SARS-CoV-2 Infection of Salivary Glands Compromises Oral Antifungal Innate Immunity and Predisposes to Oral Candidiasis

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39 **Short title:** SARS-CoV-2 Infection of Salivary Glands Predisposes to Oral Candidiasis
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41 **ABSTRACT**
42 Saliva contains antimicrobial peptides considered integral components of host innate
43 immunity, and crucial for protection against colonizing microbial species. Most notable is
44 histatin-5 which is exclusively produced in salivary glands with uniquely potent antifungal
45 activity against the opportunistic pathogen *Candida albicans*. Recently, SARS-CoV-2 was
46 shown to replicate in salivary gland acinar cells eliciting local immune cell activation. In

47 this study, we performed mechanistic and clinical studies to investigate the implications
48 of SARS-CoV-2 infection on salivary histatin-5 production and *Candida* colonization. Bulk
49 RNA-sequencing of parotid salivary glands from COVID-19 autopsies demonstrated
50 statistically significant decreased expression of histatin genes. *In situ* hybridization,
51 coupled with immunofluorescence for co-localization of SARS-CoV-2 spike and histatin
52 in salivary gland cells, showed that histatin was absent or minimally present in acinar cells
53 with replicating viruses. To investigate the clinical implications of these findings, salivary
54 histatin-5 levels and oral *Candida* burden in saliva samples from three independent
55 cohorts of mild and severe COVID-19 patients and matched healthy controls were
56 evaluated. Results revealed significantly reduced histatin-5 in SARS-CoV-2 infected
57 subjects, concomitant with enhanced prevalence of *C. albicans*. Analysis of prospectively
58 recovered samples indicated that the decrease in histatin-5 is likely reversible in mild-
59 moderate disease as concentrations tended to increase during the post-acute phase.
60 Importantly, salivary cytokine profiling demonstrated correlations between activation of
61 the Th17 inflammatory pathway, changes in histatin-5 concentrations, and subsequent
62 clearance of *C. albicans* in a heavily colonized subject. The importance of salivary
63 histatin-5 in controlling the proliferation of *C. albicans* was demonstrated using an *ex vivo*
64 assay where *C. albicans* was able to proliferate in COVID-19 saliva with low histatin-5,
65 but not with high histatin-5. Taken together, the findings from this study provide direct
66 evidence implicating SARS-CoV-2 infection of salivary glands with compromised oral
67 innate immunity, and potential predisposition to oral candidiasis.
68

AUTHOR SUMMARY

Saliva contains antimicrobial peptides part of host innate immunity crucial for protection against colonizing microbial species. Most notable is the antifungal peptide histatin-5 produced in salivary glands cells. SARS-CoV-2 was shown to replicate in salivary gland cells causing tissue inflammation. In this study, we showed decreased expression of histatin genes in salivary glands from COVID-19 autopsies, and co-localization studies of SARS-CoV-2 spike and histatin revealed absence or minimal presence of histatin in acinar cells with replicating virus. To investigate the clinical implications of these findings, we conducted studies using saliva samples from subjects with mild to severe COVID-19, matched with healthy controls. Results revealed significantly reduced histatin-5 in SARS-CoV-2 infected subjects with enhanced prevalence of *C. albicans*. Prospective analysis indicated the decrease in histatin-5 is reversible in mild-moderate disease, and salivary cytokine profiling demonstrated activation of the Th17 inflammatory pathway. The importance of salivary histatin-5 in controlling the proliferation of *C. albicans* was demonstrated using an *ex vivo* assay where *C. albicans* was able to proliferate in saliva with low histatin-5, but not with high histatin-5. Collectively, the findings provide direct evidence implicating SARS-CoV-2 infection of salivary glands with compromised oral innate immunity and predisposition to oral candidiasis.

INTRODUCTION

The oral cavity remains an underappreciated site for SARS-CoV-2 infection despite the evident myriad of oral conditions observed in COVID-19 patients, and the presence of the virus in saliva (1-6). The homeostasis of the oral cavity is maintained by saliva, an

92 extracellular fluid produced by salivary glands possessing a wealth of protective
93 properties (7). Specifically, saliva is enriched with antimicrobial peptides considered to be
94 part of the host Th17-type adaptive immune response that play a vital role in innate
95 immune defenses against microbial species (7-10). Most notable are histatins, a family of
96 peptides exclusively produced in the acinar cells of the salivary glands and secreted into
97 saliva (11, 12). There are two genes for histatins, *HTN1* and *HTN3* which encode
98 histatins-1 and 3, respectively (13). Histatin-5, a proteolytic product of histatin-3, is the
99 most abundant and unique as it exhibits potent antifungal activity against the fungal
100 pathogen *Candida albicans* (*C. albicans*) (12, 14-18). Although *C. albicans* is a
101 commensal colonizer of the oral cavity, any changes in the host microenvironment
102 favoring its proliferation allows this opportunistic species to transition into a pathogen
103 causing oral candidiasis (thrush) (19, 20). As a commensal colonizer of the oral mucosa,
104 the host immune response to *Candida* is oriented toward a more tolerogenic state and,
105 therefore, local innate immune defenses and specifically histatin-5 play a central role in
106 maintaining *Candida* in its commensal state preventing development of oral candidiasis
107 (8, 21, 22).

108 Recently, the salivary glands were shown to be a potential target for SARS-CoV-2
109 infection as several studies demonstrated the concomitant expression of
110 ACE2/transmembrane serine proteases 2 (TMPRSS2) in salivary glands epithelial cells
111 (23-27). In fact, virus entry into salivary glands cells was found to be higher, as compared
112 with entry into lung cells (25). Most notable are findings from a landmark study by Huang
113 and Perez *et al.* (2021) (28) which comprehensively demonstrated that salivary gland
114 acinar and ductal cells are robust sites for SARS-CoV-2 infection and replication, eliciting

115 local immune cell activation. Additionally, architectural distortion, atrophy, fibrosis, and
116 ductal rupture were also revealed, establishing that the minor and major salivary glands
117 are susceptible sites for infection, replication and local immune cell activation. Moreover,
118 the authors demonstrated that saliva from acutely infected patients could infect and
119 replicate in Vero cells, *ex vivo*, indicating that the source of virus in saliva is likely derived
120 from infected cells in the salivary glands (28). In fact, clinical observations are in support
121 of SARS-CoV-2 mediated damage to the salivary glands as COVID-19 patients frequently
122 present with gustatory dysfunction and xerostomia, and cases of inflamed salivary glands
123 have been reported in this population (26, 29).

124 Unlike other antimicrobial peptides, histatins are exclusively expressed and secreted
125 by acinar cells of salivary glands; saliva containing histatins is secreted from the acini into
126 the lumen which transits the ductal network into the oral cavity where they can exert their
127 antimicrobial activities. Patients infected with SARS-CoV-2 have the propensity to
128 develop superimposed infection including oral candidiasis. While SARS-CoV-2 infects
129 and replicates in the acinar and ductal cells of the salivary glands, it is not known if the
130 risk of oral candidiasis is due to affects on the acinar expression of anti-candidal proteins,
131 or due to viral and immune-mediated destruction of the glands. To that end, we performed
132 mechanistic and clinical studies to investigate the implications of SARS-CoV-2 infection
133 of salivary gland tissue on oral innate immune defenses, salivary histatin-5 production,
134 and predisposition to opportunistic infections. Using *in situ* hybridization and
135 immunofluorescence, we performed co-localization studies to assess histatin production
136 in SARS-CoV-2 infected salivary gland acinar cells. To provide mechanistic insights, the
137 expression of histatin genes was comparatively evaluated in infected and uninfected

138 salivary gland tissue. The clinical implications of findings from mechanistic studies were
139 demonstrated using various cohorts of SARS-CoV-2 infected subjects to evaluate salivary
140 histatin-5 levels and oral *Candida* colonization. Collectively, the novel findings from this
141 study establish the oral cavity as a robust site for SARS-CoV-2 infection warranting
142 reassessment of the risks for oral opportunistic infections in COVID-19 patients.
143

144 **RESULTS**

145 **SARS-CoV-2 infection of the parotid glands affects the expression of histatins.** To
146 determine the effect of SARS-CoV-2 infection on the expression of anti-candidal proteins
147 in the salivary glands, RNA sequencing (RNAseq) and confirmatory hybrid *in situ*
148 hybridization and immunofluorescence microscopy, was used. First, RNAseq of SARS-
149 CoV-2 infected parotid glands showed significantly reduced expression of histatin genes
150 (*HTN1,* >100-fold lower, p=0.0022*; HTN3,* >100-fold lower, p=0.0038; Fig. 1a). Next, we
151 confirmed the loss of histatin expression and the co-localization of SARS-CoV-2 and
152 histatin protein expression in acinar cells. Hybrid *in situ* hybridization and
153 immunofluorescence microscopy co-localization studies of SARS-CoV-2 and histatins,
154 respectively, demonstrated significant (p<0.001) reduction of histatin protein expression
155 in virus infected acinar cells in parotid gland tissues obtained from deceased COVID-19
156 patients. As previously reported, the detection of SARS-CoV-2 is non-uniform across the
157 acini of the glands (28). Comparing infected vs non-infected acinar cells, histatin protein
158 expression intensity was inversely proportional to viral count (Fig.1b-f) indicating that
159 direct viral infection of the glands suppresses histatin mRNA and protein expression.
160

