

1 **Olfactory transmucosal SARS-CoV-2 invasion as port of Central Nervous System entry in COVID-19**
2 **patients**

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38

39 **Abstract**

40 The newly identified severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes
41 COVID-19, a pandemic respiratory disease presenting with fever, cough, and often pneumonia.
42 Moreover, thromboembolic events throughout the body including the central nervous system (CNS)
43 have been described. Given first indication for viral RNA presence in the brain and cerebrospinal fluid
44 and in light of neurological symptoms in a large majority of COVID-19 patients, SARS-CoV-2-
45 penetrance of the CNS is likely. By precisely investigating and anatomically mapping oro- and
46 pharyngeal regions and brains of 32 patients dying from COVID-19, we not only describe CNS
47 infarction due to cerebral thromboembolism, but also demonstrate SARS-CoV-2 neurotropism. SARS-
48 CoV-2 enters the nervous system via trespassing the neuro-mucosal interface in the olfactory mucosa
49 by exploiting the close vicinity of olfactory mucosal and nervous tissue including delicate olfactory
50 and sensitive nerve endings. Subsequently, SARS-CoV-2 follows defined neuroanatomical structures,
51 penetrating defined neuroanatomical areas, including the primary respiratory and cardiovascular
52 control center in the medulla oblongata.

53

54 Introduction

55 There is increasing evidence that the SARS-CoV-2 not only affects the respiratory tract but
56 also impacts the CNS resulting in neurological symptoms such as loss of smell and taste, headache,
57 fatigue, nausea and vomiting in more than one third of COVID-19 patients^{1,2}. Moreover, acute
58 cerebrovascular diseases and impaired consciousness have been described³. While recent studies
59 describe the presence of viral RNA in the brain and cerebrospinal fluid (CSF)^{4,5} but lack a prove for
60 genuine SARS-CoV-2 manifestation, a systematic analysis of COVID-19 autopsy brains aimed at
61 understanding the port of SARS-CoV-2 entry and distribution within the CNS is lacking⁶.

62 The neuroinvasive potential of evolutionarily-related coronaviruses (CoVs) such as SARS-CoV
63 and MERS-CoV has previously been described⁷⁻⁹. SARS-CoV including SARS-CoV-2 enter human host
64 cells primarily by binding to the cellular receptor angiotensin-converting enzyme 2 (ACE2) and by the
65 action of the serine protease TMPRSS2 for S protein priming¹⁰. Supporting evidence comes from
66 animal studies demonstrating that SARS-CoV is capable of entering the brain upon intranasal
67 infection of mice expressing human ACE2^{8,11}. However, it is not known, which cells in the olfactory
68 mucosa express these molecules in steady state or under inflammatory or septic conditions¹¹, while
69 there is first evidence for ACE2 expression in neuronal and glial cells in the CNS^{13,14}. Along that line it
70 is of note, that the immunoglobulin superfamily member CD147, which is expressed in neuronal and
71 non-neuronal cells in the CNS^{15,16}, has been shown to act as alternative cellular port for SARS-CoV-2
72 invasion¹⁷. To gain a better understanding of SARS-CoV-2 neurotropism and its mechanism of CNS
73 entry and distribution, we analyzed the cellular mucosal-nervous micro-milieu as first site of viral
74 infection and replication, followed by a thorough regional mapping of the consecutive olfactory
75 nervous tracts and defined CNS regions in 32 COVID-19 autopsy cases.

76

77 Results

78 Out of 32 COVID-19 autopsy cases, either proven to be RT-qPCR-positive for SARS-CoV-2
79 prior to death (N=29 of 32), or with clinical presentation highly suggestive of COVID-19 (N=3 of 32),
80 four patients (corresponding to 13%) presented with acute infarction due to ischemia caused by
81 (micro)thrombotic/thromboembolic events within the CNS (Supplementary Table 1). Similarly,
82 microthrombotic events were also detectable in the olfactory mucosa (Supplementary Figure 1).

