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Association between genetic variants in the tumor necrosis factor/
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syndrome in Scandinavian samples

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ABSTRACT

Objectives Lymphotoxin beta (*LTB*) has been found upregulated in salivary glands of patients with primary Sjögren's syndrome (pSS). Also, an animal model of pSS showed ablation of the lymphoid organization and a marked improvement in salivary gland function upon blocking the LTB-receptor pathway. The aim of the present study was to investigate whether single nucleotide polymorphisms (SNPs) in the lymphotoxin alpha (*LTA*)/*LTB*/tumor necrosis factor (*TNF*) gene clusters are associated with pSS.

Methods A total of 527 pSS patients and 532 controls participated in the study, all of Caucasian origin from Sweden and Norway. Fourteen SNP markers were genotyped and after quality control filtering, 12 SNPs were analysed for their association with pSS using single marker and haplotype tests, and corrected by permutation testing.

Results Nine markers showed significant association with pSS at the $p=0.05$ level. Markers rs1800629 and rs909253 showed the strongest association (p -values=1.64E-11 and 4.42E-08, respectively, after correcting for sex and country of origin. When the analysis was conditioned for the effect of rs1800629, only the association to rs909253 remain nominally significant (p -value =0.027). In haplotype analyses the strongest effect was observed for the haplotype rs909253G_rs1800629A (p -value=9.14E-17). The associations were mainly due to anti-Ro/SSA and anti-La/SSB antibody positive pSS.

Conclusions A strong association was found between several SNPs in the *LTA/LTB/TNF α* locus and pSS, some of which lead to amino acid changes. Our data suggest a role for this locus in the development of pSS. Further studies are needed to examine if the genetic effect described in this study is independent of the known genetic association between the HLA and pSS.

Key words lymphotoxin, TNF, HLA, Sjögren's syndrome, SNP, autoimmune

Primary Sjögren's syndrome (pSS) is an autoimmune chronic inflammatory disease affecting approximately 0.3% of the population [1, 2]. The disease is typically diagnosed in women during their 5th decade of life. The main target organs in pSS are the exocrine glands, and the cardinal symptoms are dry eyes and dry mouth [2]. Systemic features that may be connected with the disease are fatigue, Raynauds phenomenon, cutaneous vasculitis, arthralgia, arthritis, lymphocytic colitis and celiac disease. Ectopic germinal center-like structures develop in salivary glands of about 20% of the patients, and is a possible predictor for development of malignant lymphoma in pSS [3]. About 70% and 40% of the patients, respectively, develop autoantibodies against the ribonucleotide proteins Ro/SSA and La/SSB [4]. In Caucasians, pSS is associated with the HLA DRB1*03, DQB1*02 and DQA1*05 alleles, and the association of HLA Class II markers with pSS may concern the anti-Ro/La response rather than the disease itself [5].

pSS is regarded as a multifactorial complex disease, where several genes are influencing the disease development in concert with immunological, hormonal and environmental factors [6, 7]. Familial aggregation between pSS and other autoimmune diseases (AIDs) has been reported [8]. Most association studies based on single nucleotide polymorphism (SNP) analyses in pSS suffer from the low number of individuals enrolled [9-11], still polymorphisms in IRF5 and STAT4 have been convincingly associated and replicated as risk factors for pSS [12-14]. Gene expression studies, i.e. microarray, have demonstrated a strong interferon signature in pSS [15-17]. For instance, a very high up-regulation of the lymphotoxin beta (*LTB*) gene was identified in affected glands in pSS patients [15]. Blocking of the LT β receptor (LT β R) pathway with an immunoglobulin fusion protein (LT β R-Ig) entailed increased secretion of saliva and reduced inflammation in pSS mouse model [18]. High *LTB* gene expression levels have later been reported in other AIDs, for instance in rheumatoid arthritis synovium [19].

The lymphotoxin system is part of the tumor necrosis factor (TNF) superfamily, and has an important role in the immune response and triggering of inflammation. Signalling through LT β R is essential for lymphoid organogenesis and maintenance of tertiary lymphoid tissues. Genetic disruption of LT β leads to disorganization of T and B cells in the spleen and absence of lymph nodes [20, 21]. Mice deprived of lymphotoxin alpha (LT α) also lack lymph nodes [22, 23].

A transmembrane heterotrimeric ligand, LT α_1/β_2 binds to the LT β R, which defines the LT- LT β R signalling axis [23-26]. Soluble LT α_3 has been associated with the

proinflammatory cytokine milieu that contributes to synovitis in rheumatoid arthritis [27]. $LT\alpha_1/\beta_2$ can be cleaved from the cell membrane and by that become converted into a soluble form that acts in concert with other proinflammatory cytokines to promote chronic tissue inflammation [27]. $LT\alpha$, $LT\beta$ and also $TNF\alpha$ are encoded by genes clustered together on chromosome 6p21.3, with the close proximity of 12 kb within the HLA Class III region.

