# ORAL DISEASES

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## ORIGINAL ARTICLE

# MUC1/SEC and MUC1/Y overexpression is associated with inflammation in Sjögren's syndrome

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**OBJECTIVES:** To evaluate the expression and localization of MUC1/SEC and MUC1/Y isoforms in labial salivary glands (LSG) from Sjögren's syndrome patients (SS patients), as well as their *in vitro* expression induced by cytokines.
**SUBJECTS AND METHODS:** Labial salivary gland from 27 primary SS patients and 22 non-SS sicca subjects were studied. Relative MUC1/SEC and MUC1/Y mRNA levels were determined by qPCR and protein levels by Western blotting. Induction of mucin mRNAs was assayed *in vitro*. Immunohistochemistry was used for localization.
**RESULTS:** Relative MUC1/SEC and MUC1/Y mRNA and protein levels were significantly higher in LSG from SS patients. These mRNAs were induced by cytokines. MUC1/SEC and MUC1/Y were detected in acini apical region of control LSGs, and significant cytoplasmic accumulation was observed in acini of SS patients. MUC1/Y localized in acinar nuclei and cytoplasm of inflammatory cells of LSG from SS patients. A strong positive correlation was observed between cellular MUC1/SEC levels and glandular function determined by scintigraphy.
**CONCLUSIONS:** We show for the first time that MUC1/SEC and MUC1/Y are expressed in LSG of both SS patients and non-SS sicca subjects. The observed overexpression and aberrant localization of MUC1/SEC and MUC1/Y and their induction by pro-inflammatory cytokines may favor the perpetuation of the inflammatory environment that disrupts the salivary glandular homeostasis in SS patients.

## Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by focal infiltration of inflammatory cells into salivary and lachrymal glands, leading to dry mouth and dry eye symptoms (Garcia-Carrasco *et al*, 2006). Dryness may be the result of glandular destruction mediated by cytokines, such as IFN-γ, TNF-α, interleukins, and alterations in the composition of secretory products (i.e., mucins) (Boumba *et al*, 1995; Castro *et al*, 2013). Particularly, high levels of IFN-γ have been reported, both in SS patients and SS animal models (e.g., Aec NOD mouse). Studies in the Aec NOD mouse have shown that IFN-γ plays a role in both the pre-immune and immune phases of the disease (Cha *et al*, 2004). Moreover, the mRNA and protein of STAT-1 (an interferon-γ-inducible gene) are upregulated in labial salivary gland (LSG) from SS patients. Also, the presence of pSTAT-1α in ductal epithelium from SS patients has been reported (Wakamatsu *et al*, 2006).

The salivary glands synthesize soluble (MUC7 and MUC5B) and membrane-anchored mucins (MUC1) (Amerongen *et al*, 1995; Hanisch and Muller, 2000). An important finding has been that salivary mucin–TLR-4 interaction initiates signaling pathways leading to the induction of several pro-inflammatory cytokines, which can induce mucin gene expression (Barrera *et al*, 2014). Such mucins can also induce TLR expression, which in turn favors mucin expression, thereby generating a self-perpetuating signaling loop (Tarang *et al*, 2012).

