

1 Evidence of a Sjögren's disease-like phenotype following COVID-19

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3 Yiran Shen^{1*}, Alexandria Voigt^{1*}, Laura Goranova¹, Mehdi Abed², David E. Kleiner³, Jose O.
4 Maldonado^{2,4}, Margaret Beach², Eileen Pelayo², John A. Chiorini⁴, William F. Craft⁵, David A.
5 Ostrov⁶, Vijay Ramiya⁷, Sukesh Sukumaran⁸, Apichai Tuanyok¹, Blake M. Warner^{2#}, and Cuong
6 Q. Nguyen^{1,9,10#}

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

19 *Authors contributed equally to the study

20 #Co-correspondence authors.

21
22 Address correspondence:

23 Cuong Q. Nguyen, PhD
24 Department of Infectious Diseases and Immunology
25 PO Box 110880, College of Veterinary Medicine
26 University of Florida, Gainesville, Florida 32611-0880. USA

27 Telephone: 352-294-4180, Fax: 352-392-9704

28

29 Blake M. Warner, DDS, PhD, MPH

30 Salivary Disorders Unit

31 Building 10 Room 1A01

32 10 Center Drive

33 National Institutes of Health

34 Bethesda, MD 20895

35

36 **Key Messages:**

37 What is already known about this subject?

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

40 What does this study add?

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

48 How might this impact on clinical practice or future developments?

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

52

53 **Abstract**

54 **Objectives:**

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

61 **Methods:**

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

67 **Results:**

68 Mice infected with the virus showed a decreased saliva flow rate, elevated antinuclear
69 antibodies (ANAs) with anti-SSB/La, and lymphocyte infiltration in the lacrimal and salivary
70 glands. Sera of COVID-19 patients showed an increase in ANA, anti-SSA/Ro52, and anti-
71 SSB/La. The male patients showed elevated levels of anti-SSA/Ro52 compared to female
72 patients, and female patients had more diverse ANA patterns. Minor salivary gland biopsies of
73 convalescent COVID-19 subjects showed focal lymphocytic infiltrates in four of six subjects, and
74 2 of 6 subjects had focus scores >2. Lastly, we found monoclonal antibodies produced in
75 recovered patients can both block ACE2/spike interaction and recognize nuclear antigens.

76 **Conclusion:**

77 Overall, our study shows a direct association between SARS-CoV-2 and SjD. Hallmark features
78 of SjD salivary glands were histologically indistinguishable from convalescent COVID-19

79 subjects. The results potentially implicate that SARS-CoV-2 could be an environmental trigger
80 for SJD.

81

82

83 **Key Words:**

84

85 Sjögren's Disease (SjD), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2),

86 Coronavirus disease 2019 (COVID-19), Autoimmune disease, Autoantibodies.

87

88 Introduction

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

103 In December 2019, a novel coronavirus, severe acute respiratory syndrome-coronavirus-
104 2 (SARS-CoV-2), emerged in Wuhan, Hubei Province, China, initiating a breakout of atypical
105 acute respiratory disease, termed coronavirus disease 2019 (COVID-19). SARS-CoV-2 is a
106 *betacoronavirus* in the family of *Coronaviridae*; the virus contains four structural proteins: S
107 (spike), E (envelope), M (membrane), and N (nucleocapsid), sixteen non-structural proteins
108 (nsp1–16) and eleven accessory proteins, which support viral essential physiological function
109 and evasion from the host immune system[9]. As of May 1st, 2022, approximately one million
110 U.S. residents have died from COVID-19[10], with more than 80 million total cases. Recent
111 studies have identified the association between SARS-CoV-2 infection and autoimmune
112 response. A recent literature review[11] (n= 1176 articles and 90 case reports) revealed that the
113 primary rheumatic diseases associated with COVID-19 patients were vasculitis, arthritis,

114 idiopathic inflammatory myopathies, and systemic lupus erythematosus. Several studies have
115 found an association between antinuclear antibodies (ANAs) (35.6%) and COVID-19 infection,
116 where the leading reactive antigens include SSA/Ro (25%), rheumatoid factor (19%), lupus
117 anticoagulant (11%), and type I interferons (IFN-I) (10%)[12–14]. In 6 independent case studies,
118 COVID-19 patients were diagnosed with systemic sclerosis[15], adult-onset Still's disease[16],
119 sarcoidosis, and systemic lupus erythematosus (SLE) with 4/6 patients acutely manifesting
120 during COVID-19. An elevated level of anti-SSA/Ro52 in COVID-19 patients was linked to
121 pneumonia severity and poor prognosis[17]. The underlying mechanism for the production of
122 autoantibodies in COVID-19 patients is unknown, however, it poses a significant challenge for
123 post-COVID-19 symptoms or post-acute sequelae of SARS-CoV-2 (PASC).

124 There are reports and cases of COVID-19 patients experiencing ocular and oral
125 symptoms. Keratoconjunctivitis was observed in a few patients during a specific phase of the
126 disease[18]. One study has shown that xerostomia was observed in 29% of the patient
127 cohort[19] while another showed an increase of 30% in reporting xerostomia during
128 hospitalization[20]. While these early studies had small sample sizes, the results appeared to
129 indicate an association between COVID-19 and oral and ocular manifestations, primary
130 symptoms of SjD. Increased rates of xerostomia in this patient cohort may be explained by
131 tropism of SARS-CoV-2 for the salivary glands, resulting in host immune response and immune-
132 mediated injury[21]. Furthermore, growing evidence of autoantibody production in COVID-19
133 patients raises a critical question as to whether SARS-CoV-2 infection is a risk factor for primary
134 SjD. Therefore, the goal of this study was to determine the autoimmune response triggered by
135 SARS-CoV-2 infection. The results indicate that infection with SARS-CoV-2 recapitulated an
136 SjD-like phenotype in transgenic mice. Additionally, we show by using human sera paired by
137 sex, age, and race that not only do they contain general autoantibodies but those associated
138 with SjD, namely anti-SSA/Ro52 and anti-SSB/La.

139

140 **Methods**

141 **Human samples**

142 SARS-CoV-2 positive and healthy control (HC) sera were obtained from the CTSI
143 Biorepository at University of Florida in compliance with IRBs 202001475 and 2020000781. The
144 presence of SARS-CoV-2 was confirmed by RT-PCR for admittance into the CTSI Biorepository
145 Bank. Peripheral blood mononuclear cells (PBMC) from five post-convalescent COVID-19
146 donors were obtained from LifeSouth Community Blood Centers (Gainesville FL). The healthy
147 volunteer donors had recovered from COVID-19 and were positive for SARS-CoV2 antibodies
148 at the time of blood donation. The donors had no prior clinically diagnosed autoimmune
149 diseases. Handling of the samples was performed in a certified BSL2+ with Institutional
150 Biosafety Committee approved protocols.

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

165 (2021)[21]. After identifying SARS-CoV-2 in saliva, the protocol was amended to allow MSG
166 biopsy in acute and convalescent COVID-19 subjects[21].

167 Research and clinical records post-initiation of the global COVID-19 pandemic were
168 reviewed systematically by a rheumatology Physicians Assistant (MB). Subjects were included
169 in the histopathological analysis if they had recovered from COVID-19, had convalescent MSG
170 biopsies, and were enrolled on NIH IRB Protocols: 15-D-0051 or 20-D-0094. Subjects were
171 excluded if they were evaluated as a patient for the workup for SjD or non-SjD sicca symptoms.
172 Comprehensive investigations as described above were completed on subjects enrolled on our
173 15-D-0051 protocol, but due to constraints of the NIH SARS-CoV-2 Field Testing Facility, these
174 parameters were not able to be collected on all 20-D-0094 subjects. Clinical laboratory studies
175 at NIH include standard bloodwork, assays for antinuclear antibodies (ANA), antibodies to
176 extractable nuclear antigens (e.g., anti-SSA/SSB autoantibodies), and antibodies to pathogens
177 to assess vaccination and exposure history purposes (e.g., anti-spike, anti-nucleocapsid). In
178 one subject (subject 2), serial MSG biopsies were collected; the first was taken 5 days after the
179 first COVID-19 symptoms (reported previously as COV49[21]) and the second was taken 6
180 months later[21].

181 MSG biopsies were interpreted by a board-certified anatomic pathologist (DEK) for
182 diagnostic purposes and the histopathology was systematically reviewed by a board-certified
183 oral and maxillofacial pathologist (BMW) as previously described (PMID: 30996010). Salivary
184 gland inflammation and fibrosis were graded according to Greenspan et al. [PMID: 4589360]
185 and Tarpley et al. [PMID: 4586901]. For MSG with Greenspan grade 3 or 4 sialadenitis, a focus
186 score was calculated according to Daniels et al. [PMID: 1055974]. Hematoxylin and eosin
187 [H&E], CD20, CD3, CD4, CD8) was conducted by the Anatomic Pathology Laboratory of the
188 National Cancer Institute. Slides were scanned at x40 with a NanoZoomer S360 slide scanner
189 (Hammamatsu Photonics, Hammamatsu-city, Japan), and digital photomicrographs at x5
190 resolution were captured using NDP.view2 software (Hammamatsu Photonics).

191

192

193 **Results**

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

195 SjD patients experience xerostomia, primarily as a result of the diminished secretory
196 function of the salivary glands. In the spontaneous animal models of SjD, the secretory
197 dysfunction occurs between 15-20 weeks of age. Here, we sought to determine if SARS-CoV-2
198 can compromise saliva secretion by the glands. The homozygous K18-hACE2 mice were
199 intranasally inoculated with 860 PFU of SARS-CoV-2 WA1/2020 inoculum drop-by-drop into
200 both nostrils until fully inhaled. Saliva were collected on day 21 prior to euthanization. As
201 shown, the infected mice showed a significant loss of salivary flow rates compared to the
202 uninfected mice (infected: 6.64 ± 1.075 vs uninfected: 13.12 ± 0.532 ul/gr) (**Figure 1A**). The loss
203 of saliva was equivalent to approximately a 50% reduction in function (**Figure 1B**). The infected
204 mice showed a decrease in body weight when compared to the uninfected mice; however, the
205 decrease was not statistically significant (**Figure 1C**). The results suggest that SARS-CoV-2
206 infection has a negative effect on the secretory function of the salivary glands.

207

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

209 Seropositivity for ANA and anti-SSA/Ro is one of the major classifying criteria for SjD.
210 Here, we sought to determine if SARS-CoV-2 infection was able to induce autoantibody
211 production. As presented in **Figure 2A**, 70% of infected mice were positive and 30% were
212 negative for ANA using HEP2 cell staining. Whereas, in the uninfected mice, 70% were
213 negative, and 30% were positive for ANA. Furthermore, we examined the SjD-specific
214 autoantibodies. As indicated in **Figure 2B**, anti-SSB/La levels were highly elevated in the
215 infected group in comparison to the control group. There was a slight, though statistically
216 insignificant, increase in anti-SSA/Ro52 levels in the infected group. There was no change in

217 anti-SSA/Ro60 levels between the two groups. The results suggest that SARS-CoV-2 infection
218 in mice promotes the development of ANA and autoantibodies signature to SjD.

219

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

221 The principal targeted tissues for SjD are the lacrimal and salivary glands. The
222 inflammatory lesions are composed of a multitude of immune cell types, notably B cells, T cells,
223 and macrophages. The lacrimal glands of infected mice had multifocal apoptosis/necrosis of low
224 to moderate numbers of acinar epithelial cells characterized by cells with condensed,
225 hypereosinophilic cytoplasm and pyknotic nuclei with karyorrhexis. The apoptosis/necrosis
226 resulted in variable collapse and loss of acini. The interlobular duct epithelium was unaffected.
227 The salivary glands of infected mice showed a lymphoid nodule in the interstitium, a sign of
228 lymphocyte infiltration which was not present in control mice (**Figure 3A**). Elevated number of
229 apoptotic cells were found in both glands of infected mice (**Figure 3B**). Macrophages were
230 more consistent in the salivary glands of infected mice with a smaller frequency of T or B cells.
231 Whereas, macrophages in the lacrimal glands of infected mice were elevated drastically and
232 consistently in the infected mice in comparison to the salivary gland. Similarly, B and T cells
233 were detected at higher frequencies in the lacrimal than in the salivary glands (**Figure 3B**).
234 Lymphocytic foci were detected in both the lacrimal and salivary glands of the infected mice, but
235 not those of the control mice. Only a single infected mouse developed a focus score (FS) in the
236 salivary glands, so deviation from the control group is insignificant ($\chi^2=.27$, $p= 0.10247$).
237 However, FS were detected in the lacrimal glands of 5 infected mice ($\chi^2=13$, $p= 0.00031$) (**Table**
238 **S1**). Additionally, lymphocytic infiltration of B and/or T cells which does not qualify as a focus
239 was examined. This is an indicator of localized inflammation in the salivary ($\chi^2=11$, $p= 0.00091$)
240 and lacrimal glands ($\chi^2=24$, $p= 0.00001$). Overall, SARS-CoV-2 induced inflammation with
241 multifocal apoptosis/necrosis with more severity in the lacrimal than salivary glands.

242

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

244 As described above, mice infected with SARS-CoV-2 developed ANA and elevated anti-
245 SSB/La. Here, we sought to determine if these findings were also observed in human patients.
246 As presented in **Figure 4A**, the COVID-19 patients exhibited higher frequencies of positive ANA
247 at different sera titers compared to healthy controls. Notably, 60% patients showed positive
248 ANA with none for healthy controls at 1:160 titer. 30% patients still exhibited positive ANA at
249 1:320 titer. Further analysis of the staining patterns revealed that among the positive ANA for
250 patients, 40% were homogeneous, 15% were speckled, and 5% were centromeric (**Figure 4B**).
251 To further determine if the COVID-19 patients presented with SjD signature autoantibodies,
252 patient sera were examined for reactivity against SSA/Ro52, SSA/Ro60, and SSB/La. As
253 presented in **Figure 4C**, anti-SSA/Ro52 and anti-SSB/La were significantly elevated in COVID-
254 19 patients in comparison to healthy controls. Anti-SSB/Ro60 levels remained similar between
255 the two groups.

256 SjD has a strong predilection for females; therefore, we sought to determine whether
257 COVID-19 patients exhibited an element of sexual dimorphism in the autoantibody response.
258 Interestingly, when examining the ANA staining it was discovered that the female COVID-19
259 patients had a significantly higher percentage of positive ANA at various titers compared to
260 either the male COVID-19 patients or either sex of control patients (**Figure S1A**). Additionally,
261 the female COVID-19 patients were shown to present a more diverse ANA pattern, with 30%
262 speckled, 40% homogenous, and 10% centromeric at 1:160 titer, whereas the male patient
263 showed 10% speckled and 30% homogenous pattern at the same titer. The female patient still
264 exhibited 20% speckled with males showed 10% speckled at 1:320 titer and homogenous
265 pattern remained the same for both sexes. positive staining for all other groups only contained
266 a homogenous pattern (**Figure S1B**). To further determined if male and female COVID-19
267 patients exhibited different levels of SjD signature autoantibodies, we analyzed the samples
268 based on sex. As presented in **Figure S1C**, female and male COVID-19 patients showed

269 significantly higher levels of anti-SSA/Ro52 in comparison to their respective counterparts.
270 Interestingly, male COVID-19 patients showed elevated levels of anti-SSA/Ro52 above female
271 COVID-19 patients ($p=0.0029$). There was no statistically significant difference between male
272 and female COVID-19 patients with anti-SSA/Ro60 or anti-SSB/La. The results indicated that
273 female patients manifested more diverse patterns of ANA, however male patients exhibited
274 higher levels of anti-SSA/Ro52 than female patients.

275

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

278 It is remarkable to observe the cross-reactivity of COVID-19 patients' sera against self-
279 antigens as demonstrated. To further evaluate the B cell response of COVID-19 patients, we
280 produced and selected nine monoclonal antibodies (mAbs) from convalescent COVID-19
281 patients by isolating CD20⁺ memory B cells reactive against both the RBD and S1 of SARS-
282 CoV-2, and examined their response to self-antigens. As presented in **Figure S2A**, the mAbs
283 exhibited various degrees of inhibition against SARS-CoV-2 RBD, in which mAbs A10 and B5
284 showed the highest inhibitory activity at different dilutions. To determine if they react against
285 nuclear antigens, we tested them against HEp2 cells. As described in **Figure S2B**, seven of the
286 nine S1/RBD-reactive mAbs produced a strong homogenous staining pattern with 100% at 1:40
287 and 1:80 titers and lowered to 90% at 1:160 and 1:320 titers. Overall, the results demonstrate
288 that mAbs against the virus produced in recovered COVID-19 patients are cross-reactive and
289 capable of recognizing nuclear antigens.

290

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

293 Six generally-healthy, relatively young (Range: 19-42y; Mean: 31y) subjects who had
294 recovered from COVID-19, had convalescent MSG biopsies were identified for the study. These

295 subjects were free from evidence of pre-existing autoimmune disease or major medical
296 conditions. Subjects 1-3 were enrolled on NIH IRB Protocol: 20-D-0094 and had convalescent
297 MSG biopsies 6-13 months after recovery from COVID-19 (Table 1). In addition, Subject 2 also
298 received an initial biopsy during acute COVID-19 (Table 1, Figure 5). These subjects recovered
299 from COVID-19 without continued post-acute COVID-19 symptoms as primary clinical concerns.
300 Subjects 4-6 were enrolled on an NIH IRB Protocol: 15-D-0051 as healthy volunteers (HV) and
301 did not present with clinical complaints of Sjogren's Disease or post-acute COVID-19 syndrome
302 (Table 1). Their COVID-19 status was determined from subject interviews and serological
303 studies. In subjects with known COVID-19, their clinical course was generally mild; three of the
304 subjects reported lung involvement with shortness of breath without hospitalization and a single
305 subject reported significant gastrointestinal involvement ('mild-to-moderate COVID-19'). A single
306 subject, Subject 5 was unaware of their post-COVID-19 status and was considered
307 'asymptomatic'. Evidence of infection included clinical reports of infection in five of six subjects,
308 clinical nasopharyngeal swab PCR for SARS-CoV-2 N1 and N2 genes in four subjects (S1-3,6);
309 anti-nucleocapsid antibodies were positive in all six subjects (*data not shown*). No subjects were
310 positive for anti-nuclear antibodies (ANA) or anti-SSA/Ro antibodies. A single subject was low-
311 titre positive for anti-SSB/La antibodies. Three of the six subjects reported dry mouth during
312 acute COVID-19 which was sustained temporarily after recovery (up to 3 weeks); a single
313 subject had objective evidence of dry mouth (Subject 2) during acute COVID-19. Interestingly,
314 this subject did not produce saliva from the submandibular glands for ~3 of the 4 weeks of
315 weekly follow up after infection. Dry eye assessments could not be completed in the NIH
316 COVID-19 Testing Facility for three subjects, two of the three subjects who presented through
317 the NIH Dental Clinic had objective evidence of dry eye disease (**Table 1**) but did not have
318 clinical complaints of dry eyes.

