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# Genome-wide DNA methylation patterns in naïve CD4+ T cells from patients with primary Sjögren's syndrome

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## Abstract

**Objective—**Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with incompletely understood etiology. Very little is known about the role of epigenetic dysregulation in the pathogenesis of pSS.

**Methods—**We performed a genome-wide DNA methylation study in naïve $CD4^+$ T cells in eleven pSS patients compared to age-, sex-, and ethnicity-matched healthy controls. Cytosine methylation was quantified using the Illumina Infinium HumanMethylation450 BeadChip array and validated using bisulfite sequencing.

**Results—**We identified 553 hypomethylated and 200 hypermethylated CpG sites in naïve $CD4^+$ T cells from pSS patients compared to healthy matched controls, representing 311 hypomethylated and 115 hypermethylated gene regions. Hypomethylated genes in pSS include *LTA*, coding for Lymphotoxin α. Other relevant genes such as *CD247*, *TNFRSF25*, *PTPRC*, *GSTM1* and *PDCD1* were also hypomethylated. The interferon signature pathway was represented by hypomethylation of *STAT1*, *IFI44L*, *USP18* and *IFITM1*. A group of genes encoding for members of the solute carrier proteins were differentially methylated. In addition, the transcription factor *RUNX1* was hypermethylated in patients, suggesting a possible connection to lymphoma predisposition. Gene

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ontology (GO) analysis of hypomethylated genes demonstrated enrichment of genes involved in lymphocyte activation and immune response. GO terms for hypermethylated genes included antigen processing and presentation.

**Conclusion—**This is the first epigenome-wide DNA methylation study in pSS. Our data highlight a role for DNA methylation in pSS and identify disease-associated DNA methylation changes in several genes and pathways in naïve $CD4^+$ T cells in pSS that may be involved in the pathogenesis of this disease.

## Introduction

Primary Sjögren's syndrome (pSS) is a complex autoimmune disease characterized by production of autoantibodies against ribonucleoprotein (RNP) particles (Ro/SS-A and La/SS-B) and muscarinic acetylcholine receptor antigens, dysfunction of water transport processes, and lymphocytic infiltration of exocrine glands resulting in glandular atrophy and dysfunction (1, 2). Although, xerostomia (dry mouth) and xerophthalmia (dry eyes) are the main clinical features of pSS (3), the full spectrum of the disease encompasses involvement of different organ-systems and predisposition to lymphoproliferative disease (4). pSS predominantly affects women with a female to male ratio of 9:1 (5).

The etiology of pSS is incompletely understood, but there is growing body of evidence in diseases that often share certain clinical features with pSS, such as systemic lupus erythematosus, that epigenetic factors contribute to the pathogenesis of autoimmunity (6). Further, recent evidence suggests reduced global DNA methylation in salivary glands epithelial cells from patients with pSS (7). DNA methylation is considered the core epigenetic mechanism that regulates gene expression by altering transcriptional accessibility of regulatory regions within gene sequences (8). We have recently characterized DNA methylation changes in naïve $CD4^+$ T cells from lupus patients, revealing DNA methylation changes prior to T cell differentiation and activation, and demonstrating that interferon-regulated genes in naïve $CD4^+$ T cells from lupus patients are epigenetically poised for transcription (9). In this study, we performed a genome-wide DNA methylation study in naïve $CD4^+$ T cells from pSS and control subjects. We next validated our results using bisulfite sequencing of selected differentially methylated loci. We demonstrated differential methylation in key genes and disease associated pathways pertinent to the pathogenesis of pSS.

## Methods

### Primary Sjögren's syndrome patients and controls

We studied 11 patients with pSS and 11 healthy case controls. Patients and controls were matched for age (+/− 5 years), sex, and ethnicity. Demographic features of the study participants are shown in Table 1. Classification of pSS was based on the American-European Consensus Group 2002 revised criteria (10). The clinical features of the pSS patients included in this study are shown in Table 2. The cases were extensively characterized by a team of oral, ocular, and rheumatologic specialists for disease manifestations through the Oklahoma Medical Research Foundation (OMRF) Sjögren's

Research Clinic. Controls were recruited at OMRF and the University of Michigan. The institutional review boards at OMRF and the University of Michigan approved this study. All study participants signed a written informed consent prior to participation.