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The MUC1 gene generates several transcripts by alternative splicing (78 variants) (Zhang et al, 2013) codifying three types of MUC1 isoforms: transmembrane proteins with variable numbers of tandem repeats (VNTR), transmembrane proteins without VNTR and secreted proteins (Figure 1a) (Ligtenberg et al, 1990; Wreschner et al, 1990; Zrihan-Licht et al, 1994). The secreted MUC1 isoform (MUC1/SEC), which lacks the cytoplasmic and transmembrane domains, contains a unique 11 amino-acid peptide at the COOH terminus, not found in other isoforms (Wreschner et al, 1990; Smorodinsky et al, 1996). This sequence is referred to as the immuno-enhancing peptide (IEP) due to its ability to enhance the immune response (Herbert et al, 2004), probably by upregulation of STAT-1 (Ilkovitch et al, 2008). As such, the IEP has been proposed to modulate both the innate and adaptive immune responses (Grosso et al, 2004; Herbert et al, 2004). MUC1/SEC may induce overexpression of cytokines through its IEP and/or via formation of complexes with MUC1/Y (Herbert et al, 2004), a MUC1 transmembrane protein without VNTR (Zrihan-Licht et al, 1994; Baruch et al, 1999; Hartman et al, 1999). The interaction between MUC1/Y and MUC1/SEC can be compared to a receptor–ligand interaction that might trigger cytokine
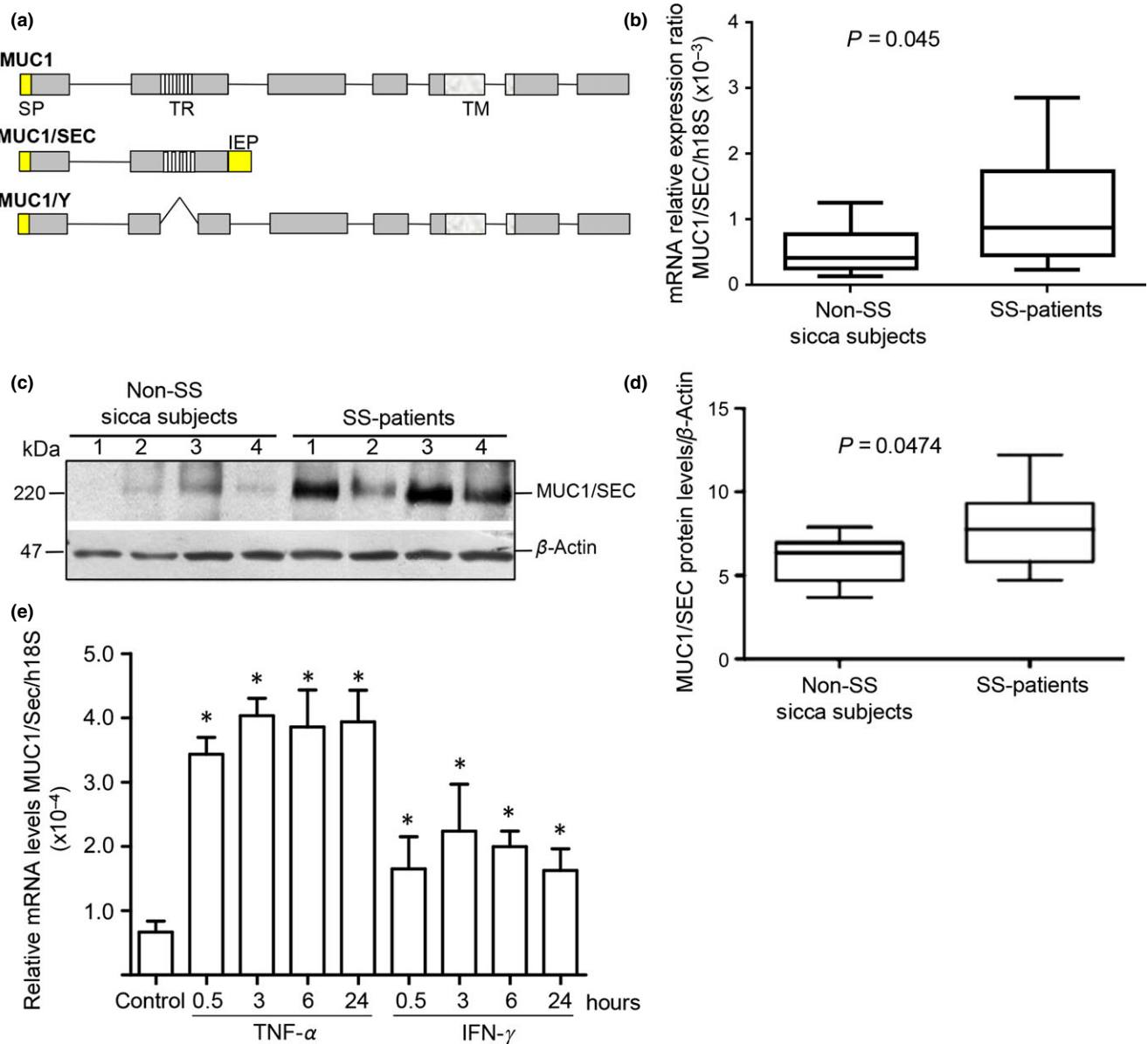


**Figure 1** MUC1 splice variants and MUC1/SEC mRNA and protein levels. (**a**) The MUC1 transmembrane, MUC1/SEC, and MUC1Y variants are depicted. SP, signal peptide; TR, tandem repeat; TM, transmembrane domain; and IEP, immune-enhancing peptide. (**b**) Relative mRNA levels of MUC1/SEC in labial salivary gland (LSG) of Sjögren's syndrome (SS) patients and control individuals. (**c**) Representative Western blots showing a single 220 kDa band for MUC1/SEC in LSG from SS patients and non-SS sicca subjects. (**d**) Relative protein levels of MUC1/SEC were averaged from triplicate assays. Data are presented as box plots, where the bottom and top sides represent the 10th to 90th percentiles and the lines within the boxes represent the median. (**e**) Relative MUC1/SEC mRNA levels in human salivary gland cells incubated with or without 1 ng ml$^{-1}$ TNF-α or IFN-γ at different times. *: $P$ values < 0.05 were considered significant

production and in doing so modulate the immune response (Baruch et al, 1999). In addition, in mammary tumors, the formation of a receptor–ligand complex between MUC1/SEC and MUC1/Y initiates a cell signaling response that alters cell morphology (Herbert et al, 2004; Ilkovitch et al, 2008). Moreover, MUC1/Y has been associated with the induction of the transcription of pro-inflammatory cytokines via NF-κB (Cascio et al, 2011).

Considering these reported findings in the literature and the relevance of MUC1 in several pathologies, we were interested in determining the expression levels of MUC1/SEC and MUC1/Y as well as their localization in the principal cell types of salivary glands of SS patients and non-SS sicca subjects. Also, we evaluated whether cytokines were able to induce their expression in vitro.

## Subjects and methods

### Patients with primary SS and non-SS sicca subjects

A total of 27 primary Sjögren's syndrome patients [mean age (± s.d.) 48.5 ± 11.1 years] were diagnosed according to the American-European Consensus Group criteria (Vitali et al, 2002). A total of 22 individuals were non-SS sicca subjects (mean age 43.1 ± 11.6 years) selected from individuals who had consulted their doctor because of oral and/or ocular dryness symptoms, but who did not fulfill the primary SS classification criteria. They did not suffer systemic diseases, and lip biopsy analysis revealed non-focal mild diffuse chronic sialadenitis. All non-SS sicca subjects were negative for rheumatoid factor, as well as antinuclear, Ro and La antibodies.

A month before the biopsies were taken, treatments of SS patients and non-SS sicca subjects on diuretics, anti-anxiety or anticholinergic drugs were suspended and the individuals were monitored during that period by the rheumatologist (Dr Sergio Aguilera) to avoid adverse effects. For those subjects without any kind of treatment, no precautions were required. Individuals who were taking medication that affected salivary function, or who consumed alcohol and/or tobacco were excluded from the study. Also, those who presented local or systemic conditions affecting salivary gland function were discarded as controls (e.g., sialadenosis, bacterial and/or viral sialadenitis, hepatitis C, HIV, sarcoidosis, active tuberculosis, graft versus host disease, etc.). Major salivary gland function was determined by scintigraphy as described previously (Schall et al, 1971). Scintigraphy results of all patients were segregated into four groups: (i) normal, (ii) mild, (iii) moderate, and (iv) severe dysfunction. Written consent was obtained from subjects according to the Declaration of Helsinki protocol. The Ethical Committee of the Faculty of Medicine, University of Chile approved this study. A detailed description of demographic, serological and histological characteristics of the SS patients and non-SS sicca subjects is summarized in Table 1.

