Evidence of a Sjögren's disease-like phenotype following COVID-19 in mice and human

Yiran Shen, … , Blake M. Warner, Cuong Q. Nguyen


OBJECTIVES. Sjögren's Disease (SjD) is a chronic and systemic autoimmune disease characterized by lymphocytic infiltration and the development of dry eyes and dry mouth resulting from the secretory dysfunction of the exocrine glands. SARS-CoV-2 may trigger the development or progression of autoimmune diseases, as evidenced by increased autoantibodies in patients and the presentation of cardinal symptoms of SjD. The objective of the study was to determine whether SARS-CoV-2 induces the signature clinical symptoms of SjD.

METHODS. The ACE2-transgenic mice were infected with SARS-CoV-2; SjD profiling was conducted. COVID-19 patients' sera were examined to detect the presence of autoantibodies. Clinical evaluations of convalescent COVID-19 subjects, including minor salivary gland (MSG) biopsies, were collected. Lastly, monoclonal antibodies generated from single B cells of patients were interrogated for ACE2/spike inhibition and nuclear antigens.

RESULTS. Mice infected with the virus showed a decreased saliva flow rate, elevated antinuclear antibodies (ANAs) with anti-SSB/La, and lymphocyte infiltration in the lacrimal and salivary glands. Sera of COVID-19 patients showed an increase in ANA, anti-SSA/Ro52, and anti-SSB/La. The male patients showed elevated levels of anti-SSA/Ro52 compared to female patients, and female patients had more diverse ANA patterns. Minor salivary gland biopsies of convalescent COVID-19 subjects showed focal lymphocytic infiltrates in four of six subjects, and 2 of 6 subjects had focus scores >2. Lastly, we found that monoclonal […]

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Evidence of a Sjögren’s disease-like phenotype following COVID-19 in mice and human

Yiran Shen¹*, Alexandria Voigt¹*, Laura Goranova¹, Mehdi Abed², David E. Kleiner³, Jose O. Maldonado², Margaret Beach², Eileen Pelayo², John A. Chiorini⁴, William F. Craft⁵, David A. Ostrov⁶, Vijay Ramiya⁷, Sukesh Sukumaran⁸, Ashley N. Brown⁹, Kaley C Hanrahan⁹, Apichai Tuanyok¹, Blake M. Warner²#, and Cuong Q. Nguyen¹,¹⁰,¹¹#

¹Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, Florida, USA; ²Salivary Disorder Unit, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland; ³Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; ⁴AAV Biology Section, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland, USA; ⁵Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, Florida, USA; ⁶Department of Pathology, Immunology & Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida, USA; ⁷LifeSouth Community Blood Centers, Gainesville Fl, ⁸Valley Children's Hospital, Madera, California; ⁹Institute for Therapeutic Innovation, Department of Medicine, University of Florida College of Medicine, Orlando, Fl; ¹⁰Department of Oral Biology, College of Dentistry; ¹¹Center of Orphaned Autoimmune Diseases, University of Florida, Gainesville, Florida, USA.

*Authors contributed equally to the study

#Co-correspondence authors.

Address correspondence:

Cuong Q. Nguyen, PhD
Department of Infectious Diseases and Immunology
College of Veterinary Medicine, University of Florida
27  2015 SW 16th Ave, V3-152
28  Gainesville, Florida 32611-0880. USA
29  Telephone:  352-294-4180, Fax:  352-392-9704
30  nguyenc@ufl.edu
31
32  Blake M. Warner, DDS, PhD, MPH
33  Salivary Disorders Unit
34  National Institutes of Health
35  Building 10 Room 1A01
36  10 Center Drive
37  Bethesda, MD 20895
38  Telephone: 301-496-4486
39  blake.warner@nih.gov
40
**Key Messages:**

What is already known about this subject?

- SARS-CoV-2 has a tropism for the salivary glands. However, whether the virus can induce clinical phenotypes of Sjögren's disease is unknown.

What does this study add?

- Mice infected with SARS-CoV-2 showed loss of secretory function, elevated autoantibodies, and lymphocyte infiltration in glands.
- COVID-19 patients showed an increase in autoantibodies. Monoclonal antibodies produced in recovered patients can block ACE2/spike interaction and recognize nuclear antigens.
- Minor salivary gland biopsies of some convalescent subjects showed focal lymphocytic infiltrates with focus scores.

How might this impact on clinical practice or future developments?

- Our data provide strong evidence for the role of SARS-CoV-2 inducing Sjögren's disease-like phenotypes.
- Our work has implications for how patients will be diagnosed and treated effectively.
Abstract

Objectives:

Sjögren's Disease (SjD) is a chronic and systemic autoimmune disease characterized by lymphocytic infiltration and the development of dry eyes and dry mouth resulting from the secretory dysfunction of the exocrine glands. SARS-CoV-2 may trigger the development or progression of autoimmune diseases, as evidenced by increased autoantibodies in patients and the presentation of cardinal symptoms of SjD. The objective of the study was to determine whether SARS-CoV-2 induces the signature clinical symptoms of SjD.

Methods:

The ACE2-transgenic mice were infected with SARS-CoV-2; SjD profiling was conducted. COVID-19 patients' sera were examined to detect the presence of autoantibodies. Clinical evaluations of convalescent COVID-19 subjects, including minor salivary gland (MSG) biopsies, were collected. Lastly, monoclonal antibodies generated from single B cells of patients were interrogated for ACE2/spike inhibition and nuclear antigens.

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Mice infected with the virus showed a decreased saliva flow rate, elevated antinuclear antibodies (ANAs) with anti-SSB/La, and lymphocyte infiltration in the lacrimal and salivary glands. Sera of COVID-19 patients showed an increase in ANA, anti-SSA/Ro52, and anti-SSB/La. The male patients showed elevated levels of anti-SSA/Ro52 compared to female patients, and female patients had more diverse ANA patterns. Minor salivary gland biopsies of convalescent COVID-19 subjects showed focal lymphocytic infiltrates in four of six subjects, and 2 of 6 subjects had focus scores >2. Lastly, we found that monoclonal antibodies produced in recovered patients can block ACE2/spike interaction and recognize nuclear antigens.