Taken together, these reports led us to hypothesize that genetic variants, which could influence the function or expression of *LTA*, *LTB* and *TNF*, could be strong candidates as risk factors for pSS. The aim of the present study was therefore to examine if SNPs within this cluster of genes are associated with the presence of pSS in a large sample of Norwegian and Swedish cases and controls of Caucasian origin.

METHODS

Study Cohorts and Phenotypes

The study cohort is described in Table 1. A total of 605 patients and 596 controls were included in the study. After genotype quality control where individuals with a genotype success rate of < 90% were removed, as well as individuals with non-Caucasian origin, 527 cases and 532 controls remained for analysis.

The patients were recruited from rheumatology clinics at Haukeland University Hospital, Bergen (n = 125) and Stavanger University Hospital (n = 68) in Norway, and from Stockholm (n = 59), Uppsala (n = 64), Malmö (n = 150) and Linköping (n = 61) University hospitals in Sweden. All patients were Caucasians and fulfilled the American European Consensus Criteria for Sjögren's syndrome [2]. Patients with secondary SS were excluded. Data on antibody profiles and extraglandular manifestations were obtained from medical records (Tables 1 and S1).

The controls were healthy blood donors recruited from the same geographical areas as the patients from Bergen (n = 142), Uppsala (n = 70) and Linköping (n = 18), and population based controls from Stockholm (n = 88) and Malmö (n = 143). In Stavanger the controls (n = 71) were volunteers recruited as healthy control subjects for participation in a controlled pSS study. The study protocol was approved by the local Committees of Ethics in both Norway and Sweden, and the investigation has been conducted according to the principles expressed in the Declaration of Helsinki. All patients gave their written informed consent.

Genotyping and Quality Control

Fourteen tag-SNPs in the genomic region on chromosome 6p21 containing the *LTA*, *TNF*, *LTB* and *LST1* genes were selected and genotyped in the pSS patients and controls using the GoldenGate assay from Illumina Inc. (San Diego, CA, USA). The criteria for the tag-SNPs was an Illumina design score > 0.4 , minor allele frequency > 0.05 , a minimum of 60 bp between SNPs (the limit for the GoldenGate assay) and $r^2 > 0.8$. With this design the 14 SNPs tagged 17/19 SNPs in the region = 89%. Later, genotyping assays were developed for the ABI PRISM 7900HT Sequence Detection System using TaqMan technology (Applied Biosystems, CA, USA), for the markers rs1800629 and rs361525. The ID and genomic positions of the genotyped SNPs according to the GRCh37 (hg19) database UCSC (University of California, Santa Cruz) [28] are given in Table 2. Given the design of the study, the point was to cover the overall cluster of these genes to capture as much of the variation as possible in this region. Since there are no SNPs in the LTB region with a minor allele frequency > 0.01 , we expanded the search to the surrounding region. Figure 1 illustrates the location of the genes *LTA*, *TNF*, *LTB* and *LST1* on chromosome 6p21. The positions of the SNPs genotyped in this study are indicated by their rs numbers.

The genotypes were filtered under the following criteria: DNA samples with genotyping call rate greater than 90%; SNP call rate > 0.8 , Hardy Weinberg Equilibrium p-value > 0.001 , minor allele frequency (MAF) > 0.01 . After quality control filtering, 12 SNPs and 1059 DNA samples remained in the study. The reproducibility was 100% for all SNPs as estimated from duplicated genotyping of 2.5% of the samples.

Statistical analyses

The SNPs were analysed for allelic association with logistic regression test (where the case versus control status was the outcome predicted by the genotypes) and genotypic association with a logistic regression on the 3 genotypes coded under an additive model (dd= 0, dD= 1 and DD=2, where D is the minor allele). The result of the Single Marker Association and Logistic Regression Analysis (Allelic Test) of pSS patients and controls is shown in Table 2. The result of the Genotypic logistic regression is shown in Table 3. Analyses were performed using the SVS software (version 7.4.1, HelixTree Genetics Analysis Software, Golden Helix Inc., Bozeman, MT, USA, http://www.goldenhelix.com/SNP_Variation/HelixTree/index.html). For the haplotype analyses we used the EM method with a maximum iteration of 1,000 and a convergence tolerance of 0.0001, and a minimum haplotype frequency of 1% as implemented in SVS. A logistic regression per haplotype was then calculated.

RESULTS

Patients were examined for autoantibodies against ribonuclear proteins and for the extraglandular manifestations noted in Table 1 and S1. In all, 75.7 % of the patients were antinuclear antibody (ANA) positive, 71.7 % were anti-Ro/SSA positive and 45.1 % anti-La/SSB antibody positive (Table 1). The most frequent extraglandular manifestation, hypergammaglobulinemia, was present in 50.5 % of the patients (Table S1).