### Naïve CD4$^+$ T cell isolation and purity

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples obtained from patients and controls using density gradient centrifugation (Ficoll-Paque, GE Healthcare, Life Sciences, New Jersey, USA). Naïve CD4$^+$ T cells were separated from PBMCs using either the naïve CD4$^+$ T Cell Isolation Kit II (Miltenyi Biotec, Cambridge, MA) that allows for indirect isolation of naïve CD4$^+$ T cells, or by sorting the CD3$^+$CD4$^+$CD45RA$^+$ naïve T cell population on a FACSAria instrument (BD Biosciences, San Jose, CA). Naïve CD4$^+$ T cell purity was confirmed by flow cytometry using fluorochrome-conjugated antibodies against CD4 and CD45RA. Isolated naïve CD4$^+$ T cell purity was consistently > 95% regardless of the method used. DNA was isolated using the DNeasy Kit (Qiagen, Valencia, CA) as described in the manufacturer's protocol.

### DNA methylation studies and array validation

Genome-wide DNA methylation in naïve CD4$^+$ T cells from pSS patients and controls included in this study was assessed using the Illumina Infinium HumanMethylation450 BeadChip array, which allows for the interrogation of over 485,000 methylation sites within the entire genome. This array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene across the promoter region, 5′ untranslated region (5′-UTR), first exon, gene body, and 3′-UTR. It also covers 96% of CpG islands. Non CpG methylated sites recently identified in human stem cells are also covered as well as microRNA promoter regions.

Validation of the array data was performed using bisulfite DNA sequencing in selected hypermethylated and hypomethylated regions. The primers for *HDAC4* were forward primer 5′-TGGTTTTATTTTTTGTAGTTAAAAA 3′ and reverse primer 5′ ATAAAACCTCTATACCTCACTCAAC 3′; for *SLC38A4* were forward primer 5′ TTTGGATTTTTTAATTAAGTTGTTA3′, and reverse primer 5′ TCTACAATATTAATACTCCTACAAACC 3′; for *DUSP22* were forward primer 5′TTATTTGTTTTTTTAGGGTAGGGAG 3′ and reverse primer 5′AATCTCCAAATCCCCCTTTAAC 3′; for *GSTM1* were forward primer 5′ GTTAGGATTTGGTTGGTGTTTTAAG 3′ and reverse primer 5′ ATCCCAATACCCCAATATCATAAAC 3′; for *RUFY1* were forward primer 5′ GTAGGAGAGGTTTTGAGTTGGATT 3′ and reverse primer 5′ TCCTCCATCATCTAACACTTAAAAA 3′; for *NAPRT1* were forward primer 5′ TATGGTGGTTTGGTAGAGGTTAGTG 3′ and reverse primer 5′ ACTAATCTATCCTCCACCCTTTCC 3′; and for *SLC9A* were forward primer 5′ GTTTTTTTATTTAGAGAGGGGTAGG 3′ and reverse primer 5′ AACCAAAAAAAAACTACAACTAAACC 3′. We used a Biorad T100 Thermocycler with the following protocol: 1 cycle at 94 °C for 5 minutes, followed by 40 cycles (94°C for 45 sec, annealing temp for each primer set for 45 sec, 72°C for 90 sec), and then 74°C for 10 minutes. The annealing temperatures for *HDAC4, SLC38A, DUSP22, GSTM1, RUFY 1, NAPRT1,* and *SLC9A1* were 54°C, 57°C, 53°C, 54°C, 56°C, 56°C, 56°C and 53°C,

respectively. We confirmed the presence of PCR product by using 1.6 % agarose gel electrophoresis. DNA was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) as specified by the manufacturer. Bisulfite treated DNA was sequenced using Sanger sequencing. DNA methylation level on each CpG following bisulfite sequencing was quantified using the ESME software package (Epigenomics AG, Berlin).