Conclusion:
Overall, our study shows a direct association between SARS-CoV-2 and SjD. Hallmark features of SjD salivary glands were histologically indistinguishable from convalescent COVID-19 subjects. The results potentially implicate that SARS-CoV-2 could be an environmental trigger for SjD.
Key Words:

Sjögren's Disease (SjD), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Coronavirus disease 2019 (COVID-19), Autoimmune disease, Autoantibodies.
**Introduction**

Sjögren's Disease (SjD) is an autoimmune disease generally categorized by sicca symptoms in the mouth and eyes, autoantibodies, and lymphocytic infiltration into the salivary gland(1, 2). It is estimated that approximately 4 million Americans are affected, making SjD the second most common autoimmune disease after rheumatoid arthritis(3–5). SjD has the most skewed sex distribution (9:1 ratio of women to men), when compared to rheumatoid arthritis (RA), multiple sclerosis (MS) and myasthenia gravis(6). SjD is most closely associated with symptoms of dryness, particularly of the mouth and eyes; however, a wide variety of extraglandular manifestations have been reported involving virtually any organ or tissue(4, 7). The extraglandular manifestations of SjD have been subdivided into visceral (gastrointestinal tract, lungs, heart, central and peripheral nervous system) and non-visceral (muscles, joints, skin) involvement, indicating the wide variety of tissues that may be involved in the disease. While both men and women at any age can be affected by SjD, it is most commonly diagnosed in women in the fourth or fifth decade of life(7, 8). The pathological framework of SjD pathogenesis remains elusive, however, studies have suggested the primary drivers are genetic susceptibility, hormonal factors, and environmental triggers.

In December 2019, a novel coronavirus, severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), emerged in Wuhan, Hubei Province, China, initiating a breakout of atypical acute respiratory disease, termed coronavirus disease 2019 (COVID-19). SARS-CoV-2 is a betacoronavirus in the family of Coronaviridae; the virus contains four structural proteins: S (spike), E (envelope), M (membrane), and N (nucleocapsid), sixteen non-structural proteins (nsp1–16) and eleven accessory proteins, which support viral essential physiological function and evasion from the host immune system(9). As of May 1st, 2022, approximately one million U.S. residents have died from COVID-19(10) with more than 80 million total cases. Recent studies have identified the association between SARS-CoV-2 infection and autoimmune response. In point of fact, a recent literature review(11) (n= 1176 articles and 90 case reports) revealed that
the primary rheumatic diseases associated with COVID-19 patients were vasculitis, arthritis, idiopathic inflammatory myopathies, and systemic lupus erythematosus. Several studies have found an association between antinuclear antibodies (ANAs) (35.6%) and COVID-19 infection, where the leading reactive antigens include SSA/Ro (25%), rheumatoid factor (19%), lupus anticoagulant (11%), and type I interferons (IFN-I) (10%)(12–14). In 6 independent case studies, COVID-19 patients were diagnosed with systemic sclerosis(15), adult-onset Still's disease(16), sarcoidosis, and systemic lupus erythematosus (SLE), with 4/6 patients acutely manifesting during COVID-19. An elevated level of anti-SSA/Ro52 in COVID-19 patients was linked to pneumonia severity and poor prognosis(17). The underlying mechanism for the production of autoantibodies in COVID-19 patients is unknown. However, it poses a significant challenge for post-COVID-19 symptoms or post-acute sequelae of SARS-CoV-2 (PASC).

There are, additionally, reports and cases of COVID-19 patients experiencing ocular and oral symptoms. Keratoconjunctivitis was observed in a few patients during a specific phase of the disease(18). One study has shown that xerostomia was observed in 29% of the patient cohort(19) while another showed an increase of 30% in reporting xerostomia during hospitalization(20). While these early studies had small sample sizes, the results indicated an association between COVID-19 and oral and ocular manifestations, primary symptoms of SjD. Increased rates of xerostomia in this patient cohort may be explained by the tropism of SARS-CoV-2 in the salivary glands, resulting in host immune response and immune-mediated injury(21). Furthermore, growing evidence of autoantibody production in COVID-19 patients raises a critical question of whether SARS-CoV-2 infection is a risk factor for primary SjD. Therefore, the goal of this study was to determine the autoimmune response triggered by SARS-CoV-2 infection. The results indicate that infection with SARS-CoV-2 recapitulated an SjD-like phenotype in transgenic mice, and patients exhibited lymphocytic sialadenitis. Furthermore, COVID-19 patients’ sera showed an increased frequency of anti-nuclear autoantibodies (ANA) and levels of anti-SSA/Ro52 and anti-SSB/La compared to healthy controls.
Results

**SARS-CoV-2 triggered the decrease in the salivary secretory function.**

SjD patients experience xerostomia, primarily due to the salivary glands' diminished secretory function. In the spontaneous animal models of SjD, the secretory dysfunction occurs between 15-20 weeks of age. Here, we sought to determine if SARS-CoV-2 can compromise saliva secretion by the glands. The homozygous K18-hACE2 mice were intranasally inoculated with 860 PFU of SARS-CoV-2 WA1/2020 inoculum drop-by-drop into both nostrils until fully inhaled. Saliva was collected on day 21, prior to euthanasia. The infected male and female mice displayed a significant loss of salivary flow rates compared to the uninfected male and female mice (infected: $6.64 \pm 1.075$ vs. uninfected: $13.12 \pm 0.532$ ul/gr). The infected males appeared to lose more saliva flow than infected females; however, the loss of saliva between infected males and females was not statistically significant (**Figure 1A**). To eliminate the probability that the change in the salivary flow rate was due to the change in weight as opposed to the saliva production, the weight change was calculated as well. The infected mice showed a decrease in body weight compared to the uninfected mice; however, the decrease was not statistically significant (**Figure 1B**). The results suggest that SARS-CoV-2 infection has a negative effect on the secretory function of the salivary glands in both males and females.

**SARS-CoV-2 induced the production of autoantibodies.**

SjD patients generally develop ANA, including anti-SSA/Ro. Here, we sought to determine if SARS-CoV-2 infection induced autoantibody production. As presented in **Figure 2A**, 70% of all infected mice were positive, and 30% were negative for ANA using HEp2 cell staining. In the inverse, of the uninfected mice, 70% of both sexes were negative, and 30% were positive for ANA. Interestingly, males increased from 20% positive for ANA to 80% positive at post-infection, and females only changed from 40% positive for ANA to 75% positive for ANA. Furthermore, we examined the SjD-associated autoantibodies. As indicated in **Figure 2B**, anti-SSB/La levels were
highly elevated in the combined infected group compared to the control group. There was no difference in anti-SSA/Ro52 and anti-SSA/Ro60 levels between the control and infected groups. Similar patterns were observed when we compared the males and females separately. The results suggest that SARS-CoV-2 infection in mice promotes the development of ANA with higher frequency in males and specific autoantibodies associated with SjD.

