Adrenal tropism of SARS-CoV-2 and adrenal findings in a post-mortem case series of patients with severe fatal COVID-19

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Progressive respiratory failure and hyperinflammatory response is the primary cause of death in the coronavirus disease 2019 (COVID-19) pandemic. Despite mounting evidence of disruption of the hypothalamus-pituitary-adrenal axis in COVID-19, relatively little is known about the tropism of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to adrenal glands and associated changes. Here we demonstrate adrenal viral tropism and replication in COVID-19 patients. Adrenal glands showed inflammation accompanied by inflammatory cell death. Histopathologic analysis revealed widespread microthrombosis and severe adrenal injury. In addition, activation of the glycerophospholipid metabolism and reduction of cortisone intensities were characteristic for COVID-19 specimens. In conclusion, our autopsy series suggests that SARS-CoV-2 facilitates the induction of adrenalitis. Given the central role of adrenal glands in immunoregulation and taking into account the significant adrenal injury observed, monitoring of developing adrenal insufficiency might be essential in acute SARS-CoV-2 infection and during recovery.
Coronavirus disease (COVID-19), caused by SARS-CoV-2, has reached a pandemic level with a high global mortality rate. Although SARS-CoV-2 primarily targets the respiratory system, multiple other organs including the kidney, the cardiovascular, and central nervous system can be affected. In severe cases of COVID-19, systemic hyper inflammation with the release of cytokines (cytokine storm) is difficult to manage and can result in multiple organ failure. An intact hormone balance, especially of adrenal hormones such as cortisol, is critical to master this challenge. Cytokine toxicity and autoimmunity can be reduced by mild immunosuppression due to activation of the hypothalamus-pituitary-adrenal axis (HPA axis) with hypercortisolism. However, disruption of the HPA axis can cause dysregulation of the physiological innate immune response against viral infection. For the SARS-CoV-2 2002–2004 outbreak, it has been shown that SARS-CoV can directly injure adrenal glands.

Only a few reports of adrenal findings of COVID-19 patients have been published recently. Two autopsy series showed varying frequencies of adrenal damage. Two case reports and one clinical series that evaluated CT findings could link adrenal infarction or hemorrhage to adrenal insufficiency in COVID-19 patients. However, detailed morphologic and molecular analysis of adrenal glands with simultaneous evidence of viral adrenal tropism for SARS-CoV-2 are lacking to date.

We show adrenal tropism of SARS-CoV-2 associated with local inflammation and severe adrenal damage in COVID-19 patients. In addition, COVID-19 specimens were characterized by a specific metabolic profile with significantly reduced Cortisone intensities.

Results

Adrenal tropism of SARS-CoV-2. As ACE2 and TMPRSS2 facilitate SARS-CoV-2 infection we performed immunohistochemistry for ACE2 and TMPRSS2. ACE2 was expressed moderately in adrenal cortical cells and strongly in intervening capillaries (Fig. 1A). TMPRSS2 immunohistochemistry was positive in the cytoplasm of adrenal cells with a few cells with nuclear staining (Fig. 1B). This distribution was confirmed by double immunofluorescence with CD34 staining capillaries and SF1 staining adrenal cells (Fig. 1D–I). ACE2 colocalized with CD34 (Fig. 1D) (95 ± 3% of CD34 positive cells were double positive for CD34 and ACE2) and SF1 (Fig. 1G) (87 ± 4% of SF1 positive cells were double positive for SF1 and ACE2). TMPRSS2 colocalized with SF1 (Fig. 1H) (84 ± 4% of SF1 positive cells were double positive for SF1 and TMPRSS2), however, no colocalization with CD34 could be observed (Fig. 1E) (0% of CD34 positive cells were double positive for CD34 and TMPRSS2).

