al., after genotype and haplotype analysis of 5 BAFF variants (rs1224141, rs12583006, rs9514828, rs1041569 and rs9514827) in high and low risk pSS patients, specific associations were reported, suggesting that BAFF may be involved in lymphoma development [109]. Similarly, the rs9514828 BAFF polymorphism was correlated with anti-Ro and anti-La antibodies or increased BAFF serum levels [52,110]. Recently, the role of the BAFF-R His159Tyr mutation was investigated in 247pSS patients, of whom 70 patients had pSS associated lymphoma [111]. This specific mutation was indentified in higher frequencies in both lymphoma and non-lymphoma pSS patients compared to normals ($p = 0.02$ and $p = 0.04$, respectively), while frequencies were found relatively low in SLE and RA patients who were also included in the study as disease controls. Interestingly, almost 70% of pSS associated MALT lymphoma patients of the third decade harbored this mutation. After measuring mRNA and protein levels of NF-kB1 and NF-kB2 in B cells of pSS lymphoma patients carrying the Hys159Tyr mutation, an upregulation of NF-kB2 but not NF-kB1 at both levels was found compared to healthy subjects.

5. Conclusions and future directions

The clinical picture of pSS is characterized by heterogeneity, and the disease outcomes differ according to the clinical phenotype. The majority of patients present with glandular manifestations and disabling symptoms but follow a benign course with low morbidity and impaired quality of life. Some of them are expected to develop extraglandular manifestations such as lung, liver and kidney involvement. In this case, the lesions evolve slowly and infrequently may lead to organ impairment. The histological hallmark that unifies glandular and extraglandular manifestations is the periepithelial lymphocytic infiltration, which is dominated by T lymphocytes. On the contrary, almost 15% of pSS patients constitute the systemic form of the disease with immune complex mediated extraepithelial complications including palpable purpura, glomerulonephritis and peripheral neuropathy. This subset carries a less favorable prognosis with higher morbidity and mortality due to the tendency to develop lymphoma. This clinical phenotype appears to be driven by a polyclonal B cell hyperactivity which early at disease onset evolves into an oligoclonal/monoclonal B cell expansion with a tendency to transform into malignancy. Inevitably, systemic manifestations and progression to lymphoma have drawn clinical and research interest to identify reliable markers for early diagnosis and prediction of lymphoma development.

As mentioned previously, based on immunopathologic studies, it became evident that T and B cell responses define the distinct clinical phenotypes of pSS. The recent advances in molecular biology and microarray technology offer the opportunity to measure many different variables at the same time and therefore to understand the biology of these

Table 1
Clinical, serological and hematological markers as predictors of systemic involvement, lymphoma and mortality in pSS.

	Systemic Form	Lymphoma	Mortality	Specific Manifestations
Clinical				
PGE	−	+	−	−
Purpura	+	+	+	−
LN	−	+	−	−
Splenomegaly	−	+	−	−
GN	+	+	+	−
PN	+	+	−	−
Serological				
MMC	+	+	−	−
Low C4	+	+	+	−
LowC3	−	+	+	−
Autoantibodies	−	−	−	+
Hematological				
Lymphopenia	−	+	−	−
Neutropenia	−	+	−	−

Abbreviations: PGE = parotid gland enlargement, LN = lymphadenopathy, GN = glomerulonephritis, PN = peripheral neuropathy, MMC = mixed monoclonal cryoglobulins.

Table 2
Biomarkers as predictors or indicators of B cell evolution in pSS.

	Polyclonal B cell	Monoclonal B cell or MALT
Histological		
FS	+	+
EGC	+	+
Chemokines	−	+
AID	+	+
B cell predominance	+	+
Macrophages	−	+
Dendritic cells	−	+
Molecular		
Salivary proteins	+	−
miRNAs	+	+
BAFF	+	+
Ftl3	−	+
Genetic		
Specific genes	+	+
SNPs	+	+

Abbreviations: FS = focus score, EGC = ectopic germinal centers, AID = activation-induced cytidine deaminase.

responses. This transition from immunopathology to the cellular and molecular level provides new perspectives regarding the diagnosis, prognosis and treatment of pSS. Although well-established clinical and serological markers for systemic involvement and lymphoma development are currently available (Table 1), cumulative data highlight important aspects of the molecular and genetic events that determine cell function and subsequent interactions. The discovery of novel molecules, subcellular pathways and gene alterations are expected to reveal major
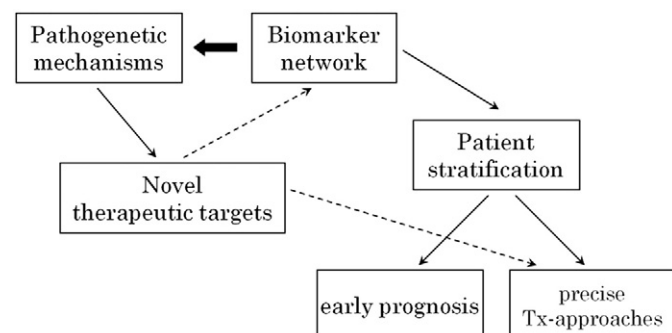

Fig. 1. The biological significance and clinical utility of biomarkers in pSS.

pathogenetic mechanisms and identify new treatment targets. Researchers have used different biological specimens, including saliva, serum and tissue from salivary glands, to apply the new technologies, and many molecular, histological and genetic biomarkers have been already described in the literature enriching the biomarker network (Table 2). Although some of them are promising, further studies are needed to confirm their reliability. Biomarkers are expected to contribute to early diagnosis establishing precise medicine and drive toward a more sophisticated patient stratification based on treatment response. Currently, the attempt to create a predictive model combining various biomarkers from different fields and biological specimens appears a more realistic approach than using single biomarkers (Fig. 1).

Take-home messages

- Primary Sjögren's syndrome has distinct clinical phenotypes with diverse outcomes.
- The systemic form is associated with immune complex mediated manifestations and lymphoma.
- Biology studies have revealed numerous histological, molecular and genetic biomarkers.
- Biomarkers are useful for early diagnosis, prognosis and patient stratification for biologic treatments.

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