161 **SARS-infected patients secrete significantly less histatins potentiating oral**
162 **candidiasis.** Clinical studies were conducted using two COVID-19 patient cohorts
163 (hospitalized [UMD] and outpatient [NIDCR]) with ranging disease severity, and matched
164 healthy controls. In the hospitalized cohort, a histatin-5 specific immunoassay to measure
165 salivary histatin-5 concentrations was used and significant (p=0.0303) differences in
166 values between the COVID-19 patients and matched healthy controls were seen. Average
167 concentrations for the control group was 21.3 μg/ml (11.1 μg/ml-34.5 μg/ml), and 18.4
168 μg/ml (1.4 μg/ml-53.5 μg/ml) for the COVID-19 group. Strikingly, 14 of the COVID-19
169 patients had concentrations below 10 μg/ml (Fig. 2a), and although unclear why, 3
170 subjects had concentrations higher than any of the controls. The difference in
171 concentrations between the two groups was also seen in age- and race-matched
172 comparison between each COVID-19 patient and their matched control subject (Fig. 2b).
173 *Candida* colonization was assessed by culturing samples; where no *Candida* was
174 recovered from any of the healthy controls, 45% (9/20) of COVID-19 patients sampled
175 were positive for *C. albicans*, some heavily colonized (Fig. 2a).
176 To corroborate these findings, a separate outpatient cohort collected by the
177 National Institute of Dental and Craniofacial Research (NIDCR) (28) of serially sampled
178 acutely infected patients was analyzed for histatin-5 expression. Compared to healthy
179 control samples collected prior to the pandemic (n=9), the acute phase COVID-19 whole
180 unstimulated saliva (n=23) had significantly lower histatin-5 concentrations (3.63 vs 10.41
181 μg/ml, $p<0.0001$; Fig. 2c). *Candida* colonization was assessed as above; no *Candida* was
182 recovered from the healthy controls, yet 17% (4/23) of COVID-19 patients sampled were
183 positive for *C. albicans.* Despite high variability in the expression of histatin-5 across the

184 cohort, prospective analysis of available serially-collected samples (n=8; 3-15 days from
185 symptom onset, 6-months, and 1-year from initial infection) from these COVID-19
186 subjects indicated a slight, but statistically significant trend in the restoration histatin-5
187 concentration from the post-acute to the chronic phase (R^2=0.30, *p*=0.0046; Fig. 2d).
188 However, some patients exhibited persistence of low salivary histatin concentration for up
189 to 1 year after recovery in the chronic phase. No statistical differences (p values > 2.0) in
190 histatin-5 concentrations were seen between subjects based on disease severity or other
191 variables (gender, race, age).

192 **Significant changes in histatin-5 concentrations over the course of COVID-19**
193 **disease in prospectively sampled subjects.** A total of 5 subjects with mild-moderate
194 disease were prospectively sampled and salivary histatin-5 was measured to monitor
195 changes in concentrations. Two of the subjects were longitudinally sampled up to 39 and
196 80 days, respectively (Fig. 3). For these subjects, saliva and histatin-5 concentrations
197 prior to COVID-19 infection were available. The baseline histatin-5 concentration
198 measured prior to COVID-19 disease for *Subject #1* was 15.7 μg/ml. However, the
199 concentration in the first sample recovered during the acute phase of the disease was 5.6
200 μg/ml (64.33% drop) and 8.8 μg/ml for the second sample recovered 2 days later. The
201 concentration gradually began to increase during the post-acute phase with a spike noted
202 on Day 15 (25.3 μg/ml) returning to baseline level in the last sample analyzed (Fig. 3a).
203 For *Subject #2*, the histatin-5 concentration measured prior to COVID-19 disease was
204 32.8 μg/ml; however, the concentration in the first sample recovered during the post-acute
205 phase of the disease was 15.65 μg/ml (52.29% drop) which gradually increased over
206 subsequent days with a spike noted on Day 45 (43.6 μg/ml) before returning to pre-

207 COVID-19 levels on last day sampled (Fig. 3c). Significantly, *C. albicans* was recovered
208 from the initial 6 samples recovered from *Subject #2*, but not from the last 3 samples that
209 followed the restoration of histatin-5 to pre-COVID-19 level (Fig. 3c). Three additional
210 subjects (Subjects 3, 4, and 5) were also prospectively sampled (Fig. 4), however, for this
211 group, pre-COVID-19 saliva was not available. For these subjects, the histatin-5
212 concentrations in the first samples recovered during the acute phase of the disease were
213 2.4, 4.1 and 5.6 μg/ml, respectively which increased during the post-acute phase to 10.9,
214 13.2, and 12.5 μg/ml, respectively in the last samples tested (Fig. 4). No *Candida* was
215 recovered from any of the samples from these 3 subjects.

216 **Activation of Th17 inflammatory pathway concomitant with changes in histatin-5**
217 **concentrations.** Salivary cytokine profiling was performed on samples recovered from
218 the 5 prospectively sampled subjects. Comparative analysis of samples from *Subject #1*
219 demonstrated a notable increase in the Th17 associated inflammatory cytokines;
220 compared to baseline pre-COVID-19 sample (IL-17A, IL-17F, IL-6, IL-10, TNF-α, IFN-**γ**,
221 IL-22, GMCSF and IL-23); concentrations gradually increased with a spike on Day 15,
222 decreasing over subsequent days and returning to baseline level in the last sample
223 measured (Day 39) (Fig. 3b). Although IL-1β followed the same trend, the spike in
224 concentration was seen in the first sample recovered during the acute phase of the
225 disease. Cytokine levels in *Subject #2* similarly demonstrated an increase in the Th17
226 associated inflammatory cytokines; compared to baseline pre-COVID-19 sample,
227 concentrations of all cytokines (IL-17A, IL-17F, IL-6, IL-10, TNF-α, IFN-**γ**, IL-22, GMCSF,
228 IL-23 and IL-1β) spiked on Day 38, gradually decreasing and returning to baseline level
229 on Day 45. IL-17E was not detectable in all samples analyzed for *Subject #1* and *Subject*

230 *#2* (Fig. 3b, 3d). For the remaining 3 subjects, increase in Th17-associated inflammatory
231 cytokines was similarly observed during the post-acute phase for *Subject #3* (Fig. 4). Only
232 the first and last samples recovered from these 3 subjects were subjected for cytokine
233 analysis.

234 ***C. albicans* proliferates in saliva from COVID-19 positive subjects with low histatin-**
235 **5.** Saliva recovered from subjects (n=3) during acute COVID-19 infection (low histatin-5)
236 and post-acute recovery (high histatin-5) phases were separately pooled and histatin-5
237 concentrations determined. Samples were subsequently tested *in vitro* for ability to control
238 *C. albicans* proliferation (Fig. 5a). Histatin-5 concentration in the pooled saliva sample
239 obtained during acute COVID-19 was 0.5 µg/ml (low), and in the pooled sample recovered
240 during post-recovery concentration was 11.5 µg/ml (high) (Fig. 5b). Based on *C. albicans*
241 growth (CFUs) following *in vitro* inoculation of saliva with $1x10^4$ cells/ml *C. albicans*, an
242 average of $2.55x10^4$ cells/ml *C. albicans* was recovered from the low histatin-5 sample
243 and $1.29x10^4$ cells/ml from the high histatin-5 sample (Fig. 5c).

244

245 **DISCUSSION**

246 To date, little is known about the physiological mechanisms of oral manifestations in
247 COVID-19 disease and the impact of SARS-CoV-2 infection on salivary gland function.
248 Clinical observations are in support of SARS-CoV-2 mediated damage to the salivary
249 glands, as COVID-19 patients frequently present with gustatory dysfunction
250 and xerostomia, and clinical cases of inflamed salivary glands have been reported in this
251 patient population (26).

252 Oral candidiasis is the most common opportunistic infection, particularly in
253 immunocompromised individuals, although immunocompromization due to COVID-19 is
254 ardently debated (1). However, the onset of oral candidiasis early in HIV disease indicates
255 that immunity to *Candida* is site-specific and involves secondary local innate defenses
256 (21, 22). In fact, innate immunity not only represents the first line of defense providing the
257 initial host response to pathogens, but also activates the adaptive immunity (8). These
258 systems exhibit coordinated regulation and response to establish and maintain tissue
259 homeostasis (8). Host-produced salivary antimicrobial peptides are considered to play a
260 vital role in innate immune defenses against microbial species, specifically histatins which
261 are localized in the serous acinar cells and secreted into saliva (7, 12, 14).