319 Overall, seven MSG biopsies were collected from 6 subjects - a single subject had serial
320 biopsies. One biopsy occurred during acute COVID-19 5 days after symptom debut, and the

321 second 6 months after recovery. Generally, biopsies exhibited mild chronic sialadenitis (**Table**
322 **1**). However, 5 of the 7 biopsies (from four of the six subjects) had multiple foci (>50
323 lymphocytes) of inflammation (e.g., focal lymphocytic sialadenitis, FLS; **Table 1**, **Figure 5**,
324 **Figure S3**). Most foci were small, although several glands exhibited multiple medium-sized and
325 coalescing foci. Mild fibrosis and atrophy of the glands were seen in three subjects (Subject 1-
326 3). It is noteworthy that Subject 2's follow-up biopsy exhibited evidence of sustained immune
327 insult as evidenced by an increased focus score (FS:1 → FS:2) and the elaboration of fibrosis
328 and atrophy of the glands (**Figure 5**). Histopathological evidence of injury included ductal injury
329 and mucous inspissation, immune infiltration of the acini with injury, perivascular infiltrates, and
330 granuloma. In some subjects, the histopathological features in four of six subjects (five biopsies)
331 are reminiscent of the range of histopathological features found in the MSG of SjD patients.

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

341

342 Discussion

343 Increasing evidence has supported the associations between viral/bacterial infections
344 and autoimmune diseases. An early study demonstrated that murine cytomegalovirus induced
345 an SjD-like disease in C57Bl/6-lpr/lpr mice with sialadenitis, severe salivary gland inflammation,
346 and production of anti-SSA/Ro and anti-SSB/La[28]. Recent studies suggested that the virus
347 has a tropism for the SG, including SARS-CoV-2[21,29]. Here, we sought to determine if SARS-
348 CoV-2 infection could also trigger SjD-like phenotypes in a murine model. The results indicate
349 that SARS-CoV-2 infection recapitulates several signature disease phenotypes, specifically,
350 diminished salivary flow rates, salivary and lacrimal gland inflammatory lesions, and elevated
351 autoantibodies. Similar findings were also observed in COVID-19 patients, in which, significantly
352 elevated levels of anti-SSA/Ro52 and anti-SSB/La were seen. Additionally, female patients
353 manifested more diverse patterns of ANA, and male patients exhibited higher levels of anti-
354 SSA/Ro52 than female patients. In summary, the data suggest that SARS-CoV-2 infection
355 triggered a SjD-like disease in a murine model as well as in human patients.

356 SARS-CoV-2 primarily uses ACE2 as a receptor[30,31], which is broadly expressed by
357 endothelial and epithelial cells, including those of the aerodigestive tract and the salivary
358 glands[21,32–34]. It has now been shown that salivary glands can robustly support infection and
359 replication of SARS-CoV-2, and that saliva is potentially infectious and transmissible[21]. Intra-
360 individual spread of SARS-CoV-2 initiates from the epithelial cells of the upper respiratory tract
361 (e.g., acinar and ductal cells of the salivary glands) by active replication and egress of offspring
362 viruses subsequently infecting ACE2-expressing cells in downstream organs, including the
363 heart, kidneys, gastrointestinal tract, and vasculature[21,35,36]. The hACE2 transgenic model
364 expressed high levels of hACE2 in the lacrimal glands and a lesser amount in the salivary
365 glands. The tissue and cellular tropism of SARS-CoV-2 were not noticeably different between
366 the two glands at the study endpoint. The more severe lacrimal gland inflammation and cell
367 death could be attributed to the higher expression of hACE2, which allows for active viral

368 infection and replication, however, the endpoint timeline was not able to capture these temporal
369 infectious changes (**Figures S4 and S5**). ACE2 is expressed in squamous epithelial cells of the
370 dorsal tongue, gingiva, and buccal tissue, and TMPRSS2 is expressed in taste bud cells and
371 submandibular glands. SARS-CoV-2 was detected in SGs with higher levels in the minor SGs.
372 In addition, saliva is a natural reservoir for viruses as one of the major fluids for viral detection.
373 Therefore, it is not surprising that SARS-CoV-2 was found in the SGs and facilitated the
374 inflammatory response.

375 The severity of COVID-19 is mediated by unregulated inflammation. During the later
376 stage of the disease, immune-mediated damage leads to a progressive increase in
377 inflammation. And patients with life-threatening pneumonia had neutralizing autoantibodies
378 against IFN- ω and IFN- α [14]. As demonstrated, mice infected with SARS-CoV-2 developed
379 higher levels of ANA and anti-SSB/La levels. Similarly, patients developed elevated levels of
380 ANA, specifically anti-SSA/Ro52 and anti-SSB/La. In a study analyzing the sera and plasma
381 from 64 COVID-19 patients, approximately 25% of patients exhibited an autoantibody response
382 on average 12.3 days post-diagnosis and the reactivity was primarily to nuclear antigens,
383 including RNP (n=8), SSA, SSB, dsDNA, chromatin, or centromere[37] Chang et al. showed that
384 autoantibodies are present in approximately 15% of healthy controls and 50% of COVID-19
385 patients against commonly recognized antigens in an array of autoimmune disorders, including
386 SSA/Ro52[38]. However, Burbelo, et al., 2022 demonstrated that a considerable fraction of the
387 autoantibody positivity in severe COVID-19 subjects may be related to receipt of intravenous
388 immunoglobulins (IVIG)[39]. Thus, these results suggest the need for longitudinally sampled
389 and controlled serosurveillance need to be performed. A metaanalysis revealed that the
390 development of primary rheumatic diseases associated with COVID-19 patients were vasculitis,
391 arthritis, idiopathic inflammatory myopathies, and systemic lupus erythematosus; overall, the
392 association between ANAs (35.6%) and COVID-19 infection was 35.6% and the reactive
393 antigens were found at the following rates: SSA (25%), rheumatoid factor (19%), lupus

394 anticoagulant (11%), and IFN-I (10%)[11]. Autoantibody responses in COVID-19 patients can be
395 influenced by sex, with men exhibiting an autoantibody response after an infection defined as at
396 least mildly symptomatic, whereas women were prone to produce this response following an
397 asymptomatic infection; thus autoantibody profiles are highly variable between the sexes and
398 dependent on the disease severity[40]. It is unknown how SARS-CoV-2 infection could induce a
399 plethora of autoantibodies, specifically autoimmune-specific antibodies. One hypothesis is that
400 tropism of SARS-CoV-2 to vulnerable cells triggers a robust immune response that damages
401 virally-infected cells leading to the presentation of anti-viral proteins-viral particle-antibody
402 immune complexes to antigen presenting cells in the interstitium. A study showed that
403 heptapeptide sharing exists between SARS-CoV-2 spike glycoprotein and human proteins, an
404 indication of the molecular mimicry mechanism[41]. However, the spike protein does not share
405 any homology with SjD-specific autoantigens. Therefore, it remains to be determined the
406 underlying mechanism of autoantibody response triggered by SARS-CoV-2.

407 We, and others, have confirmed that salivary glands are exquisitely supportive of
408 infection and replication of SARS-CoV-2, and saliva is an ideal secretion for inter and intra-
409 individual spread of de novo virus[21,42]. Because of the long-hypothesized connection
410 between viral infection and the initiation of autoimmune diseases, we examined available clinical
411 data and minor salivary gland biopsies from convalescent COVID-19 subjects with mild-to-
412 moderate infections. While no patients satisfied strict 2016 ACR/EULAR classification criteria,
413 focal lymphocytic sialadenitis or clinical signs and symptoms of SjD were found in most of the
414 available subjects[43]. In select patients, the histopathological features of inflammation in the
415 salivary glands are indistinguishable from SjD and in the proper clinical context would be
416 supportive of the diagnosis.

417 The most prevalent and persistent oral symptoms associated with COVID-19 include
418 taste dysfunction, in addition to that, dry mouth (xerostomia) is often overlooked in COVID-19
419 patients and was identified as another highly prevalent (43%) oral manifestation of COVID-

420 19[44]. A review of 12 studies, including patients diagnosed with SARS-CoV-2 infection from
421 different countries with reported oral symptoms associated COVID-19 infection showed that
422 xerostomia occurs in the early stages of COVID-19 with a prevalence ranging from 20% to
423 61.9%, and can persist for at least 8 months after recovery[45]. The percentage is higher in
424 patients with mild symptoms, as a study in Israel showed 61.9% of 97 confirmed non-
425 hospitalized patients reported xerostomia[46]. This is consistent with our data showing a
426 markedly diminished salivary secretion after SARS-CoV-2 infection in mice. The precise etiology
427 of gland dysfunction requires further investigation. As demonstrated, the influx of inflammatory
428 cells in the glands, concomitantly with the rapid increase of acinar cell apoptosis/necrosis, may
429 contribute to the diminished gland function. We did not measure tear secretion, mainly to avoid
430 further physical stress on the mice as a result of the drug side effect and handling.

431 In summary, our study underpins the pathogenic role of SARS-CoV-2 in SjD. SARS-
432 CoV-2 induced gland inflammation leading to the loss of saliva in mice. It triggered the
433 production of SjD-specific autoantibodies in mice and human patients. This study raises the
434 prospect of managing SjD in long COVID-19. Further studies are needed to examine the
435 pathoetiology of SARS-CoV-2 in SjD.

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440 **Competing interests**

441 All authors have no competing interests.

442

443

444 **Data availability statement**

445 Data are available upon reasonable request

446

447 **Ethics statement**

448 **Patient consent for publication**

449 Not required

450 **Ethics approval**

451 Subjects were consented to National Institutes of Health (NIH) Central Institutional
452 Review Board (IRB)-approved protocols (15-D-0051: *Characterization of Salivary Gland*
453 *Disorders* [PI-Warner]; 20-D-0094: *Transmissibility and Viral Load of SARS-CoV-2 in Oral*
454 *Secretions* [PI-Warner]). Convalescent samples were collected under the University of Florida
455 approved protocol (IRB202000781). Participants gave informed consent to participate in the
456 study before taking part.

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459

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466 patients and collection of research data and tissues.

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473 **Figure legends**

474 **Figure 1: Decrease in saliva secretion by salivary glands by SARS-CoV-2. A.** Saliva flows
475 were collected as described in the materials and methods section. The data shown represent
476 the saliva flow rate (ul/gram). The mice were randomly selected for saliva collection at the
477 endpoint (control/uninfected n=8 and infected n=8). To minimize the exposure of working in
478 BSL-3 mouse colony, the smaller number of mice was chosen for saliva collection. **B.** The
479 mean percentage loss of saliva flow rates (SFR) in comparison to the control mice, which was
480 set at 100. **C.** The weight of the mice in grams (control n=11, infected n=8) Data were
481 presented as mean \pm SEM. One-tailed Mann-Whitney t-tests were performed where *** p<
482 0.001, ns: not significant.

483
484 **Figure 2: Autoantibody profile of mouse sera. A)** ANA profile was determined using HEp2
485 cells. A Chi-squared test was performed on the control (n=10, 5 females, 5 males) and SARS-
486 CoV-2 infected samples (n=13, 6 males, 7 females), with a value of 32, p <0.00001. Sera were
487 diluted as described and positive signals were detected at 1:40-1:320 titers. **B)** Anti-SSB/La,
488 anti-SSA/Ro52, and anti-SSA/Ro60 were determined using ELISA. Welch's t-test was
489 performed to determine the significance of these results, where ***p= 0.0003, ns: not significant.

490
491 **Figure 3: Histological examination of lymphocytes in the salivary and lacrimal glands. A)**
492 H&E staining of the salivary and lacrimal glands of the control and SARS-CoV-2 infected mice.
493 Inlets with red arrows showed the apoptotic/necrotic acinar cell bodies (control, n=5 females:
494 infected, n=26, 13 males, 13 females). **B)** Elevated number of apoptotic cells in the salivary and
495 lacrimal glands of the infected mice. **C)** Increase in the frequency of macrophage in salivary
496 and lacrimal glands of the infected mice. Enumeration of immune cells using
497 immunofluorescent staining. Identification of infiltrating cells in the salivary glands and lacrimal
498 glands, where immunofluorescent staining of CD3⁺ T cells, B220⁺ B cells and CD68⁺

499 macrophages are displayed in yellow, red, and green, respectively, with blue DAPI nuclei
500 staining. One-tailed Mann-Whitney t-tests were performed where ** $p < 0.01$, *** $p < 0.001$ **** $p <$
501 0.0001 . Con: Control, SG: salivary glands, LG: lacrimal glands.

502
503 **Figure 4: Autoantibody induction in COVID-19 human sera.** A) ANA profile was determined
504 using HEp2 slides at various sera titers. B) A breakdown by specific ANA staining pattern is
505 presented. C) Anti-SSB/La, anti-SSA/Ro52, and anti-SSA/Ro60 were determined using ELISA
506 Welch's t-test was performed to determine the significance of these results, where ** $p = 0.0015$
507 and * $p = 0.0415$.

508
509 **Figures 5: Representative minor salivary glands H&E photomicrographs of health (HV),**
510 **Sjogren's Disease (SjD), and two representative subjects recovered from COVID-19.**
511 Convalescent glands exhibit a range of inflammation severity ranging from normal to mild-to-
512 moderate sialadenitis with focal lymphocytic sialadenitis reminiscent of inflammation found in
513 SjD. The histopathological findings from three patients (P2 & P5) exhibit inflammation consistent
514 with findings observed in SjD salivary glands (e.g., focal lymphocytic sialadenitis with focus
515 scores >1.0). However, P1, P3, and P6 exhibited FLS but did not reach the threshold of >1.0
516 focus per 4mm^2 of tissue.

517
518 **Figures 6: Representative immunophenotyping studies examining CD3, CD4, CD8, and**
519 **CD20 on a minor salivary gland biopsy during infection (D5 post first symptom; FS: 1)**
520 **and post (6 Months) COVID-19 infection (P2).** Immunophenotyping demonstrates diffuse
521 mild-to-moderate chronic sialadenitis with focal lymphocytic sialadenitis. The infiltrate is
522 strikingly similar to infiltrates found in SjD with a predominant T cell and B cell in the ducts and
523 acini associated with inflammation.

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

Sub.	Sex	Patient type	Histopathological Diagnosis	Focus Score ^a	Fibrosis/ Atrophy	Other Features	Oral Symptoms	Oral Signs	Ocular symptoms	Ocular Signs	COVID-19 Severity	Biopsy Post COVID-19
1 ^d	F	Conv.	FLS with Mild Chronic Sialadenitis	0 ^b	N		N	N/A	N	NA	Mild	8
2 ^d	F	Conv.	FLS with Mild Chronic Sialadenitis	1	Y	Duct injury and dilatation	Y	Y	N/A	N/A	Mild	0 ^c
			FLS with Mild-to-Moderate Chronic Sialadenitis	2	Y	GC	Y	N	N/A	N/A		6
3 ^d	M	Conv.	FLS with Mild Chronic Sialadenitis	0 ^b	Y	Inflam. infiltrating acini	Y	N/A	N/A	N/A	Mild-to-Moderate	13
4 ^e	F	HV	Mild Chronic Sialadenitis	0	N		N	N	N	Y	Mild-to-Moderate	21
5 ^e	M	HV	FLS with Mild-to-Moderate Chronic Sialadenitis	2	N	GC, granuloma	N	N	N	N	Asymp.	UNK
6 ^e	M	HV	FLS with Mild Chronic Sialadenitis	0 ^b	N	Perivascular infiltrates	Y	N	N	Y	Mild-to-Moderate	7
SjD ^e	F	SjD	FLS with Mild-to-Moderate Chronic Sialadenitis	2	Y		Y	Y	Y	Y		
CTL ^e	F	HV	Normal Histology	0	N		N	N	N	N		

^aThe focus score (FS) is the number of inflammatory infiltrates of at least 50 cells present in 4 mm² of salivary gland area.

^bThe subjects has focal lymphocytic sialadenitis (FLS) but with less than 1 per 4 mm² of tissue. P2 has 8 foci per 37 mm² of tissue (FS: 0.9) and P6 has 5 foci per 25 mm² (FS: 0.8), and thus are borderline. Subject 3 has 4 foci per ~47 mm² (FS: 0.3).

^cThe 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).

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

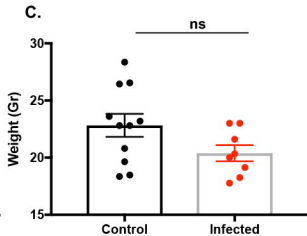
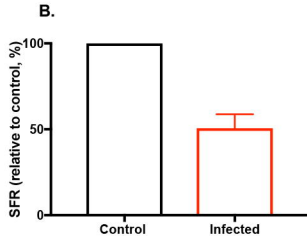
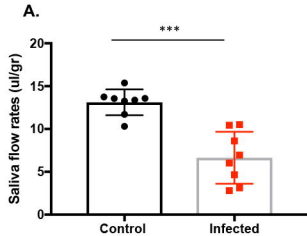
^eThe subjects were enrolled on 15-D-0051 as either affected subjects ("SjD") or healthy volunteers (HV) (Subjects 4-6 and Ex: HV).

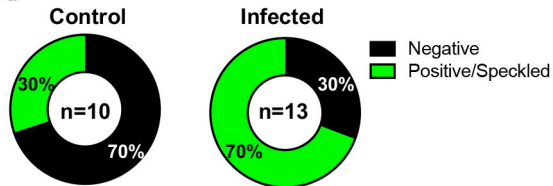
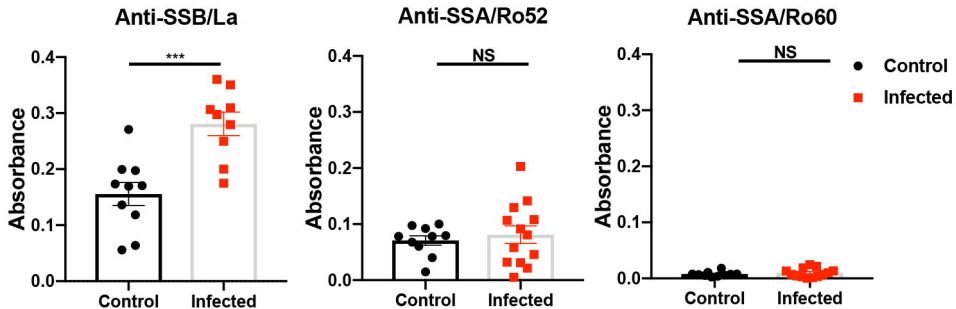
Age range of subjects and comparators was 19-42 years.