SARS-CoV-2 caused apoptosis and inflammation in the lacrimal and salivary glands of mice.

The principal targeted tissues for SjD are the lacrimal and salivary glands. The inflammatory lesions are composed of a multitude of immune cell types, notably B cells, T cells, and macrophages. As presented in Figure 3A, the lacrimal glands of infected mice had multifocal apoptosis by caspase-3 staining of low to moderate numbers of acinar epithelial cells characterized by cells with condensed, hypereosinophilic cytoplasm and pyknotic nuclei with karyorrhexis. The apoptosis/necrosis resulted in variable collapse and loss of acini. The interlobular duct epithelium was unaffected. The salivary and lacrimal glands of infected mice occasionally had small interstitial lymphocytic infiltrates, characteristic of immune stimulation and response. Salivary and lacrimal gland interstitial lymphocytes were not present in the non-infected mice. Examining the infiltrate areas revealed that both males and females showed an increase in the lacrimal and salivary glands, with more severe lacrimal infiltrates in the infected females than in males (Figure 3B). We further examined the apoptotic levels using TUNEL and caspase-3. As indicated in Figures 3C and D, infected female mice showed a significant increase in TUNEL⁺ and caspase-3⁺ cells in both glands. Interestingly, infected females exhibited higher levels of salivary TUNEL⁺ cells compared to infected male mice.

To further quantify the lymphocytes as a response to inflammation, we performed staining for CD3⁺ T cells, B220⁺ B cells, and CD68⁺ macrophages (Figures 3E, 3F, Figure S1A, S1B). A few infected female mice showed increased CD3⁺ T cells and B220⁺ B cells in the salivary
glands; however, both sexes showed a substantial uptick in macrophages. In contrast, only infected males showed an increase in CD3+ T cells and B220+ B cells in the lacrimal glands, with a significant increase of macrophages in both males and females. Remarkably, infected males showed elevated lacrimal macrophage populations compared to infected females (Figure 3F). Lastly, we correlated these data with the presence of lymphocytic foci. Only a single infected mouse developed a focus score (FS) in the salivary glands, so deviation from the control group is insignificant ($\chi^2=.27$, $p=0.10247$). However, FS were detected in the lacrimal glands of 5 infected mice ($\chi^2=13$, $p=0.00031$) (Table S1). Additionally, lymphocytic infiltration of B and/or T cells which do not qualify as a focus, was examined. This indicates localized inflammation in the salivary ($\chi^2=11$, $p=0.00091$) and lacrimal glands ($\chi^2=24$, $p=0.00001$). Overall, SARS-CoV-2 induced inflammation with multifocal apoptosis with more severity in the lacrimal than salivary glands.

COVID-19 is associated with higher autoantibody levels in a sex-specific manner.

As described above, mice infected with SARS-CoV-2 developed ANA and elevated anti-SSB/La. Here, we sought to determine if these findings were also observed in human patients. As presented in Figure 4A, the COVID-19 patients exhibited higher frequencies of positive ANA at different sera titers compared to healthy controls. Notably, 60% of patients showed positive ANA, with none for healthy controls at 1:160 titer. 30% of patients still exhibited positive ANA at 1:320 titer. Further analysis of the staining patterns revealed that among the positive ANA for patients, 40% (7) were homogeneous, 15% (4) were speckled, and 5% (1) were centromeric (Figure 4B). To further determine if the COVID-19 patients presented with SjD signature autoantibodies, patient sera were examined for reactivity against SSA/Ro52, SSA/Ro60, and SSB/La. As presented in Figure 4C, anti-SSA/Ro52, and anti-SSB/La were significantly elevated in COVID-19 patients compared to healthy controls. Anti-SSB/Ro60 levels remained similar between the two groups.
SjD has a strong predilection for females; therefore, we sought to determine whether COVID-19 patients exhibited an element of sexual dimorphism in the autoantibody response. Interestingly, when examining the ANA staining, it was discovered that the female COVID-19 patients had a significantly higher percentage of positive ANA at various titers than the male COVID-19 patients or either sex of control patients (Figure S2A). Additionally, the female COVID-19 patients were shown to present a more diverse ANA pattern, with 30% speckled, 40% homogenous, and 10% centromeric at 1:160 titer, whereas the male patients showed 10% speckled and 30% homogenous pattern at the same titer. The female patients still exhibited 20% speckled, with males showing 10% speckled at 1:320 titer, and the homogenous pattern was 10% for both sexes. Positive staining for both sexes of the control group only contained a homogenous pattern, but were unsustained past 1:80 titer (Figure S2B). To further determine if male and female COVID-19 patients exhibited different levels of SjD-associated autoantibodies, we performed ELISAs on the sera based on sex. As presented in Figure S2C, female and male COVID-19 patients showed significantly higher levels of anti-SSA/Ro52 than their counterparts. Interestingly, male COVID-19 patients showed elevated levels of anti-SSA/Ro52 above female COVID-19 patients (p=0.0029). There was no statistically significant difference between male and female COVID-19 patients with anti-SSA/Ro60 or anti-SSB/La. The results indicated that female patients manifested more diverse patterns of ANA; however, male patients exhibited higher levels of anti-SSA/Ro52 than female patients.

Monoclonal antibodies produced by COVID-19 patients are reactive against nuclear antigens.

