Review

One year in review 2017: primary Sjögren's syndrome

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ABSTRACT

Primary Sjögren's syndrome (pSS) is a complex and heterogeneous disease. Last year, a great deal of basic and clinical research was carried out in pSS. Following the previous reviews of this publishing series, we will herewith provide a critical digest of the most recent literature on pSS pathogenesis, clinical manifestations and treatment. More specifically, we will focus on the heterogeneity of the disease, on the underlying pathogenetic pathways and on the possible new targeted treatments on the horizon.

Introduction

Primary Sjögren's syndrome (pSS) is a complex and heterogeneous disease at a crossroad of systemic autoimmune disorders and lymphoprolipherative conditions (1-4). The Big Data Sjögren Project Consortium exploring the influence of geolocation and ethnicity on the clinical presentation of pSS has emphasised the eclectic glandular and extraglandular manifestations of the disease all over the world (5). Recently, a great deal of basic and clinical research has been carried out in pSS, providing novel insights into disease pathogenesis, clinical subsets and treatment, especially focusing on the characterisation of homogeneous disease subtypes.

Following the previous annual reviews of this publishing series (6, 7), we will here provide a critical overview of the recent literature on pathogenesis, clinical features and novel treatments of pSS. We performed a Medline search of English language articles published in the PubMed database from 1st January 2016 to 31st December 2016. All the articles were critically analysed in order to select the most relevant contributions with regard to classification, epidemiology, pathogenesis, management and treatment of pSS.

Novel insights into pSS pathogenesis

Genetics and epigenetics

Primary SS (pSS) is a multifactorial disease resulting from a complex interplay between genetic, environmental factors, innate and adaptive immunity. This year great efforts have been made in the attempt of elucidating genetic and non-genetic factors contributing to disease heterogeneity. The impact of genetic and ethnicity cannot be neglectable, considering that the Big Data Sjögren Project Consortium (5), an international registry collecting data from 8310 patients in five countries, has shown that the disease presentation is different in the different areas of the world. For example, the female-to-male ratio was highest in Asian patients whereas the prevalence of sicca symptoms was lowest in these patients.

Quoting some recent important genetic studies in pSS, Liu *et al*. (8) have shown that the prevalence of trisomy X (47,XXX) was increased in pSS female with respect to general population. It is well known that pSS has a higher incidence in female patients (9:1) and a prevalence of ~0.5% in the general population (2). The results of the study by Liu *et al*. therefore supported the hypothesis that an X chromosome gene-dose effect might explain the powerful female bias in SS with a mechanism that appears to be at least partially independent of circulating sex hormones (8).

The vast majority of the latest genetic association studies carried out in pSS, however, have been focused on genes encoding proteins involved in both innate and adaptive immunity. Vlachogiannis *et al*. (9), in particular, investigated the association between the PTPN22W* variant and type I Interferon (IFN) responses in 352 pSS patients and 482 healthy controls. The authors found that only the low but not the high type

I IFN pSS subgroup displayed higher PTPN22W* rates compared to healthy controls, thus implying the presence of distinct genetic backgrounds among low and high type I IFN pSS patients.

Another candidate gene of particular importance in pSS pathogenesis is TNFAIP3, which encodes ubiquitin-editing enzyme A20, a critical inhibitor of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling. Johnsen *et al.* (10) found that among patients with pSS, those with lymphomas showed absent or weak protein A20 staining of lymphocytes in MSG biopsies compared to those without lymphomas. Nocturne *et al.* (11) demonstrated that rs2230926 exonic variant of TNFAIP3 was associated with an increased risk for pSS complicated by lymphoma. The same authors this year have confirmed their previous observation in two independent cohorts from UK (590 pSS patients, 31 cases of lymphoma) and France (589 pSS patients, 47 cases of lymphoma) (12). Sisto *et al.* (13) showed in 24 minor salivary gland biopsies from pSS patients that SS-specific deregulation of A20 results in excessive ectodysplasin-A1-induced NFκB signalling in SS, thus contributing to disease pathogenesis.

A growing body of evidence has implicated epigenetic factors, in particular, altered patterns of DNA methylation, miRNA and long non-coding RNA in the pathogenesis of pSS (14). More specifically, the general idea is that epigenetic regulation of gene expression may exert a key role in normal immune function and autoimmunity processes. Williams *et al.* (15) sought to profile for the first time miRNAs expression in pSS monocytes, focusing on their potential role in pSS pathogenesis. The authors found that pSS-associated monocyte miRNAs preferentially target TGFβ signalling pathways. Since intact TGFβ signalling mechanisms has been reported as crucial in controlling autoimmunity, the authors hypothesised that a defective regulatory signalling may be implied in the increased pSS inflammatory responses.

Over the last decade, changes in DNA methylation in minor salivary gland biopsies, whole blood, B- and T-cells have appeared to play a key role in pSS pathogenetic pathways. This year novel insights have been provided on this topic. Konsta *et al.* (16) have found that the global DNA hypomethylation in minor salivary glands was associated to lymphocyte infiltration. Moreover, DNA methylation was reduced in pSS patients with positivity for anti-SSB Ab. On the basis of their results, in their elegant paper the authors therefore suggested that DNA methylation changes may influence SSB gene overexpression and anti-SSB Ab production. Cole and coworkers (17), analysing changes in DNA methylation in pSS minor salivary gland biopsies, described an extended region of hypomethylation surrounding PSMB8 and TAP1, consistent with an increased frequency of antigen-presenting cells in pSS glandular tissue. Miceli-Richard *et al.* (18) investigated DNA methylation in CD4$^+$ T-cells and in CD19$^+$ B cells, detecting more frequent alterations in B cells rather than in T-cells especially in some specific pathways including interferon regulated genes. Moreover, genes with differentially methylated probes were over-represented in B cells from patients who were autoantibody positive and with active disease.

Similarly Imgenberg-Kreuz *et al.* (19) demonstrated the role of DNA methylation changes in the epigenetic regulation of IFN-induced genes in pSS minor salivary gland biopsies, whole blood and CD19$^+$ B cells.

Finally, Braekke-Norheim (20) described in pSS whole blood distinct functional pathway of genes with differently methylated CpG sites in subjects with high *versus* low fatigue shedding new lights on the possibility of using epigenetic for the differentiation of specific disease subsets.