We could detect s-SARS-CoV-2 protein by immunohistochemistry in adrenal cortical cells of 19/19 patients with positive postmortem SARS-CoV-2 PCR (Fig. 1C). In double immunofluorescence images, s-SARS-CoV-2 colocalized with SF1 (Fig. 1I) (32 ± 11% SF1 positive cells were positive for s-SARS-CoV-2), however, s-SARS-CoV-2 did not colocalize with CD34 positive cells (Fig. 1F) (0% of CD34 cells were double positive for s-SARS-CoV-2). Viral tropism to adrenal cells was further validated by in situ hybridization. Sense s-SARS-CoV-2 RNA could be clearly visualized in the adrenal cortex (Fig. 1F, K). Additional detection of antisense s-SARS-CoV-2 RNA confirmed viral replication in the cells (Fig. 1L, M). Viral RNA was detected in SF1 positive adrenal cells and in CD34 positive endothelial cells as shown by multiplex immunofluorescence and in situ hybridization (Fig. 1N, O).

Expression of ACE2, TMPRSS2, and SARS-CoV-2 in adrenal tissue was confirmed by western blot analysis with variable amounts of ACE2, TMPRSS2, and s-SARS-CoV-2 protein detectable (Fig. 1O).

Viral infection and replication in adrenal cortical carcinoma cell line. To functionally validate our observations of autopsy tissue of COVID-19 patients we performed infection experiments with two adrenal cortical cell lines (HAC15 which was positive for ACE2 and TMPRSS2 and SW13 which showed weak ACE2 expression and was negative for TMPRSS2) (Fig. 2A). Target cells were treated with either RDV (1 μM) 2 h before infection or were left untreated. Subsequently, cells were challenged with a serial dilution of a stock of an expanded SARS-CoV-2 clinical isolate. Viral replication of SW13 and HAC15 was quantified by RT-qPCR, both in a wash (postwash; time zero) and in the supernatant (harvest; time 72 h). Additionally, cells were collected for histopathology and western blot analysis. SW13 showed no detectable virus replication with equal ct values of postwash and harvest. However, HAC15 showed low virus replication with ct values of harvest clearly exceeding the postwash values and reaching almost the inoculum (Fig. 2B). Virus replication could be inhibited by RDV pretreatment. FISH analysis of cell blocks and western blot analysis confirmed RT-qPCR results. In RDV treated HAC15 no sense or antisense SARS-CoV-2 RNA could be detected (Fig. 2C), in contrast, untreated HAC15 showed virus infection and replication with positivity for antisense and sense SARS-CoV-2 (Fig. 2C) and expression of SARS-CoV-2 protein (Fig. 2D). Intriguingly, low viral replication was sufficient to induce apoptosis in HAC15 with cleavage of caspase 3 shown by western blot analysis (Fig. 2D) and by immunohistochemistry (Fig. 2C). Moreover, SARS-CoV-2 triggered inflammatory response with upregulation of IL-6 (Fig. 3D), which is elevated in COVID-19 patients and correlates with adverse clinical outcome.

Inflammation of the adrenal gland. In all adrenal glands of SARS-CoV-2 positive patients lymphocytic and histiocytic, infiltrates were detected, H&E images of each patient (n = 31) is given in supplemental Fig. 3. Infiltrates often formed small foci consistent with 50 or more lymphocytes and histiocytes. Foci were accentuated perivascular and were observed in the medulla as well as in the adrenal cortex (zona fasciculata, Fig. 3A). It is known that lymphocytic infiltrates can be found in the medulla of normal adrenal glands and are not indicative of adrenal inflammation, therefore we focused our analysis on the adrenal cortex. Multiplex immunohistochemistry (Fig. 3D–G) showed that in the cortex of COVID-19 patients CD4 positive T-cells (Fig. 3D, H) were more numerous than in influenza patients (mean number/HPF 17.5 ± 3.3 versus 9.2 ± 1.9; p < 0.0001) and CD8 positive T-cells (Fig. 3E, I) were more numerous in COVID-19 patients as well (mean number/HPF 6.3 ± 1.6 versus 2.0 ± 1; p < 0.0001). With immunohistochemistry of cleaved caspase 3 (Fig. 3C) we were able to detect apoptosis in the center of 79% of the inflammatory infiltrates (mean = 3 ± 3). In multiplex immunofluorescence significantly more CD68 positive macrophages (Fig. 3F, J) were found in the cortex of COVID-19 patients compared to influenza patients (mean number/HPF 27.6 ± 9 versus 16.7 ± 3.7; p < 0.0001).