It is remarkable to observe the cross-reactivity of COVID-19 patients' sera against self-antigens, as demonstrated here. To further evaluate the B cell response of COVID-19 patients, we produced and selected nine monoclonal antibodies (mAbs) from convalescent COVID-19 patients by isolating CD20+ memory B cells reactive against both the RBD and S1 of SARS-CoV-
2, and examined their reactivity against self-antigens. As presented in Figure S3A, the mAbs exhibited various degrees of inhibition against SARS-CoV-2 RBD, in which mAbs A10 and B5 showed the highest inhibitory activity at varying dilutions using pseudovirus. To further support the infection inhibition capacity of same mAbs, we performed a PRNT with SARS-CoV-2 USA-WA1/2020. We found that C9, A10, B5 and C7 showed high inhibition activity especially at higher concentrations compared to the negative control (Figure S3B). Lastly, we tested them against HEP2 cells to determine their reactivity against nuclear antigens. As described in Figure S3C, seven of the nine S1/RBD-reactive mAbs produced a strong homogenous staining pattern at 1:40 and 1:80 titers and lowered to 67% (6 out of 9) at 1:160 and 1:320 titers. Examining the CDR3 sequences indicated that most of mAbs share one or two serine and glycine in the heavy chain and one tyrosine in the light chain. Clones A10 and B5 displayed the longest CDR3 regions (20aa and 23aa, respectively) in the heavy chain (Table S2). Overall, the results demonstrate that mAbs against the virus produced in recovered COVID-19 patients are cross-reactive and capable of recognizing nuclear antigens.

Convalescent COVID-19 subjects demonstrate inflammation of the salivary glands and clinical signs and symptoms of Sjögren's Disease.

Six generally-healthy, relatively young (Range: 19-42y; Mean: 31y) subjects who had recovered from COVID-19 and had convalescent MSG biopsies were identified for this study. These subjects were free from evidence of pre-existing autoimmune disease or major medical conditions. Subjects 1-3 were enrolled on NIH IRB Protocol: 20-D-0094 and had convalescent MSG biopsies 6-13 months after recovery from COVID-19 (Table 1). In addition, Subject 2 also received an initial biopsy during acute COVID-19 (Table 1, Figure 5). These subjects recovered from COVID-19 without continued post-acute COVID-19 symptoms as primary clinical concerns. Subjects 4-6 were enrolled on an NIH IRB Protocol: 15-D-0051 as healthy volunteers (HV) and did not present with clinical complaints of SjD or post-acute COVID-19 syndrome. Their COVID-
19 status was determined from subject interviews and serological studies. In subjects with known
COVID-19, their clinical course was generally mild; three subjects reported lung involvement with
shortness of breath without hospitalization, and one subject reported significant gastrointestinal
involvement (‘mild-to-moderate COVID-19’). A single subject, Subject 5, was unaware of their
post-COVID-19 status and was considered 'asymptomatic.' Evidence of infection included clinical
reports of infection in five of six subjects, clinical nasopharyngeal swab PCR for SARS-CoV-2 N1
and N2 genes in four subjects (S1-3,6); anti-nucleocapsid antibodies were positive in all six
subjects (data not shown). No subjects were positive for anti-nuclear antibodies (ANA) or anti-
SSA/Ro antibodies. A single subject was low-titer positive for anti-SSB/La antibodies. Three of
the six subjects reported dry mouth during acute COVID-19, sustained temporarily after recovery
(up to three weeks); a single subject had objective evidence of dry mouth (Subject 2) during acute
COVID-19. Interestingly, this subject did not produce saliva from the submandibular glands for
about three of the four weeks of weekly follow-up after infection. Dry eye assessments for three
subjects could not be completed in the NIH COVID-19 Testing Facility. Two of the three subjects
who presented through the NIH Dental Clinic had objective evidence of dry eye disease (Table
1) but did not have clinical complaints of dry eyes.

Overall, seven MSG biopsies were collected from 6 subjects - a single subject had serial
biopsies. One biopsy occurred during acute COVID-19 5 days after symptom debut, and the
second 6 months after recovery. Generally, biopsies exhibited mild chronic sialadenitis (Table 1).
However, 5 of the 7 biopsies (from four of the six subjects) had multiple foci (>50 lymphocytes) of
inflammation (e.g., focal lymphocytic sialadenitis, FLS; Table 1, Figure 5, Figure S4). Most foci
were small, although several glands exhibited multiple medium-sized and coalescing foci. Mild
fibrosis and atrophy of the glands were seen in three subjects (Subjects 1-3). It is noteworthy that
Subject 2's follow-up biopsy exhibited an increased focus score (FS:1 → FS:2) and the
elaboration of fibrosis and atrophy of the glands (Figure 5). Histopathological evidence of injury
included ductal injury and mucous inspissation, immune infiltration of the acini with injury,
perivascular infiltrates, and granuloma (examples illustrated in Figures 5 and S4). In some
subjects, the histopathological features in four of six subjects (five biopsies) are reminiscent of
the range of histopathological features found in the MSG of SjD patients.

To understand the composition of the immune infiltrates, clinical immunophenotyping was
performed on four biopsies from three subjects. The infiltrates are generally composed of varying
proportions of T and B cells, with small foci predominantly composed of T cells and larger foci
exhibiting a shifted balance towards B cell predominance. CD8 T cells were found scattered
throughout the gland and in the inflammatory foci (Figure 6, Table 2). These
immunohistochemical studies are highly similar to the inflammatory infiltrates found
characteristically in SjD. In the single subject (S2) with follow-up MSG, the amount of inflammation
and the shift to B cell predominance can be appreciated in the areas of FLS at six months.
Discussion

Increasing evidence has supported the associations between viral/bacterial infections and autoimmune diseases. An early study demonstrated that murine cytomegalovirus induced an SjD-like disease in C57Bl/6-lpr/lpr mice with sialadenitis, severe salivary gland inflammation, and production of anti-SSA/Ro and anti-SSB/La (22). Recent studies suggested that SARS-CoV-2 has a tropism for the salivary gland, including SARS-CoV-2 (21, 23). Here, we sought to determine if SARS-CoV-2 infection could also trigger SjD-like phenotypes in a murine model. The results indicate that SARS-CoV-2 infection recaptulates several signature disease phenotypes, specifically, diminished salivary flow rates, salivary and lacrimal gland inflammatory lesions, and elevated autoantibodies. Similar findings were also observed in COVID-19 patients, in which significantly elevated levels of anti-SSA/Ro52 and anti-SSB/La were seen. Additionally, female patients manifested more diverse patterns of ANA, and male patients exhibited higher levels of anti-SSA/Ro52 than female patients. In summary, the data suggest that SARS-CoV-2 infection triggered an SjD-like disease in a murine model and in human patients.