262 The recent demonstration of SARS-CoV-2 replication in salivary glands and
263 destruction of gland integrity including at sites where histatins are produced. These
264 findings indicate that histatin production may be compromised in infected individuals. This
265 hypothesis was validated using transcriptional analysis where the expression of both
266 histatin genes (*HTN1* and *HTN3)* were shown to be downregulated in SARS-CoV-2
267 infected salivary gland tissue (Fig. 1a). Importantly, SARS-CoV-2 and histatin co-
268 localization studies indicated an inversely proportional association where little or no
269 histatin signal was detected in virus infected acinar cells compared to uninfected cells
270 (Fig. 1b-f). The clinical implications of these findings were subsequently revealed using
271 various cohorts of SARS-CoV-2 infected subjects. Overall, analysis of saliva samples
272 demonstrated significant reduction in histatin-5 levels in infected subjects compared to
273 healthy controls (Fig. 2). However, despite an association between infection and reduced
274 histatin expression, the exact temporal relationship could not be perfectly gleaned. To

275 better understand the temporal relationship between infection and histatin expression, we
276 included a panel of prospective samples from moderately symptomatic COVID-19
277 subjects, which allowed us to glean some insights (Fig. 3, 4). Specifically, analysis
278 indicated that the decrease in histatin-5 during COVID-19 is likely reversible as
279 concentrations tended to increase during the post-acute phase of the disease. However,
280 it is important to iterate that co-localization studies exhibited an inversely proportional
281 association between histatin and viral counts in infected acinar cells (Fig. 1c-f), and some
282 patients experienced long-term deficits in salivary histatin-5 levels (42 days to 1 year).
283 Therefore, in severe COVID-19, a robust salivary infection and the ensuing adaptive
284 immune response may irreparably damage the salivary gland parenchyma. Thus, due to
285 pathologically lower histatin-5 secretion (and likely reduced saliva secretion overall),
286 affected individuals may remain predisposed to both recurrent and recalcitrant to
287 antifungal therapy oral candidiasis as part of the Long COVID syndrome (30)
288 (https://www.covid.gov/be-informed/longcovid/about#term). It is important to note here
289 that there are no known set "*normal*" values for salivary histatin-5 concentrations and
290 what is considered physiological is dependent on many factors including: host factors
291 (e.g, age, sex), time of collection, the collection method, and post-processing. Moreover,
292 histatin-5 is expression is secreted primarily by serous acinar cells; these cells
293 predominate in the parotid glands but are half the acinar cells in the submandibular
294 glands, and almost entirely absent from the sublingual glands. The relative contributions
295 of each gland to the total saliva collected is also host specific. Therefore, it is expected to
296 see a wide range of histatin-5 concentrations among the healthy control subjects.
297 However, based on our experience using our assay, prior clinical studies, and evaluations

298 of anti-candidal activity, we have set arbitrary concentrations as a general guidelines for
299 what is physiological (31).

300 With limited available clinical information on the hospitalized cohort population
301 (including presence of oral lesions), we recognize that we cannot fully account for
302 confounding factors that may influence histatin-5. However, our study focuses on the
303 presence of SARS-CoV-2 virus rather than disease severity; importantly, on average we
304 observed lower histatin-5 levels for infected subjects in all cohorts studied (hospitalized,
305 outpatient and individual case). In our analysis, we did not find strong association
306 between histatin-5 concentrations and *Candida* colonization regardless of disease status
307 or demographics. One intriguing observation in the samples from the hospitalized cohort
308 is the exceptionally high histatin-5 concentrations seen for 3 of the subjects with values
309 higher than that of any of the healthy controls (Fig. 2a). Although unclear as to why, it is
310 important to note that in the two prospectively sampled subjects with known pre-COVID-
311 19 histatin-5 values, a spike in histatin-5 and inflammatory cytokines was noted over the
312 course of the disease progression prior to return to baseline levels (Fig. 3). Therefore, we
313 speculate that these three hospitalized subjects may have been sampled during these
314 transient spikes. Interestingly, all 3 subjects with high histatin-5 levels were also positive
315 for *C. albicans* (Fig. 2a), and it is tempting to also speculate that these high values may
316 be part of the host immune response to *Candida* presence.

317 Importantly, our findings demonstrated that changes in histatin-5 concentrations were
318 concomitant with activation of the Th17 inflammatory pathway. Antimicrobial peptides,
319 including histatins, are part of the host Th17-type adaptive immune response (8).
320 Interleukin-17 (IL-17) is a pro-inflammatory cytokine that regulates multiple immune

321 events (32). In the tissues of the oral cavity, including the salivary glands, the IL-17/Th17
322 signaling pathway is essential for host protection against *C. albicans* infection (32, 33).
323 Therefore, it was not surprising that cytokine profiling of saliva samples from prospectively
324 sampled subjects displayed a trend for the Th17 associated cytokines similar to that for
325 histatin-5 (Fig. 3). Interestingly, in the subject heavily colonized with *C. albicans*, the spike
326 in cytokine concentrations coincided with increase in salivary histatin-5, and more
327 significantly, with the clearance of *C. albicans* (Fig. 3c). The activation of the Th17 immune
328 response in COVID-19 disease is expected as immunophenotyping and microscopic
329 analysis of SARS-CoV-2 infected salivary gland tissues demonstrated chronic, focal
330 lymphocytic sialadenitis with a T-lymphocytic infiltrates (28, 29). As IL-17 antifungal
331 activity involves regulation of the expression of antimicrobial peptides, including histatins
332 (8, 32, 33), the gradual increase in histatin-5 concomitant with clearance of *C. albicans*
333 may also be mediated by activation of Th17 response. Therefore, based on these
334 collective findings, we presume that decreased histatin-5 salivary levels due to SARS-
335 CoV-2 infection of salivary gland serous acini permits proliferation of colonizing *C.*
336 *albicans* leading to the development of oral candidiasis.

337 The importance of salivary histatin-5 in controlling the proliferation of *C. albicans* was
338 shown using an *ex vivo* anti-candidal assay where saliva with low histatin-5 was able to
339 allow *C. albicans* proliferation *in vitro* (Fig. 5). These findings provide experimental
340 evidence establishing the importance of salivary histatin-5 in preventing *Candida*
341 proliferation by maintaining colonizing *Candida* in the commensal state. Further, in
342 addition to anti-candidal activity, given the established anti-inflammatory and wound
343 healing properties of histatins (34-36), it is possible that compromised levels of histatins

344 (and other antimicrobial proteins) may contribute to other immune- and dysbiosis-
345 mediated mucosal conditions observed in COVID-19 patients such as burning mouth,
346 blisters and non-specific ulcerations, and aphthous-like lesions, all of which have been
347 associated with COVID-19 (5). With the current lack of knowledge on the implications of
348 SARS-CoV-2 infection on oral health, the findings from this study provide mechanistic
349 insights underscoring the oral cavities' diverse susceptibilities to SARS-CoV-2 infection,
350 prompting a reassessment of oral opportunistic infection risks and their potential long-
351 term impacts on oral health.
352

353 **METHODS**

354 **Co-localization of SARS-CoV-2 and histatin in salivary gland tissue using *in situ***
355 **hybridization and immunofluorescence, respectively.** To investigate the expression
356 of histatin during SARS-CoV-2 infection, we employed the RNAscope Multiplex
357 Fluorescent v2 assay with antigen co-detection, adhering to the manufacturer guidelines.
358 Studies were performed on parotid FFPE (formalin-fixed, paraffin-embedded) biopsies
359 from uninfected donors (n=4) and from COVID-19 subjects (n=8) at autopsy with high and
360 low copy virus number. Uninfected donors were procured from the Human Cooperative
361 Tissue Network, and COVID-19 autopsy tissues were procured by the National Institutes
362 of Health COVID-19 Autopsy Consortium, as described previously (28, 37). Initially, slides
363 were dried in an air oven at 60°C for 30 min and subsequently rehydrated, and target
364 retrieval was performed using 1X Co-Detection Target Retrieval for 20 min at 99°C. Slides
365 were then rinsed briefly in water and washed in PBS-T (1X PBS, 0.1% Tween-20, pH 7.2),
366 and a hydrophobic barrier was established using an ImmEdge pen. Primary antibodies

367 against histatin-3 (Biorbyt; diluted 1:300 in Co-detection Antibody Diluent from ACD) and
368 AQP5 (SantaCruz; diluted 1:300 in Co-detection Antibody Diluent from ACD) were applied
369 and slides were incubated overnight at 4°C. Following washing in PBS-T, tissues and
370 antibodies were fixed in 10% Neutral Buffered Formalin (NBF) for 30 min at room
371 temperature, followed by another wash in PBS-T. Tissue sections were then treated with
372 RNAscope Protease Plus for 30 min at 40°C and immediately followed by probe
373 hybridization using either a positive control probe (320861), negative control probe
374 (320871), or the V-nCoV2019-S probe (848561). The V-nCoV2019-S probe is antisense
375 and specifically targets the spike protein sequence of viral RNA, providing insights into
376 infection and viral load within the tissue. Following hybridization, signals were amplified
377 and visualized using fluorescence Tyramine signal amplification. The primary antibodies
378 were detected using secondary antibodies conjugated to AF488 (histatin-3), and AF568
379 (AQP5; aquaporin-5) and counterstaining was performed with DAPI. Images were
380 captured using a Zeiss Axio Scan Z1 slide scanner light microscope equipped with a
381 40x/0.95 N.A. objective, and image files were imported into Visiopharm software version
382 2017.2. We first segmented the cells using the AI app from Visiopharm, once the cells
383 were segmented, using the APP Author function we designed an algorithm that detects
384 the dots of ISH and counts the number of dots per cell, and quantify the mean intensity
385 of histatin-3 per cell. The data was exported and analyzed in Prism 10. Control
386 experiments for *in situ* hybridization and immunofluorescence studies were performed
387 prior to sample analysis (Supp. Fig. 1).
388