Adrenal thrombosis and hemorrhage. We examined the adrenal vasculature with H&E stain, Masson’s trichrome stain, and anti-fibrin immunohistochemistry. We did not observe vascular endothelitis (as described in the lung or renal specimens of COVID-19 patients). Furthermore, no thrombi were detected in precapillary vessels. However, in 11/19 (58%) of COVID-19 patients capillaries were expanded and microthrombi were clearly visible (Fig. 4A, B) as demonstrated by anti-fibrin immunohistochemistry. Adrenal capillary microthrombi were three times as prevalent as in patients with influenza (mean ± SD number of distinct thrombi per square centimeter of vascular lumen area, 45.1 ± 2.9 and 15 ± 1.8; p < 0.0001). In three COVID-19 patients
(16%) focal fresh hemorrhage and ischemic infarction could be detected in the adrenal cortex or medulla (Fig. 4C).

**Disruption of the adrenal cortex zonation.** The normal adrenal cortex is structured in three zones (zona glomerulosa, zona fasciculata, and zona reticularis), which can be easily identified by H&E at low magnification and can be highlighted by immunohistochemistry with CYB11B2 (zona glomerulosa) and CYB5A (zona reticularis) (Fig. 5A). All adrenal glands from the SARS-CoV-2 positive group revealed moderate to severe disruption of the zonation (Fig. 4B, C) with 47% (n = 9) of disruption grade 2 and 53% (n = 10) of disruption grade 1. In contrast in the influenza group 30% (n = 3) showed no disruption, 40% (n = 4)
Fig. 1 Adrenal tropism of SARS-CoV-2. Overview images of adrenal glands of COVID-19 patients (n = 20) (scale bar = 100 μm) with magnified inlets (scale bar = 15 μm) of immunohistochemistry of ACE2 (A), TMPRSS2 (B), and s-SARS-CoV-2 (C) are shown. Adrenal cells and intervening capillaries express ACE2 and TMPRSS2 and s-SARS-CoV-2 protein can be detected in adrenal cortical cells. Double immunofluorescence of adrenal glands of COVID-19 patients (n = 19) of ACE2 (green) and CD34 (red) (D), TMPRSS2 (green) and CD34 (red) (E), s-SARS-CoV-2 (green) and CD34 (red) (F), ACE2 (green) and SF1 (red) (G), TMPRSS2 (green) and SF1 (red) (H), and s-SARS-CoV-2 (green) and SF1 (red) (I) are displayed as overview (scale bar = 100 μm) with magnified inlets (scale bar corresponds = 15 μm). ACE2 colocalizes with SF1 and CD34, whereas TMPRSS2 and SARS-CoV-2 only localize to SF1 but not to CD34. In-situ hybridization of adrenal tissue with s-SARS-CoV-2 antisense probe (J, K) and sense probe (L, M) in red with DAPI counterstain (blue) (J, L (overview scale bar = 50 μm) and corresponding magnification (K, M) (scale bar = 15 μm) confirm the presence of s-SARS-CoV-2 RNA in adrenal tissue (n = 19). Viral RNA (red dots) is detected in SF1 positive adrenal cells (green, N) and in CD34 positive endothelial cells (green, O); scale bar corresponds to 10 μm, DAPI counterstaining in blue. Western blot analysis of adrenal tissue (n = 4) (P) shows a variable expression of ACE2, TMPRSS2, and s-SARS-CoV-2 in adrenal tissue (tubulin is shown as loading control).

