

Immunohistochemical and Transcriptional Analysis of SARS-CoV-2 Entry Factors and Renin-Angiotensin-Aldosterone System Components in Lethal COVID-19

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Keywords

Coronavirus disease 2019 · Severe acute respiratory syndrome coronavirus-2 · Renin-angiotensin-aldosterone system · Angiotensin converting enzyme-2 · Transmembrane protease serine subtype-2

Abstract

Introduction: Since angiotensin converting enzyme-2 (ACE2) was discovered as an essential entry factor of SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), there has been conflicting evidence regarding the role of renin-angiotensin-aldosterone system (RAAS) in COVID-19. This study elucidates pulmonary expression patterns SARS-CoV-2 entry factors (ACE2 and transmembrane protease serine subtype 2, TMPRSS2) and RAAS components in lethal COVID-19. **Methods:** Lung tissue from COVID-19 autopsies ($n = 27$) and controls ($n = 23$) underwent immunohistochemical staining for RAAS components (angiotensin receptors 1 and 2, ACE2 and Mas-receptor) and bradykinin receptors 1 and 2. Staining of individual cellular populations (alveolar pneumocytes [ALV], desquamated cells [DES] and endothelium [END]) was measured by a binary scale (positive/negative).

SARS-CoV-2 was detected using immunohistochemistry against nucleocapsid protein, *in-situ* hybridization and quantitative reverse transcriptase polymerase chain reaction. Gene expression profiling for *ACE2*, *ACE* and *TMPRSS2* was performed. **Results:** Subtle differences were observed when comparing COVID-19 patients and controls not reaching statistical significance, such as a higher incidence of ACE2-positivity in END (52% vs. 39%) but lower positivity in ALVs (63% vs. 70%) and an overall downregulation of *ACE2* gene expression (0.25 vs. 0.55). However, COVID-19 patients with RAAS inhibitor (RAASi) intake had significantly shorter hospitalization times (5 vs. 12 days), higher viral loads (57,517 vs. 15,980/10⁶ RNase P-gene copies) and decreased *ACE*/*ACE2*-expression ratios (4.58 vs. 11.07) than patients without. *TMPRSS2* expression was significantly (1.76-fold) higher in COVID-19 patients than controls. **Conclusion:** Our study delineates the heterogeneous expression patterns of RAAS components in the lungs, which vary amongst cellular populations, and implies that COVID-19 patients with RAASi-intake present with a more rapid disease progression, although this requires further investigation.

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Introduction

Shortly after the emergence of the COVID-19 pandemic, angiotensin converting enzyme-2 (ACE2) and transmembrane protease serine subtype-2 (TMPRSS2) were identified as requisite cellular entry factors for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [1]. This promptly led to speculation regarding the role of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of COVID-19 and raised questions whether RAAS inhibitor (RAASi) therapy, such as angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB) and aldosterone antagonists, predisposed to SARS-CoV-2 infection [2]. RAASi therapy [3] and underlying cardiovascular disease [4] have been previously shown to lead to an increased ACE2 expression, thus implying an increased susceptibility of viral entry in patients with cardiovascular comorbidities. Indeed, previous studies have shown a higher propensity of severe COVID-19 in patients with hypertension and underlying cardiovascular disease [5–9], further underscoring the need to analyze the pathophysiological link between SARS-CoV-2 and RAAS.

ACE2, a monocarboxypeptidase homologous to ACE, promotes the conversion of angiotensin II (Ang-II) to Ang-(1-7), both ligands of plasma membrane receptors AGTR1 and AGTR2 (Ang-II receptors 1/2) and MasR (Mas-receptor) which act as central regulators of blood pressure homeostasis [10]. In the lung, AGTR1 activation by Ang-II leads to vasoconstriction, fibrotic remodeling, angiogenesis and increased vascular permeability, whereas AGTR2 elicits vasodilation and downregulates fibrosis [11]. MasR activation by Ang-(1-7), concordantly to AGTR2, results in nitric oxide-dependent vasodilation, anti-fibrotic and antiangiogenic effects [10–12]. ACE2 thus acts as an antagonist to ACE, negatively regulating vascular tone and remodeling [13], and has been shown to inhibit acute lung injury/diffuse alveolar damage (ALI/DAD) in the pulmonary microenvironment [14, 15].