Fourteen Tag-SNPs covering the *LTA/LTB/TNF/LST1* (*LST 1*; Leucocyte-specific transcript 1) locus were selected to capture most of the genetic variation in the region. After quality control filtering of the genotype data, a total of 1059 individuals and 12 SNPs remained for analysis. None of the SNPs tested showed pairwise LD $r^2 > 0.75$ in the controls. At the allelic level, nine of the 12 SNPs showed significant associations with pSS using logistic regression analysis of the individual SNPs (Table 2). The SNP rs1800629 upstream of *TNF* showed the lowest *P*-value in both the Norwegian and the Swedish cohorts, $P = 2.30E-06$ and $P = 1.26E-05$, respectively. The strength of the association signal increased when the two cohorts were analysed together (P -value = $2.48E-10$ and odds ratio (OR) 2.00 [95% CI: 1.61 – 2.49] for the minor allele A. The genotypic additive tests of the same SNP underscored the highly significant association of the markers with pSS in both populations (Table 3). Several other SNPs show strong association with pSS, notably rs909253 which reached a *p*-value of $1.25E-07$ in the total sample (Table 2). In order to determine how many independent signals of association there were, we performed a conditional regression analysis, by using the rs1800629 genotypes as covariates. As shown in Table 3, only the association to rs909253 remains significant, which probably reflects that these two markers are in low linkage disequilibrium ($D' = 0.89$ and $r^2 = 0.39$).

At the haplotype level, we performed trend regression analyses on 2- and 3- markers sliding windows (see Table 4). We observed several significant associations. Most of them, but not all, involved the 2 most associated SNPs rs909253 and rs1800629. Considering that after conditional regression for rs1800629 the signal of association to rs909253 remained significant, we performed also haplotype association for these 2 markers. We observed a very significant association for the haplotype rs909253.G_rs1800629.A (P -value = $9.14E-17$) which is reflected by a frequency of this haplotype nearly doubled in cases compared to controls (0.29 versus 0.15 respectively) and a size effect of 2.14 (95% CI: 1.72 – 2.65).

The SNP rs1800629 does not show strong LD ($r^2 = 1$) to other SNPs within 250kb, but rs909253 is in complete LD ($D' = 1$, $r^2 = 1$) with rs1041981, which introduces a missense polymorphism in exon 3 of the *LTA* gene (threonine to asparagine, T60N, referred to by some

as T26N), the same association would therefore be observed with rs1041981. We investigated also the LD between the associated SNPs and the HLA haplotypes, especially the B*0801/DRB1*0301/DQA1*0501/DQB1*0201 (aka the COX haplotype, defined by the rs3129763.C_rs4639334.C haplotype [29]) which has been associated with pSS previously. At the single marker all pairwise comparisons between rs1800629 and rs909253 and the two COX haplotype markers show and $r^2 < 0.33$. At the haplotype level, the global multi-allelic D' is 0.72. Only sixty five percent of the rs909253.G_rs1800629.A are also on the COX haplotype which means the rs909253.G_rs1800629.A is not an extension of the COX haplotype but is not completely independent of it. When we investigated the region for a marker that could be in LD with the rs909253.G_rs1800629.A haplotype, we identified a complete LD between this haplotype and the marker rs2857595, which is located 11.8 kb from *LST1*, which would need further investigation in later high density genotyping of the region.

Two of the other SNPs analysed in this study are located in exons, causing missense polymorphism: rs2229094 in exon 2 of *LTA* (cysteine to arginine), and rs2229092 in exon 3 of *LTA* (histidine to proline), only the SNP rs2229094 demonstrated a nominally significant association with pSS in the allelic and genotypic tests (Tables 2 and 3, and to autoantibodies Table 4), where the effect is associated to the major allele.

We stratified the patients with pSS into groups according to their status for the autoantibodies anti-Ro/SSA, anti-La/SSB or both. When we analysed the association between the SNPs in antibody positive patients ($n = 381$) compared with controls ($n = 532$) (Table 5), we found a stronger signal of association than in the complete set of patients for six of the SNPs with antibody positive pSS patients. The SNPs rs1800629, rs909253, rs915654 and rs2229094 gave the lowest P -values (P -values = $3.78E-18$, $1.14E-10$, $1.42E-08$, $2.37E-06$, respectively). When comparing the risk allele frequencies between the group of antibody positive patients ($n = 381$) with the risk allele frequencies in the group of antibody negative patients ($n = 138$), a suggestive association with antibody positivity was observed for several of the SNPs (Table 5).

In addition, we tested the association of the eleven SNPs with extraglandular phenotypic characteristics such as hypergammaglobulinemia, hypothyroidism, arthritis, leucopenia and lymphoma (not shown). Several signals of association with nominal p -values < 0.05 were observed, but none of them were stronger than the association with pSS or antibodies.

DISCUSSION

In this study, SNP markers covering the *LTA*, *TNF*, *LTB* and *LST1* gene loci were examined for their association with pSS in Norwegian and Swedish samples of pSS patients. Significant associations were demonstrated with several of SNPs, and an especially strong association signal was obtained with the SNP rs1800629 (P -value=2.48E-10), and for the haplotype rs909253.G_rs1800629.A (P -value = 9.14E-17).