### Biopsies

Labial salivary glands were obtained according to the method described by Daniels (1984). Samples were immediately frozen in liquid nitrogen and stored at −80°C or processed for immunohistochemical analysis.

### Cell culture

Human salivary gland (HSG) cells were cultured as previously described (Barrera et al, 2014). Differentiated HSG cells were incubated with or without 1 ng ml$^{-1}$ recombinant human TNF-α or IFN-γ (Biolegend Inc, San Diego, CA, USA) for 30 min, 3, 6 or 24 h. Cells were processed to extract RNA as described below.

### RNA extraction from LSG and HSG cells

Total RNA was extracted from LSG and HSG cells using the RNeasy Mini Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. RNA integrity was verified by electrophoresis in 1% agarose-formaldehyde gels. DNA contamination was assessed in a PCR experiment without reverse transcriptase (RT). RNA concentrations and

**Table 1** Demographic and serological characteristics of the patient and control groups

| | Non-Sjögren's syndrome sicca subjects | Patients with primary Sjögren's syndrome |
|---|---|---|
| No. of individuals | 22 | 27 |
| Sex, no. of female/no. of male | 19/3 | 26/1 |
| Age, mean (range), years | 43.1 (21–67) | 48.5 (27–73) |
| Xerophthalmia, n (%) | 13 (59%) | 26 (96%) |
| Xerostomia, n (%) | 12 (55%) | 24 (89%) |
| Focus score[a] | | |
| 1 | 0 | 21 |
| 2 | 0 | 1 |
| 3 | 0 | 3 |
| USWSF, mean ± s.d. | 2.6 ± 1.7 | 1.1 ± 1.2 |
| ml 15 min$^{-1}$ (range) | (0.2–4.9) | (0–5.3) |
| Scintigraphic data score[b] | | |
| 1 | 7 | 0 |
| 2 | 10 | 9 |
| 3 | 5 | 6 |
| 4 | 0 | 12 |
| Ro antibodies | 0/22 | 18/27 |
| Ro/La antibodies | 0/22 | 13/27 |
| Antinuclear antibodies | 0/22 | 22/27 |
| Rheumatoid factor | 0/22 | 6/27 |
| Cumulative ESSDAI, median; IQR (25–75) | – | 4; 3 (2–5) |

USWSF, Unstimulated whole salivary flow; s.d., standard deviation; n, number; %, percentage; ESSDAI, EULAR Sjögren syndrome disease activity index; EULAR, European League against Rheumatism; IQR, interquartile range.
[a]Number of foci per 4 mm$^2$ of tissue.
[b]1 = normal salivary gland function, 2 = mild impairment of salivary gland function, 3 = moderate impairment of salivary gland function and 4 = severe impairment of salivary gland function.

purity were assessed by measuring the absorbance at 260 and 280 nm. Samples with A260/A280 ratios ranging from 1.8 to 2.0 were used in this study.

### Quantitative RT-PCR assays

One microgram of total RNA was reverse-transcribed to cDNA using random primers, oligo-dT and reverse transcriptase (SuperScript II; Invitrogen, San Diego, CA, USA). Primer sequences for the genes h18S, MUC1/SEC and MUC1/Y were designed with the Amplifix 1.4 software (Nicolas Jullien: CNRS, Aix-Marseille Université, France) (Table S1). The conditions employed in MUC1/SEC qPCR assay have been described previously (Bahamondes et al, 2011). The same qPCR conditions were used for amplification of MUC1/Y. However, the primers recognized simultaneously two splice variants, MUC1/Y and MUC1/Z (amplicons of 209 and 263 bp, respectively). For this reason, conventional PCR assays were performed, and conditions were chosen to ensure that the products were analyzed in the exponential amplification phase. The amplicons were analyzed by electrophoresis in 2% agarose gel and quantified by densitometry. Values were normalized to those h18S.

### Protein extraction and Western blotting

Labial salivary gland samples were homogenized using RIPA buffer and the complete Protease Inhibitor Cocktail EDTA-free Mini Tablets (Roche, Mannheim, Germany). Proteins were quantified using the Bradford method (Bradford, 1976). Protein aliquots of 25 μg (MUC1/SEC) or 20 μg (MUC1/Y) were separated by 7% (MUC1/SEC) or 8% (MUC1/Y) SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Headquarters Hercules, CA, USA) for 2 h at 4°C. Blots were blocked for 1 h at room temperature in a 7% (MUC1/SEC) or 5% (MUC1/Y) fat-free milk solution prepared in Tris-buffered saline–Tween buffer [10 mM

Tris HCl (pH7.5), 150 mM NaCl, 0.1% Tween 20], and then incubated with chicken anti-human MUC1/SEC (kindly donated by Dr. Sandra Gendler) or MUC1/Y (BOS6E6 monoclonal antibody) antibodies using dilutions of 1:50 000 and 1:8 000, respectively. After rinsing, blots were incubated either with a 1:50 000 dilution of the horseradish peroxidase (HRP)-conjugated anti-chicken antibody for MUC1/SEC or a 1:30 000 dilution of the HRP-conjugated anti-mouse antibody for MUC1/Y. DA3/SR9 cells transfected with MUC1/Y were used as positive controls. MUC1/SEC and MUC1/Y bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Inc. (Pierce), Waltham, MA, USA), quantified by densitometry, and normalized to $\beta$-actin levels.