83 Assessment of viral load by means of RT-qPCR in regionally well-defined tissue samples
84 including olfactory mucosa (region (R1), olfactory bulb (R2), oral mucosa (uvula; R3), trigeminal
85 ganglion (R4) and medulla oblongata (R5) demonstrated highest levels of SARS-CoV-2 copies per cell
86 within the olfactory mucosa sampled directly beneath the cribriform plate (N=13 of 22; Figure 1).
87 Lower levels of viral RNA were found in the cornea, conjunctiva and oral mucosa, highlighting the
88 oral and ophthalmic routes as additional potential sites of SARS-CoV-2 CNS entry (Figure 1). Only in
89 few cases the cerebellum was also positive (N=2 of 21), while the wall of the internal carotid artery,
90 which served as an internal negative control, was found to be negative in all investigated cases
91 (N=10). The assessment of subgenomic (sg) RNA as surrogate for active virus replication yielded a
92 positive result in 4 out of 13 SARS-CoV-2 RNA-positive olfactory mucosa samples and in 2 out of 6
93 SARS-CoV-2 RNA-positive uvulae, but in none of the other tissues analyzed in this study
94 (Supplementary Table 1). Patients with shorter disease duration were more likely to be tested
95 positive for viral RNA in the CNS tissue (Supplementary Table 1). The anatomical proximity between
96 neurons, nerve fibers and mucosa within the oro- and nasopharynx (Figure 2) and the reported
97 clinical-neurological signs related to alteration in smell and taste perception suggest that SARS-CoV-2
98 exploits this neuro-mucosal interface as port of CNS entry. On the apical side of the olfactory
99 mucosa, dendrites of olfactory receptor neurons (ORNs) project into the nasal cavity, while on the
100 basal side axons of olfactory receptor neurons merge into fila, which protrude through the cribriform
101 plate directly into the olfactory bulb (Figure 2), thereby also having contact with the CSF¹⁸.

102 Further evidence and support for a site-specific infection and inflammation by SARS-CoV-2
103 was provided by immunohistochemistry (Figure 3). Cells of the olfactory mucosa showed strong
104 immunoreactivity in a characteristic perinuclear pattern when an antibody against the SARS-CoV
105 spike protein was used. Furthermore, early activated macrophages formed small cell clusters in the
106 epithelium expressing myeloid-related protein 14 (MRP14) (Supplementary Figure 2), initiating and
107 regulating an immune cascade, which e.g. upon influenza virus infection, has been shown to
108 orchestrate virus-associated inflammation by acting as endogenous damage-associated molecular
109 pattern (DAMP), ultimately initiating TLR4-MyD88 signalling¹⁹.

110 Additional support for SARS-CoV-2 persistence was provided by ultrastructural analyses of
111 ultrathin sections. We found Coronavirus-like particles (Figure 3) – despite subtle ultrastructural
112 differences compared to coronavirus derived from infected cell cultures providing a somewhat
113 different milieu (Supplementary Figure 3) - fulfilling the criteria of size, shape, substructure
114 (membrane, surface projections and internal electron dense material, resembling ribonucleoprotein)
115 and intracellular localization of Coronavirus particles, while being clearly distinct from intrinsic
116 cellular structures resembling Coronavirus particles^{20–25}.

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119 Discussion

120 We provide first evidence that SARS-CoV-2 neuroinvasion occurs at the neuro-mucosal
121 interface by transmucosal trespassing via regional nervous structures followed by a transport along
122 the olfactory tract of the CNS, thus explaining some of the well-documented neurological symptoms
123 in COVID-19 patients including alterations of smell and taste perception. Further studies are required
124 to identify the precise cellular and molecular entry mechanism as well as receptors in the olfactory
125 mucosa, where also non-neuronal pathways may play a role²⁶. This will include a precise
126 characterization of ACE2, TMPRSS2 and of CD147 expression, which have been implicated in enabling
127 SARS-CoV-2 invasion in cells. Moreover, in line with recent clinical data demonstrating
128 thromboembolic CNS events described in few patients²⁷, we found in 13% of the 32 investigated
129 cases also the histopathological correlate of microthrombosis and territorial brain infarcts.

130 The presence of genuine virus in the olfactory mucosa with its delicate olfactory and
131 sensitive – partially axonally damaged (Supplementary Figure 1) nerves in conjunction with SARS-
132 CoV-2 RNA manifestation preferentially in those neuroanatomical areas receiving olfactory tract
133 projections (Figure 1) may speak in favor of SARS-CoV-2 neuroinvasion occurring via axonal transport.
134 However, several other mechanisms or routes, including transsynaptic transfer across infected
135 neurons, infection of vascular endothelium, or leukocyte migration across the blood-brain barrier
136 (BBB), or combinations thereof, be it in addition or exclusive, cannot be excluded at present¹⁴.