SARS-CoV-2 utilizes its spike protein to bind to ACE2. The spike protein is cleaved by a transmembrane serine protease, TMPRSS2, which facilitates membrane fusion [16], ultimately leading to ACE2 internalization and shedding [13, 14]. Decreased membranous ACE2 leads to a relative accumulation of Ang-II, which then binds AGTR1, inducing vascular permeability and neutrophil accumulation; this induces noncardiogenic pulmonary edema and DAD [14, 15], a readily detected finding in

COVID-19 [9, 17]. Furthermore, decreased ACE2 prevents the degradation of des-Arg bradykinin, which results in the activation of bradykinin receptor 1 (BDKRB1), leading to hypotension, natriuresis and vasodilation due to the release of proinflammatory chemokines [18]. This “bradykinin storm” may be one of the underlying mechanisms for the inflammatory response in severe COVID-19 [19]. Thus, decreased membranous ACE2 may reduce susceptibility for viral entry but facilitates tissue damage, whereas increased ACE2 increases the likelihood for viral entry but simultaneously prevents ALI. These concepts demonstrate the complex, dynamic role of ACE2 in COVID-19 pathophysiology. To study the putative effects of RAAS and viral entry factors in severe COVID-19, we performed an immunohistochemical (IHC), viral *in-situ* analysis and gene expression profiling on RAAS components, bradykinin receptors, ACE2 and TMPRSS2 on lung tissue from patients with lethal COVID-19 versus age-matched controls.

Materials and Methods

Patient Selection

Twenty-seven COVID-19 autopsy cases and 23 controls were enrolled [9]. All COVID-19 patients tested positive for SARS-CoV-2 per antemortem nasopharyngeal swab. The cause of death in all cases was COVID-19-associated respiratory failure. Age-matched SARS-CoV-2 negative autopsy controls were selected in line with a range of etiologies (histomorphologically unremarkable lungs, $n = 5$; pneumococcus pneumonia, $n = 4$; influenza pneumonia, $n = 5$; non-COVID-19 DAD, $n = 9$). Causes of non-COVID-19 DAD included infections by other pathogens (*E. faecalis*, *C. albicans*, *K. pneumoniae* and *Pseudomonas spp.*), postoperative/paraneoplastic and post-stem cell transplant/drug-induced. Controls with pneumococcus and influenza pneumonia did not have histological signs of DAD. ACEi, ARB, renin inhibitors and aldosterone antagonists were considered as RAASi.

Tissue Sampling, Processing, Immunohistochemistry, and in situ Hybridization

A lung tissue sample measuring $1.5 \times 1 \times 0.3$ cm was placed in 4% phosphate-buffered formalin at autopsy for 48 h and embedded in paraffin. Tissue microarrays (TMAs) were generated of all samples using the TMA Grand Master (3DHitech Ltd., Budapest, Hungary). Representative regions of histological sequelae of COVID-19 (i.e., characteristic changes in the vascular compartment, DAD) were annotated by expert staff pathologists on corresponding hematoxylin and eosin-stained sections. These regions were transferred to a recipient block, generating three 1.5 mm-diameter cores per patient, analogous to previous protocols [20]. Slides were stained with hematoxylin and eosin and for AGTR1&2, ACE2, MasR, BDKRB1&2 and SARS-CoV-2 nucleocapsid protein (online suppl. Table 1 for detailed protocols; for all online suppl. material, see www.karger.com/doi/10.1159/000520221). *In-situ* hybridization for SARS-CoV-2 was executed as previously described

Table 1. Clinical characteristics of COVID-19 patients, classified according to RAASi-intake, in comparison with age-matched controls