Long non-coding RNA represent a relatively new chapter in the epigenetics implied in pSS pathogenesis. Long non-coding RNAs (long ncRNAs, lncRNA) are an abundant class of endogenous, non-protein coding transcripts longer than 200 nucleotides conserved across species that are positioned near their target protein coding gene. Wang *et al.* (21) investigated the expression of lncRNA TMEVPG1 in CD4$^+$ T cells of 25 SS patients and demonstrated that it was upregulated. More specifically, the authors found that the proportion of Th1 cells and the levels of TMEVPG1 and T-bet were increased in pSS patients, and that the level of expression of TMEVPG1 was correlated with the level of anti-SSA ab, erythrocyte sedimentation rate (ESR), and IgG. Similarly, Shi H and co-authors (22) characterised the expression profile of lncRNAs in labial salivary glands (LSGs) in pSS patients, describing a total of 1243 lncRNAs that were dysregulated. Interestingly, these authors also observed a strong correlations between these lncRNAs and β2 microglobulin, ESR, disease course, rheumatoid factor (RF), IgA, IgM, visual analogue scale (VAS) of parotid swelling and VAS of dry eyes.

Innate immunity

Primary SS pathogenesis implies a dysregulation of innate and adaptive immunity pathways. In recent years a great attention has been paid to innate immunity, especially due to its involvement in the early phases of the disease. It is generally recognised that environmental triggers (*i.e.* viruses) may initiate the cascade of events leading to the inflammation and disruption of the exocrine glands as well as to the systemic pSS manifestations. Not surprisingly therefore, type 1 IFN pathway, an innate immune mechanism of antiviral host defense, Toll-like receptors (TLRs) (*i.e.* nucleic acid sensors that defend against viruses), IL-1 family cytokines, NK cells and other players of the innate immunity have been extensively studied in pSS.

Narkeviciute *et al.* (23) in this regard, described a decrease of effector CTL and cDC, accompanied by increase of transitory phenotype memory CTL in peripheral blood of pSS patients, suggesting that the observed changes in peripheral blood of patients might reflect a persistent virus infection in pSS patients. In addition, Mavragani *et al.* (24) studied the expression of long interspersed nuclear element 1 (LINE-1; L1), an autonomous family of endogenous virus-like genomic repeat elements and type I IFN in minor salivary glands of pSS patients demonstrating that L1 levels were increased and that this increase in L1 was associated with elevated type I IFN production. Increased L1 expression in MSG tissue seemed to be associated with reduced methylation of L1 promoter. Noteworthy, the authors, by transfecting plasmacytoid dendritic cells (PDCs) or monocytes with an L1-encoding plasmid or L1RNA, suggested the existence of a L1 RNA–mediated activation of pattern-recognition innate immune pathways in the induction of type I IFN.

Regarding the IFN signature in pSS, the increased activity of type I IFN has been confirmed by two further studies. In detail, Sjöstrand *et al.* demonstrated that BAFF is an interferon stimulated genes and identified IRF1 and IRF2 as positive regulators of BAFF transcription and IRF4 and IRF8 as potent repressors. Maria N. *et al.* (25) investigated another type I IFN downstream pathway, focusing on the kynurenine pathway and on Indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan catabolism, degrading tryptophan to kynurenine. The authors hypothesised that the enhanced IFN activity in pSS might result in higher IDO expression. The authors investigated IDO activity in conjunction with CD25highFoxP31 Treg cell levels and kynurenine, neuroactive metabolites, in patients with primary SS stratified according to their IFN gene expression signature. The authors found a significant increase of IDO activity and of CD25highFoxP31 Treg cells in the serum from IFN-positive pSS patients; moreover, the proapoptotic and neurotoxic downstream enzyme kynurenine 3-monooxygenase was up-regulated whereas kynurenine aminotransferase were down-regulated in IFN-positive patients when compared to healthy controls.

The production of type 1 IFN is mainly due to the activation of dendritic cells (DC) and macrophage. Nucleic acid sensors including Toll like receptors (TLR), RNA-sensing receptors DDX58/retinoic acid inducible gene-I (RIG-I) and IFIH1/melanoma differentiation associated gene-5 (MDA5) play a crucial role in recognising viral as well as self nucleic acids and in the subsequent activation of DCs cells and macrophage. Karlsen *et al.* (26) have extensively investigated the expression of TLR in pSS peripheral blood mononuclear cells both at mRNA levels and at protein levels. Patients with pSS showed significantly higher mRNA levels of TLR8 than controls, while transcript levels of TLR9 were significantly lower. At the protein level, pSS patients expressed significantly less TLR5 and significantly more TLR7 compared to healthy controls. The differential expression of various TLR in the PBMC of patients with pSS has also been described by Maria N *et al.* (27). The authors found an upregulation of endosomal TLR 7, but not TLR9, in IFN-positive pDCs and monocytes. Additionally, the downstream signalling molecules MyD88, RSAD2 and IRF7 were upregulated, as were the cytoplasmic RNA-sensing receptors DDX58/retinoic acid inducible gene-I (RIG-I) and IFIH1/melanoma differentiation associated gene-5 (MDA5). IFN-negative patients presented a distinct expression pattern with normal TLR7, and decreased TLR9, RIG-I and MDA5 (27). Moreover, TLR2 has been implicated in the released of IL-15 another pro-inflammatory cytokine that has recently demonstrated to be involved in pSS pathogenesis (28, 29). Among novel damage associated molecular pattern proteins (DAMPs) acting as endogenous ligands of Toll-like receptors, S100 A proteins A8/A9 have been recently investigated in pSS (30, 31). Serum levels of S100A8/A9 were significantly increased in pSS patients compared to healthy controls. The expression of S100A8 and S100A9, identified in professional phagocytes (neutrophils, monocytes and plasmacytoid dendritic cells), was increased in minor salivary glands of pSS patients and saliva and correlated with focus score (30, 32, 33). *In vitro*, recombinant S100A8/A9 increased the production of IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-17A and IL-22 by PBMCs (30). The latter in particular, seems to be produced essentially by NK cells and Th17 and apparently exerts important function in regulating inflammation, development, maintenance, and function of ectopic lymphoid structures and in controlling the cell proliferation (34).