### Statistical and bioinformatics analysis

DNA methylation analysis was performed using the GenomeStudio methylation analysis package (Illumina) as previously described (8). The average level of DNA methylation (β) on each CpG site was compared between pSS patients and controls. To identify differentially methylated CpG sites between pSS cases and controls, we used three data filtering criteria: (i) CpG site with an average difference in DNA methylation level of at least 1.2-fold (ii) differential methylation score of ≥ 22 ($P \leq 0.01$) after adjusting for multiple testing using a false discovery rate of 5%, and (iii) exclusion of CpG sites assessed by probes with a genetic variant located within 10bp of the 3′ end of the probe.

To systematically highlight the most over-represented biological terms, out of the differentially methylated gene, we performed gene ontology, network, and pathway analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7(11). In pathway and gene ontology analysis, we used Expression Analysis Systematic Explorer (EASE) Score threshold of <0.1, for detection of gene enrichment analysis (EASE score represent a modified Fisher Exact P-Value, which is considered a measure to examine the significance of gene-term enrichment), in addition to fold enrichment of 1.5 and FDR for correction of multiple testing < 10% (12).

## Results

We evaluated DNA methylation changes in naïve $CD4^+$ T cells from pSS patients and age-, sex-, and ethnicity-matched controls. We identified 753 differentially methylated CpG sites in naïve $CD4^+$ T cells from pSS patients (Table 3). Two hundred sites were hypermethylated and 553 were hypomethylated in patients with pSS compared to controls. A total of 426 differentially methylated unique genes were identified in naïve $CD4^+$ T cells from pSS patients, with the majority (311 genes, 75%) being hypomethylated. Bisulfite DNA sequencing was used to validate the DNA methylation array results by studying a group of hypermethylated (4 genes, 7 loci) and hypomethylated (3 genes, 3 loci) CpG sites. The average Pearson's correlation coefficient ($r^2$) value between the Illumina Infinium HumanMethylation450 array data and bisulfite sequencing data was 0.803 (Supplementary Figure 1).

Despite consistently achieving naïve $CD4^+$ T cell purity of >95%, to ensure that differential methylation patterns identified between patients and controls in our study were not influenced by potential differences in naïve $CD4^+$T cell activation status induced by the isolation procedures used, we examined the DNA methylation levels of a region in the *IL2* promoter-enhancer sequence that is known to readily demethylate upon T cell activation. Demethylation of the promoter-enhancer region of *IL2* is a sensitive indicator of $CD4^+$ T cell activation (13, 14) and is a prerequisite for *IL2* transcription (15). We detected high

DNA methylation levels in the *IL2* promoter-enhancer region, as expected in naïve $CD4^+$ T cells, and no difference between patients and controls (Supplementary Table 2). Further, we examined the DNA methylation levels in genetic loci known to demethylate in specific antigen experienced and regulatory $CD4^+$ T cell subsets, including *IFNG* (Th1 cells), *IL4*, *IL5* and *IL13* (Th2 cells), *IL17* (*IL17A*) and *IL17F* (Th17 cells) and *FOXP3* (regulatory T cells). Again, we find consistent high DNA methylation levels across these genetic loci, and no difference between patients and controls (Supplementary Table 2).

Hypomethylated genes in naïve $CD4^+$ T cells from pSS patients include *LTA*, encoding Lymphotoxin α, which is involved in the LTβ receptor signaling pathway, activation of follicular dendritic cells, and expression of interferon-α. We identified 5 differentially methylated CpG sites in *LTA* located at the 5′UTR, within 200 base pairs (bp) upstream of the transcriptional start site (abbreviated as TSS 200) and in the first exon (Figure 1). The average differential methylation score (Diff. score) of the 5 CpG sites was −35.4 (range −22.3 to −53.8; average fold change 1.82). Other relevant hypomethylated genes include *CD247*, which encodes for TCR-zeta chain (2 CpG sites located in the body of the gene; Diff. score −23.9 and −32.3; average fold change 1.96), *TNFRSF25* (1 CpG site; TSS 1500; Diff. score −23.2; fold change 1.59), *PTPRC* (2 CpG sites; TSS 1500 and body of the gene; Diff. score −49.7 and −75.5; average fold change average 1.96), *GSTM1* (2 CpG sites located in TSS 200; Diff. score −31.4 and −48.8; average fold change 1.40) and *PDCD1* (1 CpG site; TSS 1500; Diff. score −46.9; fold change 2.63). The type-I interferon pathway, which plays a major role in the pathogenesis of pSS (16), was represented by hypomethylation of *STAT1* (2 CpG sites; 5′UTR; Diff. score −23.9 and −79.2; average fold change 1.41), *IFI44L* (1 CpG site; 5′UTR; Diff. score −24.1; fold change 1.22), *IFITM1* (1 CpG site; TSS 1500; Diff. score −35.8; average fold change 1.75), and *USP18* (1 CpG site; 5′UTR; Diff. score −65.1; fold change 1.40).