	COVID-19 patients (n = 27)			Controls (n = 23)
	RAASi (n = 14)	no RAASi (n = 13)	total	
Clinical characteristics				
Age, median (IQR)	80 (21)	72 (12)	78 (33)	76 (35)
BMI, median (IQR)	27 (9)	28 (5)	27 (19)	27 (13)
Sex, male, n (%)	10 (77)	10 (77)	19 (70.4)	13 (56.5)
Hospitalization time, days, median (IQR)	5 (4)**	12 (7)**	7 (27)	7 (39)
Hypertension, n (%)	14 (100)**	9 (69)**	23 (85)	15 (65)
Cardiovascular disease, n (%)	9 (64)	9 (69)	18 (67)	14 (61)
Pathological findings				
DAD (% total)	9 (64)	8 (72)	17 (63)	11 (48)
Exudative	7 (50)	2 (15)	9 (33)	6 (26)
Proliferative	2 (14)	6 (46)	8 (27)	5 (22)
None	5 (36)	5 (38)	10 (37)	12 (52)
SARS-CoV-2 qRT-PCR viral load, mean (SD)	57,517 (64,451)**	15,980 (53,527)**	37,517 (64,161)	n/a
Expression of ACE, median (IQR)	1.99 (2.88)	2.69 (1.96)	2.48 (2.20)	2.07 (3.14)
Expression of ACE2, median (IQR)	0.33 (0.36)	0.24 (0.20)	0.25 (0.28)	0.55 (0.71)
ACE/ACE2 ratio, median (IQR)	4.59 (5.03)*	11.08 (20.72)*	6.47 (11.86)	5.16 (8.11)
Expression of TMPRSS2, median (IQR)	1.76 (2.00)	2.30 (0.84)	2.00 (1.00)*	1.30 (1.06)*

RAASi, renin-angiotensin-aldosterone system inhibitor; DAD, diffuse alveolar damage; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ACE/ACE2; angiotensin converting enzyme/angiotensin converting enzyme-2; TMPRSS2, transmembrane protease serine subtype 2. * bold = *p* values significant at the 0.05 level. ** bold = *p* values significant at the 0.01 level.

[21]. TMAs were manually assessed for IHC/*in-situ* hybridization positivity in different cellular populations (positive staining in alveolar pneumocytes [ALVs], desquamated cells [DESS] and endothelium [END]) by means of a binary scale (positive/negative) (online suppl. Methods). Results were independently controlled and corroborated by 2 pathologists (A.T. and J.D.H.) and Cohen's kappa coefficient (κ) values ranging from fair ($\kappa = 0.4$ for endothelial staining of AGTR2) to substantial agreement (e.g., $\kappa = 0.769$ for alveolar and endothelial staining of ACE2) to perfect agreement (e.g., $\kappa = 1$ for ACE2 staining DESS).

Quantitative Reverse Transcriptase Polymerase Chain Reaction
See online supplementary Methods.

Gene Expression Profiling
See online supplementary Methods.