389 **Bulk RNAseq analysis of salivary gland tissue.** Total RNA was extracted from
390 RNAlater (Invitrogen)-preserved parotid gland at autopsy from uninfected donors (n=3)
391 and COVID-19 subjects (n=8), using the RNeasy Mini (Qiagen) according to
392 manufacturer protocols. Following standard bulk RNAseq using Illumina platform,
393 normalized counts per million (CPM) were plotted for *HTN1* and *HTN3* and expression
394 levels of both genes were compared.
395
396 **Study subjects.** *Hospitalized cohort.* Adult COVID-19 patients (n=26) hospitalized at the
397 University of Maryland Medical Center and a matched control (age, race and gender)
398 group of healthy volunteers (n=26) were enrolled in the study. The University of Maryland
399 Baltimore Institutional Review Board approved this study, no patient identifiers were used
400 and informed consent was obtained from all subjects. Inclusion criteria for control subjects
401 included: healthy adults over 18 years of age with no history of oral candidiasis, any
402 predisposing factors or antifungal therapy. As the premise of the study is to investigate
403 the presence of the virus in the oral cavity regardless or disease severity, the only
404 inclusion criteria for the COVID-19 cohort was COVID-19 positivity. Of the 26 subjects, 11
405 were COVID-19 positive but asymptomatic and were hospitalized for other medical
406 reasons; the remaining 15 were symptomatic with severe disease, admitted to the
407 intensive care unit and/or receiving oxygen supplementation. The hospitalized COVID-19
408 population included 14 males and 12 females; 15 Caucasians and 11 African American
409 with age range of 29-76. *Outpatient cohort.* This cohort included 33 subjects with mild-
410 moderate disease; a total of 68 samples were prospectively collected from these subjects
411 at NIDCR (previously described) (28). *Prospective cases.* In addition to the cohorts, 5

412 otherwise healthy outpatient subjects (4 females, one male; age range 30-58) were
413 prospectively sampled at different timepoints during the course of their COVID-19
414 disease. A total of 29 samples were collected and analyzed for these subjects.
415
416 **Clinical Samples.** Saliva samples were collected from participating subjects using the
417 Salivette collection system as we previously performed (31, 38). Saliva was recovered,
418 aliquoted and immediately stored at -80°C with protease inhibitors. In addition, oral swabs
419 of the oral mucosal tissue were recovered for culturing to evaluate *Candida* recovery. Due
420 to inaccessibility to some patients the result of medical status, samples for fungal culturing
421 were only obtained from 20 of the 26 hospitalized patients. For the outpatient cohort and
422 their control subjects, whole unstimulated saliva samples were collected and processed
423 as above.
424
425 **Evaluation of salivary *Candida* colonization.** Oral swabs from all sampled subjects
426 were immediately cultured on fungal Yeast Peptone Dextrose (YPD) agar media (Difco
427 Laboratories) and incubated at 35°C for 24-48 h for fungal growth evaluation. The
428 chromogenic medium CHROMagar Candida was used for *Candida* speciation.
429
430 **Measurement of histatin-5 (Hst-5) salivary levels using ELISA.** ELISA was performed
431 as we previously described (31). A high purity Hst-5 peptide was synthesized by
432 GenScript and Hst-5 specific rabbit polyclonal antibody was produced by Lampire
433 Biological Laboratories. For measurement of Hst-5 levels, a standard curve was
434 performed with each assay using Hst-5 peptide concentrations ranging from 0.5-500

435 μg/ml. Wells of high-binding 96-well plates were coated with 100 μl of each Hst-5
436 concentration or 1/100 dilution of saliva. Following overnight incubation at 4°C, wells were
437 blocked with 0.1% dry milk in PBS for 1 h and anti-Hst-5 antibody (1/1000) (100 μl) was
438 added for 1 h at 37°C. Following washing, HRP-labeled goat anti-rabbit secondary
439 antibody (1/3000) (Abcam) was added, and plates incubated for 1 h at 37°C. Following
440 washing, 100 μl of ABTS Peroxidase Substrate (KPL, Inc.) was added and plates
441 incubated for 20 min until color develops. The reaction was stopped by the addition of 50
442 μl of Stop Solution (KPL, Inc.) and optical density (OD) was measured at 405nm using a
443 microtiter plate reader. A standard curve was plotted with each run; samples were tested
444 in triplicate on two separate occasions and the average Hst-5 concentration calculated in
445 μg/ml. As there are no set normal Hst-5 salivary concentrations, based on our experience
446 with clinical studies on Hst-5 salivary levels using our immunoassay, arbitrary
447 concentrations of approximately 9-10 μg/ml was considered the cutoff whereby lower
448 concentrations are considered in the low range (31).
449
450 **Salivary cytokines profiling.** Saliva samples from outpatient subjects were analyzed at
451 the University of Maryland Cytokine Core using the Luminex Multianalyte System. Each
452 sample was measured in triplicate and results expressed in Pg/ml. Not enough saliva was
453 available from other cohorts for cytokine analysis.
454
455 ***Ex vivo* salivary *C. albicans* inhibition assay.** In order to assess the anti-candidal
456 potency of saliva with respect to Hst-5 concentration, saliva samples were recovered from
457 3 subjects during COVID-19 and after recovery, then pooled to generate a COVID-19

458 positive and a COVID-19 negative sample. Hst-5 concentration in the pooled samples
459 was pre-determined by ELISA and samples were filter-sterilized and comparatively tested
460 for efficacy in inhibiting *C. albicans* proliferation *in vitro*. For these assays, cultures of the
461 standard *C. albicans* SC5314 strain were grown in YPD broth (Difco Laboratories)
462 overnight at 30°C with shaking and cells were washed and resuspended in sterile PBS
463 (1mM). *C. albicans* cells were added to each of the saliva samples (100 µl) at final cell
464 density of 1×10^4 cells/ml in the wells of a 96-well microtiter plate and plates were
465 incubated for 1 h at 37°C with shaking. Aliquots from reactions were diluted with PBS and
466 plated on YPD agar and incubated for 24–48 h at 35°C. The number of single colonies
467 on each plate was counted and the level of *C. albicans* proliferation was determined
468 based on CFU counts (cells/ml).
469
470 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism 10.2.1. A
471 Wilcoxon rank-sum test was used to compare histatin-5 levels between COVID-19
472 patients and healthy control subjects and a t-test was used to evaluate associations
473 between histatin-5 values and patient characteristics (disease severity, *Candida*
474 colonization, demographics). For *in vitro* assays, histatin-5 levels in pooled saliva of
475 COVID-19 positive and negative subjects and *Candida* CFU counts were determined
476 using the unpaired two-sample t-tests. Significance of *HTN1* and *HTN3* expression levels
477 in parotid glands was determined using ANOVA analysis with Bonferroni correction for
478 multiple measurement. Figures were constructed using GraphPad Prism 10.2.1 and R
479 statistical programming software.
480

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504 Consortium; M.A.J-R., A.A.A., T.W.W., P.P., B.M.W., A.S., T.F.M., analyzed data; M.A.J-
505 R., A.A.A., T.W.W., B.M.W. wrote the paper; B.M.W. led and oversaw the execution of NIH
506 clinical and molecular studies supporting this work; M.A.J-R. oversaw the entire study.
507
508 **ETHICAL APPROVAL.** *NIH.* Autopsies are exempt from NIH single institutional review
509 board (IRB), consent from families of fatal COVID-19 cases were obtained. Otherwise,
510 NIH single IRB conducts ethical reviews for human research studies as required by
511 Department of Health and Human Services regulations for the Protection of Human
512 Subjects. All patients seen at the author's (B.M.W.) institute (NIH/NIDCR) reported herein
513 provided informed consent before participation in IRB-approved research protocols (NIH
514 IRB: 20-D-0094, NCT04348240; NIH IRB: 15-D-0051, NCT02327884). Individuals on 20-
515 D-0094 had the option to receive a $50 payment per visit ($300 total) to offset the cost of
516 travel.
517
518 **REFERENCES**
519 1. Amorim Dos Santos J, Normando AGC, Carvalho da Silva RL, De Paula RM,
520 Cembranel AC, Santos-Silva AR, et al. Oral mucosal lesions in a COVID-19 patient: New
521 signs or secondary manifestations? Int J Infect Dis. 2020;97:326-8.
522 2. Salehi M, Ahmadikia K, Badali H, Khodavaisy S. Opportunistic fungal Infections in
523 the epidemic area of COVID-19: A cinical and diagnostic perspective from Iran.
524 Mycopathologia. 2020 185(4):607-11.

525 3. Salehi M, Ahmadikia K, Mahmoudi S, Kalantari S, Jamalimoghadamsiahkali S,
526 Izadi A, et al. Oropharyngeal candidiasis in hospitalised COVID-19 patients from Iran:
527 Species identification and antifungal susceptibility pattern. Mycoses. 2020; 63(8):771-8.
528 4. Riad A, Gad A, Ockova B, Klugar M. Oral candidiasis in non-severe COVID-19
529 patients: Call for antibiotic stewardship. Oral Surg. 2020;15(3): 465-466.
530 5. Amorim Dos Santos J, Normando AGC, Carvalho da Silva RL, Acevedo AC, De
531 Luca Canto G, Sugaya N, et al. Oral manifestations in patients with COVID-19: A living
532 systematic review. Dent Res. 2021;100(2):141-54.
533 6. Brandini DA, Takamiya AS, Thakkar P, Schaller S, Rahat R, Naqvi AR. Covid-19
534 and oral diseases: Crosstalk, synergy or association? Rev Med Virol. 2021; 31(6): e2226.
535 7. Vila T, Rizk AM, Sultan AS, Jabra-Rizk MA. The power of saliva: Antimicrobial and
536 beyond. Plos Pathog. 2019;15(11).
537 8. Salvatori O, Puri S, Tati S, Edgerton M. Innate immunity and saliva in *Candida*
538 *albicans*-mediated oral diseases. J Dent Res. 2016;95(4):365-71.
539 9. Sultan AS, Kong EF, Rizk AM, Jabra-Rizk MA. The oral microbiome: A lesson in
540 co-existence. PLoS Pathog. 2018;14(1):e1006719.
541 10. Peters BM, Shirtliff ME, Jabra-Rizk MA. Antimicrobial peptides: Primeval
542 molecules or future drugs? PLoS Pathog. 2010;6(10):e1001067.
543 11. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, et al.
544 Histatins, a novel family of histidine-rich proteins in human parotid secretion. J Biol Chem.
545 1988;263(16):7472-7.