### Immunohistochemical detection of MUC1/VNTR, MUC1/ SEC, and MUC1/Y

Labial salivary gland were fixed in Bouin's solution and embedded in paraffin wax for histological and immunohistochemical studies. For MUC1/Y staining, antigen detection was recovered by incubation with a 0.01 M citrate solution (pH 6.0) for 5 min at 92°C. All tissue sections were treated with 0.18% of $H_2O_2$ diluted in methanol for 20 min at room temperature to inactivate endogenous peroxidase activity. After incubation at room temperature with 0.25% casein in PBS for 1 h, slides were incubated for 22 h at 4°C with a 1:50 dilution of the VU4H5 antibody that recognizes the APDTR sequence of the MUC1 VNTR (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), a 1:75 dilution of chicken anti-human MUC1/SEC or a 1:200 dilution of the mouse monoclonal BOS6E6 anti MUC1/Y antibody. Slides were incubated at room temperature for 1 h and 15 min with peroxidase-labeled streptavidin goat-anti-chicken antibody (Southern BioTech, Birmingham, AL, USA) diluted 1:200, or with biotinylated secondary antibody and a streptavidin-peroxidase-conjugated complex (Agilent Technologies (Dako), Santa Clara, CA, USA). Slides were developed with DAB chromogen (DakoCytomation Inc, CA, USA) and counterstained with Mayer's hematoxylin. Images were captured with a Zeiss (Oberkochen, Germany) light microscope Axiostar plus. For semi-quantification of MUC1/VNTR staining intensity, 20 random acini and 20 random ducts from each biopsy were photographed. Staining intensity of three random regions of interest from each image was evaluated using the Matlab 6.5 software (The Mathworks Inc, MA, USA).

### Statistical analysis

Normalized values for MUC1/SEC and MUC1/Y mRNA and protein levels were processed to calculate mean values and s.d. The Mann–Whitney U-test and Spearman's correlations were used for statistical analysis. $P < 0.05$ was considered significant.

## Results

### Relative mRNA and protein levels of MUC1/SEC

Previous studies have shown that MUC1/SEC has the ability to enhance the immune response on its own, as well as by functioning as a ligand for MUC1/Y (Baruch et al, 1999). We measured the relative levels of MUC1/SEC mRNA and protein in acini of LSG. In SS patients, we observed significantly higher MUC1/SEC mRNA ($P = 0.045$; Figure 1b), and protein ($P = 0.0474$; Figures 1c,d) levels compared with non-SS sicca subjects. To mimic the inflammatory environment occurring in LSG from SS patients, we emulated this condition in vitro by incubating HSG cells with TNF-$\alpha$ or IFN-$\gamma$. Substantial MUC1/SEC mRNA induction was observed with both cytokines (Figure 1e).

### Cellular localization of MUC1/VNTR in LSG of non-SS sicca subjects and SS patients

The region VNTR (defined in the introduction) is shared by several MUC1 isoforms, including MUC1/SEC, but not by MUC1/Y (Wreschner et al, 1990; Ligtenberg et al, 1991; Zrihan-Licht et al, 1994). To detect this epitope, we used an antibody directed against this region. The results obtained show that MUC1/VNTR was predominantly present in the apical cytoplasm of acini and duct cells in LSG sections of non-SS sicca subjects (Figure 2a,c), while MUC1/VNTR was distributed throughout the cytoplasm
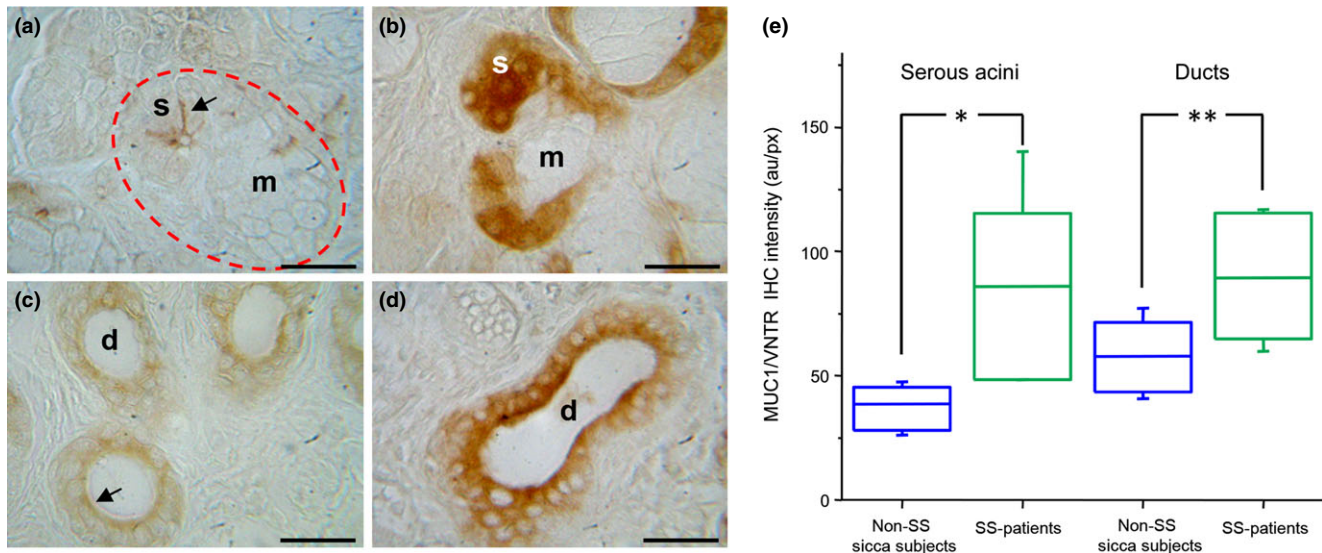