137 The detection of - compared to values measured in the lower respiratory tract
138 (Supplementary Table 1) - persistently high levels of SARS-CoV-2 RNA in the olfactory mucosa (124%
139 as mean value compared to lower respiratory tract) up to 53 days after initial symptoms
140 (Supplementary Table 1), including the detection of sgRNA suggests that olfactory mucosa remains a
141 region of continuous SARS-CoV-2 replication and persistence, thus enabling a constant viral
142 replenishment for the CNS. In line with our findings there is a comparable SARS-CoV-2 infection
143 gradient from the nose to the lungs which is paralleled by expression of the receptor molecule
144 ACE2²⁸. Although this remains to be speculation and widespread dysregulation of homeostasis of
145 cardiovascular, pulmonic and renal systems has to be regarded as the leading cause in fatal COVID-19
146 cases, previous findings of SARS-CoV infection and other coronaviruses in the nervous system²⁹ as
147 well as the herein described presence of SARS-CoV-2 RNA in the medulla oblongata comprising the
148 primary respiratory and cardiovascular control center bring to mind the possibility that SARS-CoV2
149 infection, at least in some instances, can aggravate respiratory or cardiac problems - or even cause
150 failure - in a CNS-mediated manner^{6,30}.

151

152 Even when following distinct routes upon first CNS entry and - based on our findings - in the
153 absence of clear signs of widespread distribution of SARS-CoV-2 in the CNS (i.e. no signs of
154 meningitis/encephalitis in COVID-19 cases), it cannot be excluded that the virus may spread more
155 widely to other brain regions, thus eventually contributing to a more severe or even chronic disease
156 course, depending on various factors such as the time of virus persistence, viral load, and immune
157 status, amongst others.

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159 **Methods:**

160 **Study design**

161 32 autopsy cases of either PCR-confirmed SARS-CoV-2 COVID-19 patients (N=29 of 32) or of patients
162 clinically highly suggestive of COVID-19 (N=3 out of 32) were included. Autopsies were performed at
163 the Department of Neuropathology and the Institute of Pathology, Charité - Universitätsmedizin
164 Berlin (N=25 out of 32) including one referral case from the Institute of Pathology, DRK Kliniken
165 Berlin, the Institutes of Pathology and of Neuropathology, University Medicine Göttingen (N=6 out of
166 32) and the Institute of Forensic Medicine Charité - Universitätsmedizin Berlin (N=1 out of 32). This
167 study was approved by the Ethics Committee of the Charité (EA 1/144/13 and EA2/066/20) as well as
168 by the Charité-BIH COVID-19 research board and was in compliance with the Declaration of Helsinki.
169 In all deceased patients a whole-body autopsy was performed, which included a thorough
170 histopathologic and molecular evaluation comprising virological assessment of SARS-CoV-2 RNA
171 levels as indicated in Supplementary Table 1. Clinical records were assessed for pre-existing medical
172 conditions and medications, current medical course, and ante-mortem diagnostic findings.

173 **SARS-CoV- and SARS-CoV-2-specific PCR including subgenomic RNA assessment**

174 RNA was purified from ~50 mg of homogenized tissue from all organs by using the MagNAPure 96
175 system and the MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche) following the
176 manufacturer's instructions.

177 Quantitative real-time PCR for SARS-CoV-2 was performed on RNA extracts with RT-qPCR targeting
178 the SARS-CoV-2 E-gene. Quantification of viral RNA was done using photometrically quantified *in*
179 *vitro* RNA transcripts as described previously³³. Total DNA was measured in all extracts by using the
180 Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Karlsruhe, Germany).

181 Detection of subgenomic RNA (sgRNA), as correlate of active virus replication in the tested tissue was
182 done using oligonucleotides targeting the leader transcriptional regulatory sequence and region
183 within the sgRNA coding for the SARS-CoV-2 E gene, as described previously³⁴.