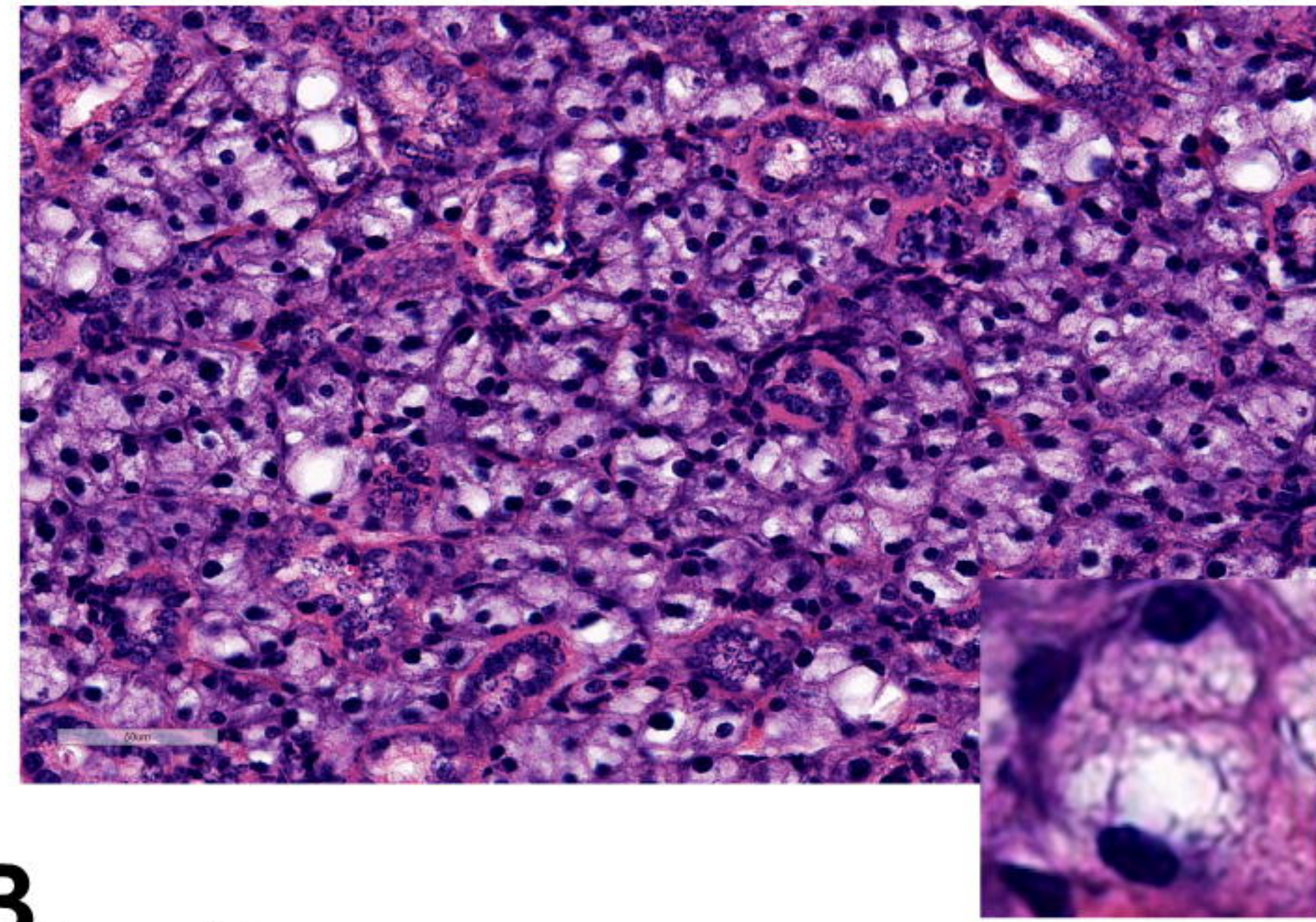
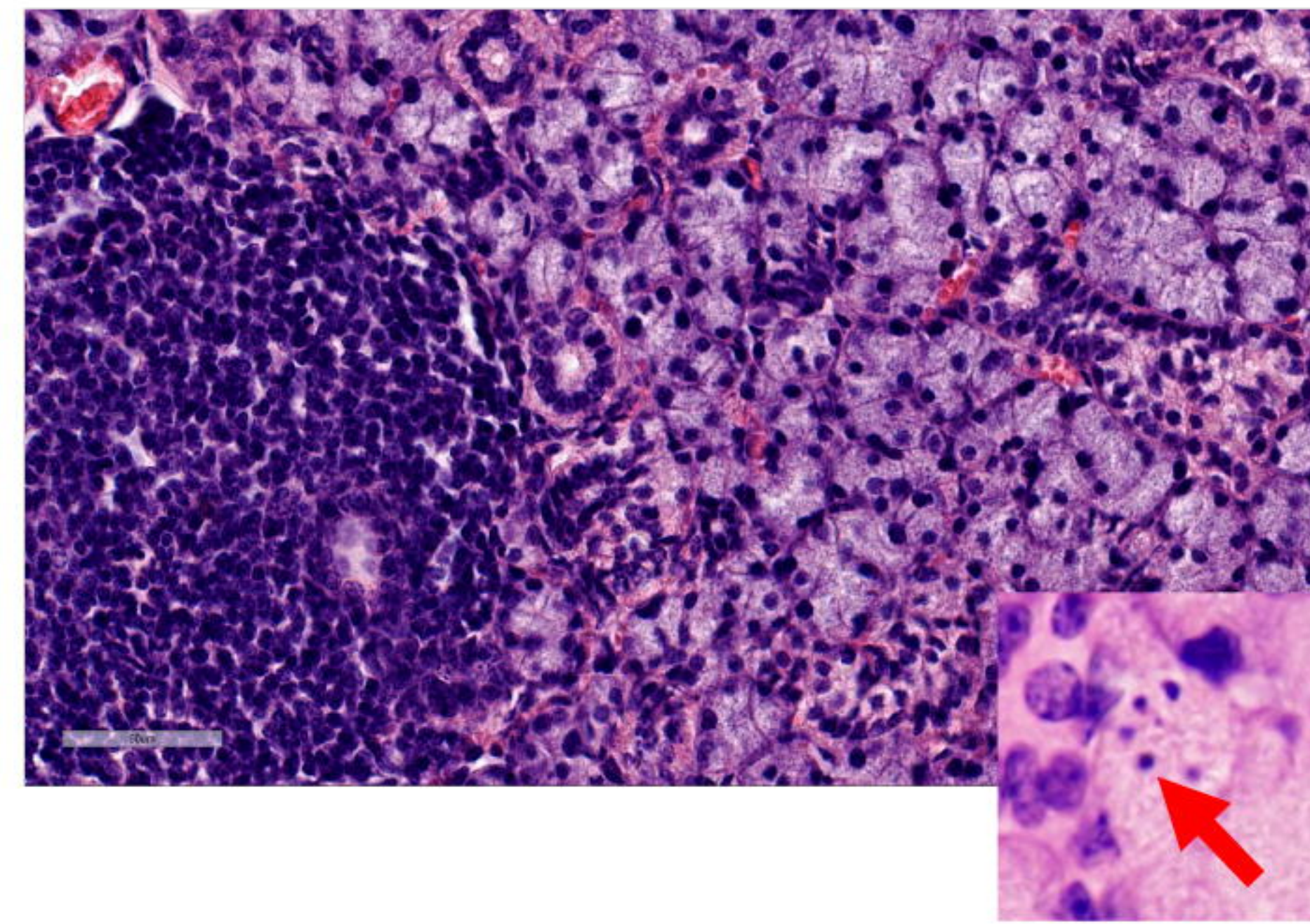
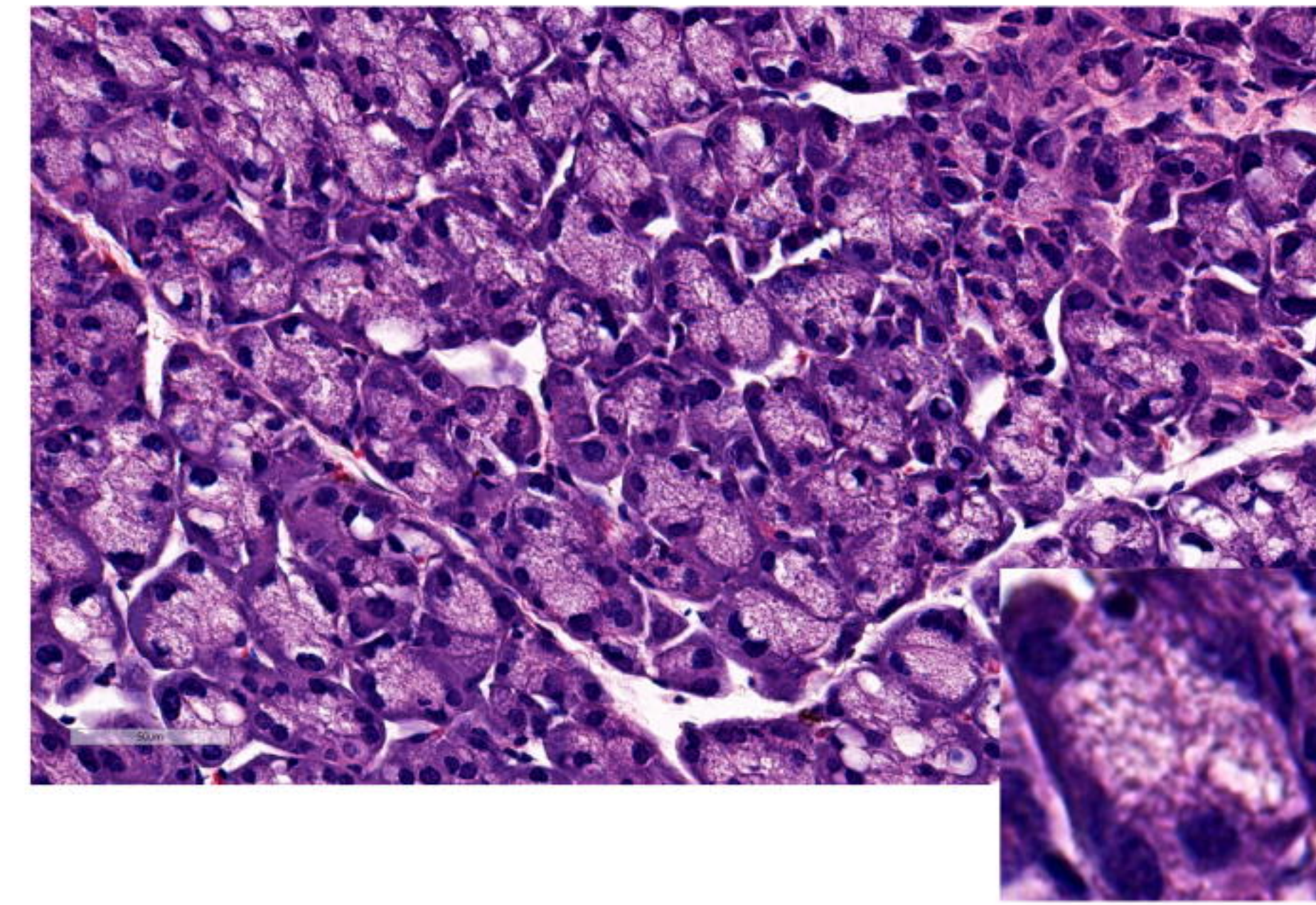
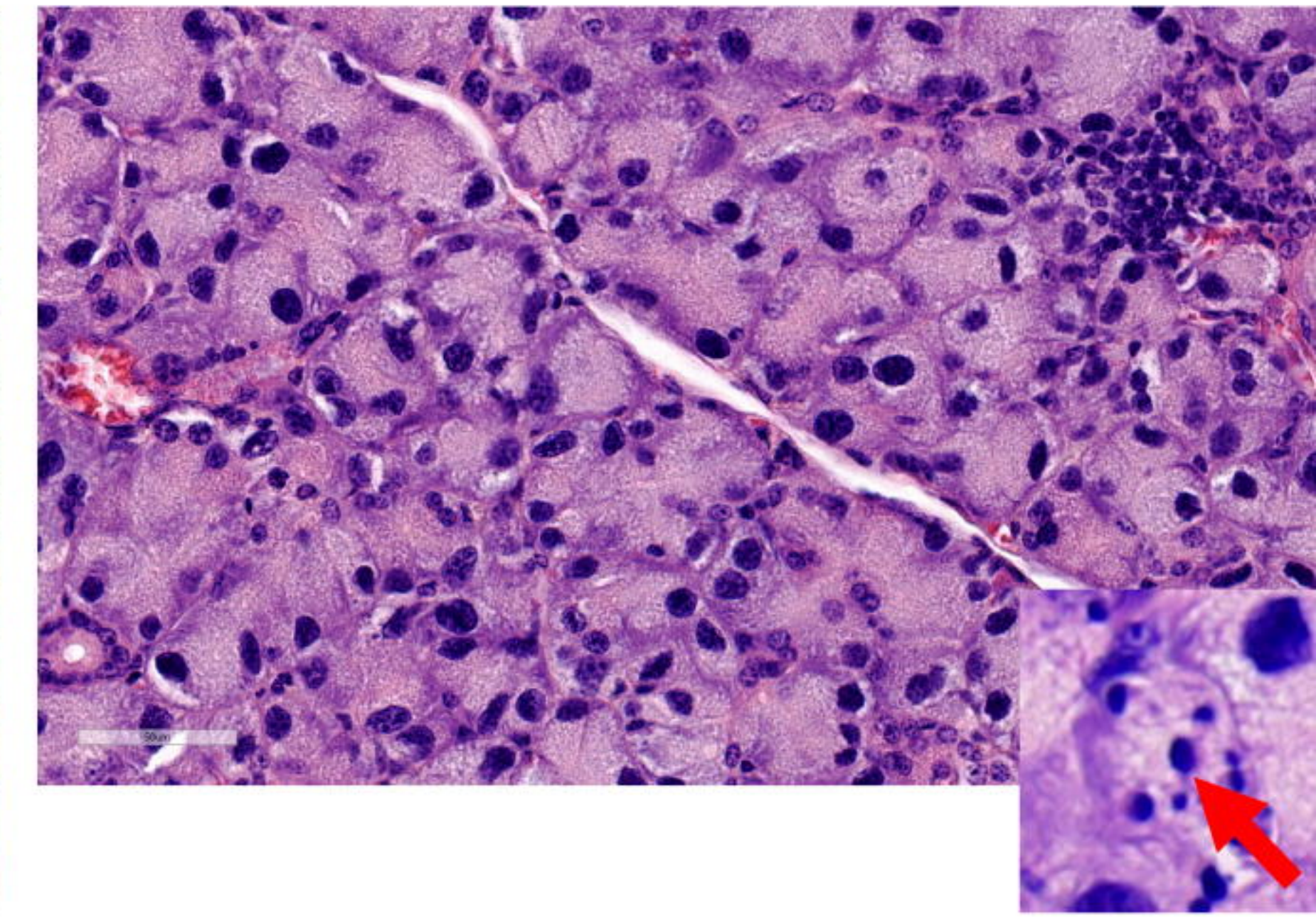
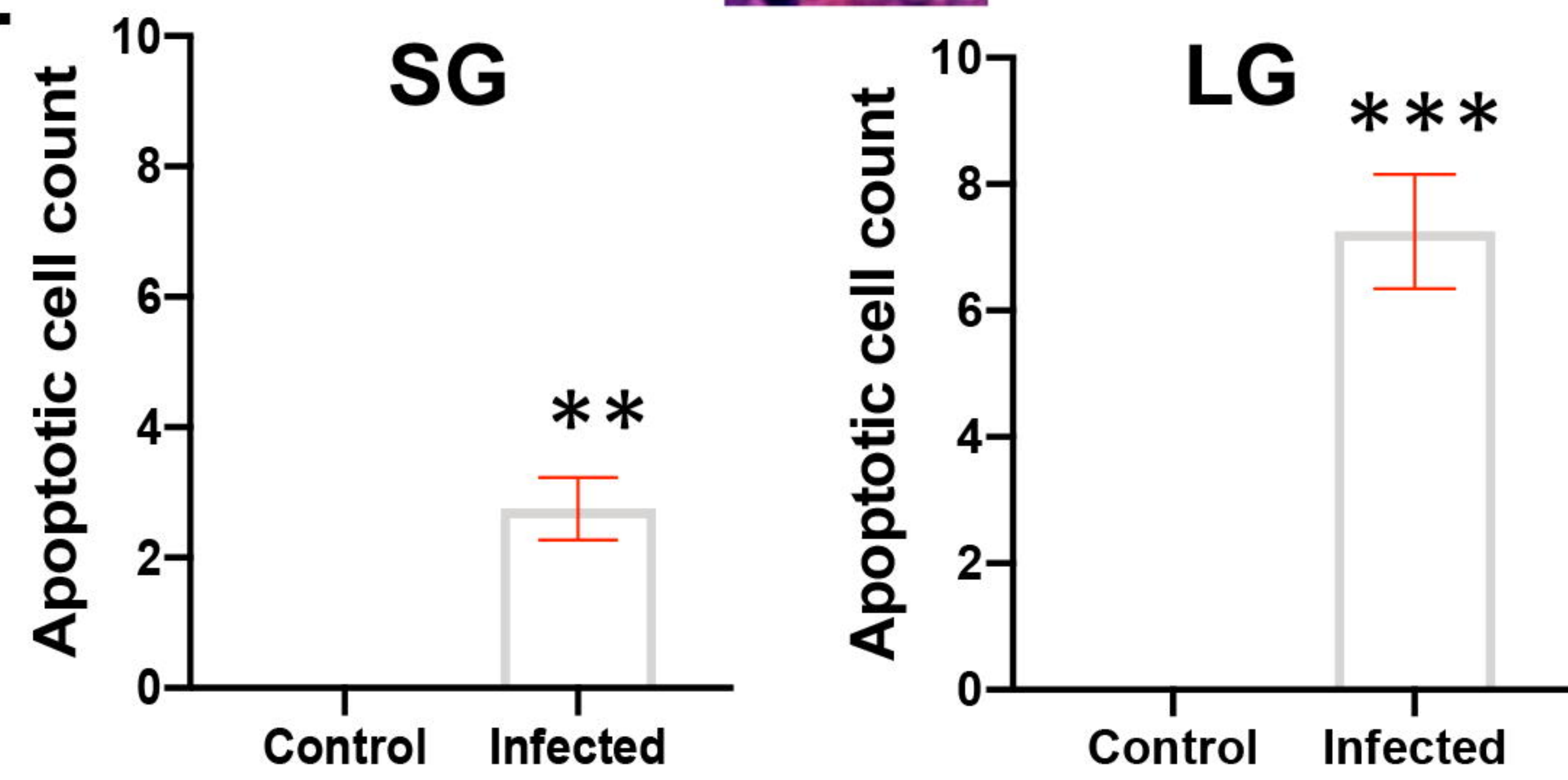
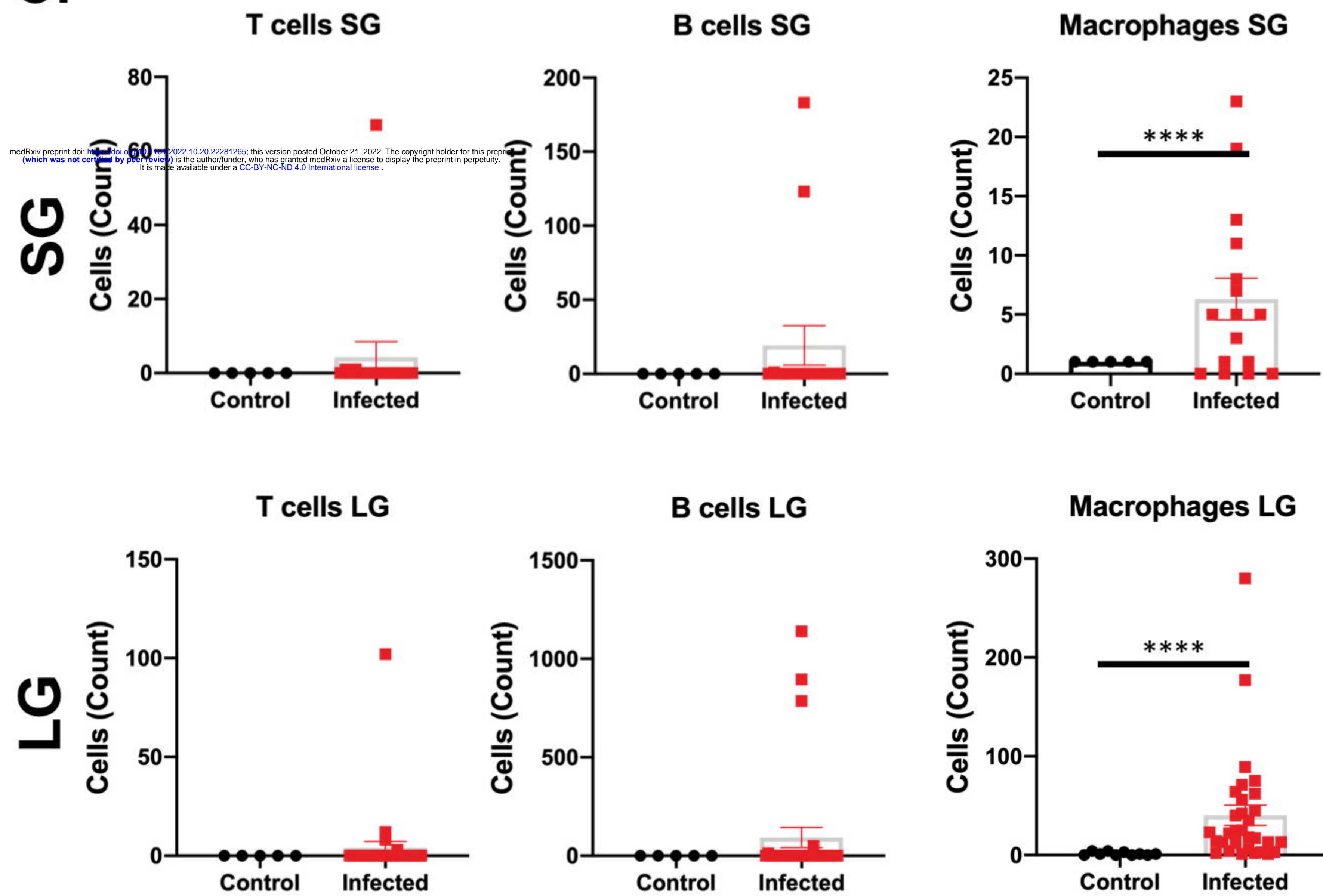
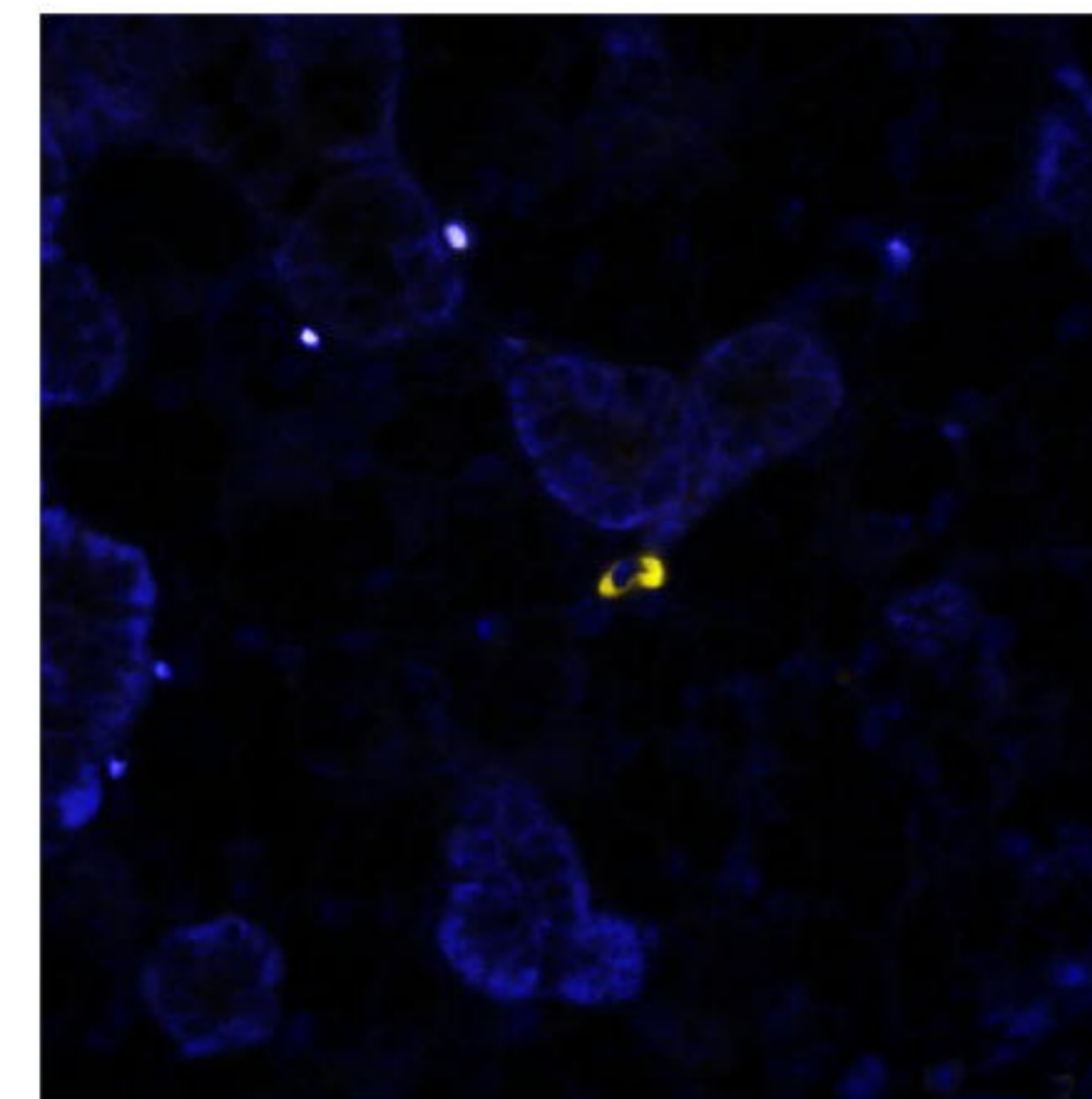
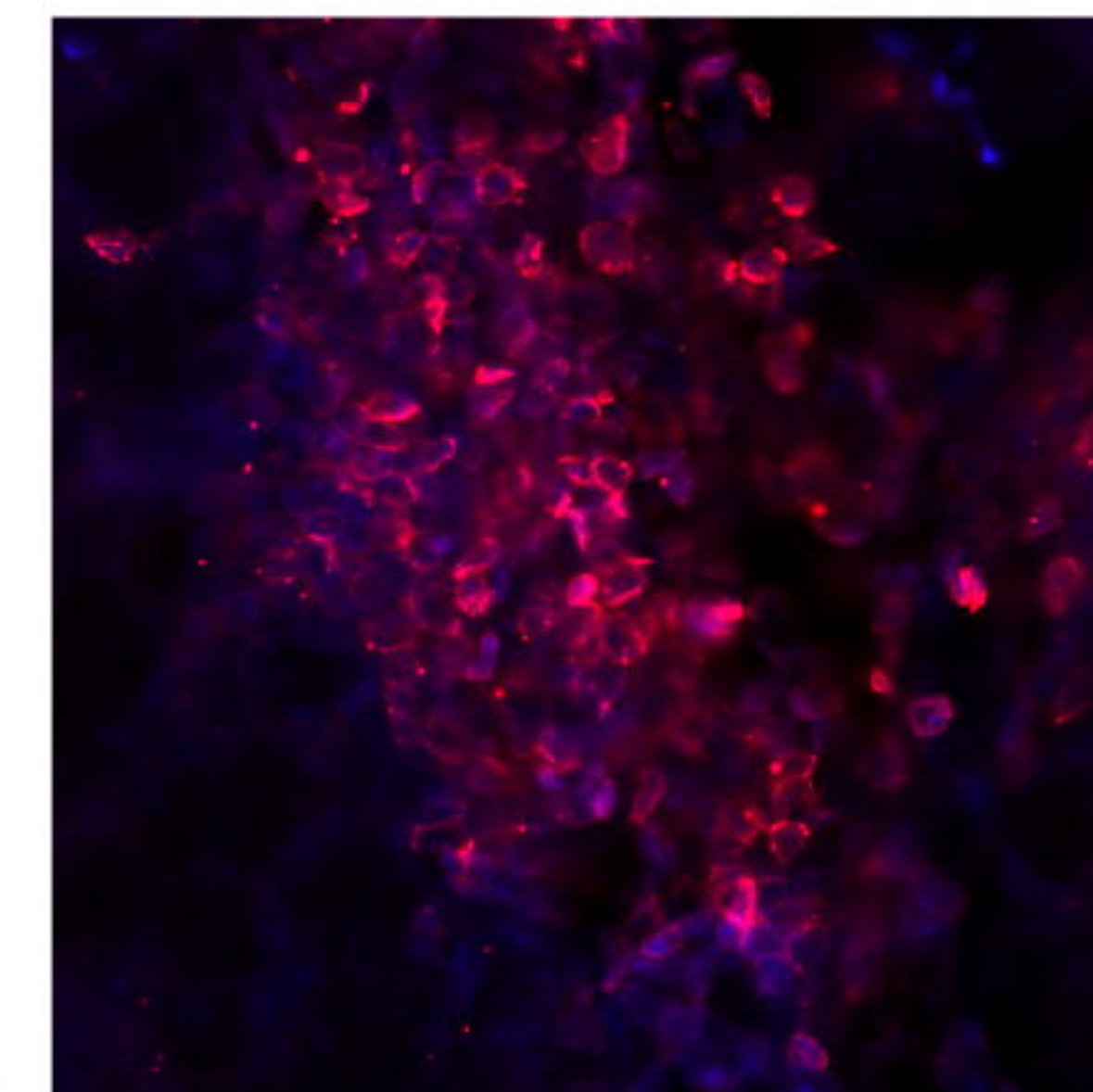
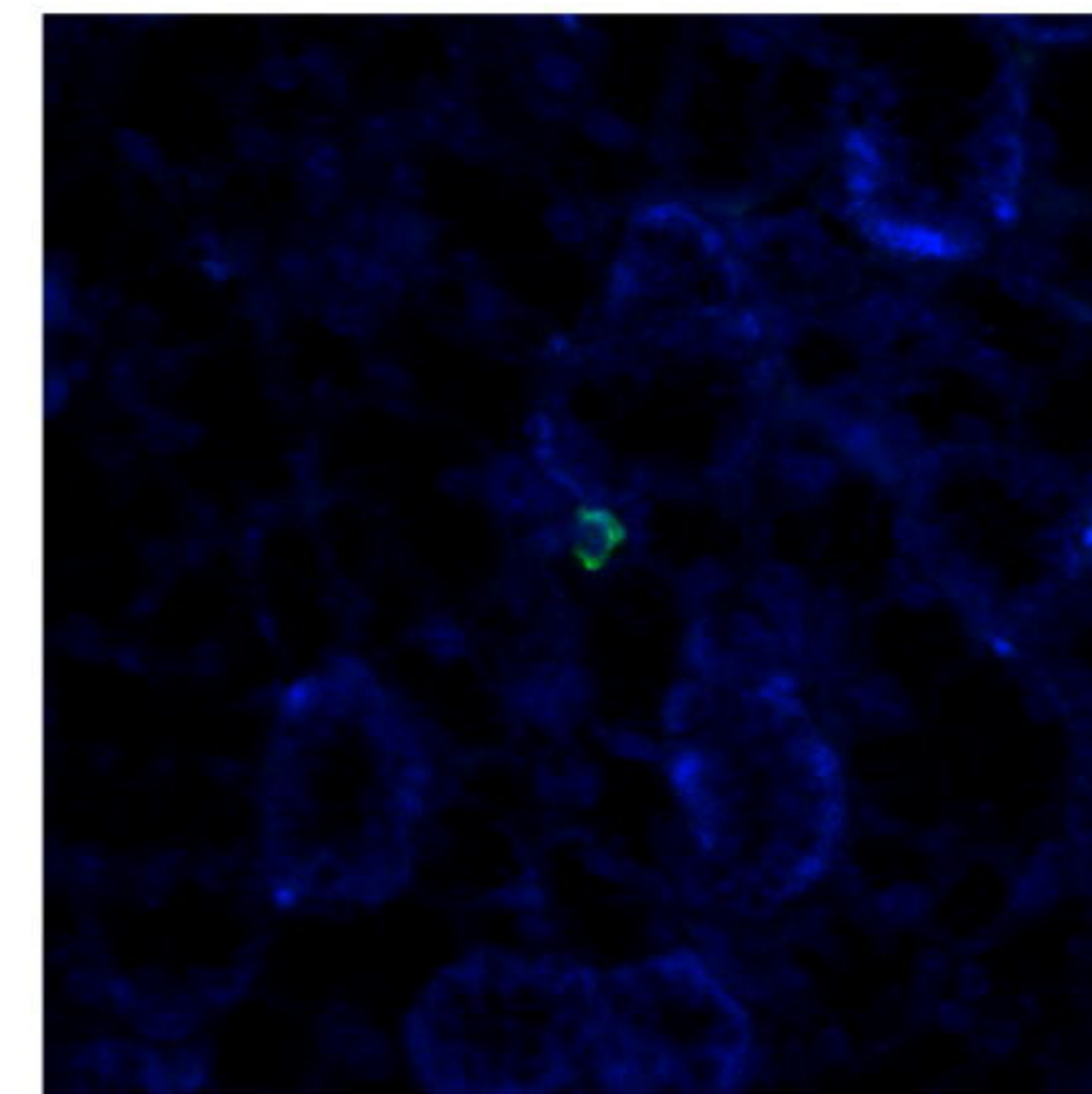