SARS-CoV-2 primarily uses ACE2 as a receptor (24, 25), broadly expressed by endothelial and epithelial cells, including those of the aerodigestive tract and the salivary glands (21, 26–28). It has now been shown that salivary glands can robustly support infection and replication of SARS-CoV-2 and that saliva is potentially infectious and transmissible (21). Intra-individual spread of SARS-CoV-2 initiates from the epithelial cells of the upper respiratory tract (e.g., acinar and ductal cells of the salivary glands) by active replication and egress of offspring viruses subsequently infecting ACE2-expressing cells in downstream organs, including the heart, kidneys, gastrointestinal tract, and vasculature (21, 29, 30). The hACE2 transgenic model expressed high levels of hACE2 in the lacrimal glands and a lesser amount in the salivary glands (Figure S5). Furthermore, lacrimal glands exhibited an elevated frequency of nucleocapsid-positive cells than the salivary glands (Figure S6). Therefore, higher expression of hACE2 and nucleocapsid-positive cells could explain the more severe lacrimal gland inflammation and cell death due to
higher viral infection and replication. The viral loads in the lungs varied among the tested mice; however, the clinical features were not affected (Figure S7). The kidneys and brains were examined and no abnormal pathology was noted (data not shown). ACE2 is expressed in squamous epithelial cells of the dorsal tongue, gingiva, and buccal tissue, and TMPRSS2 is expressed in taste bud cells and submandibular glands (21). SARS-CoV-2 was detected in SGs, with higher levels in the minor SGs (21). In addition, saliva is a natural reservoir for viruses as one of the major fluids for viral detection (31). Therefore, it is not surprising that SARS-CoV-2 was found in the SGs and facilitated the inflammatory response.

The severity of COVID-19 is mediated by unregulated inflammation (32). During the later stage of the disease, immune-mediated damage leads to a progressive increase in inflammation (33). And patients with life-threatening pneumonia had neutralizing autoantibodies against IFN-ω and IFN-α (14). As demonstrated, mice infected with SARS-CoV-2 developed higher ANA and anti-SSB/La levels. Similarly, patients developed elevated levels of ANA, specifically anti-SSA/Ro52 and anti-SSB/La. To determine whether the presence of autoantibodies that are characteristic of other autoimmune diseases, we performed the INNO-LIA ANA Update Test strips (FujireBio Diagnostics, Tokyo, Japan), which detect 13 nuclear antigens. None of the infected mice were positive for the nuclear antigens. SSA/Ro60 was positive in a single female control patient, whereas four COVID-19+ patients (1 male and 3 females) were positive. Additionally, ribosomal P was solely positive in a few patients at 1:320 serum dilution (Figure S8). A potential pitfall of the assay is that it is a qualitative multiparameter western blot immunoassay with limited sensitivity (34). In a study analyzing the sera and plasma from 64 COVID-19 patients, approximately 25% of patients exhibited an autoantibody response on average 12.3 days post-diagnosis, and the reactivity was primarily to nuclear antigens, including RNP (n=8), SSA, SSB, dsDNA, chromatin, or centromere (35). Chang et al. showed that autoantibodies are present in approximately 15% of healthy controls and 50% of COVID-19 patients against commonly recognized antigens in an array of autoimmune disorders, including...
SSA/Ro52(36). However, Burbelo et al., 2022 demonstrated that a considerable fraction of the autoantibody positivity in severe COVID-19 subjects may be related to receiving intravenous immunoglobulins (IVIG)(37). Thus, these results suggest that longitudinally sampled and controlled serosurveillance must be performed.

A meta-analysis revealed that the development of primary rheumatic diseases associated with COVID-19 patients were vasculitis, arthritis, idiopathic inflammatory myopathies, and systemic lupus erythematosus; overall, the association between ANAs and COVID-19 infection was 35.6%, and the reactive antigens were found at the following rates: SSA (25%), rheumatoid factor (19%), lupus anticoagulant (11%), and IFN-I (10%)(11). Autoantibody responses in COVID-19 patients can be influenced by sex, with men exhibiting an autoantibody response after an infection defined as at least mildly symptomatic, whereas women were prone to produce this response following an asymptomatic infection; thus, autoantibody profiles are highly variable between the sexes and dependent on the disease severity(38). It is unknown how SARS-CoV-2 infection could induce a plethora of autoantibodies, specifically those hallmarks of autoimmune diseases. One hypothesis is that the tropism of SARS-CoV-2 to vulnerable cells triggers a robust immune response that damages infected cells leading to the presentation of anti-viral proteins-viral particle-antibody immune complexes to antigen-presenting cells in the interstitium. A study showed that heptapeptide sharing exists between SARS-CoV-2 spike glycoprotein and human proteins, indicating the molecular mimicry mechanism(39). However, the spike protein does not share any homology with SjD-specific autoantigens. We found only 20% positive ANA for female patient sera and 30% for male sera at 1:320 titer, as recommended by the ACR criteria for SjD (40). Even with the small number of male and female patients and controls, the finding may have potential clinical implications in diagnostics and treatment. The cross-reactivity of mAbs and antinuclear antigens by HEp2 cells was revealing, especially in the CDR3 regions. Most highly cross-reactive mAbs shared one or two serine and glycine in the heavy chain and one tyrosine in the light chain, with some having long CDR3 regions in the heavy chain. Previous studies have
shown that these amino acids tend to be selected within CDR regions after affinity maturation, for example, serine and glycine are small neutral amino acids that allow structural flexibility in the antigen-binding site. And the large side chain of tyrosine would allow it to actively interact with residues at the antigen interface by hydrogen bonds as well as hydrophobic and attractive electrostatic interactions with positively charged groups(41). While long CDR3 regions have not been directly implicated in autoimmune diseases or SjD, studies have shown that the CDR3 lengths were similar between autoreactive and non-autoreactive immunoglobulin genes in RA patients(42) and polyreactive immunoglobulin M (IgM)(43). Theoretically, a more extended CDR3 region could provide more potential interaction sites, albeit with reduced affinity for the target antigen. Regardless, it is imperative to determine the underlying mechanism of autoantibody response triggered by SARS-CoV-2.