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187 **Electron microscopy**

188 Autopsy tissues were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, postfixated
189 with 1% osmium tetroxide in 0.05M sodium cacodylate, dehydrated using graded acetone series,
190 then infiltrated and embedded in Renlam resin. *En bloc* staining with uranyl acetate and
191 phosphotungstic acid was performed at the 70% acetone dehydration step. 500 nm semithin sections
192 were cut using an ultramicrotome (Ultracut E, Reichert-Jung) and a histo jumbo diamond knife
193 (Diatome), transferred onto glass slides, stretched at 120°C on a hot plate and stained with Toluidine
194 blue at 80°C. 70 nm ultrathin sections were cut using the same ultramicrotome and an ultra 35°
195 diamond knife (Diatome), stretched with xylene vapor, collected onto pioloform coated slot grids and
196 then stained with lead citrate. Standard TEM was performed using a Zeiss 906 in conjunction with a
197 2k CCD camera (TRS). Large-scale digitization was performed using a Zeiss Gemini 300 field-emission
198 scanning electron microscope in conjunction with a STEM-detector via Atlas 5 software at 4-6 nm
199 pixel size. Regions of interest of the large-scale datasets were saved by annotation (“mapped”) and
200 then recorded at very high resolution using 0.5-1 nm pixel size.

201 **Immunohistochemical procedures and stainings**

202 Routine histological stainings (Hematoxylin and eosin (HE), Masson-Goldner, Periodic acid-Schiff
203 reaction (PAS), and Toluidine blue) were performed according to standard procedures.
204 Immunohistochemical stainings were either performed on a Benchmark XT autostainer (Ventana
205 Medical Systems, Tuscon, AZ, USA) with standard antigen retrieval methods (CC1 buffer, pH8.0,
206 Ventana Medical Systems, Tuscon, AZ, USA) or manually using 4-µm-thick FFPE tissue sections. The
207 following primary antibodies were used: monoclonal mouse anti-S100 (DAKO Z0311, 1:3000),
208 monoclonal mouse anti-AE1/AE3 (DAKO M3515, 1:200), monoclonal mouse anti-MRP14 (Acris,
209 BM4026B, 1:500, pretreatment protease), monoclonal mouse anti-CD56 (Serotec, ERIC-1, 1:200),
210 mouse monoclonal anti-SARS spike glycoprotein antibody (Abcam, ab272420, 1:100, pretreatment
211 Citrate + MW) and polyclonal rabbit anti-OLIG2 (IBL, 18953, 1:150, pretreatment Tris-EDTA + MW).
212 Briefly, primary antibodies were applied and developed either using the iVIEW DAB Detection Kit
213 (Ventana Medical Systems) and the ultraView Universal Alkaline Phosphatase Red Detection Kit
214 (Ventana Medical Systems) or by manual application of biotinylated secondary antibodies (Merck,
215 RPN1001, RPN1004), peroxidase-conjugated avidin, and diaminobenzidine (DAB, Sigma, D5637) or 3-
216 Amino-9-Ethylcarbazol (AEC). Sections were counterstained with hematoxylin, dehydrated in graded
217 alcohol and xylene, mounted and coverslipped. IHC stained sections were evaluated by at least two
218 board-certified neuropathologists with concurrence. For data handling of whole slides images an
219 OME-TIFF workflow was used³⁵.

220

221 **Acknowledgements**

222 This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research
223 Foundation) under Germany's Excellence Strategy – EXC-2049 – 390688087, as well as SFB TRR 167
224 and HE 3130/6-1 to F.L.H., SFB 958/Z02 to J.S., SFB TRR 130 to H.R., EXC 2067/1- 390729940, SFB TRR
225 274 and STA 1389/5-1 to C.S., by the German Center for Neurodegenerative Diseases (DZNE) Berlin,
226 and by the European Union (PHAGO, 115976; Innovative Medicines Initiative-2; FP7-PEOPLE-2012-
227 ITN: NeuroKine). We are indebted to Francisca Egelhofer, Petra Matylewski, Kathrein Permien, Vera
228 Wolf, Sandra Meier, René Müller, Uta Scheidt and Katja Schulz for excellent technical assistance and
229 advice. We thank the Core Facility for Electron Microscopy of the Charité for support in acquisition of
230 the data. The authors are most grateful to the patients and their relatives for consenting to autopsy
231 and subsequent research, which were facilitated by the Biobank of the Department of
232 Neuropathology – Universitätsmedizin Berlin, Germany. Furthermore, we thank the Charité
233 foundation for financial support. Cartoon images were partially created with Biorender.com.