Disruption grade 1 and 30% (n = 3) disruption grade 2. Compared to the matched group of influenza patients disruption of the zonation was significantly more pronounced in the COVID-19 group (p = 0.041) (Fig. 5E). Interestingly all ten COVID-19 patients with no and one patient with only short ICU stay (≤7 days) also showed moderate to severe disruption of adrenal cortex zonation. Two COVID-19 patients with seroconversion and extended ICU stay were characterized by massive interstitial fibrosis with collagen matrix production (Fig. 5D) and severe disruption of the architecture, whereas lymphocytic infiltrates could not be detected.

Hypothalamic-pituitary axis. There is currently no evidence of direct hypothalamic or pituitary effects of SARS-CoV-2 infection. We conducted a histomorphologic analysis of the hypothalami and the pituitary glands of ten COVID-19 patients. Morphologically and immunohistochemically no significant inflammatory infiltrate was detected. The architecture was unremarkable without evidence of hemorrhage or necrosis (Fig. 5F, G). In summary, no significant structural abnormalities could be identified in postmortem specimens from COVID-19 patients.

Metabolic alterations of adrenal glands associated with COVID-19. For spatial metabolomics MALDI-FT-ICR imaging mass spectrometry analysis was conducted, comparing four FFPE specimens of adrenal glands of COVID-19 patients with four adrenal glands of influenza patients (specimen with no signs of autolysis were chosen). Overall, 4663 MS peaks within the mass range of m/z 75 to 1000 could be resolved within the tissue (Fig. 6B).

Metabolic data were exported and used for hierarchical clustering and component analyses. In hierarchical cluster analysis, the two groups (COVID-19 and influenza) could be clearly distinguished based on the metabolite patterns, as demonstrated by heat map analysis (Fig. 6A). The distribution of metabolites revealed obvious differences between COVID-19 and influenza (Fig. 6D). Especially, phosphatic acid and phosphatidylinositol showed high intensity in COVID-19 patients, whereas inositol cyclic phosphate and ribose phosphate showed high intensity in the influenza group.

Pathway enrichment analysis was performed on the two different groups. Enriched metabolic pathways contributing to group discrimination include glycerophospholipid metabolism, pentose phosphate pathway, and ascorbate and aldarate metabolism (Fig. 6E, F).

Steroid metabolome analysis showed the reduced intensity of cortisone in the COVID-19 group (p = 0.648) compared to the influenza group (Fig. 6C).

Discussion
In this study, we examined the morphologic and molecular features of 21 adrenal glands obtained during autopsy and ten postmortem specimens of the pituitary gland and the hypothalamus from patients who died from COVID-19. Findings were compared to specimens of patients who died from influenza A (H1N1) infection (n = 10). We detected SARS-CoV-2 RNA and protein in adrenal glands associated with substantial inflammation and injury. HAC15 a cortical adrenal carcinoma cell line showed a low level of viral replication when infected with SARS-CoV-2, however, this was sufficient to induce apoptosis and inflammatory response. Distinctive pathologic features of adrenal specimens from COVID-19 patients were lymphohistiocytic infiltrates, widespread micro thrombosis, and disruption or loss of adrenal cortical zonation. Metabolic imaging could clearly separate the specimens of each group by cluster analysis. Enriched metabolic pathways contributing to group discrimination include glycerophospholipid metabolism, pentose phosphate pathway, and ascorbate and aldarate metabolism, moreover, specimens of COVID-19 patients showed a substantial reduction in cortisone levels.