Besides type 1 IFN signature, other IFNs might be involved in pSS pathogenesis. Apostolou *et al.* investigated the presence of novel type III IFNs (*i.e.* IFN-k1/interleukin (IL) 229, IFN-k2/IL-28A and IFN-k3/IL-28B) in pSS MSG tissues, peripheral blood mononuclear cells (PBMCs) and serum, as well as in long-term cultured salivary gland epithelial cells (SGEC). The authors found that in pSS patients with intermediate MSG lesions, the epithelial expression of IFN-k2/IL-28A was more intense compared to sicca controls. In peripheral blood mononuclear cells, only IFN-k1/IL-29 was detected and appeared significantly elevated in pSS patients with intermediate MSG inflammatory lesions compared to sicca controls. Interestingly, the authors found that resting SGECs did not express any of the type III IFNs that were induced by TLR3 stimulation. These findings support the hypothesis that similarly to type 1 IFN, type III IFNs may be induced in pSS by environmental factors.

Regarding innate immunity, moreover, a separate distinct brief mention should be given to invariant Natural Killer T (iNKT) cells and mucosal-associated invariant T (MAIT) cells, novel subpopulations of immunity cell subsets that bridges innate and adaptive immunity. Invariant NKT cell negatively regulate autoreactive B cells, thus inhibiting autoantibodies production. Guggino *et al.* (35) demonstrated that iNKT were undetectable in the salivary glands of pSS patient , speculating that iNKT absence in salivary glands may results in B cells activation and anti-SSA production. Wang *et al.* (36), on the other hand, reported that MAIT cells were significantly decreased in pSS peripheral blood and differently from controls, detectetable in the salivary gland tissue from pSS patients. The authors hypothesised that the altered function of MAIT cells in target tissues from pSS patients may lead to a dysregulation of mucosal immunity ultimately resulting in a subsequent initiation of autoimmune response.

Finally, other crucial effectors of innate immunity that have appeared increasingly important as possible targets for novel therapies are inflammatory cytokines. It has been well described the role of IL-1 family in pSS pathogenesis, especially in disease-related fatigue and this year, the importance of IL-33 and IL-36 has been confimed (37-41). Moreover, levels of IL-6 have been described as increased in serum and biopsy samples in patients with pSS, suggesting a specific role of IL-6 in the disease pathogenesis (42). IL-6 seems to be able to promote TH17 and Follicular T helper cells (Tfh), thus in turn linking innate to adaptive immunity.

Adaptive immunity

Undoubtedly, T cells and B cells orchestrate systemic and glandular pSS manifestations (43). Th1 cells are considered to be the predominant $CD4^+$ T cell subset infiltrating minor salivary gland biopsies whereas the presence of B cells indicates a major complexity in the infiltrates (44). Ding *et al*. (45) confirmed that glandular B cell hyperactivation, differentiation into germinal centre (GC)-like structures and plasma cell accumulation could be attributed to increased BAFF. The authors tested their hypothesis by overexpressing BAFF in a mouse model of pSS. Intriguingly, they found that BAFF overexpression enhanced lymphocytic infiltration and B cell differentiation. However, BAFF was not able to efficiently promoting ectopic GC formation, thus opening new avenues for alternative pathways. From this perspective, another subset of T cells that has recently been implicated in pSS pathogenesis is represented by Th17 cells. These cells were detected in pSS biopsies but their importance in pSS pathogenesis remains unclear. In addition, as far as clinical manifestations are concerned, no association between IL-17/Th17 cells and severity of clinical picture has been reported until now. Recently, Alunno *et al*. (46) have found that IL-17+ inflammatory cells were preferentially observed around lymphatic vessels in pSS MSGs, thus suggesting a pro-lymphangiogenic function of interleukin (IL)-17 in pSS. From this perspective, Zhao *et al*. (47) analysed retinoic acid receptor related orphan nuclear receptor (ROR)-γ expressing (*i.e.* Th-17 cells) and non-expressing subsets (non-Th17 cells) of $CD161^+$ T cells in peripheral blood mononuclear cells to determine the relevance of the Th17 pathway in pSS. The authors concluded that the increase in the ROR-γ + subset positively correlated with anti-SSA/SSB autoantibodies and hypergammaglobulinaemia, but not with disease activity. By contrast the ROR-γ negative (non-Th17) $CD161^+$ subset is the one associated with disease activity in pSS patients.

In addition to Th-17, the emerging amount of data has recently underscored the role of several homeostatic lymphoid chemokines, such as CXCL13, CCL19, CCL21, and CXCL12, and their specific receptors for formation and perpetuation of the ectopic lymphoid structures and of IL-21 (4, 48, 49). IL-21 is the main soluble factor released by Tfh and both in salivary gland tissue and in peripheral blood provides potent signalling for B-cell survival, proliferation, and differentiation (50). Moreover, Hillen *et al*. (51) investigated the expression of soluble form of IL-7 receptor in serum and salivary gland biopsies of pSS patients. The authors observed that sIL-7R was increased in serum and minor salivary gland supernatant of patients with pSS and that high local production of sIL-7R was related to increased B-cell activity, autoimmunity, and risk for lymphoid neogenesis.

Novel insights into pSS clinical manifestations, diagnosis and biomarker discovery

Systemic manifestations and the challenge of an early diagnosis

When we looked at the literature published in 2016, a sort of common thread could be recognised, focusing on the early characterisation of pSS different subsets and, eventually, in their targeted treatments with the ultimate aim of optimising management of pSS patients in the near future. Specific subgroups of patients have been investigated, comparing for example those presenting prominent glandular manifestations versus those presenting systemic involvement, those at higher risk for lymphoma *versus* those characterised by a more benign disease course etc. From this perspective, Brito-Zeron *et al*. in their systematic review have highlighted the importance of an early diagnosis for pSS producing recommendations for the early recognition of the disease, especially oriented in recognising organ-specific "occult" systemic disease presentations (52).