The SNP rs1800629 (TNF α -308 G>A) is a polymorphism of the TNF- α gene promoter that has been associated with TNF- α protein levels, inflammation and susceptibility to several AIDs [30-32], and also to non-Hodgkins lymphoma, specifically the subtype diffuse large B-cell lymphoma (DLBCL). This is of special interest since non-Hodgkins lymphoma is increased in the presence of autoimmune conditions such as Sjögren's syndrome [3, 33-36] [37] up to 16 fold according to Theander et al. [38]. Also, a large-scale consortial study provided evidence that the rs909253(LTA252A>G)/rs1800629(TNF α -308G>A) haplotype containing the *LTA*/TNF variant alleles, which we found strongly associated in the present study to pSS, was strongly associated with DLBCL ($P=2.9\times 10^{-8}$) [36]. In addition, it was reported recently that the AA genotype of *LTA*804C>A, ie rs1041981 which causes a missense mutation in the *LTA* gene and is in LD with rs909253, and the GG genotype of receptor-interacting kinase 1 gene (RIPK1, rs2272990) may correlate with shorter time to progression in patients with DLBCL with a combination chemotherapy [39]. However, the SNP rs909253 was not associated with increased risk of lymphoma in our study, maybe due to the low number of lymphomas in our cohort and consequently, lack of power in our case-case analysis. Another possibility could be that they constitute different types of lymphomas. It should also be mentioned that though there are several reports of associations between SNP rs1800269 and several diseases and protein levels, direct functional consequences of the TNF- α promoter polymorphism on a cellular level are at present not clear [40]. Mutations in the *TNF* and *LTA* genes have been reported to be correlated with the outcome of patients during serious infections [41]. The SNP rs909253 was reported to enhance the transcriptional level of *LTA* and the plasma levels of LT α [41]. A strong association of this SNP with susceptibility to myocardial infarction has been reported [42], and an increased risk for lethal infections during treatment of childhood acute leukemia [41]. LT α was also expressed in atherosclerotic lesions in mice, and interestingly, loss of LT α reduced the size of those lesions with 62%, whereas loss of TNF did not alter lesion development compared with wild-type mice [43]. This demonstrated a role for LT α in promoting lesion growth.

Interestingly, the association between several of the twelve SNPs analyzed in the *LTA/LTB/TNF* locus and pSS was almost entirely due to the antibody positive pSS patients. The SNPs most strongly associated with pSS in the whole cohort, rs1800629 and rs909253, showed an even higher association signal when only antibody-positive patients with pSS were compared with the controls (P -values=3.78E-18, OR=3.09 and 1.14E-10, OR 1.90, respectively), despite a lower number of patients. It is noteworthy that the minor allele frequency among the antibody negative patients was similar to the controls (0.19 versus 0.20 for rs1800629, and 0.40 versus 0.37 for rs909253), in contrast to the frequencies in antibody-positive patients (0.52 and 0.39). A stronger association between anti-SSA and anti-SSB antibody positive pSS patients and HLA-DRB1*03 compared with antibody negative patients has been described previously [5], and our present results confirm genetic differences between antibody-positive versus antibody-negative patients with pSS.

By linkage disequilibrium, the region tested in the present study encompasses also *LST1*. The specific function of *LST1* is not known, although expression analysis and functional data suggest an immunomodulatory role with a strong inhibitory effect on lymphocyte proliferation [44]. The expression of *LST1* was reported to be increased in rheumatoid-arthritis-affected blood and synovium, and associated with more severe inflammation in the synovium [19]. *LST1* has been found significantly up-regulated in response to lipopolysaccharide, IFN- γ and bacterial infection [44], and IFN- γ and interferon induced genes have been found upregulated in pSS [15, 17].

Several genes encoding proteins involved in immune and inflammatory responses are located within the MHC Class III region. Unravelling the genes of a complex genetic disease is challenging [45], even more so in the genomic region of the MHC where the extended LD renders the interpretation of association difficult [29]. In the present study, the association observed would reflect a specific effect of genetic variant(s) on one or another gene in the cluster, but as some LD between this cluster and other HLA loci has been reported it is difficult to entangle this effect from potential long range effect of other variants in the MHC region. Association to several HLA loci, including *LTA/LTB/TNF*, and several AID is well documented and several studies have aimed at understanding the complexity of this region [46-48]. Several extended haplotypes in the regions have been associated to AIDs. In pSS, association reports to the HLA haplotypes, for instance with the COX haplotype, have been conflicting, and none has reported an effect as strong as the one we observe in the present study. In addition we found that the haplotype associated in this study is not in LD with the COX haplotype. The basis for this study was that in salivary glands of patients we observed a

strong up-regulation of *LTB*, which was in agreement with further support for a role of *LTB* in pSS. Interestingly in a recent effort to understand the effect of different HLA haplotypes on the expression of genes located in the MHC region, Vandiedock et al. [45] showed an up-regulation of *TNF* and *LTA*, and a down regulation of *LTB* in cell lines homozygous for the COX haplotype (Figure S1), thus in contradiction with the upregulation we observed in salivary glands of patients with pSS. Further work is then warranted to understand the association between the present haplotype and pSS or other AID and their effect on the genes in the region.