**Figure 2** Changes in distribution and staining intensity of MUC1/VNTR in labial salivary gland (LSG) from non-Sjögren's syndrome (SS) sicca subjects and SS patients. (**a, c**) Black arrows show MUC1/VNTR distributed in apical cytoplasm of acini and duct cells in LSG sections of non-SS sicca subjects. (**b, d**) MUC1/VNTR distributed throughout the cytoplasm in acinar and duct cells in LSG sections of SS patients. Red dashed line outlines an acinus. S: serous acini, m: mucous acini, d: duct, Bars: 50 $\mu$m. (**e**) MUC1/VNTR immunohistochemistry (IHC) semi-quantification. Relative staining intensity was expressed as arbitrary units/pixel (au/px). *: $P < 0.001$, **: $P = 0.006$

of acinar and duct cells in LSG sections of SS patients (Figure 2b,d).

*Different patterns of localization and distribution of MUC1/SEC in acini of LSG from non-SS sicca subjects and SS patients*

Alternatively, using a specific antibody directed against the 11 amino-acid peptide at the COOH terminus of MUC1/SEC, we sought to determine whether the pattern of detection was similar to that observed for MUC1/VNTR. These immunohistochemical assays were performed using the same primary and secondary antibodies as for the Western blot experiments. No differences in MUC1/SEC staining were observed in ducts of both groups. For inflammatory cells, no MUC1/SEC-specific staining was observed. However, for the acini of both groups, substantial differences in localization and distribution were readily visible and then quantified as is shown in Figure 3a–e. These samples were classified histologically according to four patterns of localization and distribution for MUC1/SEC in acini: panel A, preferential localization in the apical region; panel B, low levels of MUC1/SEC; panel C, increased staining and distribution throughout the cytoplasm; and panel D, high staining intensity distributed throughout the cytoplasm of damaged acini. The number of acini with MUC1/SEC A, B, C or D pattern was recorded by optical microscopy for each slide. The percentages (%) of acini with a particular MUC1/SEC localization pattern per sample were expressed relative to the total number of acini in each slide. In non-SS sicca subjects, a significantly higher percentage of acini with A and B patterns (Figure 3a,b,e) ($P = 0.0001$ and $P = 0.0092$, respectively) were observed. Alternatively, for samples from SS patients, a significantly higher percentage of acini with the C pattern were detectable (Figure 3c,e) ($P = 0.0362$). In SS patients, acini with the pattern D were also present (Figure 3d,e). These different

localization patterns observed for MUC1/SEC were independent of their proximity to inflammatory cell infiltrates (Figure S1A–C).

*Relative mRNA and protein levels of MUC1/Y*

With the results obtained for MUC1/SEC in mind, we then determined the mRNA and protein levels of MUC1/Y. In SS patients, we observed significantly higher MUC1/Y mRNA ($P = 0.036$; Figure 4a,b) and protein ($P = 0.0111$; Figure 4c,d) levels compared with non-SS sicca subjects, with an overall tendency that was similar to that observed for MUC1/SEC. As was mentioned in Subjects and methods, primers also recognized the MUC1/Z variant (Figure 4a). Moreover, we performed immunoprecipitation assays to determine whether MUC1/Y and MUC1/SEC form complexes. However, the two antibodies available for MUC1/Y and MUC1/SEC were not appropriate for such experiments. As described above, when HSG cells were incubated with TNF-α or IFN-γ, substantial increases in MUC1/Y mRNA levels were detected (Figure 4e).

*Cellular localization of MUC1/Y in LSG of non-SS sicca subjects and SS patients*

MUC1/Y was observed mainly in the apical region of acini and in cytoplasm of ducts in LSG from non-SS sicca subjects (Figure 5a), whereas in SS patients, MUC1/Y was distributed throughout the cytoplasm in acinar, ductal, and inflammatory cells (Figure 5b). Interestingly, for some acinar cells, nuclear MUC1/Y staining was detected in LSG from SS patients (Figure 5c).

*Correlation analysis between clinical parameters and MUC1/SEC levels in non-SS sicca subjects and SS patients*

A Spearman rank correlation analysis comparing different clinical parameters, including age, xerophthalmia, xerosto-
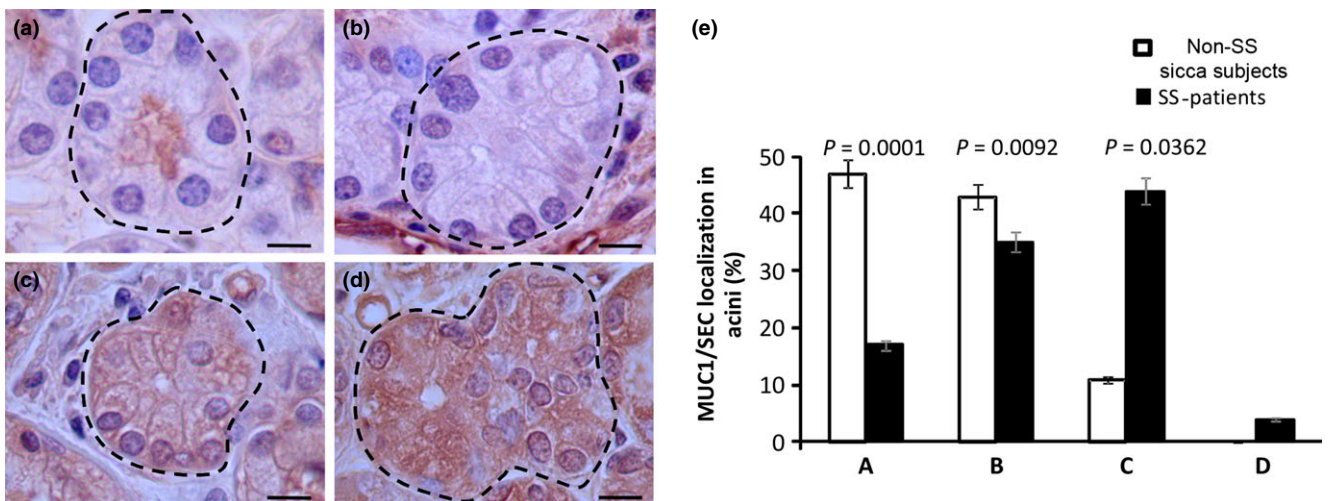