Lymphoma: risk factors and prediction rules for an early recognition of patient at lymphoma risk

Regarding pSS patients subgroups at risk for lymphoma (NHL), four studies published in 2016 deserve to be quoted. Nocturne *et al*. (53) in a retrospective study analysing 101 pSS patients with lymphoma found that rheumatoid factor and ESSDAI were independent risk factors for lymphoma development. Fragkioudaki *et al*. (54), in line with previous predicting models (55), elaborated a novel tool for predicting lymphoma risk in clinical practice. The authors identified a number of independent risk factors for lymphoma which include: salivary gland enlargement, lymphadenopathy, Raynaud's phenomenon, anti-Ro/SSA or/and anti-La/SSB positivity, RF positivity, monoclonal gammopathy, and C4 hypocomplementaemia. Based on the results of the logistic regression analysis, a predictive model was formulated and the relative risk for NHL development was calculated based on the number of independent risk factors: the probability of NHL development was 3.8% for patients presenting with 2 risk factors, 39.9% for those having 3 to 6 risk factors and reached 100% in the presence of all 7 risk factors. Despite the small number of patients (n=82) and the retrospective design of this study, this score risk represents an easy to use risk assessment tool in everyday clinical practice, allowing the definition of early preventative therapeutic strategies in high risk SS patients for NHL development. The relationship between monoclonal gammopathy and subsequent risk for lymphoma was also reinforced by the study by Tomi *et al*. (56) that described this condition as a premalignant state for both NHL and multiple myeloma in pSS. Finally, Retamozo *et al.* (57) in a multicentre study including 515 patients confirmed the strong association between cryoglobulinaemic vasculitis at the diagnosis and NHL development suggesting to check all patients at the diagnosis for cryoglobulins, RF, C3/C4 complement and serum immunoelectrophoresis, in order to monitor more closely patients with higher risk for a worse long-term outcome.

Non vasculitic clinical manifestations: renal and neurological involvement

Some other recent important literature contributions have specifically explored pSS patients subgroups presenting with neurological and renal involvement, respectively. A recent French multicentre prospective study including 395 pSS patients from the Assessment of Systemic Signs and Evolution in Sjögren's syndrome (ASSESS) cohort documented neurological manifestations in 18.9% of pSS patients (58). Frequency was 16% for peripheral nervous system (PNS) and 3.6% for central nervous system (CNS) manifestations. The most common PNS manifestation was pure sensory neuropathy, followed by sensorimotor neuropathy. Regarding CNS, cerebral vasculitis, seizures, stroke, transverse myelitis, meningitis, encephalitis and meningoencephalitis were the most frequently observed clinical manifestations. Neurological manifestations were associated with greater ESSDAI score. Interesting, the development of new neurological manifestations was more common among patients with prior neurological involvement (58). In addition, pSS patients with neuropsychiatric syndromes (NP) are more likely to have elevated levels of serum/plasma anti-NR2A/B antibodies compared to pSS patients without NP syndromes, even if anti-NR2A/B antibody positivity cannot distinguish specific NP syndromes (59). As far as renal involvement is concerned, among the recent literature has to be quoted a multicentre study by Jasiek *et al.* (60). The authors reviewed 95 biopsy-proven cases of renal disease in pSS, confirming that tubulointerstitial nephritis (TIN) is far more common than cryoglobulinaemia-related membranoproliferative glomerulonephritis in pSS (97.5% *vs.* 2.5%). Intriguingly, the latter received an earlier diagnosis and had a better prognosis, in contrast with TIN patients who present with less defined clinical features. Light microscopy examination of kidney biopsy of patients with TIN revealed that the cellular infiltrate was mainly composed of lymphocytes, but contained plasma cells 68% cases. Moreover, correlations of histological findings with immunological features showed that anti-SSA/SSB antibodies were frequent in TIN and associated with worse renal prognosis.

These studies overall emphasise the actual lack of effective treatment options for SS, especially for SS-related non-vasculitic manifestations and patients at risk for lymphoma.

How to conduct clinical trials in pSS

Therefore, in parallel with multicentre observational studies, a growing interest has arisen in assessing the most effective way of conducting clinical trials in pSS for specific disease subsets thus providing novel effective therapeutic strategies for the disease. From this perspective, this year, the 2016 ACR-EULAR classification criteria for pSS have been published in order to facilitate uniform classification of patients for enrolment in clinical studies (61). These criteria represent the last step of a long and challenging journey that has started in 1965 and has led to the development of 11 criteria sets (62). The new ACR /EULAR classification criteria are applicable to any patients with least one symptom of ocular or oral dryness (based on AECG questions) or suspicion of SS due to systemic features derived from ESSDAI measure with at least one positive domain item. The criteria are based on five objective items and the individuals are classified as having primary SS if they have a total score of ≥4, derived from the weighted sum of the five items: anti-SSA/Ro antibody positivity and focal lymphocytic sialadenitis with a focus score of ≥1 foci/4 mm^2, each scoring 3; an abnormal Ocular Staining Score (OSS) of ≥5 (or van Bijsterveld (VB) score of ≥4), a Schirmer's test result of ≤5 mm/5 min. and an unstimulated salivary flow rate of ≤0.1 mL/min, each scoring 1. Traditional tests assessing major salivary gland morphology and function (*i.e.* scintigraphy and sialography) have been ruled out. It has been proposed to replace them with salivary gland ultrasonography (US) but as Jousse Joulin *et al.* stated in their systematic review, consensual US procedures and a validated US scoring system are needed before being able to evaluate this possibility (63). With respect to the AECG 2002 criteria (64), the exclusion criteria have also been updated: IgG4-related disease has been added; hepatitis C infection requires confirmation by PCR and pre-existing lymphoma is allowable since diagnosis. The 2016 ACR-EULAR classification criteria have highlighted the relevance of minor salivary gland biopsy and patients serology for pSS classification. Therefore, the scientific community has made an effort to produce guidelines for the standardisation of minor salivary gland histhopatology for clinical research. Fisher *et al.* (65) provided guidelines on how to perform and read the biopsy, specifying a number of points that have to be assessed when evaluating minor salivary gland biopsies in clinical trials. These principal points were focused on glandular tissue requirements (a minimun of 4 LSGs, minimum surface area of gland sections 8 mm^2), presence of focal lymphocytic sialoadenitis and calculation of focus score (the area of individual foci should also be summed and divided by glandular surface area), presence of germinal centre-like structure and lymphoepitelial lesions, extent of atrophy, fibrosis, duct dilatation and non specific chronic sialoadenitis, staining for CD3, CD20 and CD21 and proportion of foci with both T/Bcell segregation and follicular dendritic cell networks.