546 12. Ahmad M, Piludu M, Oppenheim FG, Helmerhorst EJ, Hand AR.
547 Immunocytochemical localization of histatins in human salivary glands. J Histochem
548 Cytochem. 2004;52(3):361-70.
549 13. Sabatini L, Azen E. Two coding change mutations in the HIS2(2) allele characterize
550 the salivary histatin 3-2 protein variant. Human Mutation. 1994;4(1):12-9.
551 14. Edgerton M, Koshlukova SE, Araujo MWB, Patel RC, Dong J, Bruenn J. Salivary
552 histatin 5 and human neutrophil defensin 1 kill *Candida albicans* via shared pathways.
553 Animicrob Agents Chemother. 2000;44(12):3310-6.
554 15. Edgerton M, Koshlukova S, Lo T, Chrzan B, Straubinger R, Raj P. Candidacidal
555 activity of salivary histatins. J Biol Chem. 1998;272(32):20438-47.
556 16. Helmerhorst EJ, Troxler RF, Oppenheim FG. The human salivary peptide histatin
557 5 exerts its antifungal activity through the formation of reactive oxygen species. Proc Nat
558 Acad Sci. 2001;98(25):14637-42.
559 17. Nikawa H, Jin C, Fukushima H, Makihira S, Hamada T. Antifungal activity of
560 histatin-5 against non-albicans *Candida* species. Oral Microbiol Immunol. 2001;16:250-
561 2.
562 18. Situ H, Bobek L. In vitro assessment of antifungal therapeutic potential of salivary
563 histatin-5, two variants of histatin-5, and salivary mucin (MUC7) domain 1. Animicrob
564 Agents Chemother 2000;44(6):1485-93.
565 19. Jabra-Rizk MA, Kong E, Tsui C, Nguyen M, Clancy C, Fidel P, et al. *Candida*
566 *albicans* pathogenesis: Fitting within the host-microbe damage response framework.
567 Infect Immun. 2016;84(10):2724-39.

568 20. Vila T, Sultan AS, Montelongo-Jauregui D, Jabra-Rizk MA. Oral candidiasis: A
569 disease of opportunity. J Fungi (Basel). 2020;6(1):pii: E15.
570 21. Fidel PJ. Immunity to *Candida*. Oral Dis. 2002;8(Suppl 2):69-75.
571 22. Fidel PL, Jr. *Candida*-host interactions in HIV disease: Relationships in
572 oropharyngeal candidiasis. Adv Dent Res. 2006;19:80-4.
573 23. Song J, Li Y, Huang X, Chen Z, Li Y, Liu C, et al. Systematic analysis of ACE2
574 and TMPRSS2 expression in salivary glands reveals underlying transmission mechanism
575 caused by SARS-CoV-2. J Med Virol. 2020 92(11):2556-66.
576 24. da Silva Pedrosa M, Sipert CR, Nogueira FN. Are the salivary glands the key
577 players in spreading COVID-19 asymptomatic infection in dental practice? J Med Virol.
578 2021;93(1):204-5.
579 25. Pascolo L, Zupin L, Melato M, Tricarico PM, Crovella S. TMPRSS2 and ACE2
580 coexpression in SARS-CoV-2 salivary glands infection. J Dent Res. 2020;99(10):1120-1.
581 26. Corchuelo J, Ulloa FC. Oral manifestations in a patient with a history of
582 asymptomatic COVID-19: Case report. Int J Infect Dis. 2020;100:154-7.
583 27. Tsuchiya H. Oral symptoms associated with COVID-19 and their pathogenic
584 mechanisms: A literature review. Dent J (Basel). 2021;9(3):32.
585 28. Huang N, Pérez P, Kato T, al. e. SARS-CoV-2 infection of the oral cavity and saliva.
586 Nat Med 2021;27(5):892-903.
587 29. Shen Y, Voigt A, Goranova L, Abed M, Kleiner DE, Maldonado JO, et al. Evidence
588 of a Sjögren's disease-like phenotype following COVID-19 in mice and humans. JCI
589 Insight. 2023;8(24):e166540.

590 30. Pisano M, Romano A, Di Palo MP, Baroni A, Serpico R, Contaldo M. Oral
591 candidiasis in adult and pediatric patients with COVID-19. Biomedicines.
592 2023;11(13):846.
593 31. Khan SA, Fidel Jr P, Al Thunayyan A, Meiller T, Jabra-Rizk MA. Impaired histatin-
594 5 level and salivary antimicrobial activity against *C. albicans* in HIV-infected individuals. J
595 AIDS Clin Res. 2013;4(2):1-6.
596 32. Gaffen SL, Moutsopoulos NM. Regulation of host-microbe interactions at oral
597 mucosal barriers by type 17 immunity. Sci Immunol. 2020;5(43):eaau4594.
598 33. Pavlova A, Sharafutdinov I. Recognition of *Candida albicans* and role of innate
599 type 17 immunity in oral candidiasis. Microorganisms. 2020;8(9):1340.
600 34. Kong E, Tsui C, Boyce H, Ibrahim A, Hoag S, Karlsson A, et al. Development and
601 *in vivo* evaluation of a novel histatin-5 bioadhesive hydrogel formulation against oral
602 candidiasis. Antimicrob Agents Chemother. 2015;60(2):881-9.
603 35. Sultan AS, Rizk AM, Vila T, Ji Y, Masri R, Jabra-Rizk MA. Digital design of a
604 universal rat intraoral device for therapeutic evaluation of topical formulation against
605 *Candida*-associated denture stomatitis Infect Immun. 2019 87(12):e00617-19.
606 36. Sultan AS, Vila T, Hefni E, Karlsson AJ, Jabra-Rizk MA. Evaluation of the antifungal
607 and wound healing properties of a novel peptide-based bioadhesive hydrogel formulation.
608 Antimicrob Agents Chemother. 2019;63(10):e00888-19.
609 37. Stein SR, Ramelli SC, Grazioli A, Chung JY, Singh M, Yinda CK, et al. SARS-CoV-
610 2 infection and persistence in the human body and brain at autopsy. Nature Biotech.
611 2022;612(7941):758-63.

612 38. Alfaifi A, Sultan A, Montelongo-Jauregui D, Meiller TF, Jabra-Rizk MA. Long-term
613 post-COVID-19 associated oral inflammatory sequelae. Front Cell Infect Microbiol.
614 2022;12:831744.