**Figure 3** Different patterns of localization and distribution of MUC1/SEC in acini of labial salivary gland from non-Sjögren's syndrome (SS) sicca subjects and SS patients. Four MUC1/SEC staining patterns were quantified in acini of non-SS sicca subjects and SS patients. (**a**) MUC1/SEC localized at the apical region, (**b**) low overall level of MUC1/SEC, (**c**) MUC1/SEC distributed throughout the cytoplasm, and (**d**) highly expressed MUC1/SEC distributed throughput the cytoplasm of damaged acini. (**e**) The graph summarizes changes in the percentage of acini showing each of the MUC1/SEC localization and distribution patterns (A–D). White and black bars correspond to non-SS sicca subjects and SS patients, respectively. Black dashed line indicates an acinus. Bars: 10 μm
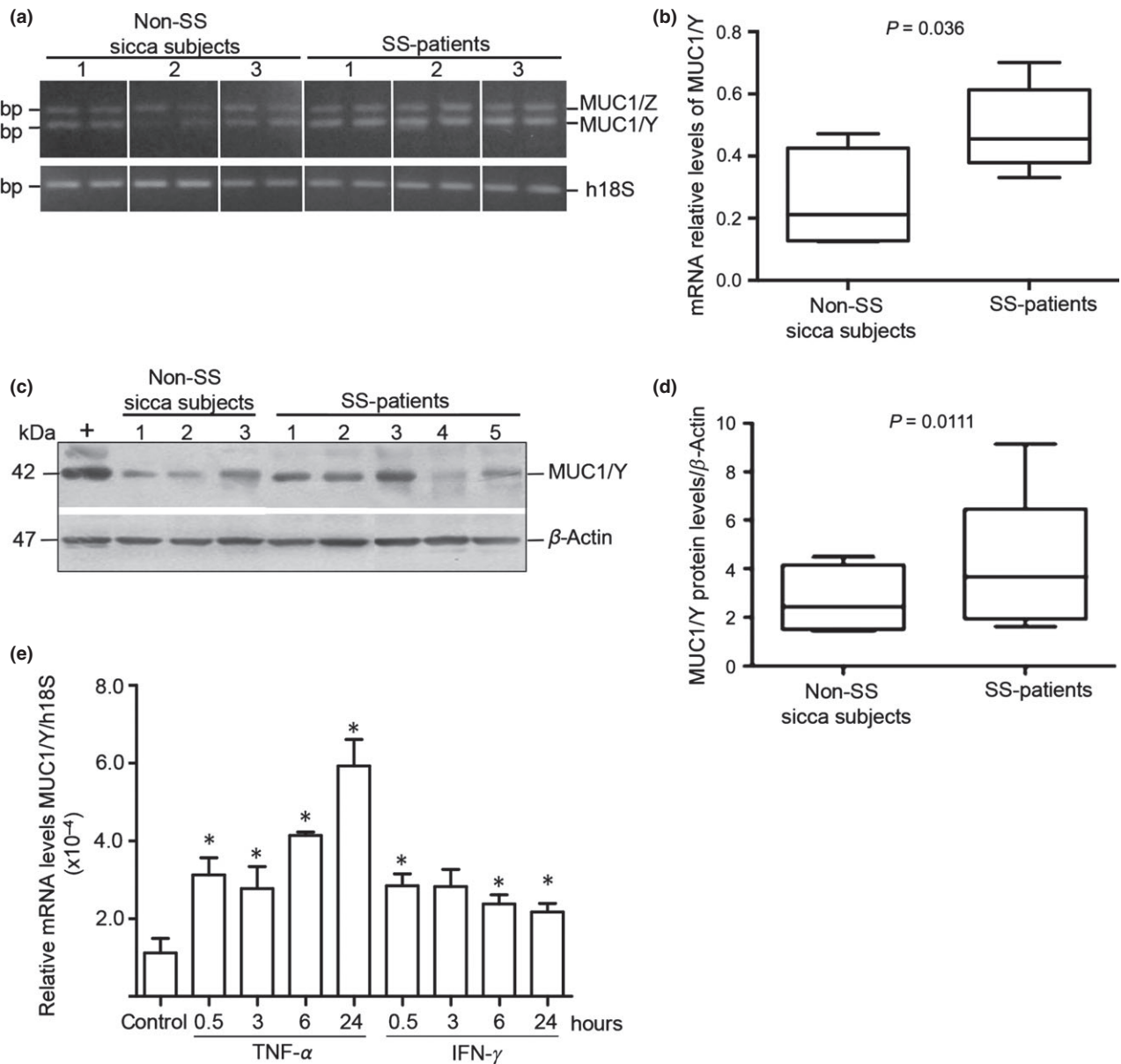
**Figure 4** MUC1/Y mRNA and protein levels. (**a**) Representative 2% agarose gel for MUC1/Y, MUC1/Z, and h18S amplicons are shown. (**b**) Values for densitometric data of MUC1/Y amplicons averaged from triplicate assays in labial salivary gland (LSG) of Sjögren's syndrome (SS) patients and non-SS sicca subjects are shown. (**c**) Representative Western blot showing a single band of 42 kDa for MUC1/Y in LSG from SS patients and non-SS sicca subjects. (**d**) Relative protein levels of MUC1/Y were averaged from triplicate assays. Data are presented as box plots, where the bottom and top sides represent the 10th to 90th percentiles and the lines within the boxes represent the median. (**e**) Relative MUC1/Y mRNA levels in human salivary gland cells incubated with or without 1 ng ml$^{-1}$ TNF-$\alpha$ or IFN-$\gamma$ at different time points. *: $P$ values $< 0.05$ were considered significant. +: DA3/SR9-cells stably transfected with MUC1/Y