In addition to ACR 2016 classification criteria, Seror *et al.* (66) in a multicentre study including 790 patients defined some important inclusion criteria and endpoints for clinical trials. The authors proposed to include patients with moderate activity (ESSDAI≥5) and define response to treatment as an improvement of ESSDAI at least three points (*i.e.* minimal clinically important improvement; MCII). It was suggested that patients with the highest ESSDAI (ESSDAI>13) that have been recognised at increased mortality risk were excluded from clinical trials for ethical reasons. In line with this, Brito-Zeron *et al.* found that high activity in at least one ESSDAI domain, a baseline ESSDAI >13 and more than one laboratory abnormalities (lymphopenia, anti-La/SSB, monoclonal gammopathy, low C3, low C4 and/or cryoglobulins) were associated with overall mortality. The activity in the constitutional (fever, weight loss, lymphadenopathy), lymphadenopathy and pulmonary ESSDAI domains at diagnosis was related to poor survival (high risk of systemic disease and/or lymphoma during the follow-up) (67).

To address patient-reported outcomes, the authors identified a patient acceptable symptom state (PASS) as an ESSPRI<5 and suggested including patients with unsatisfactory symptom state (ESSPRI≥5); a response was considered an improvement of ESSPRI of at least one point or 15%. Moreover, since the biological domain of ESSDAI, including B-cell biomarkers (elevated gammaglobulin or immunoglobulin G serum levels, low complement levels, the presence of cryoglobulinaemia and/or of a monoclonal gammopathy), may induce collinearity of data and might falsely induce or increase association between the biomarker and activity measure a clinical score without biological domain was developed (68). The so-called ClinESSDAI, derived from the ESSDAI by exclusion of the biological domain. In multivariate modelling, all 11 domains of the Clin-ESSDAI, remained significantly associated with disease activity, with slight modifications of some domain weights. The psychometric properties of the ClinESSDAI, including reliability and sensitivity to change over time in clinical trials, were similar to those of ESSDAI. Disease activity levels and minimal clinically important improvement thresholds of clinESSDAI were also similar to those of ESSDAI. This new score thus provides an accurate evaluation of disease activity independent of B-cell biomarkers. Even if ESSDAI remains the gold standard, ClinESSDAI could be also used in clinical studies to avoid data collinearity, as secondary endpoint and in clinical practice to assess systemic disease activity for visits where immunological tests have not been done.

Additional data on the role of ESSPRI in clinical trial was given from a large prospective therapeutic trial in which health-related quality-of-life (HRQoL) of patients with active pSS were evaluated by Short Form survey 36 (SF36). SF36 scores indicated marked HRQoL impairments in patients with active pSS. The cardinal symptoms of pSS (xerostomia, xeroftalmia, pain and fatigue, assessed using the ESSPRI) are stronger predictors of HR-QoL impairment than systemic manifestation (assessed by the ESSDAI). In particular, pain and ocular dryness intensity showing independent associations with HR-QoL. These findings indicate that primary endpoints for therapeutic trials should include the cardinal pSS symptoms assessed by ESSPRI, in addition to extra-glandular involvement, which is the primary endpoint in all ongoing clinical trials in pSS (69).

Fatigue represents the major contributor to the impaired quality of life in pSS. The pathogenesis of fatigue has been studied during the last decades, but is still far to be understood. Karageorgas *et al.* (70) investigated 106 pSS patients in order to identify independent contributors of fatigue. In agreement with the literature, severe fatigue was documented in approximately one-third of the patients and found to be associated with a history of arthralgias/myalgias, fibromyalgia (FM), anxiety, depression, neuroticism scores and impaired sleep patterns. However, multivariate analysis indicated only depression, neuroticism, and FM to be independently associated with pSS-related fatigue. These results suggest an active collaboration between rheumatologists and mental health professionals. No association was detected between fatigue and clinical and laboratory markers of disease activity (ESSDAI score, BAFF, IFN-induced IDO-1, anti-21(OH) antibodies). Howard Tripp *et al.* (71) showed that in 159 pSS patients some proinflammatory cytokines were significantly higher compared to non-fatigued healthy controls but, unexpectedly, the levels of 4 proinflammatory cytokine: interferon-γ-induced protein-10 (IP-10), TNF-α, lymphotoxin-α and IFN-γ were inversely related to patient-reported levels of fatigue. This data may help to explain why treating inflammation does not necessarily improve fatigue in patients with pSS and challenge the notion that proinflammatory cytokines directly mediate fatigue in chronic immunological conditions. Moreover, a regression model predicting fatigue levels in pSS based on cytokine levels, disease-specific and clinical parameters revealed IP-10, IFN-γ (both inversely), pain and depression (both positively) as the most powerful predictors of fatigue in pSS. From this perspective in the lack of effective medical treatment able to control fatigue, regular physical activity was suggested to ameliorate fatigue in pSS (72, 73).

Comorbidities

Among the novel insights on pSS clinical manifestations, a specific paragraph has to be dedicated to the studies that have analysed *comorbidities* in pSS patients exploring the relationship between pSS and systemic diseases such as cardiovascular disorders. A cross-sectional multicentre study comparing the prevalence of comorbidities in patients from SJÖGRENSER (Spanish Rheumatology Society Registry of Primary SS) and RELESSER (Spanish Rheumatology Society Registry of SLE) showed a lower prevalence of classical cardiovascular risk factor (smoking, dyslipidaemia, and arterial hypertension) and serious cardiovascular events (*i.e.* stroke or myocardial infarction) in pSS with respect to SLE (74). However, Birt *et al.* in their retrospective, real-world analysis, including 10,414 patients newly diagnosed with pSS and registered in the MarketScan Commercial Claims database from Jan. 1, 2006 to Dec. 31, 2011, revealed that in the first year after pSS diagnosis cardiovascular events increased and all-cause healthcare costs grew by 40% (75). Moreover, Balarini *et al.* (76) demonstrated that in pSS patients disease-associated risk factors including glucocorticoid use, constitutional ESSDAI-domain and use of saliva substitute were associated significantly with carotid atherosclerosis plaque. In contrast, presence of carotid atherosclerosis was not associated with ESSDAI total score or any other domain of ESSDAI, or with presence of autoantibodies or leukocyte or lymphocyte count. The study evaluated also some cytokines potentially important in the pathogenesis of atherosclerosis and found that calprotectin (which serum levels were higher in pSS patients) was independently associated with presence of carotid atherosclerosis, indicating that calprotectin may be used as a biomarker of subclinical atherosclerosis in pSS.