234 **Author Contributions**

235 J.M., J.R., R.M., J.F., O.H., M.W., L.R., H.M., W.J.S-S., C.S., S.H., M.S.W., B.T., S.E., D.H., L.O., M.T,
236 B.I.H., H.R., F.L.H. performed clinical workup and sections and/or histological analyses, Ca.D., H.H.G.,
237 M.L. and W.S. did ultrastructural analyses, J.S., S.B., Ch.D. and V.M.C. made viral RT-qPCR analyses.
238 All authors contributed to the experiments and analyzed data; H.R. and F.L.H. designed and
239 supervised the study; J.M., J.R., and Ca.D. prepared figures. All authors wrote, revised and approved
240 the manuscript.

241

242 **Competing Interests**

243 All authors declare no competing interest.

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246 **References**

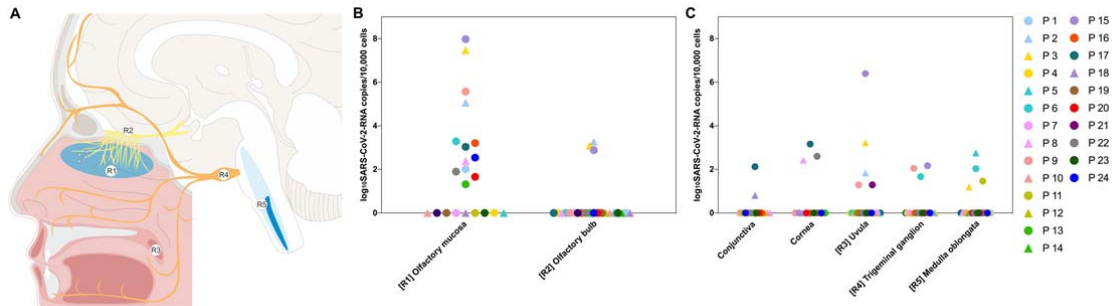
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Figures 1 - 3 (incl. Figure legends)