615

616 **FIGURE LEGEND**

617

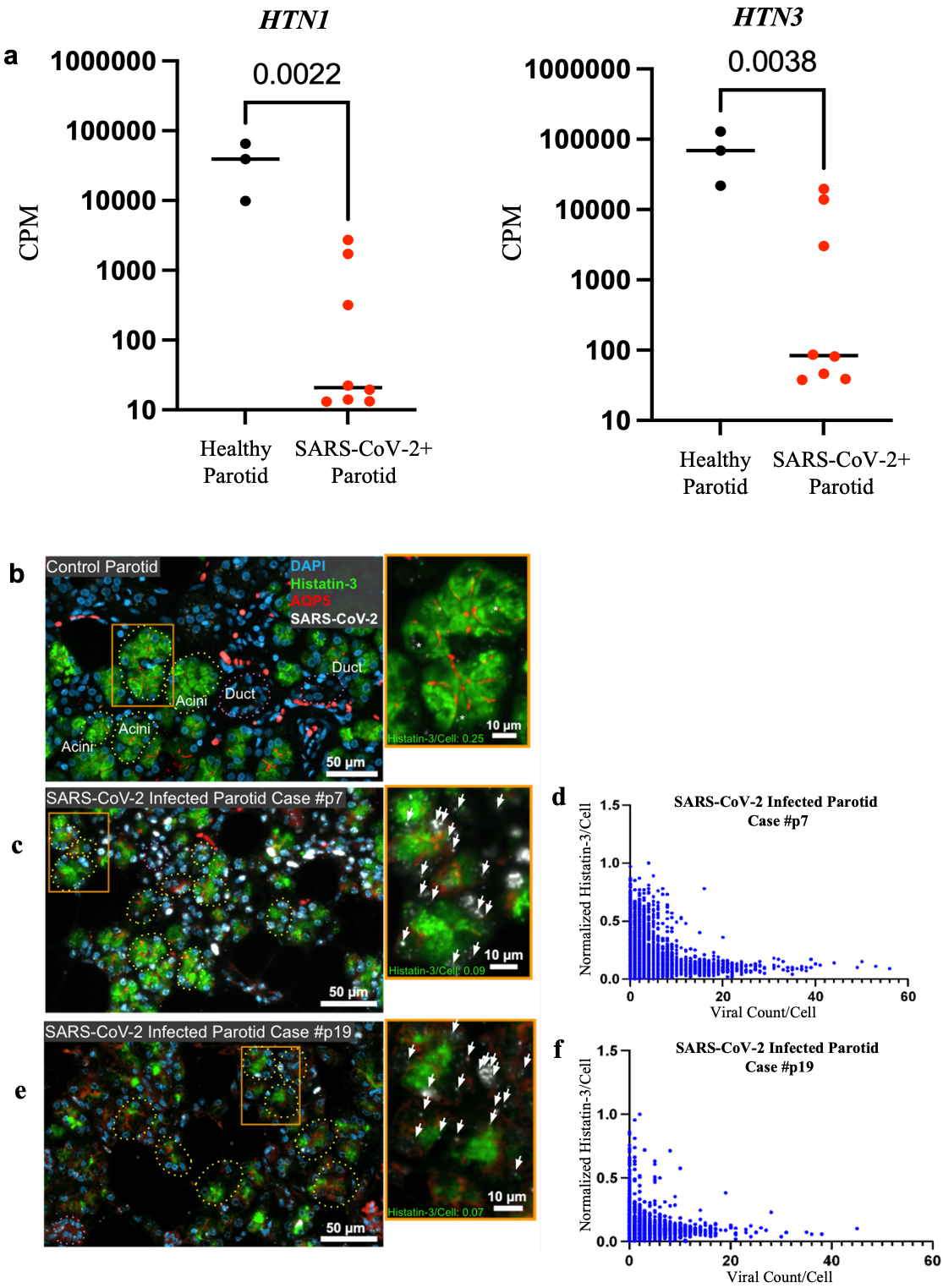
618 **Figure 1. Impact of SARS-CoV-2 salivary gland infection on histatin production,**
619 **gene expression and salivary levels. (a)** Parotid expression of histatin genes (*HTN1*
620 and *HTN3*) from deceased COVID-19 subjects (n=8) and healthy control subjects (n=3)
621 using bulk RNA sequencing. **(b-f)** Co-localization studies using *in situ* hybridization for
622 SARS-CoV-2 (*white dots* and *arrows*) and immunofluorescence for histatin-3 (*green*) with
623 quantitative correlation between the intensity of histatin and SARS-CoV-2 counts within
624 infected acinar cells of parotid tissue from **(b)** healthy subject (*white asterisk,* non-specific
625 signal) and **(c, e)** deceased COVID-19 patients. Acini (dotted circles) identified based on
626 expression of AQP5 (*red,* apical membrane); *inset* (*orange box*) shows the presence of
627 the viral genome in acinar cells of the parotid glands. **(d, f)** Per acinar cell expression of
628 histatin and the per cell viral count are inversely proportional as shown in two
629 representative COVID-19 cases (P7, P19).

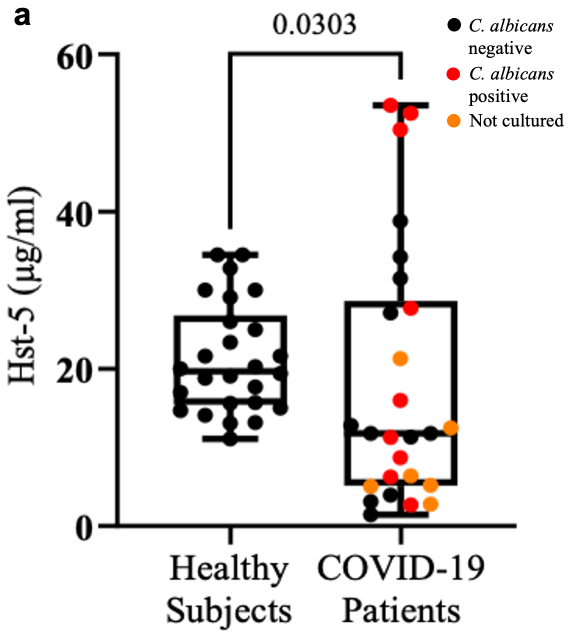
630

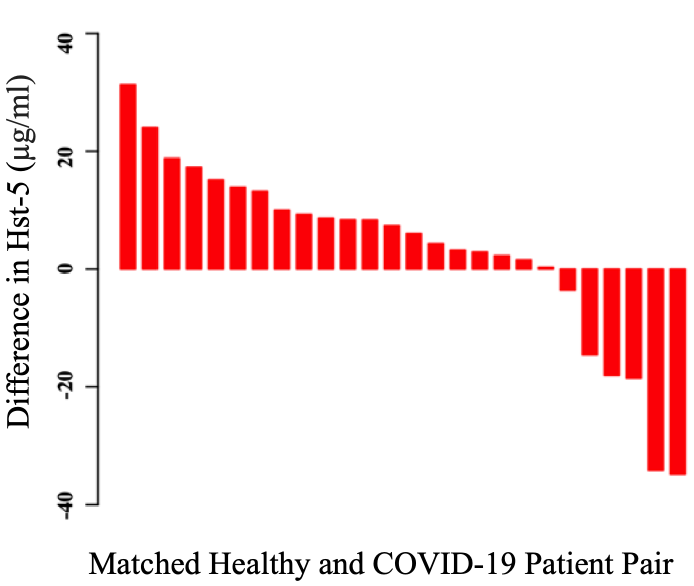
631 **Figure 2. Salivary histatin-5 (Hst-5) levels and fungal colonization in prospectively**
632 **sampled hospitalized and outpatient COVID-19 cohorts. (a)** Boxplot with Hst-5
633 concentrations and *Candida* recovery from 26 saliva samples from hospitalized COVID-
634 19 patients and matched healthy controls. **(b)** Waterfall plot depicting 26 pairs of COVID-

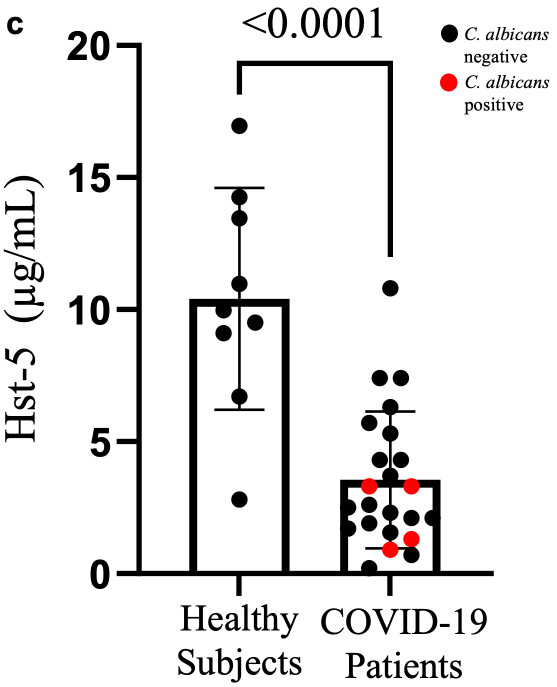
635 19 and healthy subjects matched for race and age. Bar height represents differences
636 among pairs in Hst-5 levels (μg/ml) between healthy controls and COVID-19 patients. **(c)**
637 Bar plot depicts Hst-5 concentrations and *Candida* recovery of 9 healthy subjects and 23
638 COVID-19 outpatients' saliva. Red marked dots indicate cultured saliva contained
639 candidal outgrowth **(d)** Linear regression analysis of serially sampled COVID-19
640 outpatients' saliva (n=8) shows time-dependent restoration of Hst-5 concentration from
641 the post-acute phase to the chronic phase, based on longitudinally collected samples
642 from acute phase (3-15 days) to chronic phase (6-12 months). Blue line, linear fit; light
643 blue shading, confidence interval of the linear fit.
644
645 **Figure 3. Prospective time course longitudinal analysis of saliva samples for Hst-5**
646 **from two subjects with moderate COVID-19.** Changes in Hst-5 and cytokine
647 concentrations in two longitudinally sampled subjects prior to and during the acute and
648 post-acute phases of COVID-19 disease. Timeline of sampling during COVID-19 infection
649 up to 49 and 85 days post-COVID-19 infection for subjects 1 and 2 **(a and c,**
650 **respectively)**. Measurement of Hst-5 concentrations and fungal culturing of samples
651 from the two subjects prior to and during the acute and post-acute phases of COVID-19
652 disease. Line graphs in bottom rows depict similar trends for Hst-5 and Th17 associated
653 cytokine levels for subjects 1 and 2 **(b and d, respectively)**. Upon culturing, *C. albicans*
654 was recovered from the initial 6 samples from subject 2 **(c, d)**.
655
656 **Figure 4. Prospective sampling and analysis of saliva from three subjects with**
657 **moderate COVID-19.** Timeline of sampling and measurement of salivary Hst-5 and