Although AIDs share many genetic factors resulting in similarity of disease mechanisms, there is evidence that points towards genetic differences between them. The multiple genome wide association studies (GWAS) performed across AIDs have made it possible to study the homogeneity of genetic architecture across these diseases [49], and systematically identify allele-specific relationships. Interestingly, Sirota et al. used GWAS data to compare genetic variation profiles of six AIDs and five non-AIDs [50, 51], they were able to differentiate clusters of AIDs where SNPs that make an individual susceptible to one class of AID, also protect from disease in another autoimmune class. In other words, they performed an AID classification by reverse association with SNP. Hopefully such approach will also add new information about the genetics of pSS in the future, though GWAS with large sample for pSS are still needed.

In conclusion, the present study provides strong support for the involvement of *LTA/LTB/TNF* in pSS, in particular anti-SSA and anti-SSB antibody positive pSS. Further high density genotyping, gene expression profiling or re-sequencing of the region is warranted in both patients and controls and in several tissues to better understand the complexity of this region.

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Patient consent Obtained

Ethics approval The study protocol was approved by the local Committees of Ethics in both Norway and Sweden, and the investigation has been conducted according to the principles expressed in the Declaration of Helsinki. All patients gave their written informed consent.

Competing Interests None declared

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Figure legend.

Figure 1. Region of association around the *LTA*, *TNFA*, *LTB* and *LST1* genes on chromosome 6p21, and LD plot of HapMap pSS data on the associated Class IV SNPs.

The HapMap coordinates are numbered according to the GRch37 database (hg19) [28]. Exons are depicted as blue rectangles, while introns are depicted as blue horizontal lines. The markers can be identified by their rs numbers. The y-axis in the graph corresponds to the negative log₁₀ of the *P*-values for each marker in a test of association with pSS. The lower part of the figure illustrates the degree of correlation (LD) between pairs of markers which is indicated by the observed r^2 value in the control sample (black/white scale or r^2 , with black is $r^2=1$, white is $r^2=0$, 100 x r^2 is displayed).

Figure S1. Haplotype-specific pattern of expression. Cell line transcription levels of the genes *LTA*, *TNF*, *LTB* and *LST1* of three different cell lines; PGF (HLA-A3-B7-Cw7-DR15, pink), COX (HLA-A1-B8-Cw7-DR3, yellow) and QBL (HLA-A26-B18-Cw5-DR3-DQ2, purple). Bars corresponding to the genes on the minus strand are displayed leftward, those for the plus strand rightward. (Modified from Vandiedonck et al. [45]).

Table 1 Characterization of the study cohorts.

Category	Cases (pSS patients)			P-value ^a	Healthy controls			P-value ^a
	Norway	Sweden	Total sample		Norway	Sweden	Total sample	
Subjects	193	334	527		213	319	532	
Females (%)	178 (90.8)	309 (92.5)	487 (92.4)	1.00	193 (90.6)	292 (91.5)	485 (91.2)	0.76
Age (±SD) ^b	57.4 (11.8)	58.2 (14.0)	57.9 (13.2)	0.66	49.3 (13.6)	57.7 (11.4)	54.2 (13.0)	< 0.0001
Age females (±SD) ^b	57.3 (11.8)	58.2 (14.0)	57.9 (13.2)		48.9 (13.7)	57.5 (11.2)	53.8 (13.0)	
Age males (±SD) ^b	58.7 (11.5)	57.9 (15.0)	58.2 (13.6)		56.7 (11.6)	59.3 (13.0)	58.5 (12.4)	
ANA (%)	143 (74.1)	256 (76.6)	399 (75.7)	0.53				
Anti-Ro/SSA (%)	132 (71.4)	240 (71.9)	372 (71.7)	0.43				
Anti-La/SSB (%)	82 (44.3)	152 (45.5)	234 (45.1)	0.52				
Anti-Ro/SSA- Anti-La/SSB (%)	138 (74.6)	243 (72.8)	381 (73.4)	0.76				

^aFrequencies compared by Fisher's exact test. ^bAge at inclusion in the study. Data were missing for 135 controls (47 Norwegian and 88 Swedish controls). ANA, anti-nuclear antibodies. Anti-Ro/SSA and anti-La/SSB, ribonucleoproteins.

Table 2 Allelic association of individual SNPs by Logistic Regression in pSS patients and controls.