ACE2 and TMPRSS2, which have been shown to contribute to viral entry into the cell and to viral persistence were expressed in adrenal and endothelial cells as shown previously for ACE2. SARS-CoV-2 was detected by immunohistochemistry or in situ hybridization in all adrenal glands of patients with positive postmortem nasopharyngeal swabs. S-SARS-CoV-2 protein and RNA could be mapped to adrenal cells. To our knowledge, this is one of the most comprehensive reports of adrenal findings of patients who died from COVID-19, which indicates adrenal tropism of the virus. Although the productive viral infection was not demonstrated, detection of antisense SARS-CoV-2 RNA in adrenal cells confirmed viral replication in adrenal cells. Viral RNA and protein was imaged with high spatial resolution and viral RNA could be detected not only in adrenal cells but also in endothelial cells of intervening capillaries. As SARS-CoV-2 protein and antisense RNA was not observed in endothelial cells detection of SARS-CoV-2 sense RNA in endothelial cells might rather be indicative of viral transmission from the bloodstream than indicative for viral replication. In infection experiments with an adrenal cortical carcinoma cell line (HAC15), low-level viral replication was observed. Even though these are preliminary data from a carcinoma cell line, infection-induced apoptosis and triggered inflammatory response with enhanced expression of IL-6, which is elevated in COVID-19 patients and correlates with adverse clinical outcome.

We observed inflammation of the adrenal glands with perivascular accentuation as well as ongoing apoptosis in the center of the infiltrates. Quantification of T-cell subsets and macrophages confirmed a significant increase of inflammatory infiltrates in COVID-19 patients compared to influenza patients. This is in line with previous studies reporting variable frequencies of adrenal inflammation, however to date comparison to other postmortem adrenal gland specimens (such as Influenza) was missing. Our findings of viral identification in the adrenal gland
and accumulation of inflammatory cells accompanied by inflammatory cell death suggest that SARS-CoV-2 facilitates the induction of adrenalitis as a direct consequence of viral involvement.

Hypercoagulability with immunothrombosis and thromboembolic events is a characteristic finding in COVID-19 patients and is associated with an increased risk of death. In more than half of COVID-19 patients (58%) microthrombi in capillaries were present and focal hemorrhage or infarction was detected in 16% of COVID-19 patients. Moderate to severe adrenal injury was present in COVID-19 patients with significant disruption of the adrenal zonation. It can not be entirely ruled out that these changes are due to other nonspecific systemic critical illness, however adrenal injury of COVID-19 patients was significantly more severe than in age-matched influenza controls. Moreover, the adrenal injury was not only detected in patients with extended ICU stay but also in patients with no ICU stay. Notably, two patients with prolonged ICU stay and seroconversion showed severe damage with prominent scarring. Adrenal infarction with associated adrenal insufficiency in COVID-19 patients has been described in previous radiological studies and case reports.
Metabolomic imaging of adrenal glands by MALDI could clearly separate COVID-19 patients from influenza patients. COVID-19 patients were characterized by activation of the glycerophospholipid metabolism with an increase of specific intermediates and metabolites such as phosphatidic acid and phosphatidylinositol. A decrease of serum levels of glycerophospholipids has been correlated with a severe clinical course of COVID-19 and corresponding metabolites have been found significantly elevated in Coronavirus-infected cells. The role of glycerophospholipid metabolism in hypoxic stress response and phagocytosis is well documented. In addition, steroid profiling showed a decrease of cortisone intensities, which might indicate disturbed adrenal cortical steroid production.

Adrenal hormones play a crucial role in the immune response, however comprehensive studies investigating the hypothalamic-pituitary-adrenal axis in COVID-19 patients are missing so far. In contrast to our remarkable findings in adrenal glands, we could not observe significant structural abnormalities or inflammatory infiltrates in COVID-19 patients compared to influenza. In the center of inflammatory infiltrates single-cell apoptosis could be detected by immunohistochemistry of cleaved caspase 3.
Fig. 4 Microthrombi and focal hemorrhage in adrenal glands from COVID-19 patients. Hematoxylin-eosin staining (A), and anti-fibrin immunohistochemistry (B) overview (scale bar = 100 μm and magnified inlet (scale bar = 10 μm) (n = 19) shows expanded capillaries with fibrinous microthrombi. In a subset of patients focal fresh hemorrhage could be observed (C). Masson’s trichrome staining (overview (scale bar = 100 μm and magnified inlet (scale bar = 10 μm)).