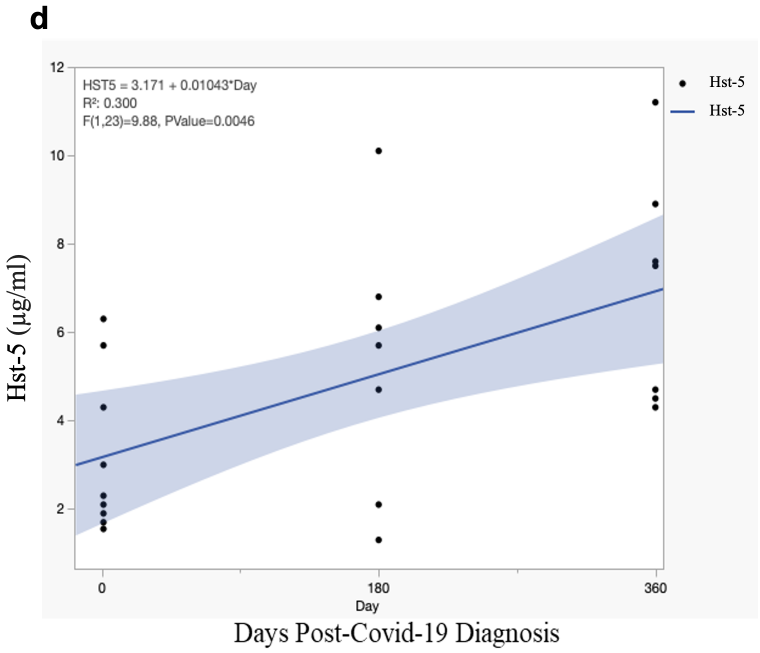
658 cytokine concentrations in samples recovered during the acute phase of the disease and
659 upon recovery. Table presents cytokine values measured in the first and last samples
660 recovered from the three subjects and the percent change in levels between the samples.
661
662 **Figure 5. *Ex vivo* proliferation assay using pooled saliva. (a)** Workflow for histatin-5
663 measurement and proliferation assay using pooled saliva samples from 3 subjects under
664 a COVID-19 infected state (PC+) and recovered state (PC-). **(b)** Bar plots depict Hst-5
665 concentrations (µg/ml) from pooled saliva samples; **(c)** recovered *C. albicans* (cells/ml)
666 following 1 h incubation in pooled saliva samples seeded with $1x10^4$ cells/ml of *C.*
667 *albicans.*
668
669
670 **SUPPLEMENTAL FIGURES**
671
672 **Supp Figure 1. *In situ* hybridization and immunofluorescence controls. (a)**
673 Immunofluorescence (IF) and *in situ* hybridization (ISH) controls (see also **Fig. 1**).
674 Positive and negative controls for IF, antibody anti-Hst-3 in control parotid shows strong
675 IF (green) in acini structures and no signal is observed in ducts. The isotype control shows
676 no signal. **(b)** Probe anti-human *PPIB* shows strong signal in all the cells (white); negative
677 control probe shows sparse, rare positive signal in the tissue.
678
679
680

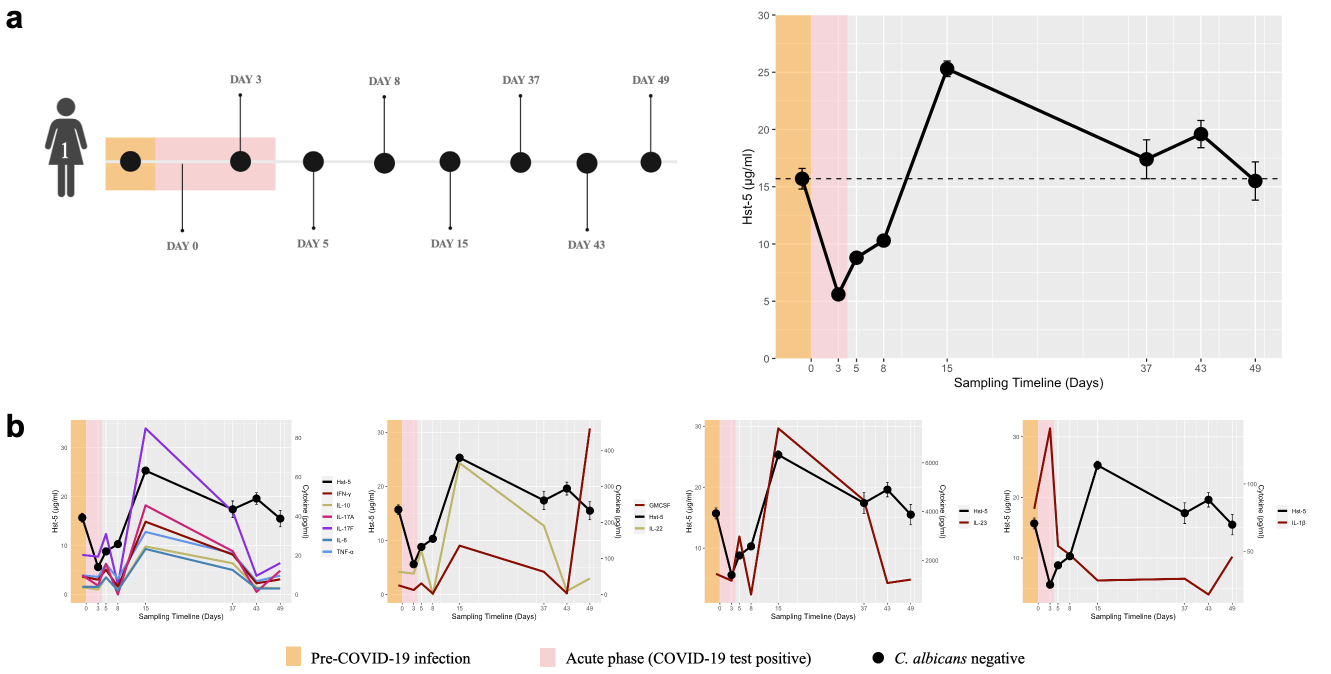

a
HTN1
0.0022
CPM
1000000
100000
10000
1000
100
10
Healthy Parotid
SARS-CoV-2+ Parotid
HTN3
0.0038
b
Control Parotid
DAPI
Histatin-3
AQP5
SARS-CoV-2
Duct
Acini
50 µm
10 µm
Histatin-3/Cell: 0.25
c
SARS-CoV-2 Infected Parotid Case #p7
Histatin-3/Cell: 0.09
d
SARS-CoV-2 Infected Parotid Case #p7
Normalized Histatin-3/Cell
Viral Count/Cell
e
SARS-CoV-2 Infected Parotid Case #p19
Histatin-3/Cell: 0.07
f
SARS-CoV-2 Infected Parotid Case #p19

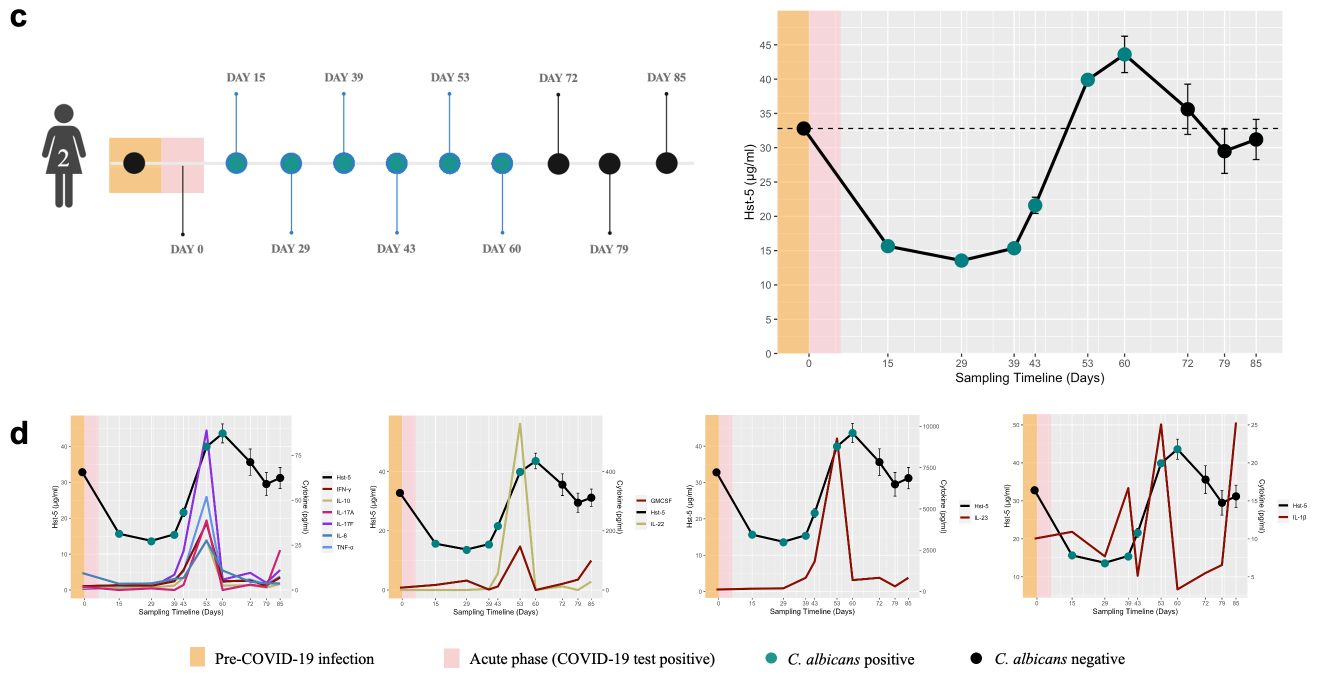

a
0.0303
C. albicans negative
C. albicans positive
Not cultured
Hst-5 (µg/ml)
60
40
20
0
Healthy Subjects
COVID-19 Patients