Recently, a cross-sectional study by Choi *et al*. (77) has focused the attention on FM, another common comorbidity in pSS. In their study including one hundred pSS patients, FM prevalence was 31.0% in pSS. The authors did not find significant difference in pSS PROs in pSS patients with FM when compared to those without FM. This study confirmed the high prevalence of depression in pSS, the severity of depression independently contributed to the increase in tender point counts and pSS PROs being positively correlated with ESSPRI. In contrast with previous literature which documented a negative correlation between pain and antibodies positivity and pSS-related extraglandular manifestations, this study showed that pSS patients with FM did not differ significantly in disease activity (assessed by ESSDAI), inflammatory markers (such as ESR and CRP), or the presence of autoantibodies. However, the prevalence of severe to moderate depression, insomnia and cognitive dysfunction was higher in pSS patients with FM. Moreover the authors evaluated the serum 25-hydroxy vitamin D3 levels and they reported that levels negatively correlated not only with ESSPRI and ESSDAI but also with the severity of depression in pSS patients. In addition, severe vitamin D deficiency in pSS patients with FM was more frequently observed than that in pSS patients without FM.

Finally, an increased prevalence of sleep disturbances has been highlighted from the recent literature (78, 79). The subjective sleep disturbances were mainly due to sicca symptoms, pain and autonomic symptoms and correlated with disease activity and damage, and the patients' quality of life.

Novel biomarkers for pSS

The last paragraph of this section on "Novel insights into pSS clinical manifestations, diagnosis and biomarker discovery" analysed the recent literature on discovery of *novel biomarkers* for pSS. A huge amount of articles has been published on this topic, partially overlapping with novel insights into the pathogenesis of the disease. Promising biomarkers for pSS and pSS subgroups are coming out from genetic, epigenetic, molecular, and omics studies. A critical reappraisal is ongoing on autoantibodies in pSS, investigating novel correlations between different serological profiles and patients clinical features. Baer *et al*. (80) in their study on 82 primary SS patients with anticentromere antibodies (ACAs), confirmed the unique phenotypic features of these patients subset including features of limited cutaneous systemic sclerosis and a lower frequency of anti-SSA/SSB antibodies, RF, and hyperglobulinaemia (81, 82). In their study, that enrolled the largest series of ACA positive pSS patients, when compared to ACA negative patients, these patients seemed have more severe salivary and lacrimal gland dysfunction, greater degrees of labial gland biopsy focus score, lower Schirmer's and salivary flow rate. Moreover, these patients had a higher frequency of clinical features commonly seen in limited cutaneous systemic sclerosis, including Raynaud's phenomenon, dilated nailfold capillary loops, and oral mucosal telangiectasia. The same group also explored novel autoantibodies in pSS. In particular, the authors investigated the significance of anti-human interferon-inducible protein-16 (anti-IFI16) antibodies (83). IFI16 is an intracellular DNA receptor that senses DNA from invading pathogens in both the nucleus and cytoplasm and is thus a key component of the innate immune response. The authors demonstrated for the first time that anti-IFI16 antibodies were associated with markers of more severe pSS including germinal centre–like structures in labial salivary gland lymphoid infiltrates, and higher focus scores. Another promising field for biomarker discovery in pSS is represented by salivary proteomics. Delaleu *et al*. (84) by using a 187-plex capture antibody-based assay, identified six clinically distinct disease salivary phenotypes in a cohort of pSS patients. Hyposalivation was associated with significant alteration in 22 out of 119 reliably detectable biomarkers and in particular with the IL-1 system activation. Pregnancy associated plasma protein A, thrombospondin 1 and peptide YY were by contrast associated with the presence of germinal centre like structures in minor salivary gland samples.

Finally, mass citometry has recently emerged as a promising tool for clinical research. Mingueneau *et al*. (85) searched for pSS novel biomarkers in both blood samples and minor salivary gland biopsies with the ultimate aim of clustering patients into subsets with distinct disease activity and glandular inflammation. The authors found a high number of activated $CD8^+$ T cells, terminally differentiated plasma cells, and activated epithelial cells in minor salivary gland biopsies of pSS patients. In blood, they identified a 6-cell disease signature defined by decreased numbers of CD4 and memory B lymphocytes, decreased plasmacytoid dendritic cell numbers, and increased representation of activated CD4 and CD8 T cells and plasmablasts.

Taken together, then great efforts have been made in characterising novel and traditional biomarkers for different pSS subsets, the real challenge today is to be able to combine single information from different fields and biological specimens in a full picture thus creating a predictive model to be used in daily practice and in clinical trials.

New insights into the treatment of Sjögren's syndrome

The recent advances in the complex pathogenesis of the disease have highlighted some pathways which may be employed as targets for a new therapeutic scenario in pSS. However, the management of pSS still lacks targeted therapies against glandular and extra-glandular manifestations. Carsons *et al*. (86) have recently published guidelines for treatment of rheumatologic manifestations of pSS; however, there are no validated guidelines for the management of the other manifestation of the disease. Recently, a real-world, population-based study on more than 10.000 SS patients demonstrated that, in the first year post diagnosis, pharmacologic therapies consisted mainly of symptomatic drugs and traditional immunosuppressive therapy, being biologic therapies prescribed in a minority of patients (75). Interestingly, immunomodulatory traditional and biologic drugs were more frequently prescribed in pSS patients with concomitant autoimmune disease, suggesting the lack of therapies specifically targeted to treat pSS (75).