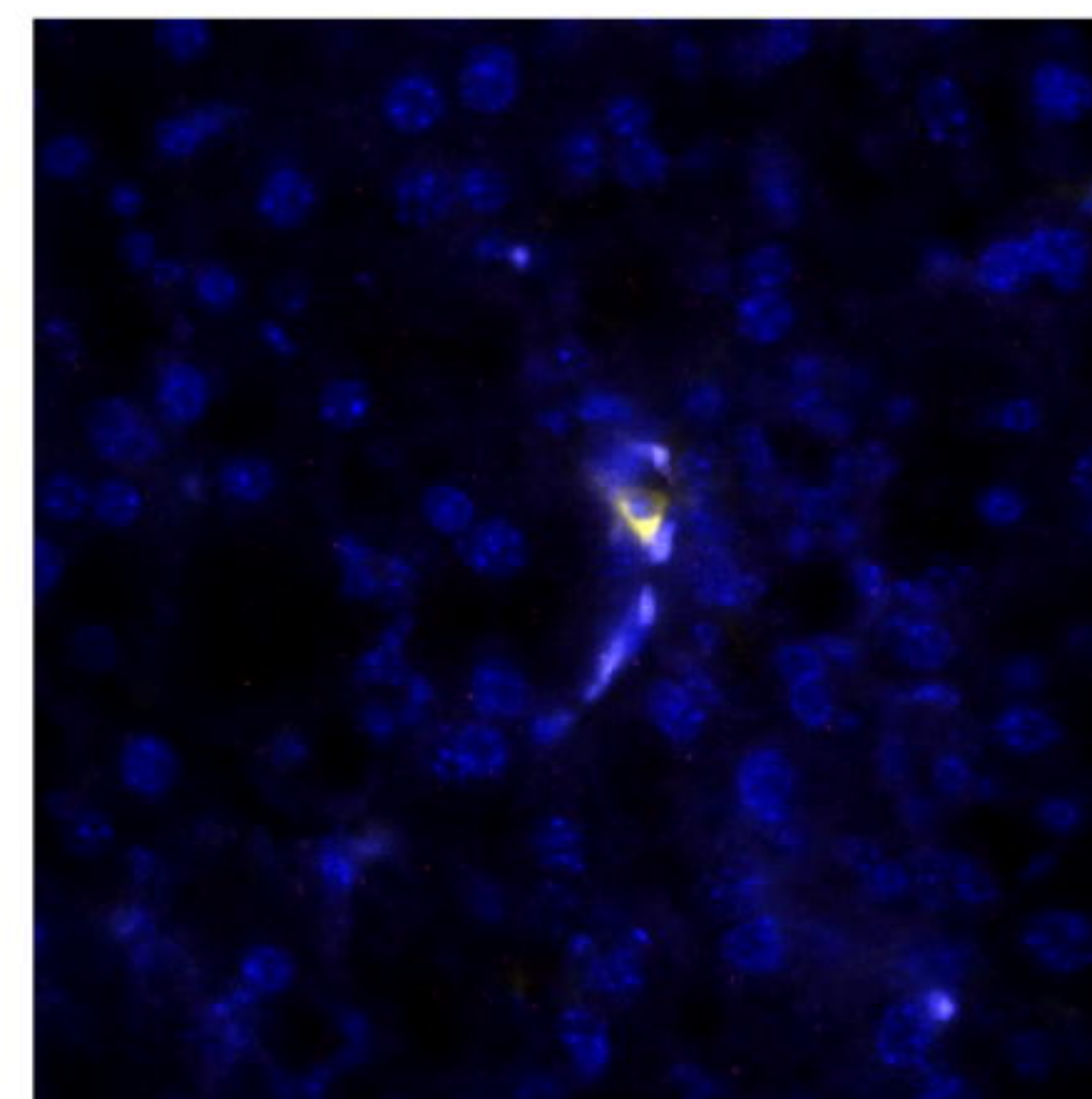
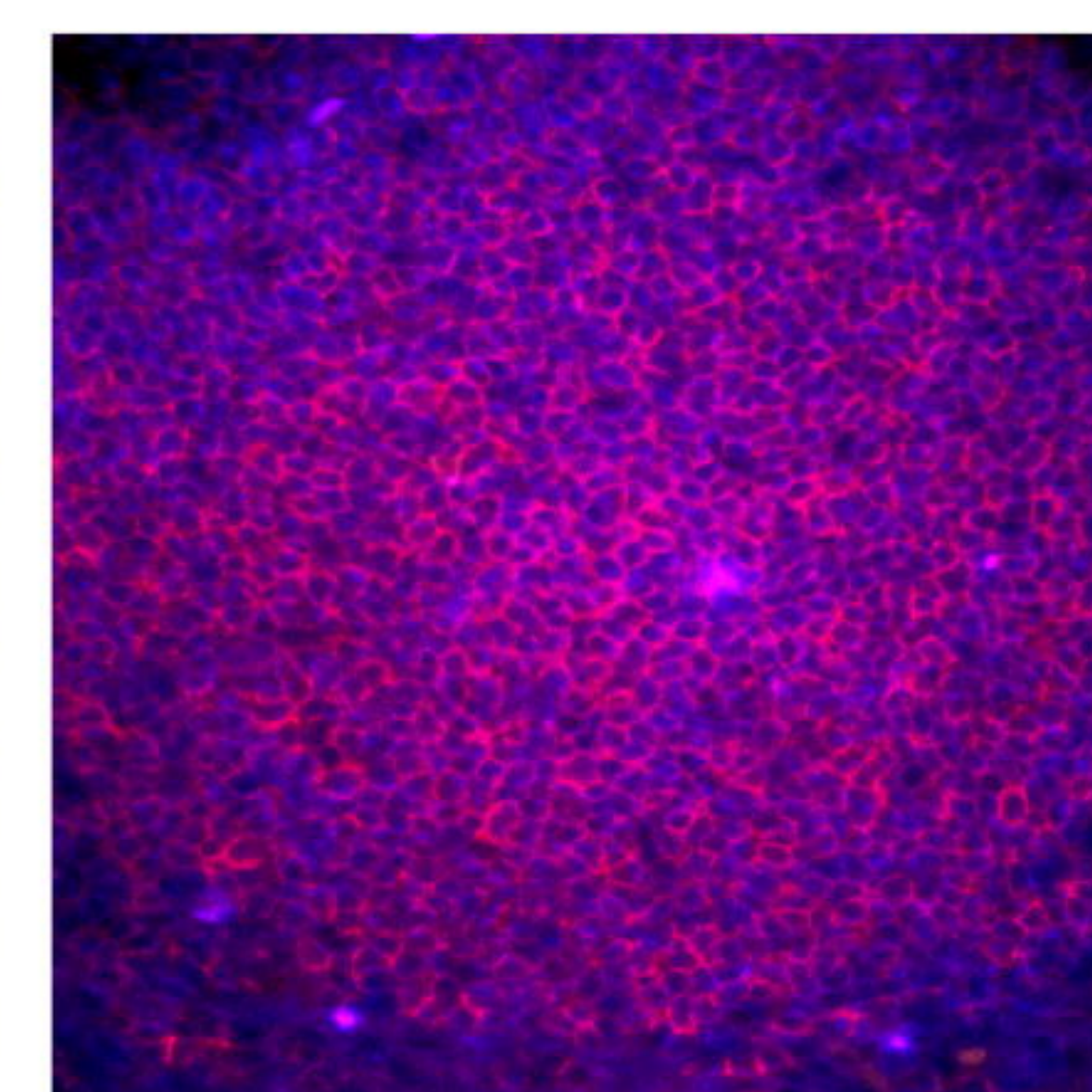
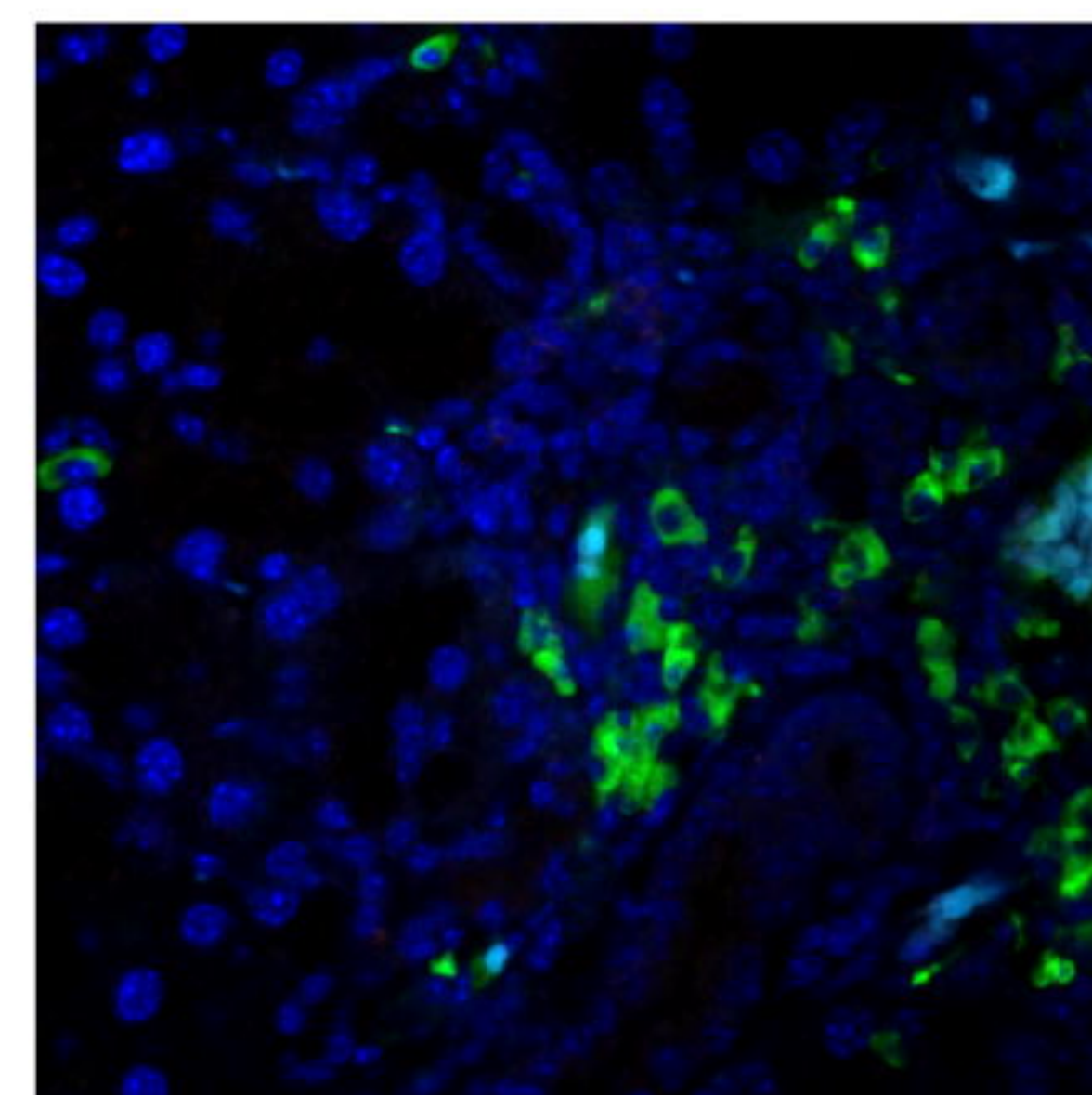
Abbreviations: F, female; M, male; Conv., convalescent; N, no; Y, yes; FLS, focal lymphocytic sialadenitis; GC, germinal center; UNK, unknown; SjD, Sjögren's Disease; HV, healthy volunteer; CTL, control gland from healthy volunteer.