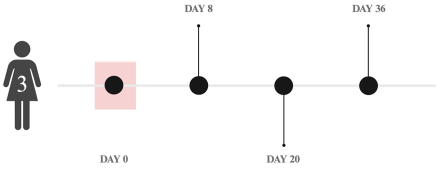

b
Difference in Hst-5 (µg/ml)
40
20
0
-20
-40
Matched Healthy and COVID-19 Patient Pair

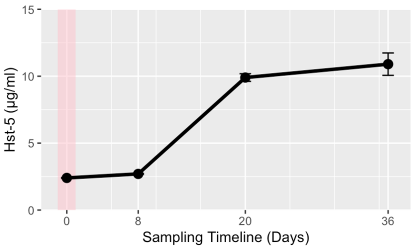

c
<0.0001
C. albicans negative
C. albicans positive
Hst-5 (µg/mL)
20
15
10
5
0
Healthy Subjects
COVID-19 Patients


d
HST5 = 3.171 + 0.01043*Day
R²: 0.300
F(1,23)=9.88, PValue=0.0046
Hst-5
Hst-5
Hst-5 (µg/ml)
12
10
8
6
4
2
0
180
360
Day
Days Post-Covid-19 Diagnosis

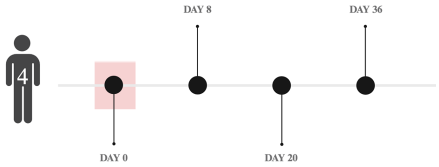

a
DAY 0
DAY 3
DAY 5
DAY 8
DAY 15
DAY 37
DAY 43
DAY 49
Hst-5 (µg/ml)
Sampling Timeline (Days)
b
Hst-5 (µg/ml)
Cytokine (pg/ml)
Sampling Timeline (Days)
Hst-5
IFN-γ
IL-10
IL-17A
IL-17F
IL-6
TNF-α
GMCSF
IL-22
IL-23
IL-1β
Pre-COVID-19 infection
Acute phase (COVID-19 test positive)
C. albicans negative

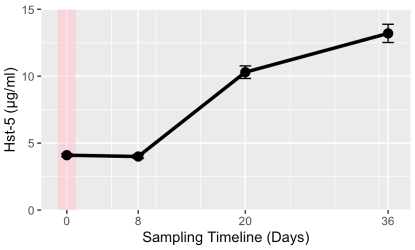

c
DAY 0
DAY 15
DAY 29
DAY 39
DAY 43
DAY 53
DAY 60
DAY 72
DAY 79
DAY 85
Hst-5 (µg/ml)
Sampling Timeline (Days)
d
Hst-5 (µg/ml)
Cytokine (pg/ml)
Sampling Timeline (Days)
Hst-5
IFN-γ
IL-10
IL-17A
IL-17F
IL-6
TNF-α
GMCSF
IL-22
IL-23
IL-1β
Pre-COVID-19 infection
Acute phase (COVID-19 test positive)
C. albicans positive
C. albicans negative



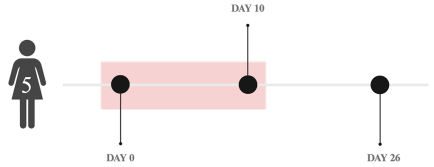


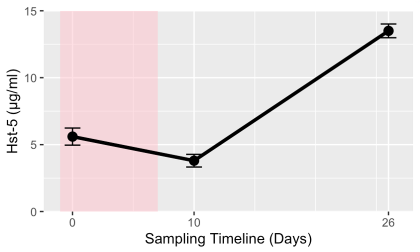
Cytokine	Day 0	Day 35	% Change
IL-1ß (pg/ml)	122.56	150.51	22.80
IL-6 (pg/ml)	0.57	0.57	0
IFN- γ (pg/ml)	2.31	2.24	-2.92
IL-10 (pg/ml)	0.94	0.80	-14.82
IL-17A (pg/ml)	5.26	2.17	-58.78
TNF-α (pg/ml)	6.31	6.63	5.14
IL-22 (ng/ml)	ND	ND	N/A
IL-23 (ng/ml)	0.41	0.13	-69.46
IL-17F (ng/ml)	0.002	0.001	-69.64
GMCSF (ng/ml)	0.011	0.005	-52.69
IL-17E (ng/ml)	ND	ND	N/A





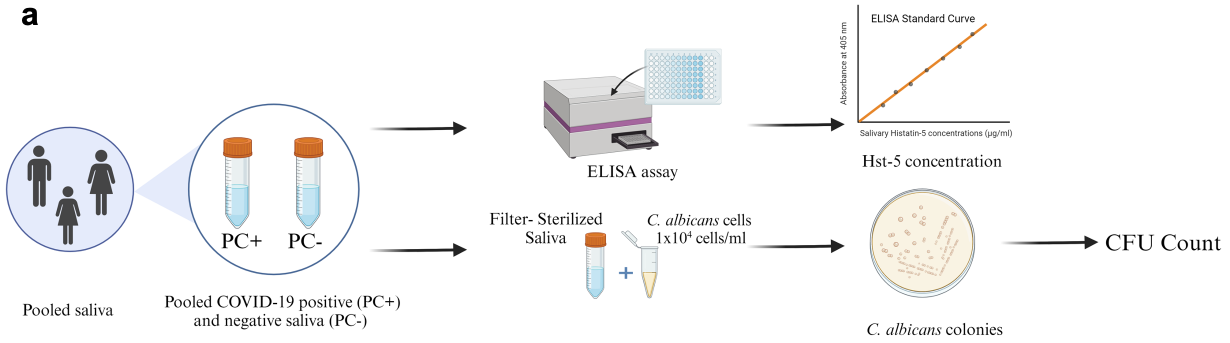
Cytokine	Day 0	Day 35	% Change
IL-1ß (pg/ml)	218.31	2991.20	1270.15
IL-6 (pg/ml)	2.02	13.80	584.19
IFN- γ (pg/ml)	4.67	4.06	-12.96
IL-10 (pg/ml)	1.28	3.53	175.77
IL-17A (pg/ml)	6.34	19.49	207.40
TNF-α (pg/ml)	7.91	38.98	392.69
IL-22 (ng/ml)	0.050	0.135	170.62
IL-23 (ng/ml)	0.60	1.54	157.01
IL-17F (ng/ml)	0.012	0.017	42.62
GMCSF (ng/ml)	0.016	0.052	221.11
IL-17E (ng/ml)	ND	ND	N/A

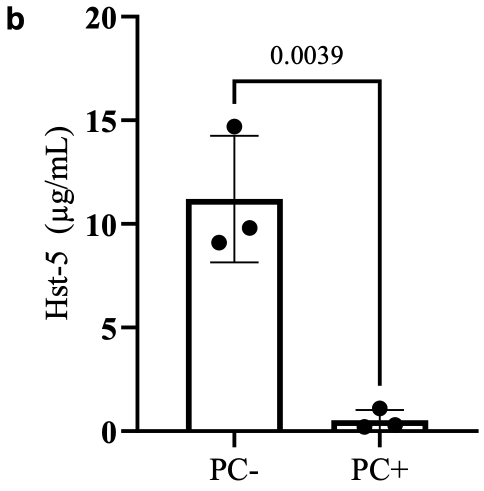


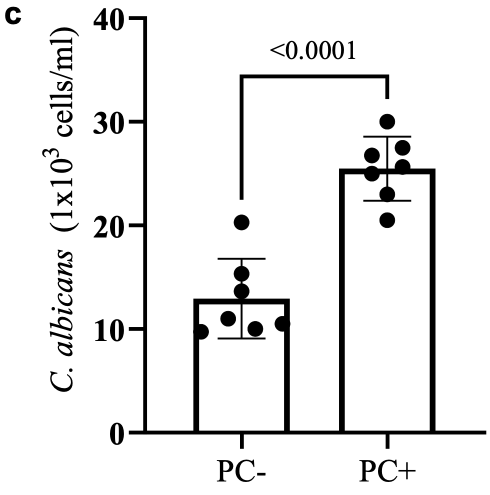


Cytokine	Day 0	Day 12	% Change
IL-1ß (pg/ml)	61.71	44.32	-28.18
IL-6 (pg/ml)	0.047	0.57	1103.07
IFN- γ (pg/ml)	1.35	2.72	100.48
IL-10 (pg/ml)	0.66	0.80	21.07
IL-17A (pg/ml)	ND	ND	N/A
TNF-α (pg/ml)	9.49	9.64	1.62
IL-22 (ng/ml)	ND	ND	N/A
IL-23 (ng/ml)	ND	0.08	N/A
IL-17F (ng/ml)	ND	ND	N/A
GMCSF (ng/ml)	ND	0.003	N/A
IL-17E (ng/ml)	ND	ND	N/A

Acute phase (COVID-19 test positive)


a
PC+
PC-
Pooled saliva
Pooled COVID-19 positive (PC+)
and negative saliva (PC-)
ELISA assay
ELISA Standard Curve
Absorbance at 405 nm
Salivary Histatin-5 concentrations (µg/ml)
Hst-5 concentration
Filter- Sterilized
Saliva
C. albicans cells
1x10⁴ cells/ml
C. albicans colonies
CFU Count


b
Hst-5 (µg/mL)
20
15
10
5
0
0.0039
PC-
PC+


c
C. albicans (1x10³ cells/ml)
40
30
20
10
0
<0.0001
PC-
PC+