A group of genes encoding members of the solute carrier proteins, which are membrane transport proteins that are important for maintenance of cell function were hypomethylated (*SLC11A1*, *SLC11A2*, *SLC22A23*, *SLC25A25*, *ALC25A3*, *SLC25A33*, *SLC6A20*), whereas, *SLC9A1*, which is important for the maintenance of pH homeostasis was hypermethylated in pSS patients compared to controls. In addition, the transcription factor *RUNX1* was hypermethylated in patients with pSS. Supplementary Table 1 provides a summary of all CpG sites that were differentially methylated in patients with pSS in our study.

Next, we used DAVID software (11) to facilitate the systematic identification and grouping of differentially methylated genes into biological networks. Canonical pathway analysis identified type I diabetes mellitus (P= 4.59E-06), allograft rejection (P= 1.66E-04), viral myocarditis (P= 2.34E-04), graft-versus-host disease (P= 2.63E-04), autoimmune thyroid disease (P= 1.15E-03), antigen processing and presentation (P= 3.22E-03) and cell adhesion molecules (P= 3.73E-03) as the most significant pathways unifying the differentially methylated genes in naïve $CD4^+$ T cells from pSS patients (Table 4).

Gene ontology (GO) analysis of hypomethylated genes demonstrated enrichment of genes involved in lymphocyte activation (P= 1.10E-04), leukocyte differentiation (P= 1.42E-03), immune response (P= 2.20E-03), chromatin organization (P= 3.90E-03), T cell

differentiation (P= 4.20E-03), homophilic cell adhesion (P= 5.80E-03), and L-amino acid transport (P= 6.10E-03). GO terms for hypermethylated genes included antigen processing and presentation ontology (P= 6.89E-06) (Table 5).

## Discussion

We performed an unbiased genome-wide DNA methylation study in naïve $CD4^+$ T cells from pSS patients and identified differentially methylated genes and involved pathways compared to healthy matched controls.

The molecular basis of pSS is not well characterized; however, there is cumulative evidence that activation of the lymphotoxin-β receptor (LTβR) pathway plays an integral role in the pathogenesis of pSS (17). We have identified five differentially hypomethylated CpG sites in *LTA* gene in naïve $CD4^+$ T cells from pSS patients compared to controls. Lymphotoxins (LTα, LTβ) and their receptors are part of the tumor necrosis factor (TNF) superfamily (18). Soluble LTα promotes production of IFNs and multiple chemokines that are important activating signals to the immune cells (19). Moreover, LTα forms a heterodimer with LTβ (LTα1β2), which in turn binds to LTβR (20). Activation of the LTβR pathway is crucial for the development, organization, and maintenance of lymphoid structures (21), and modulating expression of chemokines and adhesion molecules that aid in trafficking of lymphocytes and follicular dendritic cell activation (22). Further, murine models highlight an interesting role of LTα in the pathogenesis of Sjögren's-like disease; LTα is overexpressed in salivary gland secretions and sera of a transgenic mouse model for SS (IL14αTG mouse) (23). The IL14αTG mouse reproduces the clinical and immunological changes characteristic of SS (24). Interestingly, IL14αTG mice with deletion of the *LTA* gene did not develop disease (23). In another murine model of Sjögren's syndrome (Non-Obese Diabetic (NOD) mouse), Catumu and colleagues (22) showed that blocking the LTβR pathway results in ablation of the lymphoid organization in the NOD mouse salivary glands and an improvement in salivary gland function (22). In another study, blocking of LTβR reduced the size of leukocyte infiltrates in lacrimal glands, and improved tear production and corneal integrity (25). Additional evidence that LTα has a role in Sjögren's syndrome comes from the observation that LTα is overexpressed in the salivary gland and sera of patients with Sjögren's syndrome (23). Indeed, a clinical trial to evaluate the use of a LTβR fusion protein (Baminercept) in the treatment of pSS is currently in progress. Our results suggest that epigenetic factors may play a role in LTα overexpression in pSS, as cytosine demethylation predisposes to transcriptionally-permissive chromatin architecture.