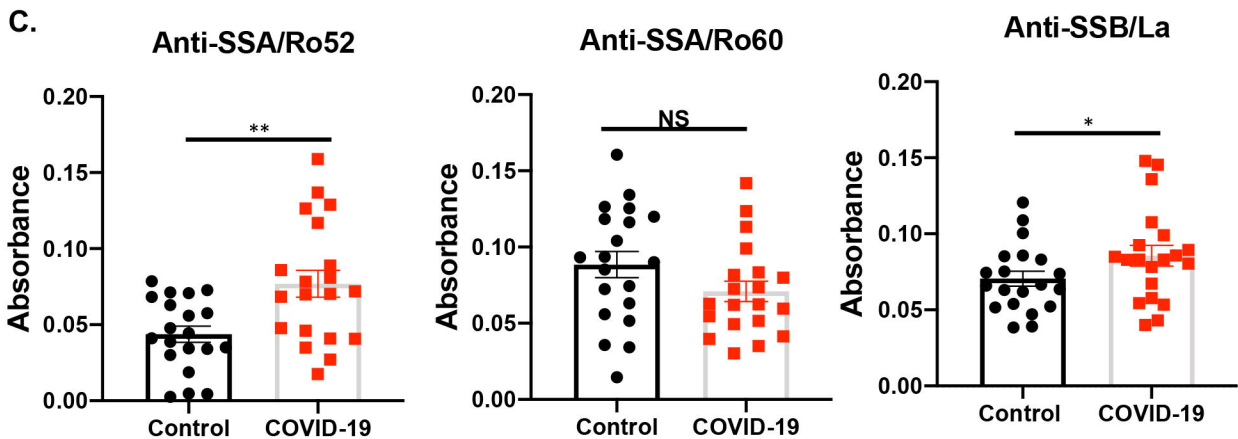
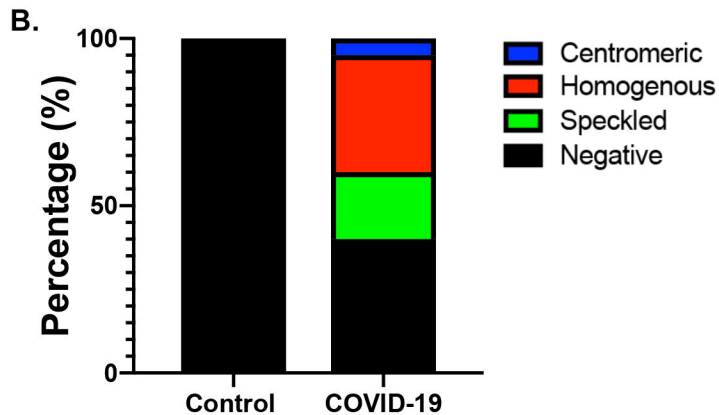
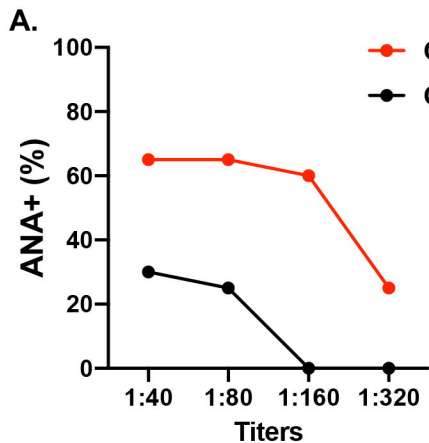
Table 2. Immunophenotyping of convalescent COVID-19 subjects salivary glands.