	Marker	Loc bp UCSC ^a	Loc gene	Minor allele	Cases			OR (95% CI)	Major allele	Regression P-value ^b
					Ctr					
Total	rs915654	31538497	1379 basis	T	0.25	0.32	0.69 (0.57 - 0.83)	A	1.13E-04	
Norway			upstream	T	0.27	0.36	0.67 (0.50 - 0.91)	A	9.32E-03	
Sweden			of <i>LTA</i>	T	0.23	0.30	0.71 (0.55 - 0.91)	A	5.86E-03	
Total	rs2844482	31539767	109 basis	A	0.11	0.15	0.71 (0.55 - 0.91)	G	6.92E-03	
Norway			upstream	A	0.13	0.17	0.72 (0.49 - 1.07)	G	1.03E-01	
Sweden			of <i>LTA</i>	A	0.10	0.14	0.70 (0.50 - 0.98)	G	3.85E-02	
Total	rs2239704	31540141	Intron 1/	A	0.32	0.37	0.80 (0.66 - 0.96)	C	1.42E-02	
Norway			5'-UTR of	A	0.31	0.36	0.79 (0.59 - 1.06)	C	1.11E-01	
Sweden			<i>LTA</i>	A	0.33	0.38	0.80 (0.63 - 1.01)	C	5.71E-02	
Total	rs909253	31540313	<i>LTA</i> intron 1	G	0.49	0.37	1.59 (1.34 - 1.89)	A	1.25E-07	
Norway				G	0.49	0.38	1.58 (1.20 - 2.09)	A	1.27E-03	
Sweden				G	0.48	0.37	1.61 (1.29 - 2.00)	A	2.52E-05	
Total	rs2229094	31540556	<i>LTA</i> exon 2	G	0.19	0.25	0.69 (0.56 - 0.85)	A	4.15E-04	
Norway			(C > R)	G	0.19	0.25	0.71 (0.51 - 0.99)	A	4.15E-02	
Sweden				G	0.18	0.25	0.68 (0.52 - 0.89)	A	4.13E-03	
Total	rs2229092	31540757	<i>LTA</i> exon 3	C	0.05	0.06	0.80 (0.55 - 1.16)	A	2.40E-01	
Norway			(H > P)	C	0.07	0.09	0.82 (0.49 - 1.37)	A	4.52E-01	
Sweden				C	0.04	0.05	0.82 (0.47 - 1.41)	A	4.66E-01	
Total	rs1799964	31542308	210 basis	G	0.15	0.20	0.74 (0.59 - 0.93)	A	9.60E-03	
Norway			downstream	G	0.17	0.21	0.79 (0.56 - 1.13)	A	1.96E-01	
Sweden			of <i>LTA</i>	G	0.14	0.19	0.72 (0.53 - 0.96)	A	2.57E-02	
Total	rs1800629	31543031	319 basis	A	0.34	0.20	2.00 (1.61 - 2.49)	G	2.48E-10	
Norway			upstream of	A	0.36	0.19	2.40 (1.65 - 3.48)	G	2.30E-06	
Sweden			TNF	A	0.33	0.21	1.81 (1.38 - 2.37)	G	1.26E-05	
Total	rs3093662	31544189	<i>TNFA</i>	G	0.04	0.05	0.83 (0.55 - 1.25)	A	3.79E-01	
Norway			intron 2	G	0.04	0.04	0.92 (0.46 - 1.84)	A	8.07E-01	
Sweden				G	0.04	0.05	0.78 (0.47 - 1.30)	A	3.44E-01	
Total	rs769178	31547514	823 basis	A	0.05	0.06	0.98 (0.66 - 1.43)	C	9.04E-01	
Norway			downstream	A	0.06	0.06	1.03 (0.56 - 1.88)	C	9.35E-01	
Sweden			of <i>LTB</i>	A	0.05	0.05	0.95 (0.58 - 1.57)	C	8.42E-01	
Total	rs2256965	31555130	<i>LST1</i>	A	0.34	0.40	0.76 (0.63 - 0.90)	G	1.90E-03	
Norway			Intron 2	A	0.31	0.39	0.71 (0.53 - 0.95)	G	2.24E-02	
Sweden				A	0.36	0.42	0.77 (0.62 - 0.97)	G	2.51E-02	
Total	rs1052248	31556581	3'UTR	T	0.20	0.25	0.76 (0.61 - 0.93)	A	6.91E-03	
Norway			<i>LST1</i>	T	0.22	0.25	0.83 (0.60 - 1.15)	A	2.60E-01	
Sweden				T	0.19	0.25	0.71 (0.55 - 0.93)	A	1.17E-02	

^aLocalizations according to GRCh37 (hg19) human genome build (as seen on UCSC genome browser <http://genome.ucsc.edu>) [28].

^b *P*-values for associations resisting the 1000 permutations test are highlighted in bold.

The analysis is based on a total of 527 pSS patients and 532 controls.