B-cell target therapy

Nevertheless, the current, developing knowledge of the pathogenesis of the disease suggests that biological treatment may be a promising opportunity to potentially control disease activity and prevent its complications. In particular, at the moment, inhibition of both B-cell and B-T crosstalk through costimulatory molecules seems to be the most promising avenues. Indeed, it is now assumed that B cells play a central role in pSS through the production of autoantibodies, including anti-Ro/SSA, anti-La/SSB, rheumatoid factor (RF), cryoglobulins, monoclonal immunoglobulins, and through glandular infiltration leading to the development of ectopic germinal centres and, potentially, lymphoproliferative disease (87). In particular, the pathways B-cells Activating Factor of the TNF Family (BAFF), B-Lymphocyte Stimulator (BLyS)/A PRoliferation-Inducing Ligand (APRIL) play a pivotal role in this mechanism. Concerning this target, rituximab, a chimeric monoclonal antibody targeting CD20, showed efficacy in improving disease activity and patient symptoms in the great majority of the open-label studies, but it failed to reach primary endpoints in randomised controlled trials (RCT). In the TRACTISS trial, preliminary data presented as an abstract at the ACR meeting in 2015 showed no improvement of disease symptoms was detected in the rituximab arm, except for a modest increase of the salivary flow. The TEAR trial included a population of 122 patients and had as primary endpoint an improvement in at least 2 of 4 visual analogic scale (VAS) score, including dryness, global assessment of disease, fatigue and pain. No significant difference in the primary end point was detected between groups at 24 weeks, although a significant improvement in the VAS fatigue score was observed in the rituximab arm at 6 weeks (88). The results of this study confirmed the lack of efficacy of rituximab, although it is noteworthy to consider that the enrolled population had a low disease activity and the primary endpoint was a very subjective measure (88). A sub-analysis of TEAR trial employing a different composite index, the SS Responder Index (SSRI) including scores on fatigue, oral and ocular dryness, unstimulated whole saliva and erythrocyte sedimentation rate, demonstrated the significant effect of rituximab in reducing SSRI of at least 30% in comparison to infliximab (89). Cornec *et al*. showed this year that rituximab was ineffective especially in patients displaying an intense BAFF-driven B-cell activation (90). The same group analysed whether response to rituximab could be influenced by high-grade salivary involvement assessed by ultrasonography and histopathology (91). Thirty-five of 122 patients enrolled in TEAR trial underwent salivary gland ultrasonography (SGUS) at inclusion. Of interest, SGUS score, evaluating echostructure of salivary glands on a 0–4 scale, was significantly higher at inclusion in non-responders according to SSRI-30 in comparison to responder patients, thus suggesting that SGUS score may be employed as potential biomarker to assess response to therapy in these patients (91). In constrast, in a double-blind, placebo-controlled trial, sequential parotid gland biopsies were taken at baseline and after 12 weeks of treatment in twenty rituximab-treated and ten controls to assess amount of lymphocytic infiltrate (stained for CD45), absolute number of T and B cells per mm2 parenchyma, focus score, number of germinal centres and of lymphoepithelial lesions per mm^2 in parotid gland parenchyma (92). A significant reduction of $CD20^+$ B cells, number of germinal centres and number and severity of lymphoepithelial lesions per mm^2 was observed in the rituximab-treated group, suggesting that baseline histopathologic features of salivary gland biopsies may be employed as biomarkers to assess response to therapy in clinical trials. In this setting, the need to identify specific biomarkers for the stratification of patients according to degree of disease activity, systemic involvement or glandular inflammation, is crucial in order to clarify the effect of biologic therapy on pSS course.

Finally, rituximab may have an effect on some systemic manifestations of the disease. Recently, a retrospective analysis of 10 primary SS patients with interstitial lung disease demonstrated a potential role of rituximab in improving functional respiratory parameters (93). Six months after the first Rituximab administration, a significant improvement in pulmonary function tests, in particular in the predicted value of carbon monoxide-diffusing capacity (DLCO) and of DLCO/alveolar volume, was observed with a concomitant reduction, although not statistically significant, of high-resolution computed tomography score. Interestingly, significant improvement in subjective symptoms on VAS scale was detected, including global disease, fatigue, shortness of breath, cough, xerophthalmia and xerostomia. Moreover, a significant reduction of validate outcome measures of disease activity and damage, European SS disease activity index (ESSDAI) and patient-reported index (ESSPRI), was achieved following rituximab infusion. The drug administration was well tolerated with only one patient developing a serious pulmonary infection requiring hospitalisation (93). Nevertheless, although an improvement of glandular symptoms may be achieved following rituximab administration, its efficacy in SS cannot be established yet, due to the different primary endpoints in the mentioned studies, the heterogeneity of study populations, the short period of follow-up after rituximab infusion and the lack of efficacy in RCTs. A recent meta-analysis and systematic reviews of RCTs involving about 150 primary SS patients demonstrated that a single rituximab administration has some effect in improving lacrimal gland function (moderate-quality evidence) while a low quality evidence supports the effect of this drug on salivary flow or oral dryness improvement, fatigue and disease activity reduction and quality of life improvement (94).

Concerning B-cell target therapy, an open-labelled trial conducted in 30 patients with SS, anti-SSA/Ro and either current systemic complications or salivary gland enlargement or early disease (<5 years) or biomarkers of B-cell activation, tested the efficacy of belimumab, a monoclonal antibody targeting BAFF (BELISS study) (95). The primary endpoint was improvement at week 28 in 2 of 5 items, including reduction of at least 30% on VAS scale of dryness, fatigue, pain, physician systemic activity and at least 25% improvement in B-cell activation biomarkers. The primary endpoint was achieved in 60% of the patients, suggesting a possible drug efficacy. Interestingly, the drug demonstrated its efficacy in improvement of some objective clinical signs and biomarkers of B cell activation. On the other hand, the improvement of patient symptoms resulted more limited and no effect was detected on objective measures of dryness, including unstimulated salivary flow and Schirmer's test. The follow-up data after belimumab discontinuation in 13 Italian patients enrolled in BELISS study have been recently published (96). The systemic disease activity, assessed by ESSDAI, significantly increased at 12 months following belimumab discontinuation. Similarly, a significant increase of RF was observed, supporting the effect of belimumab on RF-producing B-cells. Taken together, present data support a plausible efficacy of belimumab in controlling disease activity and biomarkers of B cell activation in SS patients. Interestingly, an ongoing multicentre, double-blind, RCT is testing the combination therapy of rituximab and belimumab in patients with pSS. The primary endpoint is the safety of combination therapy while ESSDAI, stimulated salivary flow, oral dryness and B-cell quantification within salivary gland biopsy are secondary endpoints (https://clinicaltrials.gov/ct2/show/ NCT02631538).