We, and others, have confirmed that salivary glands are exquisitely supportive of infection and replication of SARS-CoV-2, and saliva is an ideal secretion for inter and intra-individual spread of de novo virus(21, 44). Because of the long-hypothesized connection between viral infection and initiating autoimmune diseases, we examined available clinical data and minor salivary gland biopsies from convalescent COVID-19 subjects with mild-to-moderate infections. While no patients satisfied strict 2016 ACR/EULAR classification criteria, focal lymphocytic sialadenitis or clinical signs and symptoms of SjD were found in most available subjects(40). In select patients, the histopathological features of inflammation in the salivary glands are indistinguishable from SjD and, in the proper clinical context, would be supportive of the diagnosis. The most prevalent and persistent oral symptoms associated with COVID-19 include taste dysfunction. Furthermore, dry mouth as a result of hypofunction is often overlooked in COVID-19 patients and was identified as another highly prevalent (43%) oral manifestation of COVID-19(45). A review of 12 studies, including patients diagnosed with SARS-CoV-2 infection from different countries with reported oral symptoms associated with COVID-19 infection, showed that xerostomia occurs in the early stages of COVID-19 with a prevalence ranging from 20% to 61.9%
and can persist for at least eight months after recovery (46). The percentage is higher in patients with mild symptoms, as a study in Israel showed 61.9% of 97 confirmed non-hospitalized patients reported xerostomia (47). This is consistent with our data showing a markedly diminished salivary secretion after SARS-CoV-2 infection in mice. The precise etiology of gland dysfunction requires further investigation. As demonstrated, the influx of inflammatory cells in the glands, concomitantly with the rapid increase of acinar cell apoptosis, may contribute to diminished gland function. We did not measure tear secretion, mainly to avoid further physical stress on the mice as a result of the drug side effect and handling.

In summary, our study underpins the pathogenic role of SARS-CoV-2 in SjD. SARS-CoV-2 induced gland inflammation leading to the loss of saliva in mice. It triggered the production of SjD-associated autoantibodies in mice and human patients. Further studies are needed to examine the pathoetiiology of SARS-CoV-2 in SjD, specifically investigating the underlying mechanisms contributing to cellular damage and immunological response development. Long-term follow-up studies are necessary to assess the impact of COVID-19 and the different variants on the disease course and progression of SjD by determining if COVID-19 triggers flares or exacerbations of symptoms and investigating potential long-term complications. Future studies may focus on developing guidelines and recommendations for managing individuals with SjD and COVID-19. These guidelines may address risk assessment, preventive measures, and optimal strategies for managing both conditions concurrently.
Methods

Human samples

SARS-CoV-2 positive and healthy control (HC) sera were obtained from the CTSI Biorepository at the University of Florida in compliance with IRBs 202001475 and 2020000781. A comprehensive clinical diagnosis of the 20 controls and 20 patients was presented in Table S3. The presence of SARS-CoV-2 was confirmed by RT-PCR for admittance into the CTSI Biorepository Bank. Peripheral blood mononuclear cells (PBMC) from five post-convalescent COVID-19 donors were obtained from LifeSouth Community Blood Centers (Gainesville, FL). The healthy volunteer donors had recovered from COVID-19 and were positive for SARS-CoV2 antibodies at the time of blood donation. The donors had no prior clinically diagnosed autoimmune diseases. The samples were handled in a certified BSL2+ with Institutional Biosafety Committee-approved protocols.

NIDCR Subjects and Protocols: Subjects were consented to National Institutes of Health (NIH) Central Institutional Review Board (IRB)-approved protocols (15-D-0051: Characterization of Salivary Gland Disorders [PI-Warner]; 20-D-0094: Transmissibility and Viral Load of SARS-CoV-2 in Oral Secretions [PI-Warner]) and evaluated at either the NIH SARS-CoV-2 Field Testing Facility (20-D-0094) or the NIH Clinical Center. NIH IRB Protocol: 15-D-0051 (NCT02327884) is a cross-sectional screening protocol to evaluate subjects with a variety of disorders affecting the salivary complex and also healthy subjects (i.e., healthy volunteers [HV]). All enrolled subjects are evaluated comprehensively, including oral, sialometric, ophthalmologic, and rheumatologic evaluations; salivary gland ultrasonography, bloodwork including rheumatologic investigations, and minor salivary gland (MSG) biopsies. NIH IRB Protocol: 20-D-0094 (NCT04348240) was a short-term longitudinal study aimed at examining the potential transmissibility and viral load of SARS-CoV-2 in saliva when compared with nasal and nasopharyngeal secretions and for testing the effectiveness of masks to reduce speaking-related transmission(21). The general results of
this study are reported in Huang, et al., (2021)(21). After identifying SARS-CoV-2 in saliva, the protocol was amended to allow MSG biopsy in acute and convalescent COVID-19 subjects(21).

Research and clinical records post-initiation of the global COVID-19 pandemic were reviewed systematically by a rheumatology Physicians Assistant (MB). Subjects were included in the histopathological analysis if they had recovered from COVID-19, had convalescent MSG biopsies, and were enrolled on NIH IRB Protocols: 15-D-0051 or 20-D-0094. Subjects were excluded if they were evaluated as patients for the workup for SjD or non-SjD sicca symptoms. Comprehensive investigations as described above were completed on subjects enrolled in our 15-D-0051 protocol. Still, due to constraints of NIH SARS-CoV-2 Field Testing Facility, these parameters could only be collected on some 20-D-0094 subjects. Clinical laboratory studies at NIH include standard bloodwork, assays for antinuclear antibodies (ANA), antibodies to extractable nuclear antigens (e.g., anti-SSA/SSB autoantibodies), and antibodies to pathogens to assess vaccination and exposure history purposes (e.g., anti-spike, anti-nucleocapsid). In one subject (subject 2), serial MSG biopsies were collected; the first was taken five days after the first COVID-19 symptoms (reported previously as COV49(21)), and the second was taken six months later(21).

MSG biopsies were interpreted by a board-certified anatomic pathologist (DEK) for diagnostic purposes, and the histopathology was systematically reviewed by a board-certified oral and maxillofacial pathologist (BMW) as previously described(48). Salivary gland inflammation and fibrosis were graded according to Greenspan et al.(49) and Tarpley et al.(50). For MSG with Greenspan grade 3 or 4 sialadenitis, a focus score was calculated according to Daniels et al.(51). Hematoxylin and eosin [H&E], CD20, CD3, CD4, CD8 was conducted by the Anatomic Pathology Laboratory of the National Cancer Institute. Slides were scanned at 40X with a NanoZoomer S360 slide scanner (Hamamatsu Photonics, Hamamatsu-city, Japan), and digital photomicrographs at ×5 resolution were captured using NDP.view2 software (Hamamatsu Photonics).