Patients with pSS have an activated type I IFN response (16, 26), demonstrated by increased type I IFN activity and an "IFN signature" in peripheral blood mononuclear cells (27), saliva (28), and minor salivary gland biopsies (29, 30). Several hypomethylated genes that we identified in naïve $CD4^+$ T in pSS patients were interferon-regulated genes (*STAT1*, *IFI44L*, *IFITM*, and *USP18*). The extent of hypomethylation in interferon-regulated genes in pSS seems to be less robust in comparison to SLE (9) where we have recently identified and reproduced 21 hypomethylated interferon-regulated genes in naïve $CD4^+$ T cells.

We identified several differentially methylated genes that are important in activation of the immune system. *CD247* encodes for TCR-zeta chain, is important for signal transduction upon antigen stimulation (31) and was hypomethylated in naïve $CD4^+$ T cells in pSS. Other pertinent genes include *PTPRC*, which encodes for protein tyrosine phosphatase, receptor type C (also known as CD45 antigen or B220). PTPRC is a signaling molecule that is essential for T and B cell activation, cellular differentiation and oncogenic transformation (32). *TNFRSF25*, which plays a role in lymphocyte homeostasis and in apoptosis, was also hypomethylated in pSS. We demonstrated hypomethylation of Glutathione S-Transferase M1 (*GSTM1*), which encodes a cytoplasmic glutathione S-transferase that is important in detoxification of electrophilic compounds, including carcinogens and environmental toxins (33). Of particular interest, *GSTM1* was proposed as a genetic risk locus for pSS in one study (34), which might suggest a role for genetic-epigenetic interaction in the pathogenesis of pSS. Indeed, we have previously reported the genetic association in pSS with variants in methyl-CpG-binding protein 2 (*MECP2*), a key transcriptional regulator with a critical role in DNA methylation (35). Among the transcription factors, RUNX-1 regulates the differentiation of hematopoietic stem cells into mature cells (36) and has been linked to cancer predisposition (37, 38). *RUNX1* was hypermethylated in our study, suggesting a possible connection to lymphoma predisposition in pSS.

It is generally accepted that defects in membrane water channel proteins contribute to the exocrinopathy in pSS (39). Our study identified several differentially methylated genes encoding for members of the solute carrier proteins. The genes *SLC11A1*, *SLC11A2*, *SLC22A23*, *SLC25A25*, *ALC25A3*, *SLC25A33*, *and SLC6A20* were hypomethylated, whereas *SLC9A1*, which is expressed in the kidneys, and plays an important role in the maintenance of pH homeostasis, was hypermethylated in pSS patients compared to controls. Mutation in solute carrier proteins have been previously linked to diseases associated with acid-base disturbances, like Bartter and Gitelman syndromes (40). We hypothesize that methylation changes in these proteins may explain some pathologic aspects of pSS like defects in molecular water transport, dysfunction of the exocrine glands, and perhaps distal renal tubular acidosis in some patients with pSS (41).

We used the DAVID database to facilitate the systematic identification and grouping of differentially methylated genes into biological networks. Of particular interest, there was enrichment of ontologies involved in both the adaptive and innate immune system, underscoring involvement of the two arms of immunity in the pathogenesis of pSS (Table 6).

Two cell separation methods were used to isolate naïve $CD4^+$ T cells from patients and controls, albeit, with consistent and equal cell purity (>95%). We further confirmed the absence T cell activation or differentiation in the samples used in this study, using "epigenetic immunophenotyping" by examining the methylation status of *IL2*, *IFNG*, *IL4*, *IL5*, *IL13*, *IL17*, *IL17F*, and *FOXP3*. It is important to note that phenotypic cellular specificity is critical to accurately and reliably interpret differential methylation data in autoimmune diseases, while avoiding any perceived differences that could be related to altered cell constituents.