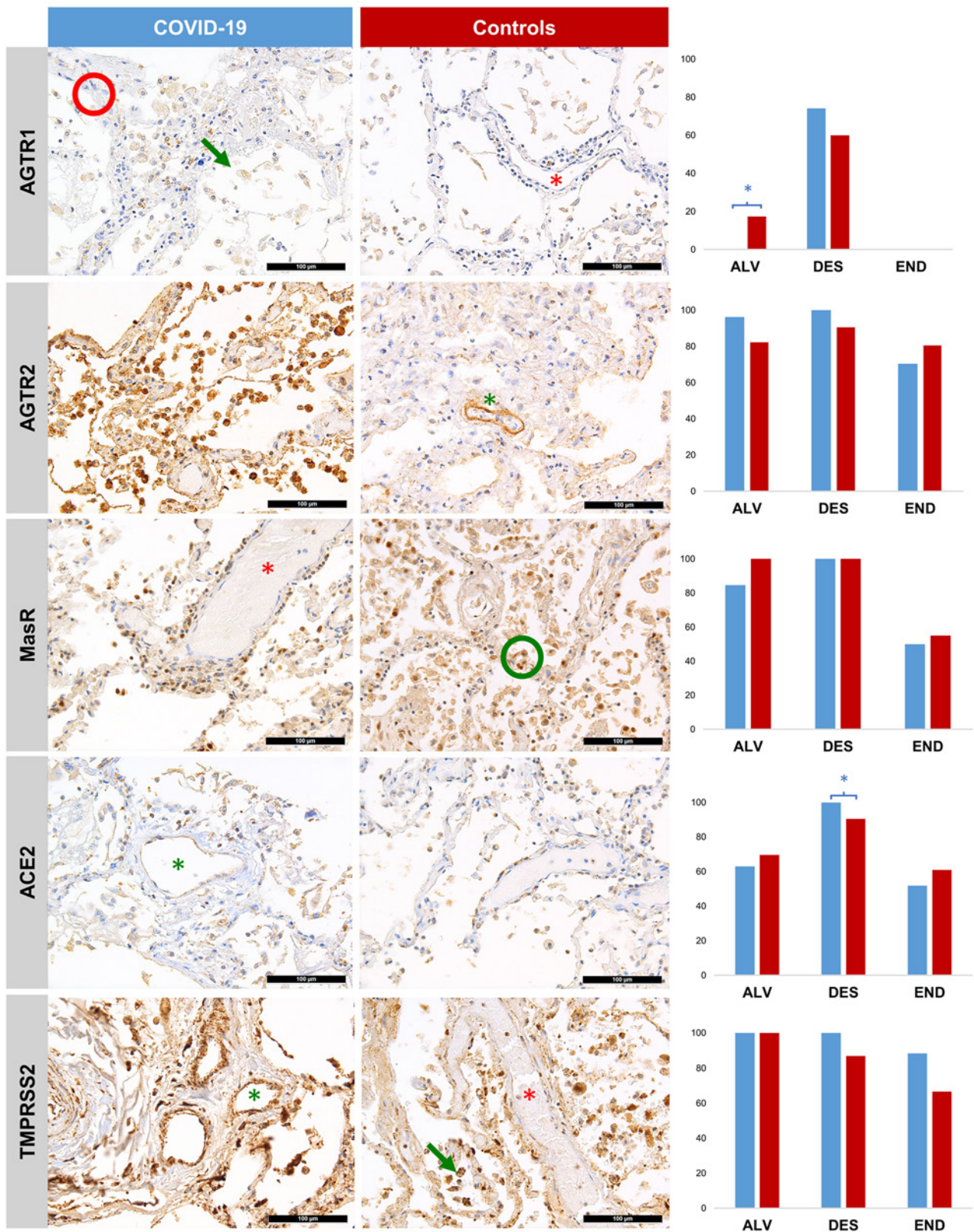
Statistical Analysis

Statistics was performed with IBM® SPSS®, version 25 (Armonk, NY, USA). 2-sided Fisher's exact tests were performed on categorical data. Nonparametric variables were computed using a Mann-Whitney U-test, while parametric variables were processed with Student's *t* test. Correlation analyses were performed with Spearman ρ . *p* values below 0.05 were considered significant. No adjustment for multiple testing was applied because the setting was considered hypothesis generating/exploratory.

Results

Clinical Characteristics and Pathology Findings

Clinical characteristics are summarized in Table 1. A higher proportion of COVID-19 patients had a history of arterial hypertension compared to controls (85 vs. 62%; *p* = n.s.); in parallel, the incidence of RAASi-intake was higher amongst COVID-19 patients (14/27; 52% vs. 9/23; 39%; *p* = n.s.). When comparing characteristics in lethal COVID-19, patients with RAASi-intake had a significantly shorter interval between admission and death (hospitalization time 5 vs. 12 days; *p* = 0.006) and, concordantly, displayed a significantly higher postmortem pulmonary viral load (57,517 vs. 15,980/10⁶ RPPH1 copies, *p* = 0.0041; Fig. 3), mirroring results on the same autopsy cohort [22]. COVID-19 patients with RAASi-intake were more likely to present with exudative DAD (50% vs. 15%; *p* = n.s.). Furthermore, amongst COVID-19 patients, hospitalization time significantly negatively correlated with pulmonary viral load ($\rho = -0.635$, *p* = 0.0004). As all COVID-19 cases stem from the first wave of the pandemic (March–June 2020), dexamethasone and antiviral treatments were uncommon (dexamethasone: 1/27, remdesivir: 3/27).



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Distribution of RAAS Components between COVID-19 Patients and Controls

Figure 1 and Table 1 display histomorphological characteristics and trends of RAAS components and SARS-CoV-2 entry factors between COVID-19 patients and controls. IHC for AGTR1 revealed weak membranous and cytoplasmatic positivity, predominantly in DESs (approximately half of epithelial and half of histiocytic origin) (up to 74% in COVID-19 lungs vs. 60% in controls, $p = n.s.$). ALVs and END did not display any AGTR1 positivity in COVID-19 patients, while sporadic positivity was observed amongst controls (4/23, $p = 0.04$). In contrast, AGTR2 displayed more widespread staining, including ALVs, DESs and END (no differences between COVID-19 patients and controls). Staining for MasR revealed lower positivity in ALVs of COVID-19 patients than controls (82% vs. 100%, $p = n.s.$), while positivity in END was equally heterogeneous in both COVID-19 and control patients (48% in both). Staining for ACE2 revealed similar positivity in ALVs in both COVID-19 patients and controls (63% vs. 70%, $p = n.s.$), while a significantly higher