mia, unstimulated salivary flow, scintigraphy, Ro, La, RF, ANA, biopsy (focus score), ESSDAI with MUC1/SEC and MUC1/Y protein and mRNA levels, was conducted. For scintigraphic data, we used lower values to indicate better glandular function, while higher values of MUC1/SEC protein are associated with poor glandular function. A strong positive correlation was observed between MUC1/SEC protein levels and poor glandular function measured by scintigraphy ($R = 0.8693$, $P = 0.0110$). No correlation was detected between clinical parameters and MUC1/Y levels.

## Discussion

This study shows for the first time that the MUC1 variants MUC1/SEC and MUC1/Y are expressed in LSG from both SS patients and non-SS sicca subjects. The MUC1/SEC isoform has been described in ocular surface tissues, endometrium and fallopian tube epithelium (Hey *et al*, 2003; Imbert *et al*, 2006), as well as breast, ovarian and cervical tumoral samples (Wreschner *et al*, 1990; Smorodinsky *et al*, 1996; Obermair *et al*, 2001; Ilkovitch *et al*, 2013). We found significantly higher mRNA and protein
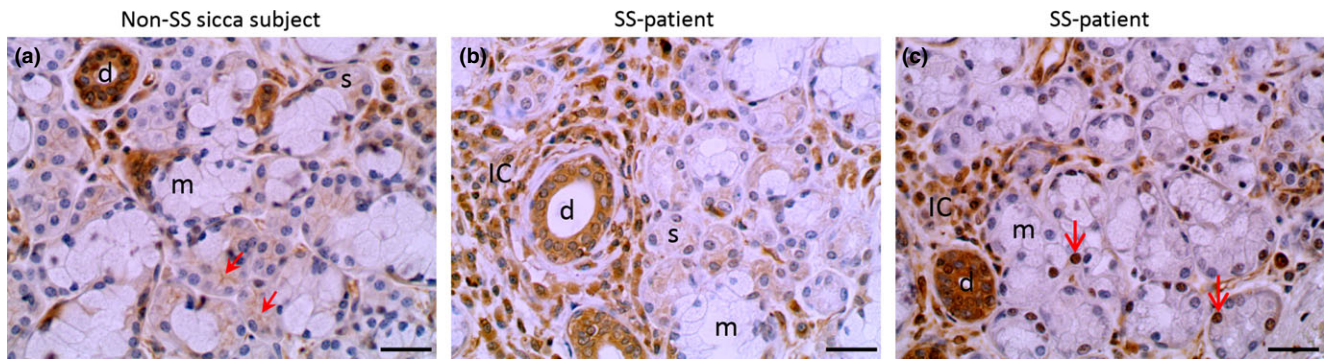
**Figure 5** MUC1/Y staining in sections of labial salivary gland (LSG) from non-Sjögren's syndrome (SS) sicca subjects (**a**) and SS patients (**b, c**). MUC1/Y was observed mainly in the apical region of acini in LSG from a non-SS sicca subject (**a**, arrows). (**b**) shows MUC1/Y distributed throughout the cytoplasm of acinar, duct, and inflammatory cells in LSG of SS patients. (**c**) shows nuclear staining of MUC1/Y in acinar cells of LSG from SS patients (arrows). m: mucous acini; s: serous acini; d: duct; and IC: inflammatory cells. Bars: 35 μm

levels of both variants in SS patients (See Figures 1b–d and 4a–d). The MUC1 gene is subject to several mechanisms of control by cytokines (IFN-γ, TNF-α, IL-7) and epigenetic factors (methylation of CpG island in the promoter, histone modification, miRNA effects) (Yamada *et al*, 2011). However, mechanisms of regulation involved in differential expression of MUC1 splice variants, specifically MUC1/SEC and MUC1/Y, have not been reported to date. MUC1/SEC has been associated with progressive inhibition of tumor development and antitumoral immune responses linked to increased STAT-1 expression (Ilkovitch *et al*, 2008). Although the precise nature of this mechanism has not yet been elucidated, it is intriguing to speculate that these effects might be the consequence of STAT-1 activation leading to the following: (i) overexpression of IFN-γ responsive signal transducer and/or (ii) activation of pro-apoptotic and pro-inflammatory genes (Ilkovitch *et al*, 2008, 2013). In addition, MUC1/SEC may induce overexpression of cytokines through its IEP and/or via formation of a MUC1/SEC-MUC1/Y complex (Herbert *et al*, 2004). On the other hand, MUC1/Y has been associated with the induction of the transcription of proinflammatory cytokines via NF-κB (Cascio *et al*, 2011). Thus, higher levels of MUC1/SEC and MUC1/Y mRNA and protein observed in salivary glands of SS patients may favor the synthesis of cytokines. Interestingly, in the present study, we demonstrate that mRNA levels of MUC1/SEC and MUC1/Y were induced by TNF-α and IFN-γ in HSG cells, supporting previous evidence from our laboratory suggesting the presence of a self-perpetuating, mucin–cytokine signaling loop in inflammatory conditions (Barrera *et al*, 2014).