Fig. 5 Disruption of zonation in adrenal glands from COVID-19 patients. Histopathological examination of adrenal glands (n = 30) reveals disruption of adrenal gland zonation in COVID-19 patients. In panel A normal zonation in zona glomerulosa, zona fasciculata, and zona reticularis is shown with hematoxylin-eosin staining (H&E) (A1) and immunohistochemistry for CYB5A (zona reticularis, A2) and for CYB11B2 (zona reticularis, A3). Panel B displays moderate disruption of the zonation and in panel C zonation of the cortex is not visible in H&E staining and is severely disrupted as visualized by immunohistochemistry. Quantification of disruption (score 0 (intact zonation, black), score 1 (moderate disruption, gray), and score 2 (severe disruption, white)) and statistical analysis (E) showed significantly more severe disruption of zonation in COVID-19 patients compared to influenza patients (p values were calculated using Chi-squared test). Source data are provided as a Source Data file. Marked interstitial fibrosis with collagen matrix production indicated in blue (D, Masson’s trichrome stain) was noticed in patients with seroconversion. Scale bars in A-C correspond to 200 μm in D to 100 μm. H&E staining of the pituitary gland and hypothalamus (F, G) shows no significant alterations (overview (scale bar = 100 μm and magnified inlet (scale bar = 10 μm)).
infiltrates in the hypothalamic or pituitary glands of COVID-19 patients. However, the severe damage of adrenal glands might lead to primary adrenal insufficiency with dysregulation of the HPA axis and hypocortisolemia in the end. Adrenal insufficiency is not a rare manifestation in COVID-19. In addition to several case reports\textsuperscript{10,25–29} some observational studies\textsuperscript{12,17} also reported this presentation. Like SARS-CoV, SARS-CoV-2 contains various amino acid sequences with homology to human adrenocorticotropic hormone (ACTH) key residues\textsuperscript{18}. Therefore, antibodies against the virus could eventually target the host ACTH and induce a relative cortisol insufficiency. In the acute setting in severely ill COVID-19 patients hypercortisolemia is prevalent\textsuperscript{30},
Methods

Study design and patient selection. The study was approved by the ethics committee of the LMU (project number 20-1039), informed consent was obtained from the legal representatives of deceased individuals and the study complies with all relevant ethical regulations.

We analysed adrenal gland autopsy specimens from 21 patients who died from COVID-19. All patients were diagnosed with COVID-19 ante mortem and PCR-tests for SARS-CoV-2 from postmortem nasopharyngeal swabs were positive except for two patients with known seroconversion. In addition, the hypothalami and pituitary glands of ten patients were analyzed. Adrenal glands were compared to those from ten patients who died from pneumonia caused by influenza A virus H1N1. Specimens from patients with influenza were archived tissues and chosen for the best possible match with respect to age, sex, and disease severity. Clinical features are detailed in Table 1. The mean duration of intensive care unit stay (ICU) was comparable between COVID-19 patients and influenza patients; in the COVID-19 group ten patients with no intensive care unit stay (ICU) were included and in the influenza group one patient did not stay on the ICU.

Histological and immunohistochemical evaluation. All adrenal glands were comprehensively analyzed by standard histopathology and histochemical methods (Hematoxylin and Eosin (H&E) and Masson’s trichrome stains). Adrenal cortical zonation was scored semiquantitatively based on the identification of the three zones ( zona glomerulosa, zona fasciculata, and zona reticularis) in the adrenal cortex at 5 x magnification. Zonation was graded as follows: 0 when the three adrenocortical zones were clearly visible, (1) when zonation was moderately disturbed and (2) when the three different zones were only distinguished by immunohistochemistry.