DNA methylation is tightly linked to chromatin accessibility, and gene expression requires both chromatin accessibility and appropriate transcription factors. Therefore, some but not all of the methylation difference we identified are expected to be associated with expression differences. Examining DNA methylation at a genome-wide level provides another level of discovering fundamental differences that might be pathogenic to the disease process at the chromatin level, which might or might not be linked directly to expression differences. For example, we recently reported wide-spread hypomethylation in interferon-regulated gene in lupus naïve $CD4^+$ T cells, before an expression difference could be detected (9). Interestingly, there are no gene expression studies in naïve $CD4^+$ T cells in pSS to our knowledge, so we do not know which genes are differentially expressed in naïve $CD4^+$ T cells in this disease. Therefore, whether or not some of the methylation changes detected in this first DNA methylome study in pSS will reflect a difference in active (or potential) expression state remains to be seen.

The recent advances in epigenetics and the emergence of “epigenome-wide association studies” resulted in successfully identifying numerous intriguing associations between epigenomic perturbations and human disease. However, this field is still in its infancy and the issue of “causality” in the epigenetic changes detected will require careful experimental examination. The “causality” inference issue in pSS is even more complicated for several reasons: first, incomplete understanding of the genetic predisposition to pSS, which might influence epigenetic variations; second, the environmental factors involved in the pathogenesis of pSS remain by large uncharacterized; and third, the retrospective case-control design of our study. The alternatives are longitudinal studies of monozygotic twins discordant for pSS, or prospective studies to evaluate epigenetic perturbations prior to the onset of the disease, both study designs are cumbersome for obvious reasons, however, such studies are warranted in the future to move this field forward (42). Further, integrating emerging genomic data in pSS with epigenomic profiling might provide an avenue to discover mechanistic pathogenic pathways to this disease. For example, we suspect that some genetic susceptibility variants that will be discovered for pSS might influence some of the methylation differences observed. A simultaneous genomic-epigenomic analysis should therefore focus on allele-specific methylation changes induced by genetic variants associated with pSS. These approaches will help to identify novel mechanisms and therapeutic targets for this disease, and might enhance our understanding for the functional consequences of some of the genetic susceptibility loci in pSS.

In summary, we identified DNA methylation changes for the first time in naïve $CD4^+$ T cells from pSS patients. These data indicate that abnormal DNA methylation exists in pSS $CD4^+$ T cells even before activation and differentiation. Therefore, our findings emphasize the potential role of DNA methylation changes in the pathogenesis of pSS. Our study demonstrates differential methylation of *LTA*, type I interferon-regulated genes, and solute carrier proteins, in addition to other key genes and pathways involved in the pathogenesis of pSS. Future studies to replicate and determine the functional consequences of the methylation changes observed upon disease pathophysiology are warranted.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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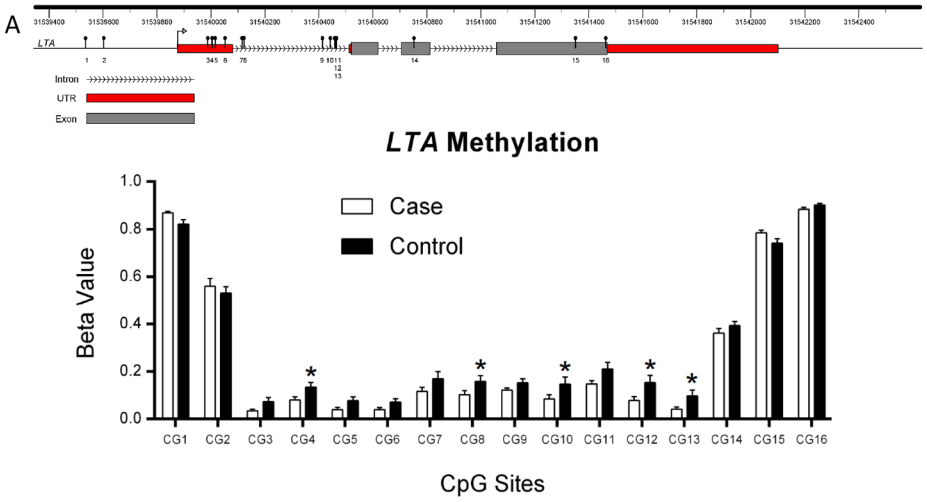