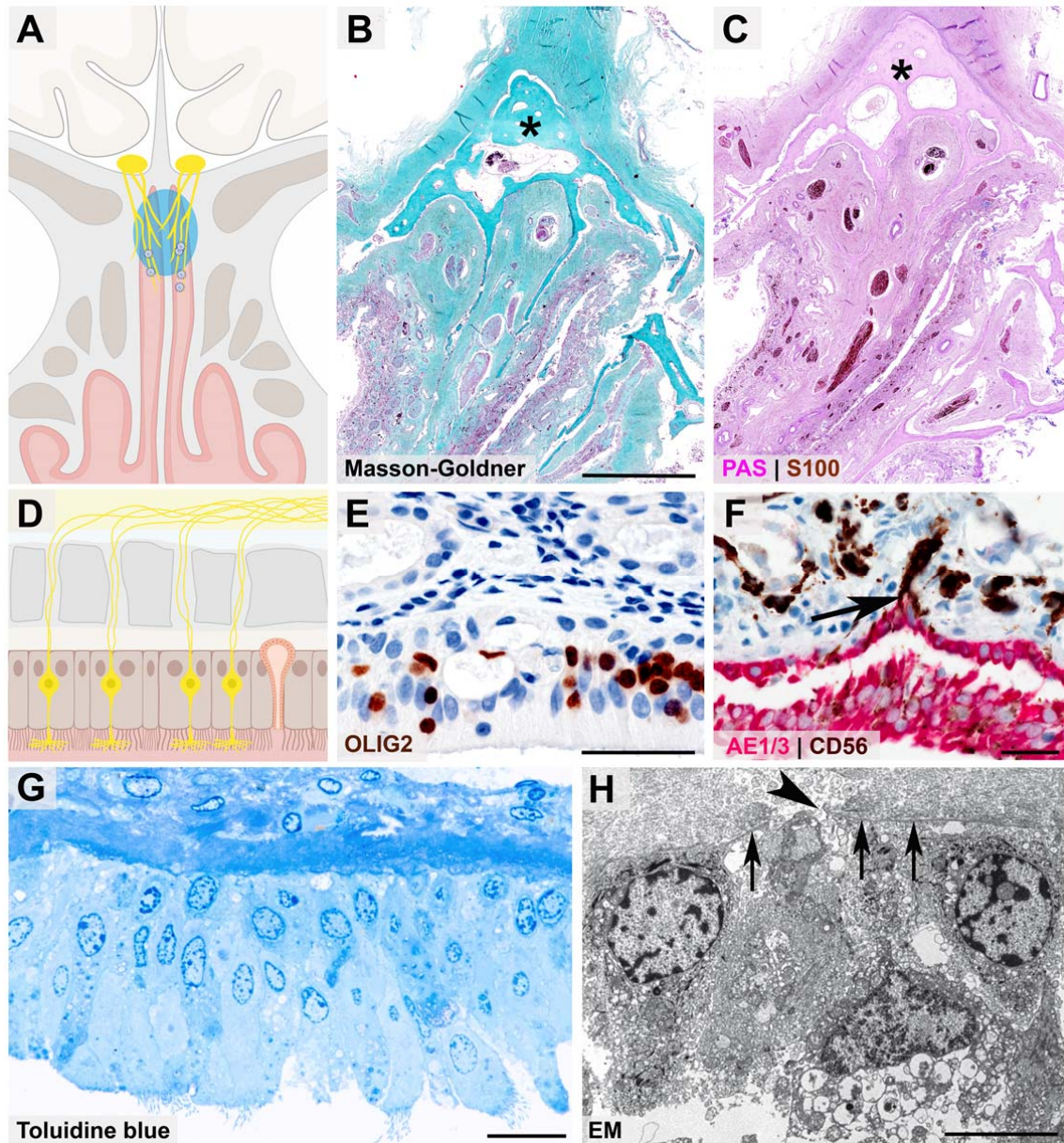


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347 **Figure 1: SARS-CoV-2 RNA levels of deceased COVID-19 patients in anatomically distinctly mapped**
348 **oro- and nasopharyngeal as well as CNS regions**

349 Cartoon depicting the anatomical structures sampled for histomorphological, ultrastructural and
350 molecular analyses including SARS-CoV-2 RNA measurement from fresh (i.e. non-formalin-fixed)
351 specimens of deceased COVID-19 patients (A). Specimens were taken from the olfactory mucosa
352 underneath of the cribriform plate (Region (R)1, blue, N=22), the olfactory bulb (R2, yellow, N=23),
353 from different branches of the trigeminal nerve (including conjunctiva (N=15), cornea (N=12),
354 mucosa covering the uvula (R3, N=20)), the respective trigeminal ganglion in orange (R4, N=20), and
355 the cranial nerve nuclei in the medulla oblongata (R5, dark blue, N=23). The quantitative results for
356 each patient are shown in a logarithmic scale normalized on 10,000 cells (B, C). Female patients are
357 displayed in triangular, male in circular symbols. P=patient.