Standard immunohistochemistry was performed for detection of adrenal cortical zonation (Cytochrome P450 family 11 subfamily B member 1 (CYB11B2) and Cytochrome B5 (CYB5A)), apoptosis (cleaved caspase 3), and expression of angiotsenin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 precursor (TMPRSS2). After antigen retrieval slides were incubated with antibodies against CYB11B2 (1:50; Proteintech, USA), CYB5A (1:120; Atlas, Sweden) cleaved caspase 3 (1:100; Cell Signaling, USA), ACE2 (1:100; Abcam, Germany), or TMPRSS2 (1:70; Bio SB, USA). Detection was carried out with InmPress anti-rabbit IgG polymer kit (Vector Laboratories, USA) or MACH 3 Mouse HRP Polymer detection (Biocare Medical, USA), respectively, using the manufacturer’s protocol. For multispectral imaging CD8 (C8/144B, dilution 1:150, Medac GmbH, Germany), CD4 (EP204, 1:200, Medac GmbH), CD34 (QEBnd-10, manufacturer’s protocol. All slides were evaluated independently by two experienced pathologists (MR, HB). For statistical analysis SPSS (IBM, New York, USA) was used. Comparison of numeric variables was conducted with Student’s t-test and for categorical variables, Fisher exact test was applied.

Immunohistochemical detection and in situ hybridization of SARS-CoV-2 protein and RNA. Antibodies and probes for detecting SARS-CoV-2 spike protein (s-SARS-CoV-2-s protein) in formalin-fixed paraffin-embedded (FFPE) tissue were first validated on SARS-CoV-2 infected and non-infected CACO2 cells (ATCC, HTB-171) that were processed to FFPE blocks (supplementary Fig. 1). For immunohistochemistry of s-SARS-CoV-2-s protein, the SARS-CoV-2-s antibody (clone 1A9, Genetex, USA) was used at a dilution of 1:80. Detection was carried out with MACH 3 Mouse HRP Polymer detection (Biocare Medical, USA) following the manufacturer’s protocol. For in situ hybridization of SARS-CoV-2 a sense probe (RNAscope CoV2019-S-sense, ACD Bio-Technie, USA) and an antisense probe (RNAscope CoV2019-S-antisense, ACD Bio-Technie) was used. Detection was carried out with Opal 570 (Akoya Bioscience) according to the manufacturer’s protocol. All slides were evaluated independently by two experienced pathologists (MR, HB).

Cell culture experiments and western blot analysis. Cell culture. SW13 cells (ATCC, CCL-105), HAC13 cells (ATCC, CRL-3301), CACO2 cells (ATCC, HTB-37), and Vero-E6 cells (ATCC, CRL-1586) were cultivated in culture medium (SW13: L15, 10% fetal calf serum (FCS)), (HAC15: COVID-19 Therapy; ClinicalTrials.gov identifier:NCT04381936)

which might be attributed to reduced protein binding, persistent suppression of metabolic degradation, and stress-induced decrease of cortisol clearance. However, preliminary results of the RECOVERY trial (Randomized evaluation of COVID-19 Therapy; ClinicalTrials.gov identifier:NCT04381936) indicate a so-far unexplained survival benefit of low dose usage of steroids in critically ill COVID-19 patients. Moreover, Leow et al. reported hypocortisolism in forty percent of patients three months after recovery from SARS. Moreover fatigue and malaise characteristics for long-COVID are common in patients with adrenal insufficiency. Therefore, considering our findings of severe impairment of adrenal glands in COVID-19 patients monitoring for adrenal insufficiency during and after SARS-CoV-2 infection seems essential to avoid adrenal crisis.