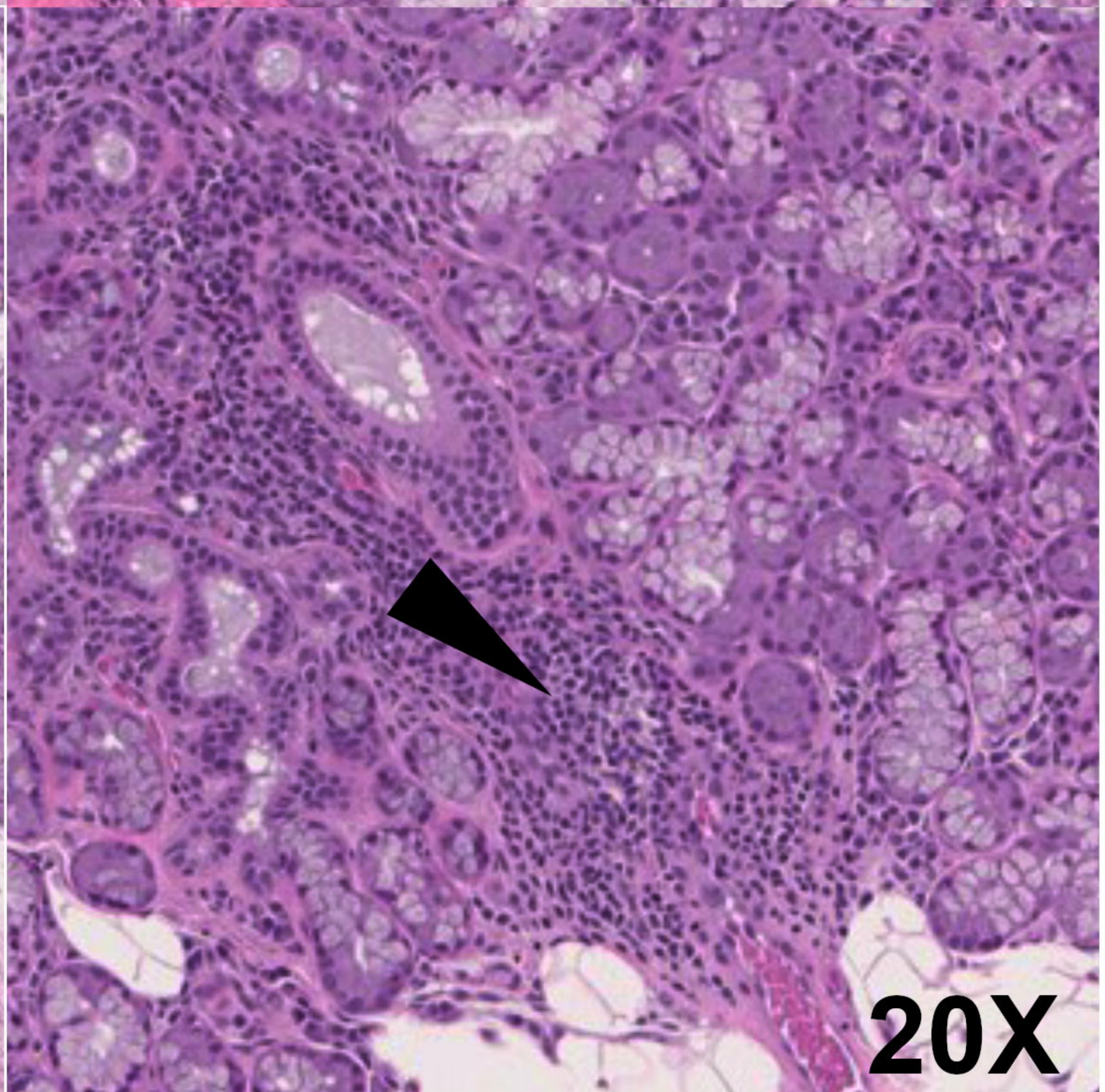
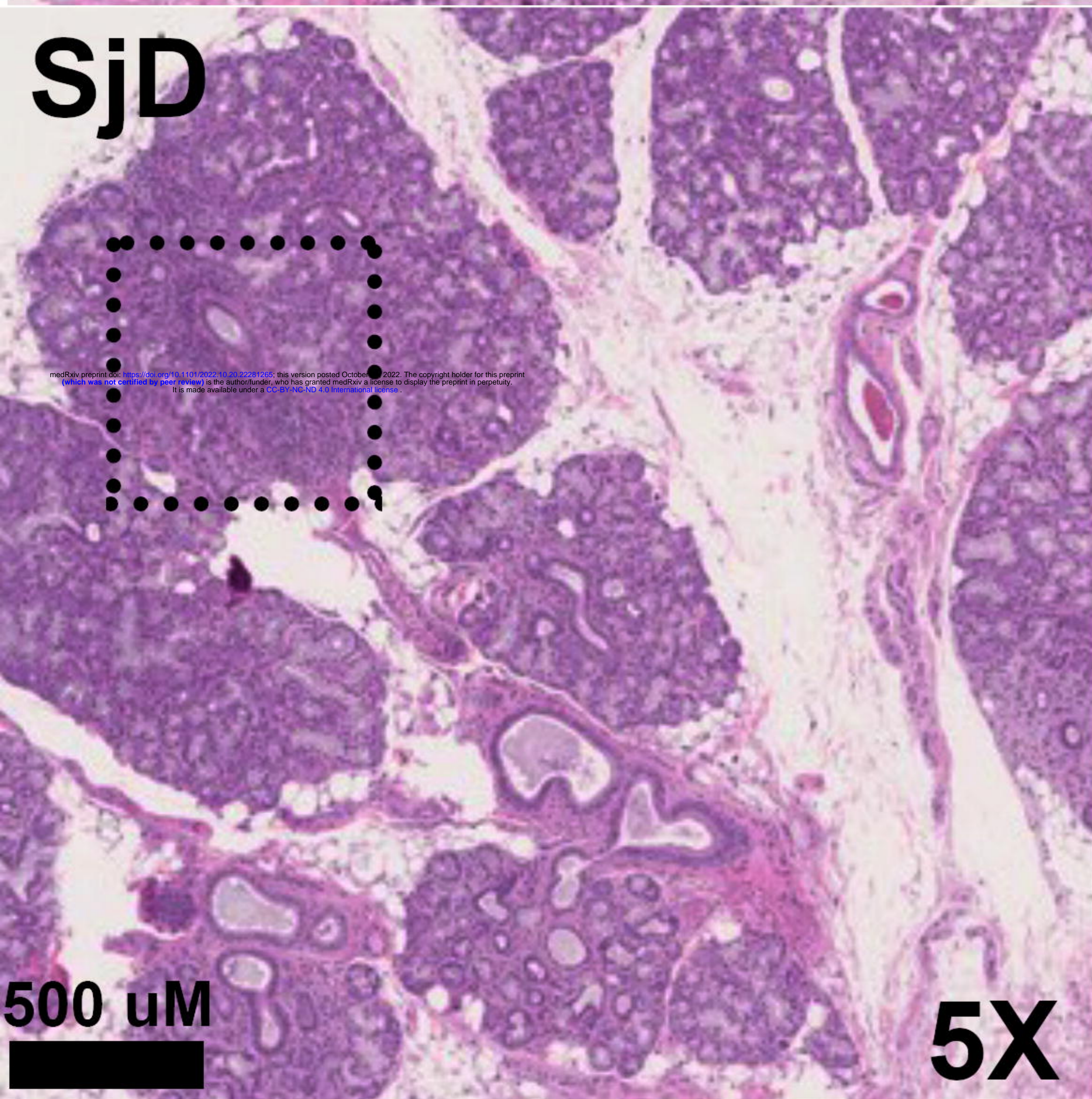
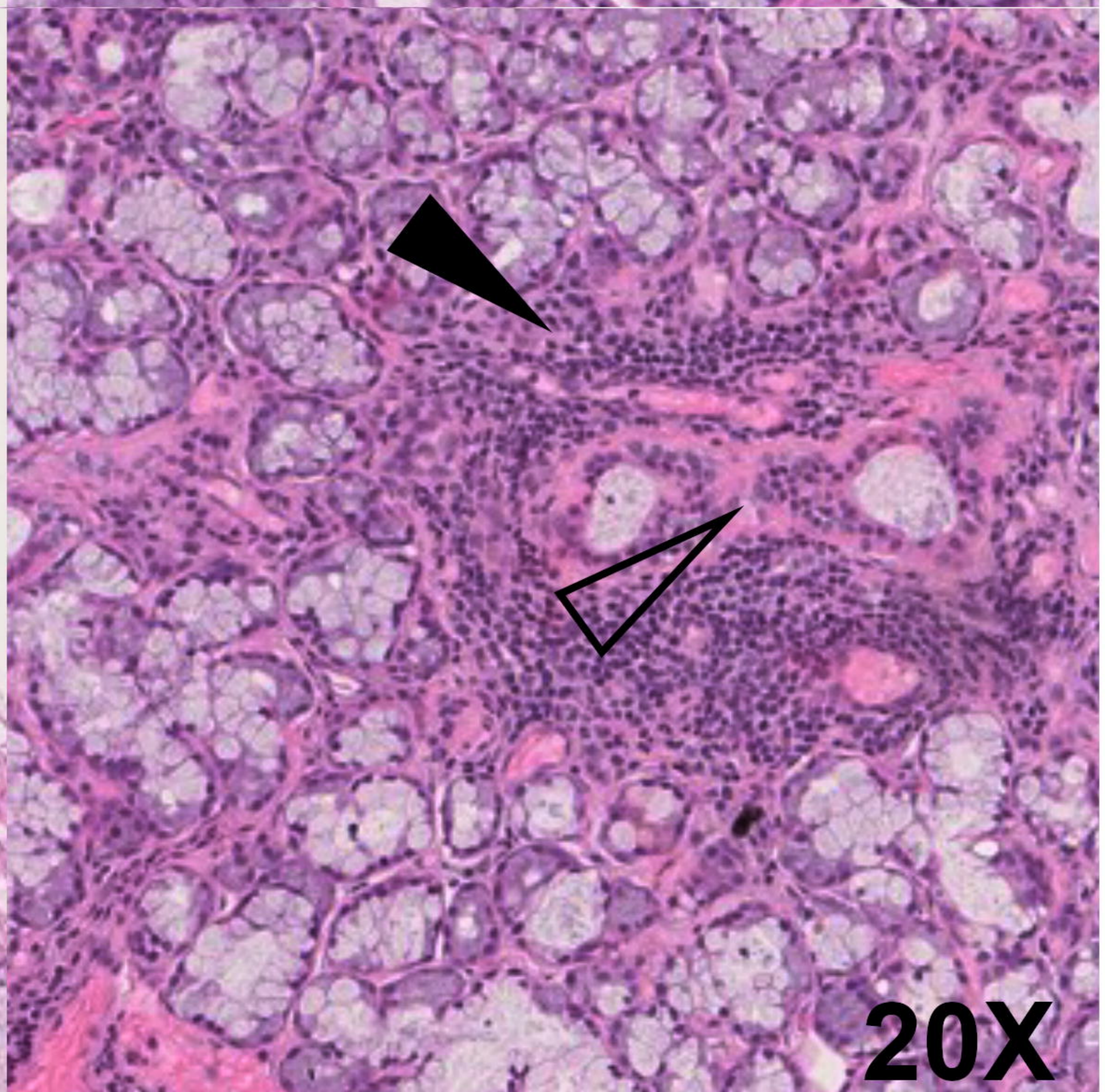
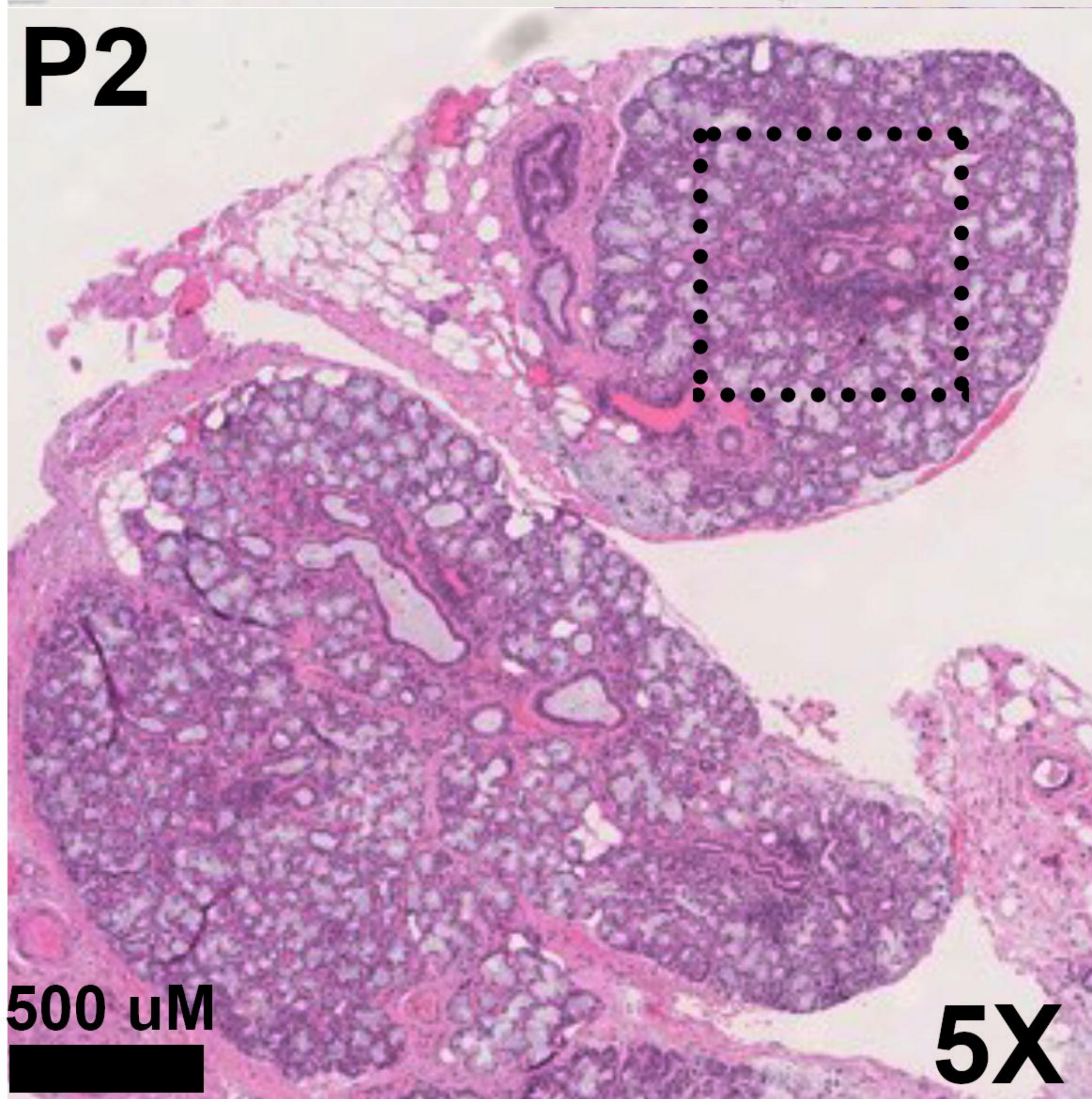
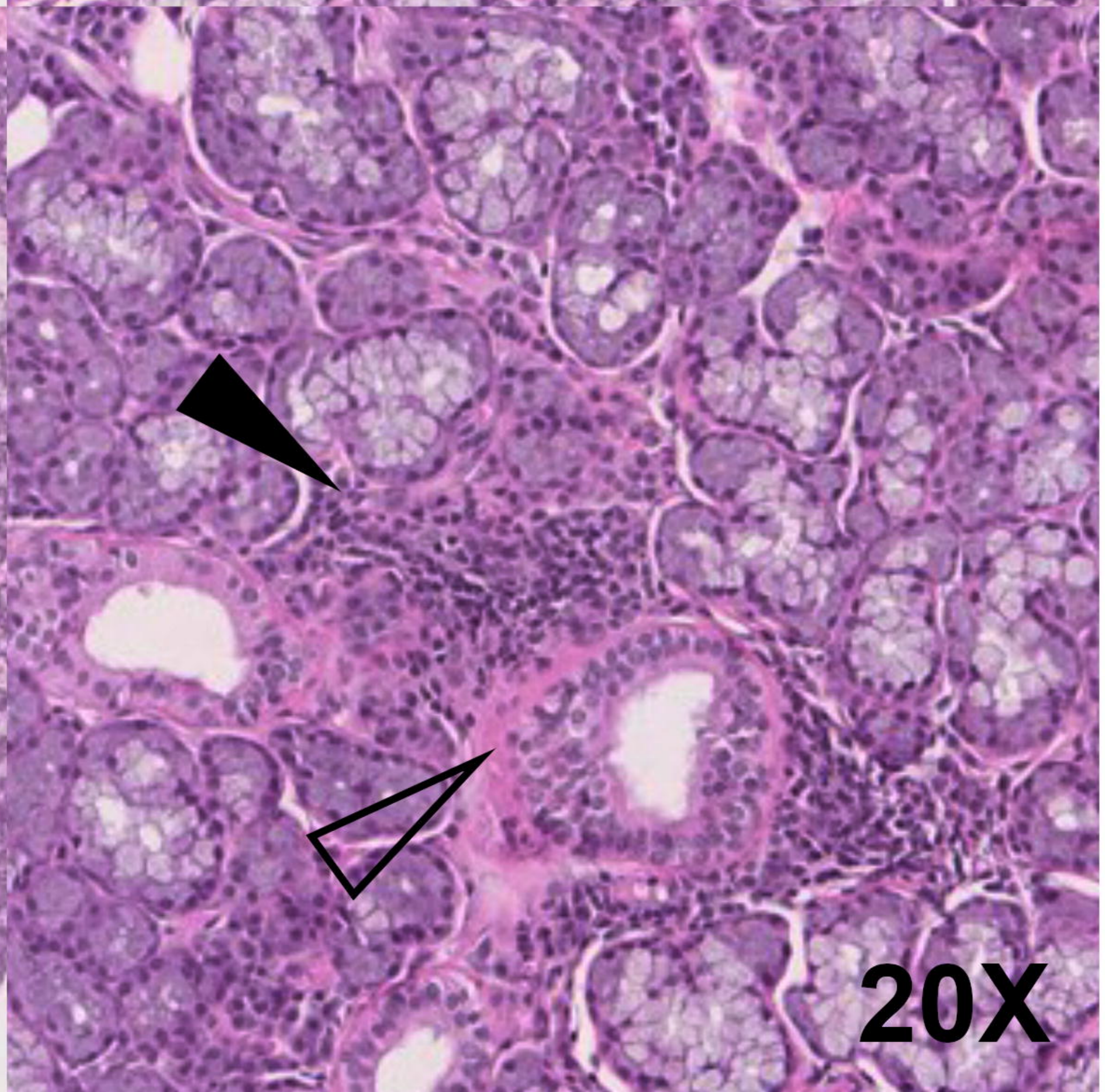
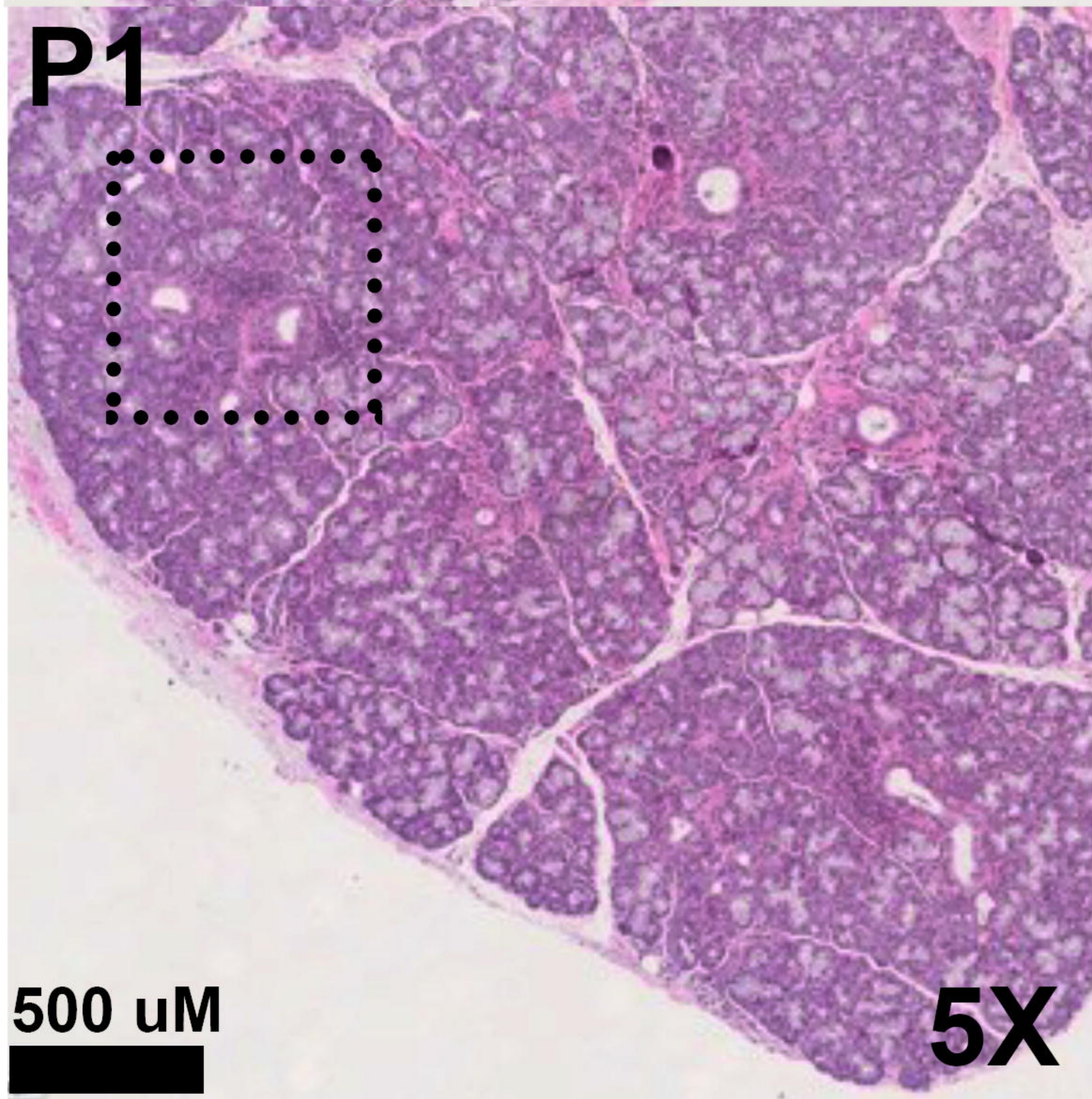
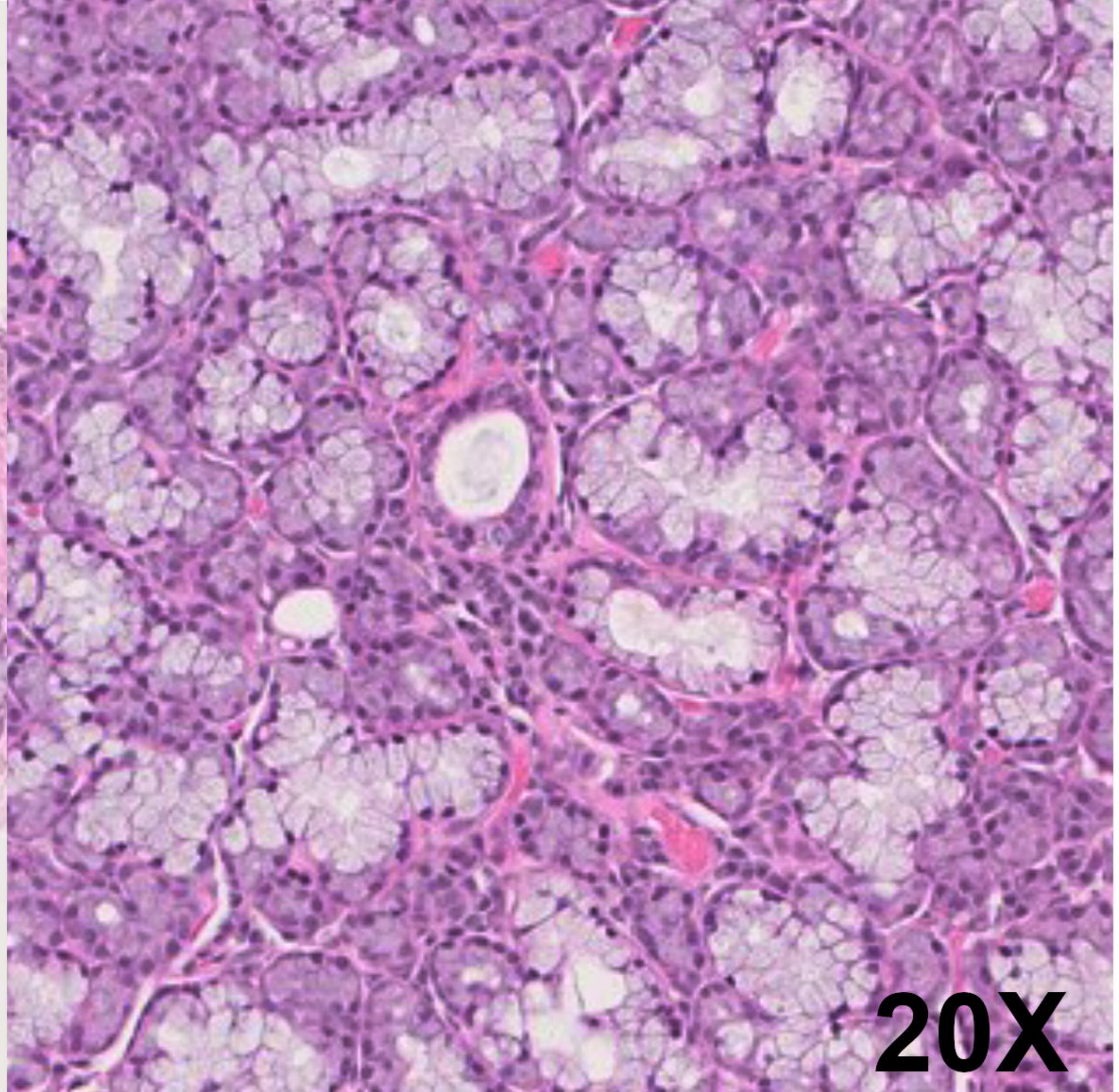
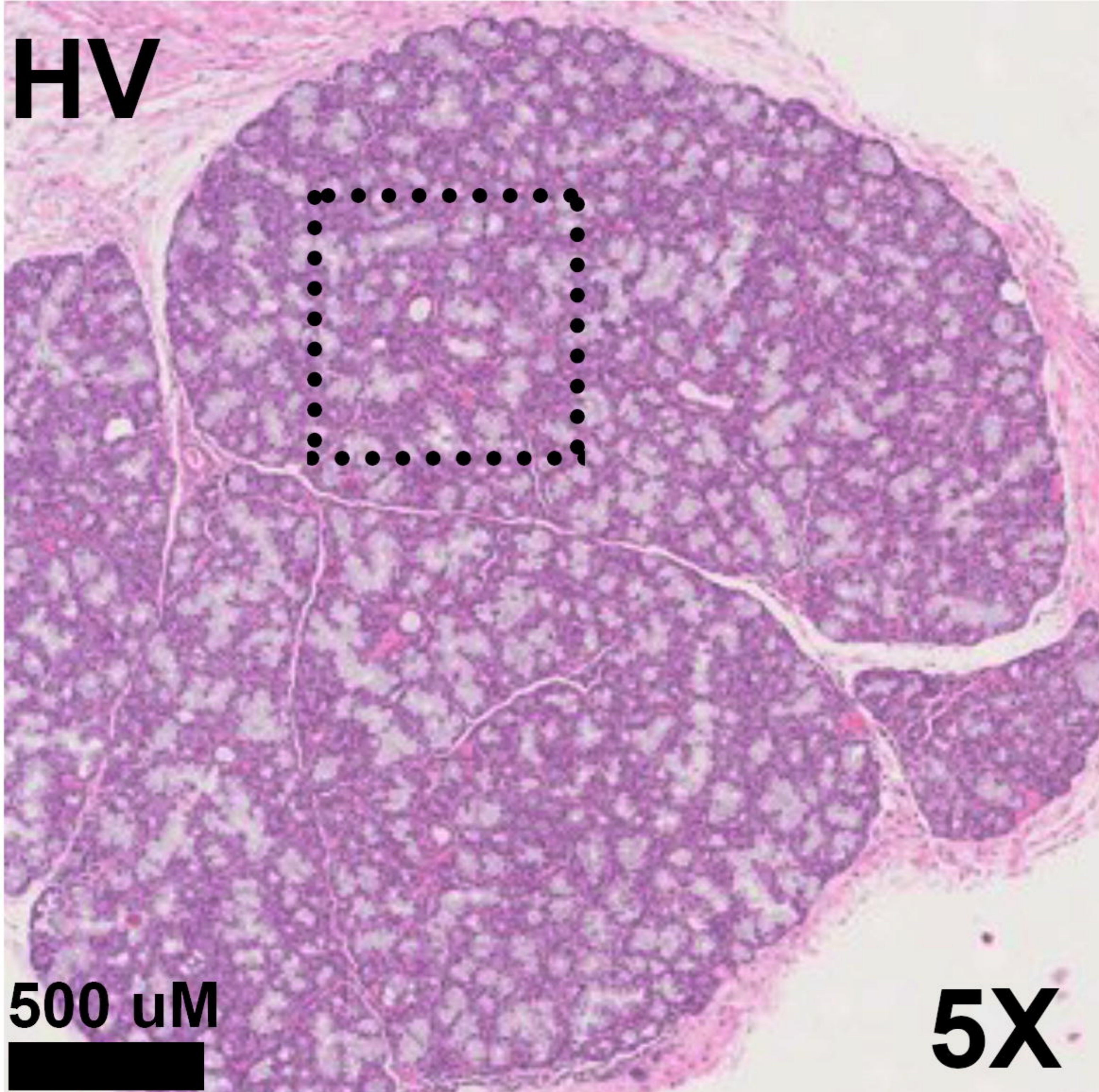
Subject	CD20	CD3	CD4	CD8	CD4:CD8
1	+	++	++	+	CD4>CD8
2-1	+	++	++	+	CD4>CD8
2-2	++	++	++	++	CD4>CD8
3	+	+	+	+	CD4>CD8