358

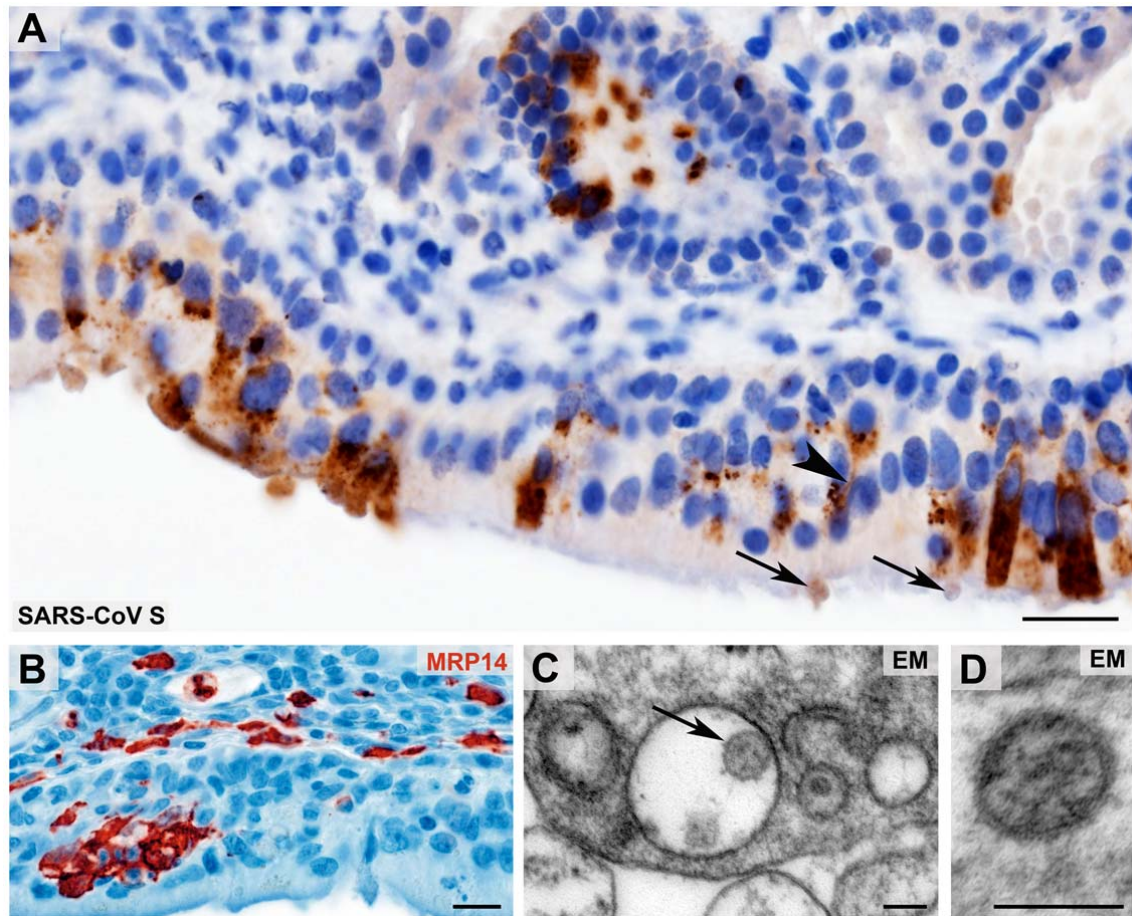


359

360 **Figure 2: Close anatomical proximity of nervous and epithelial tissues in the olfactory mucosa**

361 Cartoon (A) and histopathological coronal cross-sections (B - C) depicting the paranasal sinus region
362 with the osseous cribriform plate (turquoise color and asterisk in B, pink color and asterisk in C) and
363 the close anatomical proximity of the olfactory mucosa (green in B, purple in C) and nervous tissue
364 characterized by nerve fibers immunoreactive for S100 protein (C, brown color). Cartoon (D)
365 resembling the olfactory mucosa, which is composed of pseudostratified ciliated columnar
366 epithelium, basement membrane, and lamina propria, also containing mucus-secreting Bowman
367 glands and bipolar olfactory receptor neurons (ORNs), which coalesce the epithelial layer.
368 Immunohistochemical staining of the olfactory mucosa (E, F) showing nuclear expression of OLIG2

369 specifying late neuronal progenitor and newly formed neurons (E, brown color)³¹, which are closely
370 intermingled with epithelial cells (F, immunoreactivity for the pancytokeratin marker AE1/3, red
371 color). The basement membrane underneath the columnar AE1/3-positive epithelium (F, red color) is
372 discontinued due to CD56-positive (F, brown color) axonal projections of ORNs (F, arrow). The ORN
373 dendrite carries multiple cilia and protrudes into the nasal cavity (G, semithin section, toluidine blue
374 staining), while the axon (H, arrowhead) crosses the olfactory mucosa basement membrane (H,
375 arrows) as a precondition for ORN projection into the glomeruli of the olfactory bulb, which is readily
376 visible at the ultrastructural level). Scale bars: B: 3.5 cm; E, F: 50 μ m; G: 20 μ m; H: 5 μ m.
377



378

379 **Figure 3: Morphological evidence of SARS-CoV presence and first innate immune cell response**
380 **within the olfactory mucosa**

381 Coronavirus antigen (A, SARS-CoV Spike Protein (SARS-CoV S), brown color) exhibits a cytoplasmic
382 staining with perinuclear accentuation of infected mucosal (epithelial) cells and identifies SARS-CoV-
383 positive dendrites (arrowhead) and vesicles at the dendrite tips (arrows) of the olfactory receptor
384 neurons. Small clusters of infiltrating, early activated macrophages and granulocytes (MRP14, red
385 color) in the olfactory epithelium upon SARS-CoV-2 infection (B). Ultrastructural images of two
386 different examples of Coronavirus-like particles in the olfactory mucosa (C - D; arrow in C) fulfilling
387 the criteria of size, shape, structural features (membrane, surface structures, electron dense material
388 within the particle, resembling ribonucleoprotein) and localization (C, cytoplasmic localization within
389 a membrane compartment, sometimes with typical attachment on the inner membrane surface as
390 shown in this example; D, extracellular). Scale bars: A, B: 20 μ m; C, D: 100 nm,

391