In conclusion, we found adrenal tropism of SARS-CoV-2 in critically ill patients accompanied by adenalitis and pronounced damage of adrenal glands with disturbance of adrenal metabolism. Given the central role of the adrenal glands in stress damage of adrenal glands with disturbance of adrenal metabolism critically ill patients accompanied by adrenalitis and pronounced severe impairment of adrenal glands in COVID-19 patients steroids in critically ill COVID-19 patients Moreover, Leow and pituitary glands of ten patients were analyzed. Adrenal glands were compared to those from ten patients who died from pneumonia caused by influenza A virus H1N1. Specimens from patients with influenza were archived tissues and chosen for the best possible match with respect to age, sex, and disease severity. Clinical features are detailed in Table 1. The mean duration of intensive care unit stay (ICU) was comparable between COVID-19 patients and influenza patients; in the COVID-19 group ten patients with no intensive care unit stay (ICU) were included and in the influenza group one patient did not stay on the ICU.
Isolation and expansion of a SARS-CoV-2 clinical isolate. Caco2 cells cultivated in "virus isolation medium" (DMEM, 2% FCS, 100 U/mL penicillin-streptomycin, NEAA, 0.05 µg/mL gentamicin, and 0.25 µg/mL amphotericin B) were challenged for 2 h with a clinical isolate ([EU1, Panangol Lineage B.1.177, GISAID EPI ISL: 466888] previously obtained from a nasopharyngeal swab of a COVID-19 patient. Subsequently, cells were washed with a regular culture medium, and three days postinfection supernatant was collected and passaged onto Vero-E6 cells in the virus isolation medium. After three additional days, cell culture supernatants were harvested and stored at −80 °C. Further expansion of viruses was performed in expansion medium (DMEM containing 5% FBS, 100 U/mL penicillin-streptomycin, NEAA). Virus stocks were characterized by RT-qPCR, as reported previously, the infectious titer of virus stock was 1.79 × 10^6 IU/mL (IU = Infectious units), viral load of stock virus was 1.28 × 10^8 Geq/ml (Geq = genome equivalent). In parallel, near full-length genomes were generated from expanded stocks of SARS-CoV-2 following the ARTIC network nCoV-2019 sequencing protocol v2, as described previously.

SARS-CoV-2 infection experiments. For SARS-CoV-2 infection SW13 (5 × 10^5 cells per well) and HAC15 cells (5 × 10^5 cells per well) were plated in a 96-well plate (Sarstedt) in "virus infection medium" with low FCS (SW13: L15, 5% FCS), (HAC15: DMEM, F12, ITS, 5% FCS)). Three independent experiments with each two technical replicates were performed. Target cells were treated with either EDV (1 µM) 2 h before infection or were left untreated. Subsequently, cells were challenged with a serial dilution of a stock of an expanded SARS-CoV-2 clinical isolate (multi-NUEA, 0.5 µg/mL gentamicin, and 0.25 µg/mL amphotericin B) were challenged with a serial dilution of a stock of an expanded SARS-CoV-2 clinical isolate (multi-NSAA, 0.5 µg/mL gentamicin, and 0.25 µg/mL amphotericin B) were challenged with a serial dilution of a stock of an expanded SARS-CoV-2 clinical isolate (multi-

Nonspecific binding sites were blocked by incubation with 5% (w/v) nonfat dry milk in TTBS (0.1% Triton X-100, 20 mM Tris, 136 mM NaCl at pH 7.6) for 1 h. Subsequently, membranes were incubated with primary antibody (SARS-CoV-2 V2, Cell Signaling, USA), alpha-tubulin (Sigma-Aldrich, USA), TMPRSS2 (Bio SB, USA), Shopk, D. S. et al. (2021). J Clin Invest 131(11): 4180–4198. doi: 10.1172/JCI151022. This study investigated the role of SARS-CoV-2 TMPRSS2 in the replication of SARS-CoV-2 in Vero-E6 cells. The authors found that TMPRSS2 expression was upregulated in Vero-E6 cells following SARS-CoV-2 infection, and that inhibition of TMPRSS2 expression significantly reduced viral replication. These findings suggest that targeting TMPRSS2 may be a potential therapeutic strategy for the treatment of SARS-CoV-2 infection. 

Cell Signaling, USA), ACE2 (Abcam, Germany), cleaved caspase 3 (Cell Signaling, USA), β-Actin (Cell Signaling, USA) (1:1000) for 12 to 14 h at 4 °C. After incubation for 1 h with HRP-conjugated secondary antibody (anti-mouse IgG #7076) or anti-rabbit IgG #7074) cell signaling technologies, 1:2000) immunoreactivity was visualized with Super-Signal West Pico Chemiluminescent kit (Pierce).
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