**Figure 1.**

*LTA* gene DNA methylation in naïve $CD4^+$ T cells from primary Sjögren's syndrome patients and controls. A) *LTA* (chr6:31,539,814-–31,542,537; GRCh37/hg19) is shown depicting the location of CpG sites evaluated in this study. B) DNA methylation fractions (β value) across the CpG sites evaluated in *LTA*. We identified five hypomethylated CpG sites (*) in the *LTA* gene region in naïve $CD4^+$ T cells from primary Sjögren's syndrome patients compared to healthy controls.

**Table 1**

Demographic characteristics of the study participants. Mean age is 45.9 years and 45.54 years for primary Sjögren's syndrome patients and controls, respectively (P value = 0.95).

| pSS cases | | | | | Controls | | | |
|---|---|---|---|---|---|---|---|---|
| **ID** | **Age** | **Ethnicity** | **Sex** | **Medication** | **ID** | **Age** | **Ethnicity** | **Sex** |
| **1** | 50 | EA | Female | Methotrexate | **12** | 52 | EA | Female |
| **2** | 51 | EA | Female | Hydroxychloroquine | **13** | 47 | EA | Female |
| **3** | 30 | EA | Female | | **14** | 34 | EA | Female |
| **4** | 23 | EA | Female | Hydroxychloroquine | **15** | 23 | EA | Female |
| **5** | 62 | EA | Female | Azathioprin | **16** | 58 | EA | Female |
| **6** | 32 | EA | Female | | **17** | 36 | EA | Female |
| **7** | 35 | EA | Female | | **18** | 36 | EA | Female |
| **8** | 60 | EA | Female | | **19** | 55 | EA | Female |
| **9** | 58 | EA | Female | | **20** | 58 | EA | Female |
| **10** | 35 | EA | Female | Hydroxychloroquine | **21** | 38 | EA | Female |
| **11** | 69 | EA | Female | Hydroxychloroquine | **22** | 64 | EA | Female |

EA: European-American; pSS: primary Sjögren's syndrome

## Table 2

Clinical characteristics of the primary Sjögren's syndrome patients included in this study (n = 11).

| | Patient number (%) |
|---|---|
| Dry eyes | 11 (100) |
| Dry mouth | 11 (100) |
| Abnormal Schirmer's test | 6 (55) |
| Average Schirmer's response (mm) | 10.8 |
| Abnormal Lissamine green | 9 (82) |
| Abnormal WUSF | 5 (45) |
| Positive Anti SSA/Ro | 6 (55) |
| Positive Anti SSB/La | 6 (55) |
| MSGB consistent with pSS | 10 (91) |
| Average focus score | 4.5 |

Focus score: the number of mononuclear cell infiltrates containing at least 50 inflammatory cells in a 4 $mm^2$ glandular section. MSGB: Minor salivary gland biopsy WUSF: Whole unstimulated salivary flow.

## Table 3

Summary of the differentially methylated CpG Sites and genes in naïve $CD4^+$ T cells from primary Sjögren's syndrome patients compared to healthy controls.

| | Increased methylation | Decreased methylation | Total |
|---|---|---|---|
| CpG sites | 200 | 553 | 753 |
| Fold change (Average) | 1.20–5.20<br>1.65 | 1.20–5.22<br>1.60 | |
| Differential score (Average) | 22.0 to 342.0 (+95.6) | −22.0 to −339.8 (−66.1) | |
| Genes | 115 | 311 | 426 |

**Table 4**

Pathway analysis of the differentially methylated genes in naïve $CD4^+$ T cells from primary Sjögren's syndrome in comparison to healthy controls.