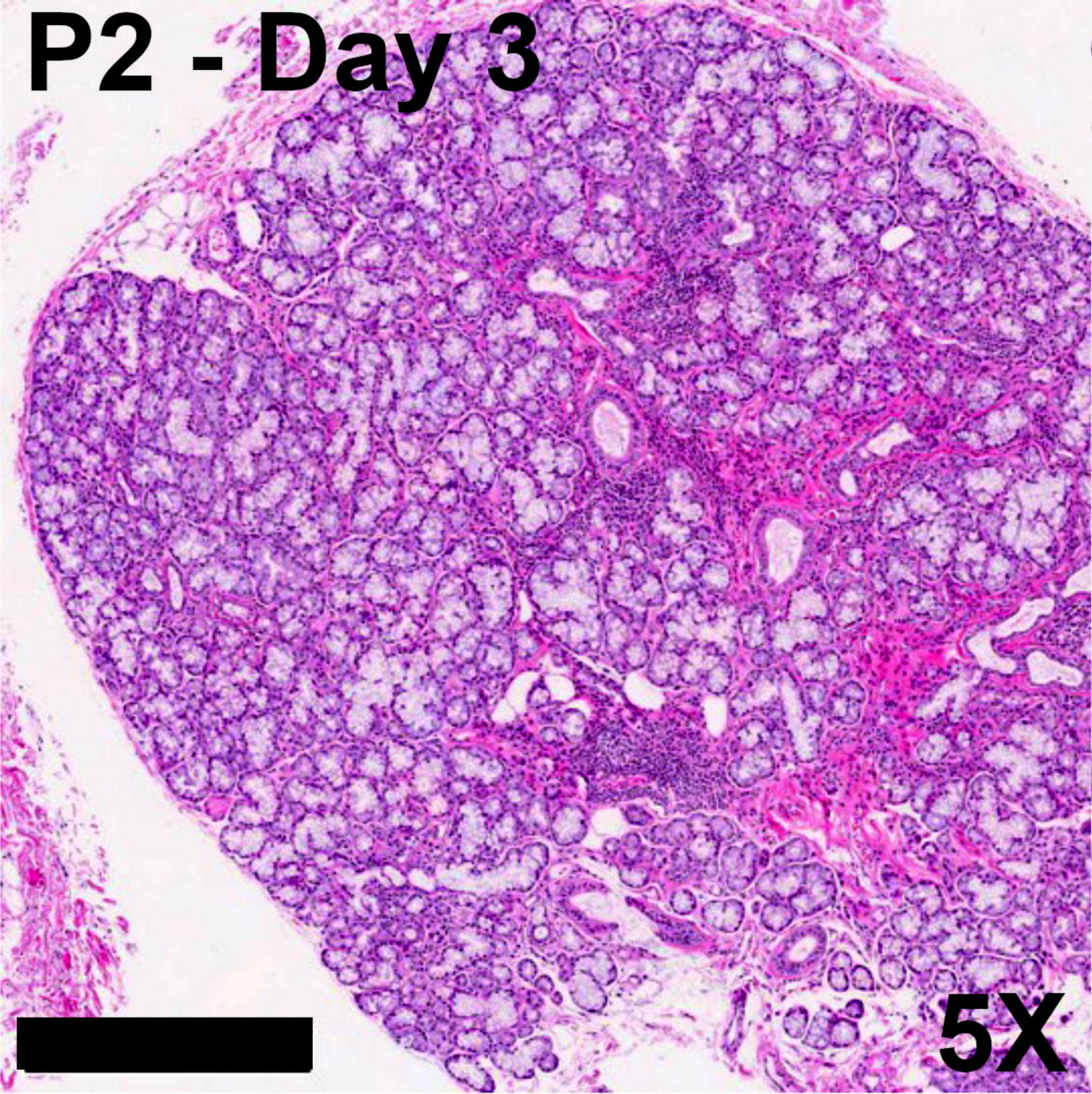
A.**B.**

A.**Con-SG****Infected-SG****Con-LG****Infected-LG****B.****C.****CD3/CD68****B220/CD68****CD68****CD3/CD68****B220/CD68****CD68**