number of cases displayed positivity in DESs (100 vs. 83%; $p = 0.04$). Endothelial positivity of ACE2 was slightly but not significantly lower in COVID-19 patients (39 vs. 52%, $p = n.s.$), which was similarly mirrored in TMPRSS2 staining (85 vs. 61%, $p = n.s.$). In other cellular populations, TMPRSS2 displayed equally widespread staining in both COVID-19 cases and controls. IHC for BDKRB1 and BDKRB2 revealed widespread nuclear and membranous positivity in ALVs and some DESs, as well as bronchial epithelium and immune cells such as granulocytes and lymphocytes, which in some COVID-19 cases revealed more extensive positivity, although this could not be quantified conclusively (Fig. 2).

Comparative Analysis of RAAS Components amongst COVID-19 Patients, Grouped by Intake of RAASi

Comparison of IHC positivity of individual cellular populations revealed characteristic trends amongst lethal COVID-19 cases dependent on the previous intake of RAASi (Fig. 3). This was predominantly discernible in the END, where there was a lower incidence of positivity

Fig. 2. Histomorphological characteristics of BDKRB1 and 2 in lethal COVID-19 cases versus controls. Annotations: green circle: membranous positivity of ALVs for BDKRB1; green arrow: membrano-cytoplasmatic positivity for BDKRB1 in a DES; red asterisk: positivity for BDKRB1 and 2 in interstitial cells including lymphocytes; green triangle: positivity for BDKRB2 in bronchial epithelium. Error bars denote 100 μm for BDKRB1 and 200 μm for BDKRB2. DES, desquamated cell; ALV, alveolar pneumocyte; BDKRB1 and 2, bradykinin receptors 1 and 2.