Statistics
Statistical analyses were performed using Prism 8 software (GraphPad, La Jolla, CA).

Where indicated, 2-way ANOVA, Welch’s t-tests, or Mann-Whitney t-tests were performed. In all cases, p values < 0.05 were considered significant. For the ANA staining, a Chi-squared test was performed.

**Study approval**

Subjects were consented to National Institutes of Health (NIH) Central Institutional Review Board (IRB)-approved protocols (15-D-0051: Characterization of Salivary Gland Disorders [PI-Warner]; 20-D-0094: Transmissibility and Viral Load of SARS-CoV-2 in Oral Secretions [PI-Warner]). Convalescent samples were collected under the University of Florida-approved protocol (IRB202000781). Participants gave informed consent to participate in the study before taking part. The University of Florida’s Institutional Animal Care approved all protocols respective to breeding and the use of animals described herein. The experimental methods were carried out in accordance with the appropriate approvals and relevant guidelines.

**Data availability**

The data sets generated and analyzed in the current study are fully available upon contact with the corresponding author.
Author contributions

For this study, A.T performed the mouse infection. Y.S. profiled the mice for SjD, generated and characterized the monoclonal antibodies. A.V and L.G performed the antinuclear antibody assays. M.A, D.E.K, J.O.M, M.B, E.P, and B.M.W recruited patients and collected samples and data for analysis. A.N.B and K.C.H performed the PRNT assay. W.F.C, Y.S, and A.V were involved with the histological examination. Y.S. A.V, J.A.C, B.M.W, and C.Q.N wrote the first draft of the manuscript. J.A.C, D.A.O, V.R, S.K, B.M.W, and C.Q.N conceptualized the study, involved in data analysis, reviewed, and edited the manuscript.
Acknowledgments

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References


Figure 1: Decrease in saliva secretion by salivary glands by SARS-CoV-2. A) Saliva flows were collected as described in the materials and methods section. The data shown represent the saliva flow rate (ul/gram). The mice were randomly selected for saliva collection at the endpoint (control/uninfected n=8 and infected n=8). To minimize the exposure of working in a BSL-3 mouse colony, a smaller number of mice was chosen for saliva collection. B) The weight of the mice in grams (control n=11, infected n=8). Data were presented as mean ± SEM. One-tailed Mann-Whitney t-tests were performed where *** p< 0.001, ns: not significant.
A. Control Infected

- Male: n=10, n=13
- Female: n=5, n=5

B. Graphs showing Abs (anti-SSB/La) for Control and COVID-19+ groups, stratified by gender (M/F). Statistical significance indicated by asterisks: *** for Control-M vs. COVID-19+M, ** for Control-F vs. COVID-19+F, * for Control-M vs. Control-F, and NS for other comparisons.
**Figure 2: Autoantibody profile of mouse sera.** A) ANA profile was determined using HEP2 cells, where M/F indicates a combined ANA profile, as opposed to male and female mice listed separately. A Chi-squared test was performed on the all control (n=10, 5 females, 5 males) and SARS-CoV-2 infected mice (n=13, 6 males, 7 females), with a value of 32, p < 0.00001, females: $X^2=25.0639$, p < 0.00001, and males: $X^2=72$; p < 0.00001. Sera were diluted at 1:40.

B) Anti-SSB/La, anti-SSA/Ro52, and anti-SSA/Ro60 were determined using ELISA. Welch's t-test was performed to determine the significance of these results, where *p= 0.026, **p= 0.01, and ***p= 0.0003, ns: not significant. On the left, a combined profile is provided (M/F), and on the right, results are separated by sex.
Caspase-3+ cells

A. Con-SG
   Infected-SG
   Con-LG
   Infected-LG

B. Control-M Infected-M Control-F Infected-F
   ** ** ** ***

C. Control-M Infected-M Control-F Infected-F
   **** **** ****

D. Control-M Infected-M Control-F Infected-F
   * **  ****

E. Control-M Infected-M Control-F Infected-F
   * **** ****

F. Control-M Infected-M Control-F Infected-F
   **** **** ****
Figure 3: Increase in inflammation and apoptosis detected in salivary and lacrimal glands of infected mice. A) Representative H&E staining of the salivary and lacrimal glands of the control and SARS-CoV-2 infected mice with caspase-3+ cells. Yellow arrows indicate lymphocytic infiltrates in the interstitium. B) Larger areas of lymphocytic infiltration are present in the exocrine glands of infected mice. The lymphocytic focal areas of 52 lacrimal glands and 26 salivary glands were counted using Aperio ImageScope [v12.4.6.5003], with each point representing one countable focus in the salivary or lacrimal gland (control, n=5 females: infected, n=26, 13 males, 13 females). C) Elevated glandular apoptosis detected by TUNEL staining (salivary glands: control males n=5, control females n=4, infected males n=4, and infected females n=5; lacrimal glands: control males n=10, control females n=5, infected males n=7, and infected females n=8). D) Elevated glandular apoptosis detected by caspase-3 staining (salivary glands: control males n=5, control females n=4, infected males n=4, and infected females n=5; lacrimal glands: control males n=10, control females n=5, infected males n=7, and infected females n=8). E) Increase in CD68+ macrophage frequency in salivary and F) lacrimal glands of the infected mice. Representative immunofluorescent staining of CD68+ macrophages are displayed in green with blue DAPI nuclei staining at 40X magnification. The statistical significance was calculated using one-tailed Mann-Whitney tests where error bars indicate SEM *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. SG: salivary glands, LG: lacrimal glands, M: Male, F: Female.
A. B. C.
Figure 4: Autoantibody induction in COVID-19 human sera. A) ANA profile was determined using HEp2 slides at various sera titers (n=20 COVID-19+ patients with 10 males and 10 females, n=20 healthy individuals with 10 males and 10 females. B) A breakdown by specific ANA staining pattern at 1/320 serum titer is presented showing the percentages (left) and number (right) of subjects. C) Anti-SSB/La, anti-SSA/Ro52, and anti-SSA/Ro60 were determined using ELISA. Welch's t-test was performed to determine the significance of these results, where *p= 0.0415, **p= 0.0015, and NS=not significant.
Figure 5: Representative minor salivary glands H&E photomicrographs of healthy volunteer (HV), SjD, and two representative subjects recovered from COVID-19. Convalescent glands exhibit a range of inflammation severity ranging from normal to mild-to-moderate sialadenitis with focal lymphocytic sialadenitis reminiscent of inflammation found in SjD. The histopathological findings from two patients (P2 & P5) exhibit inflammation consistent with findings observed in SjD salivary glands (e.g., focal lymphocytic sialadenitis with focus scores >1.0). However, P1, P3, and P6 exhibited FLS but did not reach the threshold of >1.0 focus per 4mm² of tissue. Black arrows point to foci of inflammation, outlined arrows point to areas of fibrosis, a: ductal injury, b: mucous inspissation, c: immune infiltration of the acini with injury, and d: perivascular infiltrates.
Figures 6: Representative immunophenotyping studies examining CD3, CD4, CD8, and CD20 on a minor salivary gland biopsy during infection (D5 post first symptom; FS: 1) and post (6 Months) COVID-19 infection (P2). Immunophenotyping demonstrates diffuse mild-to-moderate chronic sialadenitis with focal lymphocytic sialadenitis.