Table 3 Genotype association analysis

Marker	Additive model		Conditional regression - rs1800629		Dominant	Recessive
	<i>P</i> -values	OR (95% CI)	<i>P</i> -values	OR (95% CI)	<i>P</i> -values	<i>P</i> -values
rs915654	1.53E-04	0.69 (0.57-0.84)	0.09	0.82 (0.66-1.03)	2.51E-04	2.42E-02
rs2844482	5.46E-03	0.69 (0.53-0.90)	0.18	0.81 (0.60-1.10)	5.75E-03	3.75E-02
rs2239704	1.36E-02	0.79 (0.66-0.95)	0.75	0.96 (0.77-1.20)	3.33E-01	3.32E-04
rs909253	4.42E-08	1.65 (1.37-1.98)	0.03	1.32 (1.03-1.69)	4.05E-10	3.63E-02
rs2229094	3.91E-04	0.69 (0.56-0.85)	0.12	0.83 (0.65-1.05)	3.60E-03	3.47E-03
rs2229092	2.87E-01	0.82 (0.56-1.19)	0.78	0.94 (0.62-1.43)	4.04E-01	4.64E-02
rs1799964	8.80E-03	0.74 (0.59-0.93)	0.47	0.91 (0.70-1.18)	1.15E-02	2.11E-01
rs1800629	1.64E-11	2.22 (1.75-2.82)			6.61E-11	1.98E-03
rs3093662	3.49E-01	0.82 (0.54-1.24)	0.87	0.96 (0.61-1.52)	4.25E-01	3.2E-01
rs769178	9.10E-01	0.98 (0.66-1.45)	0.90	1.03 (0.66-1.61)	9.01E-01	n.a.
rs2256965	1.08E-03	0.74 (0.61-0.89)	0.23	0.87 (0.70-1.09)	7.11E-02	1.74E-04
rs1052248	7.36E-03	0.76 (0.62-0.93)	0.32	0.88 (0.70-1.13)	2.78E-02	2.05E-02

Genotypes were recoded under an additive, dominant and recessive model dd=0, dD=1, DD=2 where D is the minor allele. Logistic regression using sex and cohort as covariates was used for analysis.

P-values resisting permutation are highlighted in bold.

n.a.; not applicable because there were not enough homozygotes of the minor allele to run the analysis.



Table 4 Haplotype analyses. 2 - 3 sliding windows.

	Frequencies Case - Controls	Odds Ratio (95% CI)	Regression P-value
<u>2 Markers Sliding Window Haplotypes</u>			
rs915654.A_rs2844482.G	0.75 / 0.68	1.47 (1.21-1.77)	1.30E-04
rs2844482.G_rs2239704.C	0.55 / 0.46	1.44 (1.22-1.72)	8.28E-06
rs2239704.C _ rs909253G	0.48 / 0.36	1.61 (1.36-1.92)	2.42E-08
rs2239704.C _ rs909253.A	0.19 / 0.25	0.68 (0.56-0.84)	3.33E-04
rs909253.G_rs2229094.A	0.48 / 0.37	1.59 (1.34-1.89)	5.79E-08
rs909253.A_rs2229094.G	0.18 / 0.25	0.69 (0.56-0.85)	4.58E-04
rs2229094.A_rs2229092.A	0.81 / 0.75	1.45 (1.18-1.80)	4.59E-04
rs2229094.G_rs2229092.A	0.13 / 0.19	0.68(0.54 – 0.86)	1.37E-03
rs1799964.A_rs1800629.A	0.31 / 0.19	1.87 (1.52-2.30)	5.63E-14
rs1800629.A_rs3093662.A	0.31 / 0.18	1.90 (1.54-2.34)	7.21E-15
rs2256965.G_rs1052248.A	0.48 / 0.37	1.54 (1.29-1.84)	2.48E-07
<u>3 Markers Sliding Window Haplotypes</u>			
rs915654.A_rs2844482.G_rs2239704.C	0.43/0.31	1.68 (1.40-2.00)	3.90E-10
rs2844482.G_rs2239704.C_rs2239704.G	0.48/ 0.36	1.6 (1.37 - 1.94)	2.14E-08
rs2239704.C _ rs909253.G_rs2229094.A	0.49 / 0.37	1.69 (1.34-1.90)	5.10E-08
rs2239704.C _ rs909253.A_rs2229094.G	0.19 / 0.25	0.69 (0.56 -0.86)	5.33E-04
rs909253.G_rs2229094.A_rs2229092.A	0.49 / 0.37	1.59 (1.34 - 1.89)	5.79E-08
rs909253.A_rs2229094.G_rs2229092.A	0.13 / 0.19	0.68 (0.54 –0.86)	1.37E-03
rs2229094.A_rs2229092.A_rs1799964.A	0.81 / 0.75	1.45 (1.18 - 1.79)	4.59E-04
rs2229092.A_rs1799964.A_rs1800629.A	0.31 / 0.19	1.87 (1.52-2.29)	5.65E-14
rs1799964.A_rs1800629.A_rs769178.A	0.31 / 0.18	1.87 (1.52-2.30)	4.89E-14
rs1800629.A_rs769178.A_rs2256965.C	0.30 / 0.17	1.87 (1.51-2.31)	4.83E-14
rs769178.A_rs2256965.C_rs1052248.A	0.46 / 0.36	1.52 (1.27-1.81)	1.52E-06
rs2256965.G_rs1052248.A	0.48 / 0.37	1.54 (1.30-1.83)	2.48E-07
<u>rs909253 rs1800629</u>			
rs909253.G_rs1800629.A	0.29 / 0.15	2.14 (1.72-2.65)	9.14E-17