| Category | Term | Molecules | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|---|
| KEGG_PATHWAY | Type I diabetes mellitus | PTPRN2; CD28; LTA; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB5 | 4.59E-06 | 9.005 | 0.005 |
| KEGG_PATHWAY | Allograft rejection | CD28; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB5 | 1.66E-04 | 8.171 | 0.191 |
| KEGG_PATHWAY | Viral myocarditis | MYH13; CD28; CASP9; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB5 | 2.34E-04 | 5.327 | 0.268 |
| KEGG_PATHWAY | Graft-versus-host disease | CD28; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB5 | 2.63E-04 | 7.543 | 0.301 |
| KEGG_PATHWAY | Autoimmune thyroid disease | CD28; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB-5 | 1.15E-03 | 5.768 | 1.315 |
| KEGG_PATHWAY | Antigen processing and presentation | LTA; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB-4; HLA-DRB5; TAP2 | 3.22E-03 | 4.051 | 3.637 |
| KEGG_PATHWAY | Cell adhesion molecules (CAMs) | NCAM1; SELL; PDCD1; CD28; HLA-A; HLA-B, HLA-C; HLA-F; HLA-DMA; HLA-DRB1, HLA-DRB4; HLA-DRB5 | 3.73E-03 | 3.184 | 4.201 |

FDR: False Discovery Rate; KEGG: Kyoto Encyclopedia of Genes and Genomes.

**Table 5**

Gene ontology (GO) analysis was performed on hypomethylated and hypermethylated genes in pSS. Statistically significant GO terms are shown.

| Category | Term | GO ID | Genes | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|---|---|
| **GO terms of Hypomethylated genes** | | | | | | |
| GOTERM_BP_FAT | lymphocyte activation | GO:0046649 | BCL11B; CD3G; CD28; CD7; CHD7; FOXP1; HDAC4; ITPKB; JMJD6; RHOH; SLC11A1; SKAP2; UNC13D | 1.10E-04 | 3.859 | 0.185 |
| GOTERM_BP_FAT | leukocyte differentiation | GO:0002521 | BCL11B; CEBPE; CD28; CHD7; FOXP1; HDAC4; ITPKB; JMJD6; RHOH | 1.42E-03 | 4.168 | 2.361 |
| GOTERM_BP_FAT | immune response | GO:0006955 | BTLA; CD28; CD300A; CD7; FAIM3; ST6GAL1; C4BPB; FOXP1; IFI44L; IL36G; IL16; LY86; LTA; HLA-A; HLA-B; HLA-H; HLA-DRB5; PDCD1; SLC11A1; TCF12; TCF7; UNC13D; ETS1 | 2.20E-03 | 1.969 | 3.646 |
| GOTERM_BP_FAT | chromatin organization | GO:0006325 | BCOR; CREBBP; DNMT3A; H2AFV; SATB1; SMARCB1; APBB1; CHD7; HDAC4; JMJD6; KDM2B; RBBP7; TSPY4; TSPY1; TSPY2; TSPY3; TSPY7P; TLK1 | 3.90E-03 | 2.344 | 6.356 |
| GOTERM_BP_FAT | T cell differentiation | GO:0030217 | BCL11B; CD28; CHD7; ITPKB; JMJD6; RHOH | 4.20E-03 | 5.453 | 6.855 |
| GOTERM_BP_FAT | homophilic cell adhesion | GO:0007156 | PCDHAC2; PCDHA4; PCDHA2; PCDHA3; PCDHA5; PCDHA7; PCDHA8; PCDHA9 | 5.80E-03 | 3.608 | 9.360 |
| GOTERM_BP_FAT | L-amino acid transport | GO:0015807 | CACNA1A; HTT; SLC11A1; SLC7A8 | 6.10E-03 | 10.274 | 9.687 |
| **GO terms of Hypermethylated genes** | | | | | | |
| GOTERM_BP_FAT | antigen processing and presentation | GO:0019882 | TAP2; HLA-B, HLA-C; HLA-F; HLA-H; HLA-DMA; HLA-DRB4; HLA-DRB5 | 6.89E-06 | 14.817 | 0.010 |

GOTERM_BP_FAT: Gene Ontology term biological process