Table 1. Clinical and histopathological features of convalescent COVID-19 subjects and comparators.

<table>
<thead>
<tr>
<th>Sub.</th>
<th>Age</th>
<th>Sex</th>
<th>Patient type</th>
<th>Histopathological Diagnosis</th>
<th>Focus Score</th>
<th>Fibrosis/Atrophy</th>
<th>Other Features</th>
<th>Oral Symptoms</th>
<th>Oral Signs</th>
<th>Ocular symptoms</th>
<th>Ocular Signs</th>
<th>COVID-19 Severity</th>
<th>Biopsy Post COVID-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23</td>
<td>F</td>
<td>Conv.</td>
<td>FLS with Mild Chronic Sialadenitis</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N</td>
<td></td>
<td>N</td>
<td>N/A</td>
<td>N/A</td>
<td>NA</td>
<td>Mild</td>
<td>8</td>
</tr>
<tr>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25</td>
<td>F</td>
<td>Conv.</td>
<td>FLS with Mild Chronic Sialadenitis</td>
<td>1</td>
<td>Y</td>
<td>Duct injury and dilatation</td>
<td>Y</td>
<td>Y</td>
<td>N/A</td>
<td>N/A</td>
<td>Mild</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FLS with Mild-to-Moderate Chronic Sialadenitis</td>
<td>2</td>
<td>Y</td>
<td>GC</td>
<td>Y</td>
<td>N</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38</td>
<td>M</td>
<td>Conv.</td>
<td>FLS with Mild Chronic Sialadenitis</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Y</td>
<td>Inflamm. infiltrating acini</td>
<td>Y</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Mild-to-Moderate</td>
<td>13</td>
</tr>
<tr>
<td>4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>42</td>
<td>F</td>
<td>HV</td>
<td>Mild Chronic Sialadenitis</td>
<td>0</td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Mild-to-Moderate</td>
<td>21</td>
</tr>
<tr>
<td>5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>19</td>
<td>M</td>
<td>HV</td>
<td>FLS with Mild-to-Moderate Chronic Sialadenitis</td>
<td>2</td>
<td>N</td>
<td>GC, granuloma</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Asymp.</td>
<td>UNK</td>
</tr>
<tr>
<td>6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>39</td>
<td>M</td>
<td>HV</td>
<td>FLS with Mild Chronic Sialadenitis</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N</td>
<td>Perivascular infiltrates</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Mild-to-Moderate</td>
<td>7</td>
</tr>
<tr>
<td>SjD&lt;sup&gt;e&lt;/sup&gt;</td>
<td>37</td>
<td>F</td>
<td>SjD</td>
<td>FLS with Mild-to-Moderate Chronic Sialadenitis</td>
<td>2</td>
<td>Y</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTL&lt;sup&gt;e&lt;/sup&gt;</td>
<td>35</td>
<td>F</td>
<td>HV</td>
<td>Normal Histology</td>
<td>0</td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The focus score (FS) is the number of inflammatory infiltrates of at least 50 cells present in 4 mm$^2$ of salivary gland area.

*The subjects have focal lymphocytic sialadenitis (FLS) but with less than 1 per 4 mm$^2$ of tissue. P2 has 8 foci per 37 mm$^2$ of tissue (FS: 0.9) and P6 has 5 foci per 25 mm$^2$ (FS: 0.8), and thus are borderline. Subject 3 has 4 foci per 47 mm$^2$ (FS: 0.3).

*The patient had a biopsy 5 days after first symptom of COVID-19. Patient 2 clinical D5 case was reported in Huang, Perez, et al., Nature Med. 2021 (ref).

*The subjects were enrolled on 20-D-0094 and biopsied as convalescent subjects (Subjects 1-3).

*The subjects were enrolled on 15-D-0051 as either affected subjects (“SjD”) or healthy volunteers (HV) (Subjects 4-6 and Ex: HV).

**Abbreviations**: F, female; M, male; Conv., convalescent; N, no; Y, yes; FLS, focal lymphocytic sialadenitis; GC, germinal center; UNK, unknown; SjD, Sjögren’s Disease; HV, healthy volunteer; CTL, control gland from healthy volunteer.
### Table 2. Immunophenotyping of convalescent COVID-19 subjects salivary glands.

<table>
<thead>
<tr>
<th>Subject</th>
<th>CD20</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4/CD8 ratio</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>+(^c)</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>CD4&gt;CD8</td>
</tr>
<tr>
<td>2(^a)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>CD4&gt;CD8</td>
</tr>
<tr>
<td>2- longitudinal(^b)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>CD4&gt;CD8</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CD4&gt;CD8</td>
</tr>
</tbody>
</table>

\(^a\)subject 2 had a biopsy 5 days after first symptom of COVID-19
\(^b\)the same subject 2 has a longitudinal biopsy 6 months after COVID-19+ diagnosis
\(^c\)the magnitude of positive staining