Table 5 Association analysis of the 12 SNPs in the TNF/*LTA* locus in antibody positive^a pSS patients versus controls and antibody positive^a pSS patients versus antibody negative pSS patients.

SNP	Minor allele ^b	Minor allele frequency		<i>P</i> -value ^c	OR (95% CI) ^c	Minor allele frequency		OR (95% CI) ^d
		Ab positive n = 381	Controls n = 532			Ab negative n = 138	<i>P</i> -value ^d	
rs915654	T	0.20	0.32	1.42E-08	0.53 (0.42 - 0.67)	0.36	1.35E-06	0.47 (0.34 - 0.64)
rs2844482	A	0.09	0.15	6.14E-05	0.54 (0.4 - 0.74)	0.17	5.08E-04	0.47 (0.31 - 0.72)
rs2239704	A	0.32	0.37	1.49E-02	0.78 (0.64 - 0.95)	0.33	8.53E-01	0.97 (0.7 - 1.34)
rs909253	G	0.52	0.37	1.14E-10	1.9 (1.56 - 2.33)	0.40	1.88E-04	1.79 (1.31 - 2.45)
rs2229094	G	0.16	0.25	2.37E-06	0.57 (0.45 - 0.72)	0.26	1.48E-04	0.51 (0.36 - 0.72)
rs2229092	C	0.04	0.06	4.32E-02	0.64 (0.41 - 1.00)	0.08	1.65E-02	0.47 (0.26 - 0.86)
rs1799964	G	0.13	0.20	1.97E-04	0.61 (0.47 - 0.8)	0.22	1.15E-03	0.54 (0.38 - 0.78)
rs1800629	A	0.39	0.20	3.78E-18	3.09 (2.36 - 4.04)	0.19	1.52E-10	3.39 (2.27 - 5.05)
rs3093662	G	0.04	0.05	3.26E-01	0.80 (0.5 - 1.26)	0.05	3.65E-01	0.75 (0.41 - 1.37)
rs769178	A	0.05	0.06	5.66E-01	0.88 (0.56 - 1.37)	0.07	1.36E-01	0.63 (0.34 - 1.14)
rs2256965	A	0.33	0.40	1.05E-03	0.71 (0.58 - 0.87)	0.37	3.12E-01	0.85 (0.62 - 1.16)
rs1052248	T	0.18	0.25	3.92E-04	0.66 (0.52 - 0.83)	0.25	1.75E-02	0.66 (0.47 - 0.93)

^aAnti-Ro/SSA positive or anti-Ro/SSA-La/SSB positive patients. Data was not available from eight patients.

^bMinor allele based on all patients, positive and negative.

^c*P*-value, odds ratio (OR) and 95% confidence interval (CI) for differences in allele frequencies between Ab positive patients and controls.

^d*P*-value, odds ratio (OR) and 95% confidence interval (CI) for differences in allele frequencies between Ab positive patients and Ab negative patients.

Association analysis was performed using a logistic regression on genotypes coded after additive model (see Table 4) and using sex and cohort as covariate.

Significant *P*-values are highlighted in bold.

Supplementary material

Table 1S Prevalence of extraglandular manifestations in pSS patients.

Category Extraglandular manifestation	Sweden n=334		Norway n=193		Sweden and Norway n=527	
Arthritis	60/334	18.0%	35/193	18.1%	95/527	18.0%
Dermal vasculitis	52/334	15.6%	10/193	5.2%	62/527	11.8%
Hypothyroidism ^a	46/238	19.3%	54/193	28.0%	100/431	23.2%
Raynaud's phenomenon ^a	92/333	27.6%	73/193	37.8%	165/526	31.4%
P-IgG > 15 g/L ^a	160/280	57.1%	73/181	40.3%	233/461	50.5%
Leucopenia < 4 x 10 ⁹ /L ^a	96/306	31.4%	55/193	28.5%	151/499	30.3%
Lymphadenopathy ^a	36/323	11.1%	13/193	6.7%	49/516	9.5%
Lymphoma ^a	16/333	4.8%	4/193	2.1%	20/526	3.8%
Major salivary gland swelling ^a	114/331	34.4%	58/128	45.3%	172/459	37.5%

^a Data were not available in all patients.