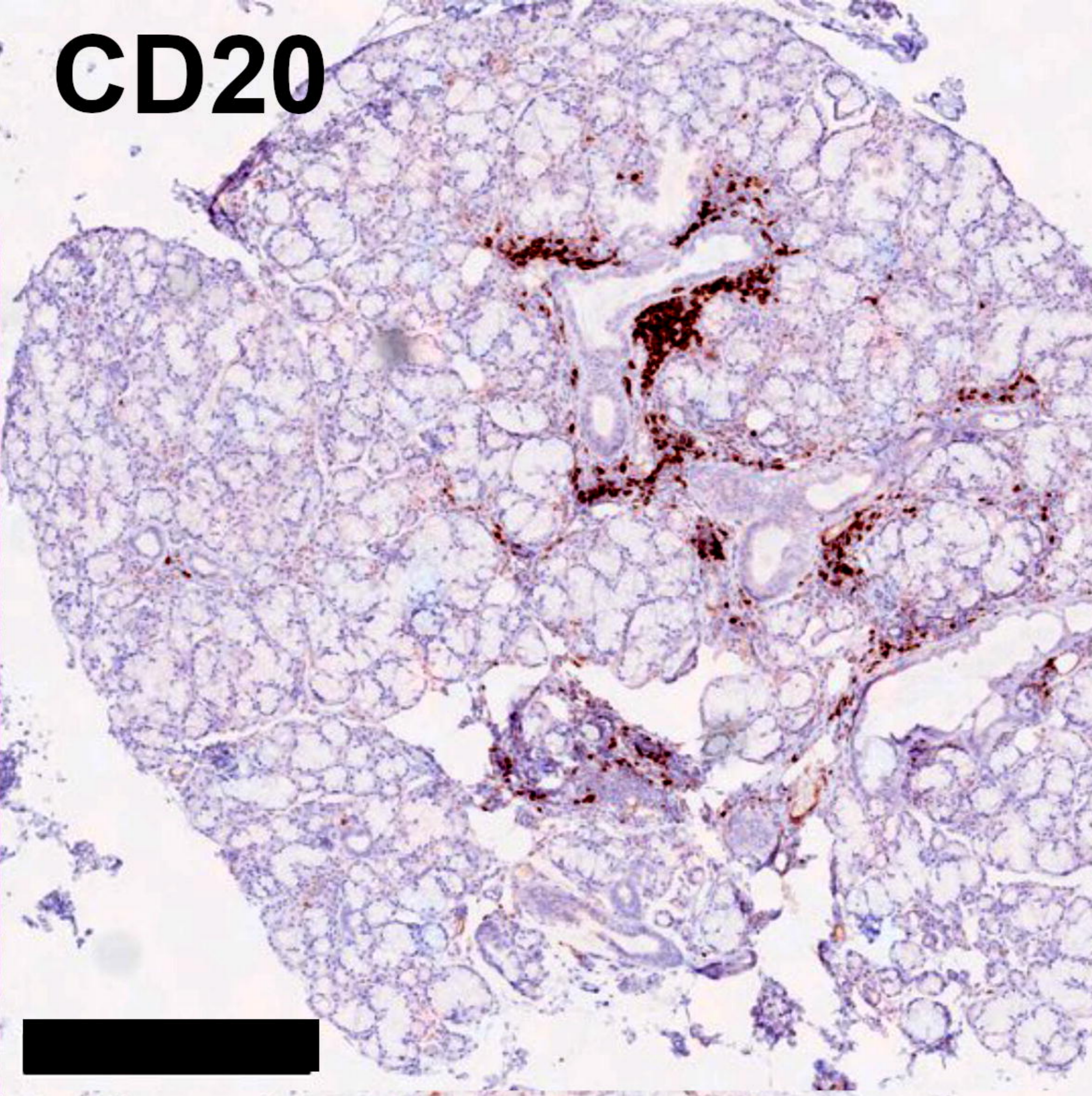




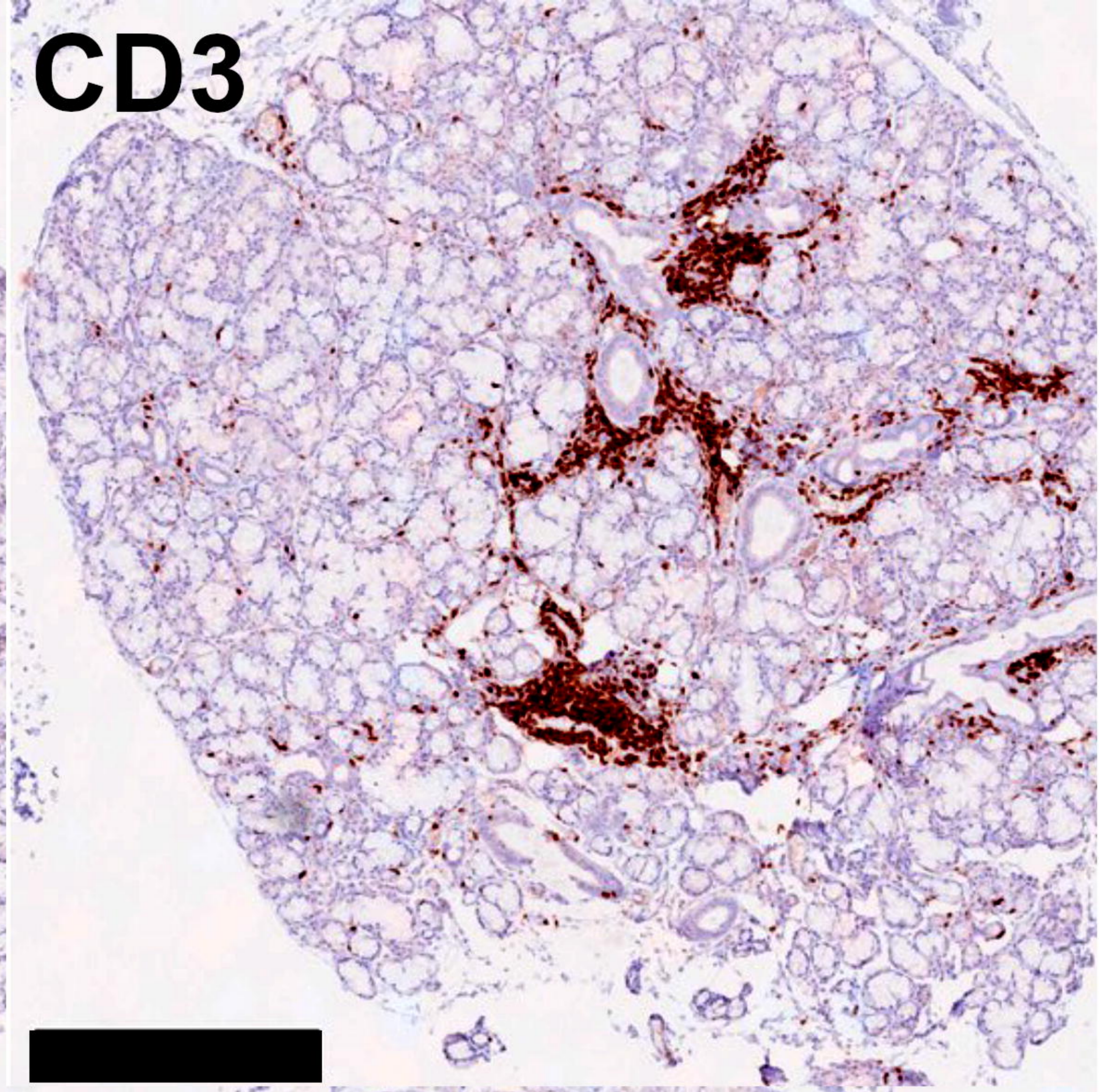
P2 - Day 3



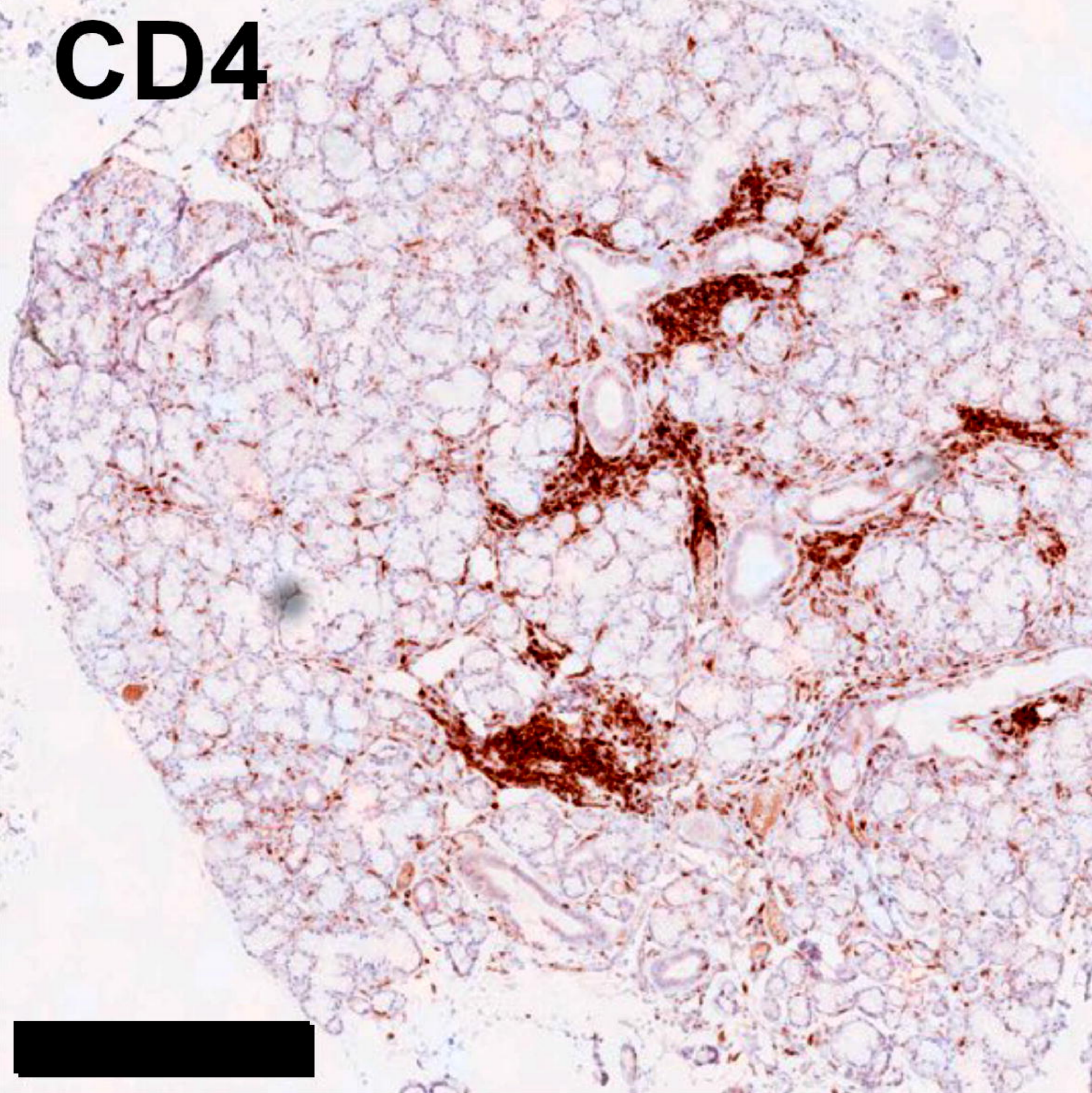
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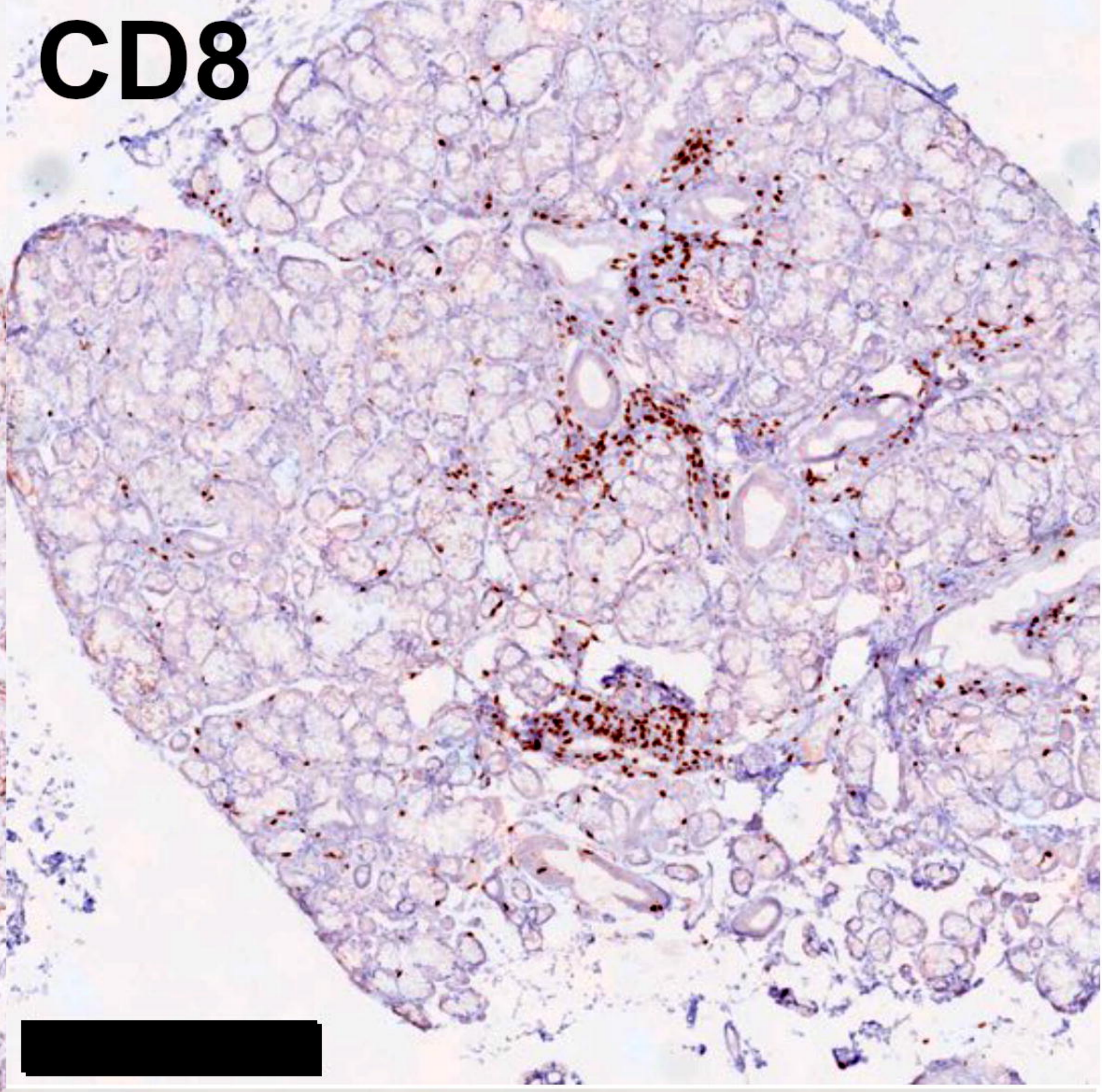
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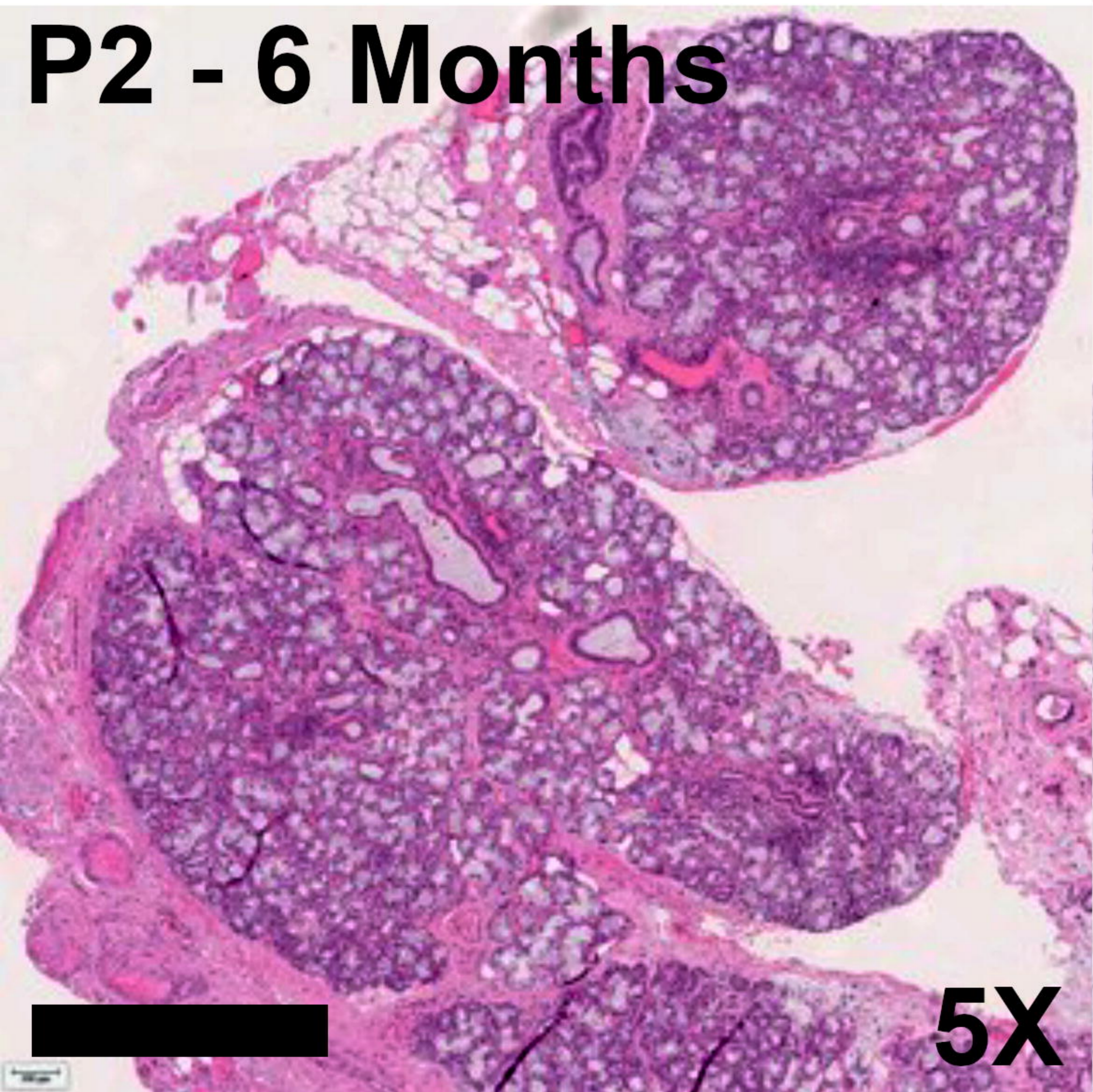
CD4



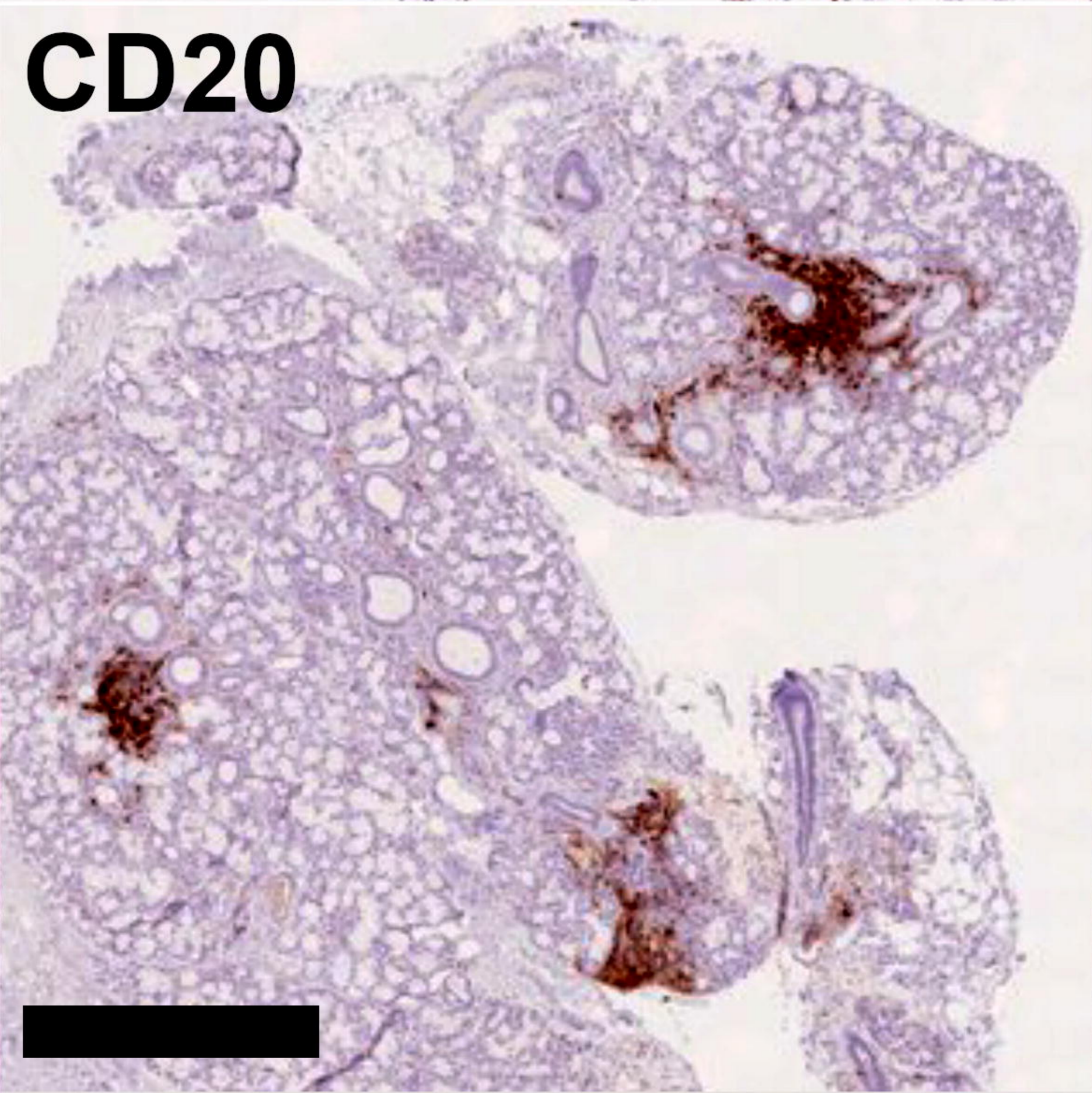
CD8



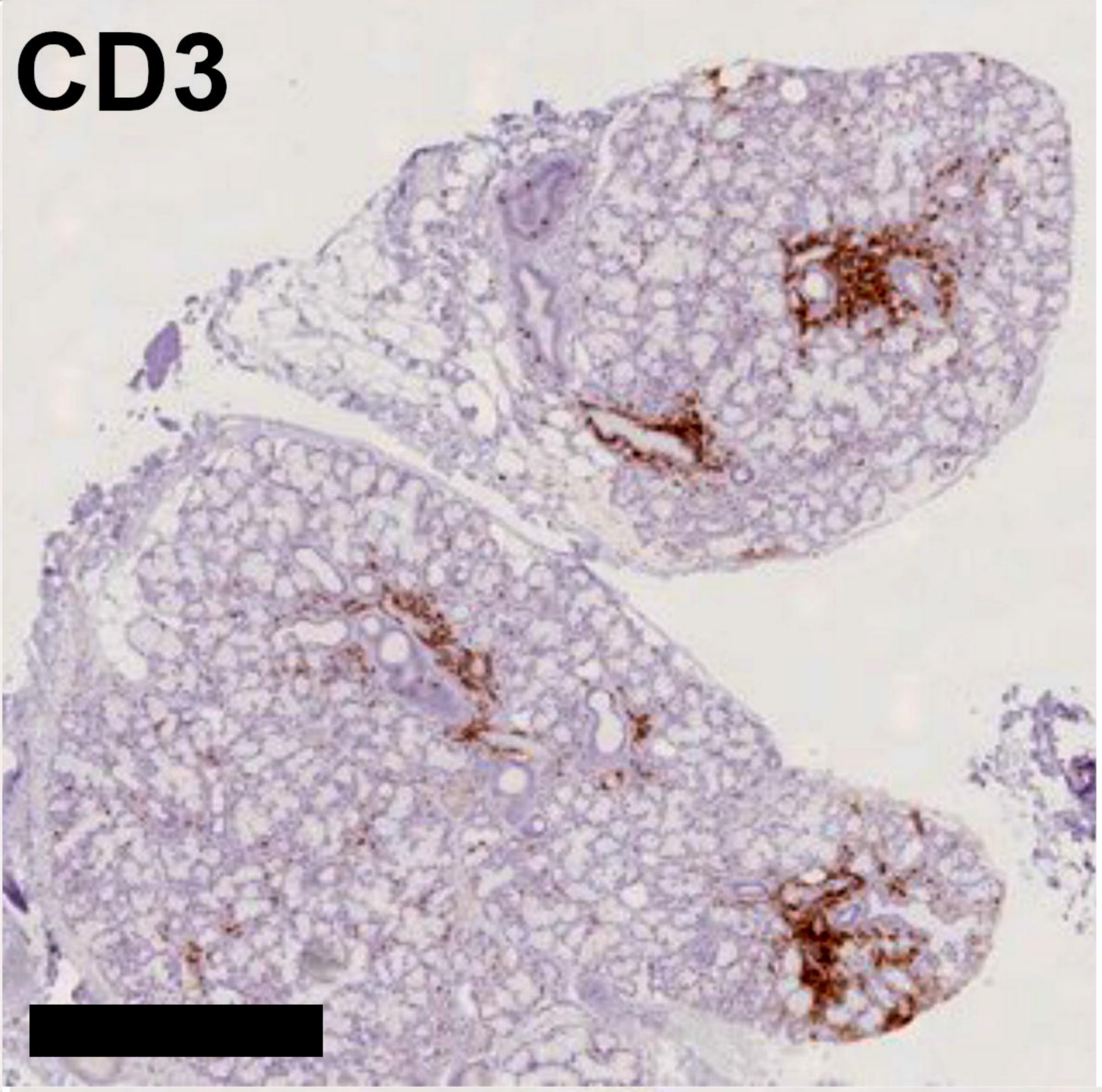
P2 - 6 Months



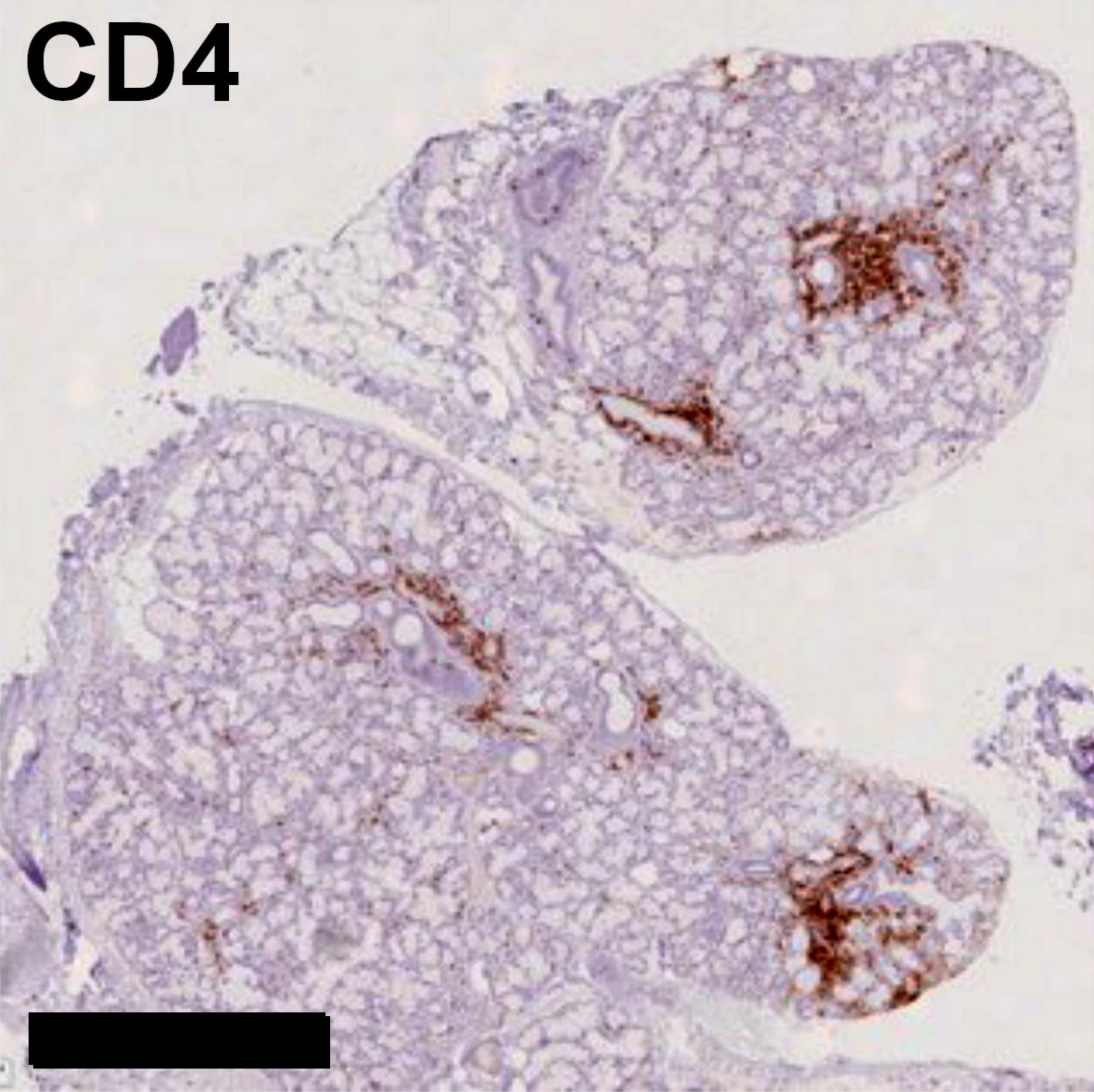
CD20



CD3



CD4



CD8