Importantly, although our control group contains mostly individuals with mild chronic diffuse gland infiltration; our findings indicate that the changes in expression and localization reported here for MUC1 in LSG of SS patients are attributable to the elevated presence of proinflammatory cytokines in these glands. Salivary acinar, ductal, and inflammatory cells from SS patients synthesize cytokines (Fox *et al*, 1999). Previous studies from our laboratory reported on the disruption of tight junctions of salivary acinar and ductal cells from SS patients and showed *in vitro* that specific aspects could be recapitulated by

exposing cells to TNF-α and IFN-γ (Ewert *et al*, 2010). These cytokines promote macropinocytosis of tight junction proteins, such as occludin, JAM-1 and claudin-1 via early endosomes, leading to reorganization of the cytoskeleton and changes in cell polarity (Utech *et al*, 2005) that impair cell–cell and cell–extracellular matrix (ECM) communication in the acini, which in turn alter salivary gland homeostasis (Barrera *et al*, 2013). Studies evaluating MUC1 function in salivary glands are not available. However, in other tissues, such as lung, bowel, and brain, studies using knockout mice for MUC1 suggest an antiinflammatory role of the protein (Choi *et al*, 2011; Sheng *et al*, 2013; Yen *et al*, 2013). In those studies, a complete loss of all isoforms of MUC1 was reported, and for the bowel, the anti-inflammatory function of MUC1 was linked to the protection of the mucous provided by MUC1 anchored to membrane (Sheng *et al*, 2013). These results have been reproduced in knockdown studies using siRNA targeting a sequence shared by all known MUC1 variants. However, it is important to emphasize that those studies do not shed light on the specific function of particular MUC1 isoforms.

The immunohistochemical analysis revealed a significantly higher proportion of acini with MUC1/SEC in the cytoplasm of SS patients (Figure 3c, $P = 0.0362$), while for non-SS sicca subjects, a significantly higher percentage of acini with MUC1/SEC in the apical region (Figure 3a, $P = 0.0001$) and low presence in the cytoplasm (Figure 3b, $P = 0.0092$) were observed. This cytoplasmic distribution of MUC1/SEC observed in the acini of SS patients was associated with loss of cell polarity, where increased intensity of cytoplasmic MUC1/SEC staining (Figure 3d) was coincident with increased acinar alterations. These results confirmed observations showing alterations in the distribution and accumulation of MUC1/VNTR in the cytoplasm of acinar and ductal cells from LSG of SS patients (Figure 2). MUC1/Y is redistributed from the apical region of acini in LSG of non-SS sicca subjects to the cytoplasm and nuclei in LSG from SS patients (Figure 5). Nuclear localization of some MUC1 isoforms has been previously described, but the mechanism involved is still unknown, while the nuclear function has been related with increased transcription of pro-inflammatory cytokines (Cascio *et al*, 2011). Previous

studies in SS patients had shown aberrant accumulation of secretory granules throughout the cytoplasm, extending to the basal region close to the nucleus (Goicovich *et al*, 2003). This may be due to the following: (i) aberrant targeting of secretory granules as a result of altered expression or localization of the GTPase Rab3D (Bahamondes *et al*, 2011), (ii) cytoskeletal alterations (Kelly, 1990) and (iii) alterations in the fusion of secretory granules with the apical plasma membrane (Barrera *et al*, 2012). In the acini of SS patients, apical–basolateral relocalization of proteins of the exocytic molecular machinery has been observed, which may explain glandular hyposecretion and/or changes in the quality of their secretory products. Recently, we found that fusion receptors (SNARE proteins), which normally localize to the apical membrane region, were redistributed to the basolateral region in salivary acinar cells from SS patients (Barrera *et al*, 2012). These changes were accompanied by ectopic exocytosis of MUC7 and MUC5B to the ECM of LSG from SS patients, despite the integrity of the acinar basal lamina. Ectopic mucins induced the expression of pro-inflammatory cytokines, via Toll-like receptor 4, which may disrupt gland organization (Barrera *et al*, 2012, 2014). Perhaps, such alterations in exocytosis may help explain cytoplasmic accumulation of MUC1/SEC and MUC1/Y in the acini of SS patients. In this context, the analysis correlating MUC1/SEC protein levels with clinical parameters revealed a highly significant positive correlation with acinar damage ($R = 0.8693$, $P = 0.0110$). In addition, high staining of cytoplasmic MUC1 has been reported in tumor cells, where the loss of the cell polarity and tissue organization is very common (Ceriani *et al*, 1992).

In summary, the observed overexpression and aberrant localization of MUC1/SEC and MUC1/Y in LSG of SS patients and their overexpression induced *in vitro* in HSG cells by pro-inflammatory cytokines are consistent with previous evidence from our laboratory pointing toward the existence of a self-perpetuating mucin–cytokine signaling loop that may facilitate the maintenance of an inflammatory environment leading to disruption of salivary glandular homeostasis in SS patients.

## Competing interests

The authors have declared no conflict of interests.

## Author contributions

H. Sung, I. Castro, M. J. González, and S. González participated in study design, acquisition of data, analysis and interpretation of data, statistical analysis, and manuscript preparation. S. Aguilera and N. Smodorinsky helped with the design of the study. V. Bahamondes, C. Alliende, J. Cortés, U. Urzúa, M. J. Barrera, C. Molina, and S. Aguilera participated in the acquisition of data. S. Aguilera, M. Hermoso, C. Molina, C. Leyton, U. Urzúa, A. Quest, and L. Herrera helped in analysis and interpretation of data. A. Quest, U. Urzúa, S. Aguilera, and N. Smodorinsky helped in manuscript preparation. C. Molina participated in statistical analysis. M. J. González had full access to the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

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738

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** MUC1/SEC distribution patterns are independent from the proximity of the inflammatory focus.

**Table S1** Sequences of primers used in this study.