Costimulatory molecules

Concerning the role of T-cell co-stimulation as potential target in pSS, the efficacy of abatacept, a fully human soluble fusion protein consisting of the extracellular domain of human cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) linked to the modified Fc portion of human IgG1 and that modulates the CD80/86:CD28 costimulatory signal required for full T-cell activation, has been assessed in two open studies. The first study by Adler *et al*. enrolled 11 patients with SS who received 8 infusions of abatacept (97). Abatacept administration was demonstrated to be effective in reducing glandular inflammation, as assessed by evaluation of lymphocytic foci and FoxP3 cells in minor salivary gland biopsy, in increasing B-cell, lymphocytes and CD4 T-cells in peripheral blood and in increasing saliva production. The second study (ASAP study) evaluated the efficacy of 8 infusions of abatacept in 15 pSS patients (98). Disease activity, as assessed by ESSDAI, and patient subjective symptoms, as assessed by ESSPRI, significantly reduced during the 24 weeks of treatment and increased at 36 and 48 weeks following drug discontinuation. Moreover, no change in measures of salivary and glandular functions was observed. A recent study reported the analysis of salivary gland biopsies taken within 12 months and 24 weeks after abatacept administration in all 15 patients enrolled in ASAP study (99). Interestingly, number of germinal centres per mm^2 was reduced by abatacept administration. In particular, germinal centres were detected in the parotid gland biopsy of five patients and were absent in all the same patients at the end of treatment. Moreover, the number of germinal centres per mm^2 at baseline was associated with improvement in the ESSDAI glandular domain, but not with other ESSDAI domains. Abatacept treatment did not reduce focus score, lymphoepithelial lesions, area of lymphocytic infiltrate, and numbers of $CD3^+$ T-cells or $CD20^+$ B cells, suggesting that this therapy may reduce parotid gland germinal centre formation in SS by co-stimulation of activated follicular-helper T-cells and inhibition memory B-cells. To further suggest a potential role of abatacept in improvement of salivary function, an open-label, 1-year, prospective, observational and multicentre study was recently conducted in order to evaluate the efficacy and safety of abatacept in 36 rheumatoid arthritis patients with secondary SS (100). About one third of patients achieved the primary endpoint, consisting of remission assessed by Simple Disease Activity index (SDAI) at 52 weeks. Interestingly, a significant increase in both salivary and lacrimal flow was achieved. In particular, saliva volume was significantly increased by abatacept therapy only in patients with Greenspan grade 1 or 2 of labial salivary glands biopsy but not in those with grade 3 or 4, thus suggesting that early administration of abatacept may recovery of secretory function in SS patients. Finally, the ASAPIII study, a phase III RCT, is currently recruiting SS patients with active disease, as defined by ESSDAI ≥5. The primary end point of the study is to evaluate efficacy of weekly subcutaneous administration of abatacept on disease activity assessed by difference in ESSDAI score at 24 weeks. Secondary endpoints are clinical, functional, laboratory, subjective, and histological parameters and safety (https://clinicaltrials.gov/ct2/show/NCT02067910).

CD40-CD40 ligand (L) interaction is important for B-cell development, antibody production, germinal centre formation and T-cell–dependent antibody responses. Increased expression of CD40L has been demonstrated in SS patients and a phase II study assessing an anti-CD40 monoclonal antibody (CFZ 533) is ongoing in SS patients with active disease (ESSDAI ≥6) (https://clinicaltrials.gov/ct2/show/NCT02291029).

Interleukin targeted therapy and novel drugs

Regarding other potential therapeutic targets, phosphoinositide 3-kinase (PI3K) may be an interesting target in SS patients. It plays a key role in the regulation of the immune response and PI3K delta is crucial for mature B-cell development. A trial enrolling active SS patients (ESSDAI ≥5) with anti-SSA/SSB positivity and salivary flow >0 is currently assessing the efficacy of a new PI3K delta inhibitor in reducing disease activity at 12 weeks (https://clinicaltrials.gov/ct2/show/NCT02610543).

Finally, a recent phase II, placebo-controlled, RCT aimed to investigate the effect of baminercept, a lymphotoxin-beta receptor (LTbR) Fusion Protein, failed to achieve the primary end point (change from baseline in stimulated whole salivary flow at week 24) (https://clinicaltrials.gov/ct2/show/NCT01552681). The LTbR signalling is crucial for secondary and ectopic/tertiary lymphoid tissue organisation. Pharmacologic modulation of the LTbR signalling pathway by treating a mouse model of SS with LTbR-Immunoglobulin demonstrated that not all T $CD4^+$ T cell subsets infiltrating the salivary gland were equally affected by this treatment, thus providing a plausible explanation for the negative results of the trial (101).

Moreover, IL-6, a cytokine that determines the activation of the immune system with recruitment of mononuclear cells, inhibition of T-cell apoptosis, Th17 differentiation and polyclonal activation of B cells, has been depicted at higher levels in serum and saliva of SS patients in comparison to healthy control and correlated directly with disease activity. Thus, inhibition of IL-6 by tocilizumab, a recombinant humanised monoclonal antibody that acts as an IL-6 receptor antagonist, could be considered in SS. The ETAP trial, a phase III placebo-controlled RCT designed to evaluate tocilizumab efficacy in reducing disease activity (improvement of ESSDAI ≥3) in active SS patients (ESSDAI ≥5) with positive anti-SSA/SSB, is currently ongoing (https://clinicaltrials.gov/ct2/show/NCT01782235).

Finally, looking at new frontiers for the future treatment for pSS, Tahara *et al*. recently analysed the efficacy and mechanism of action of retinoic acid-related orphan receptor-gamma t (RORγt) antagonist A213 in murine autoimmune sialadenitis (MIS) (102). RORγt is involved in the differentiation of Th17 cells, which exert a pivotal role in the pathogenesis of several autoimmune diseases, including SS, through the production of IL-17 (103). A M3 muscarinic acetylcholine receptor (M3R)-induced sialadenitis murine model has been employed to analyse the effect of RORγt antagonist A213. Pre-transfer A213 treatment maintained salivary volume, significantly improved MIS and reduced interferon (IFN) gamma and IL-17 production, while post-transfer treatment with A213 increased salivary volume, suppressed MIS and reduced IL-17 production. These findings suggest that A213 can be potentially useful in the treatment of SS through suppression of IL-17 and IFN-γ production by M3R-specific T cells. The importance of interferon signature in the pathogenesis of the disease and of glandular ectopic lymphocytic aggregates, defined as tertiary lymphoid structures, in worst disease prognosis and lymphoma suggests these biomarkers as potential future therapeutic target (4).

In summary, even though the treatment of SS still remains an unmet clinical need, research is progressing in many fields of disease pathogenesis, in order to find targeted therapies for disease-specific manifestation and novel biomarkers to identify treatment-responsive patients.

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