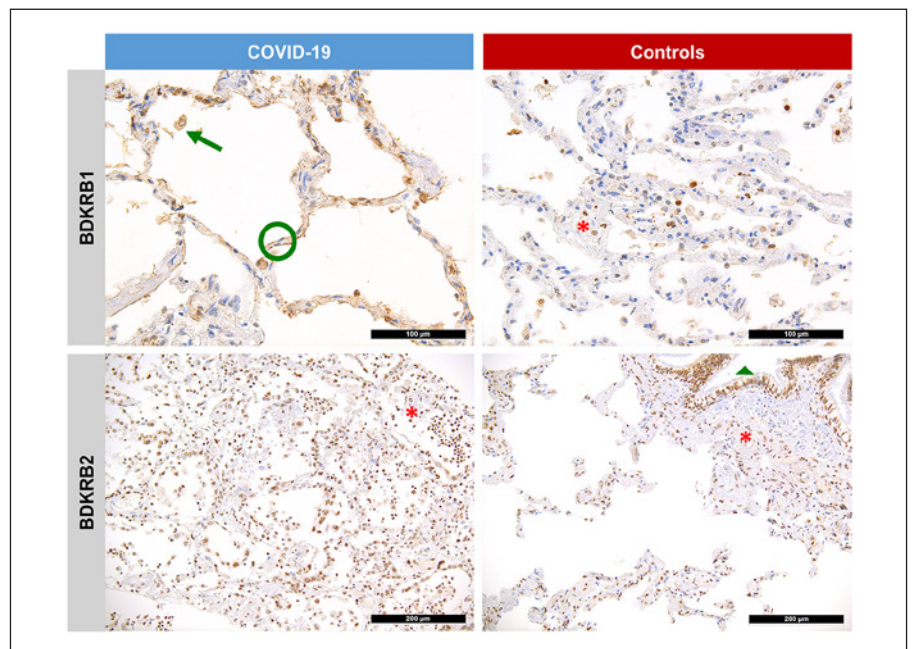
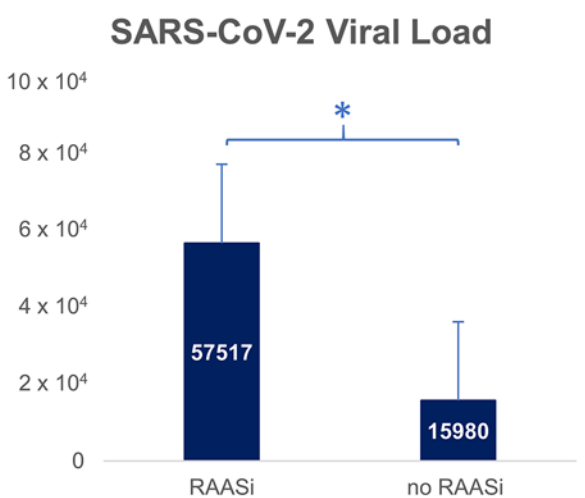
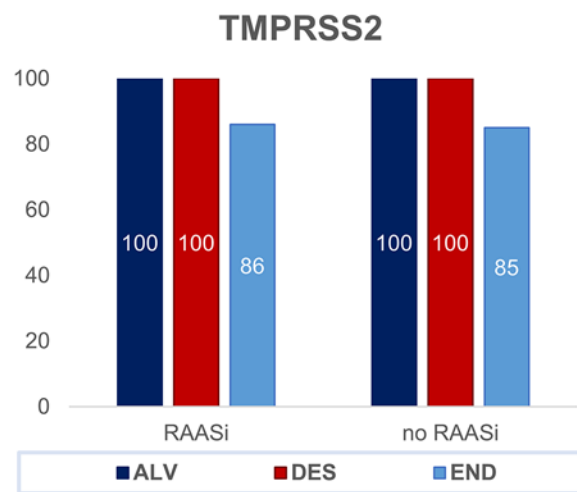
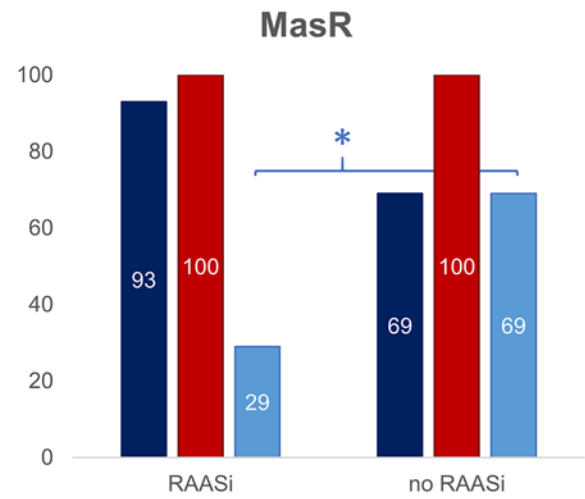
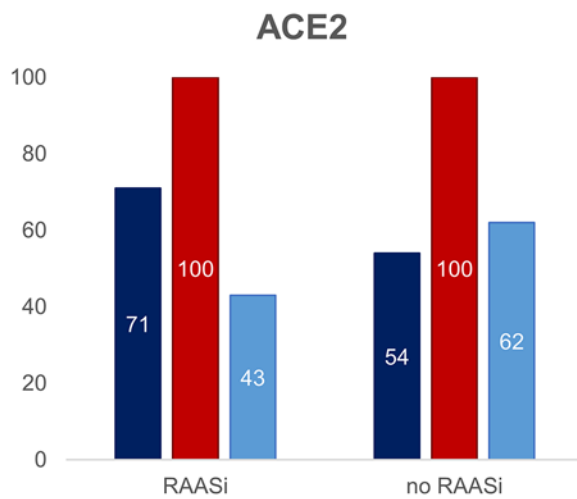
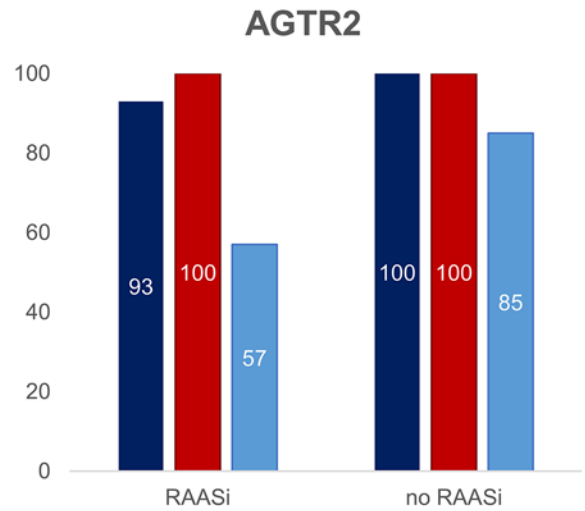
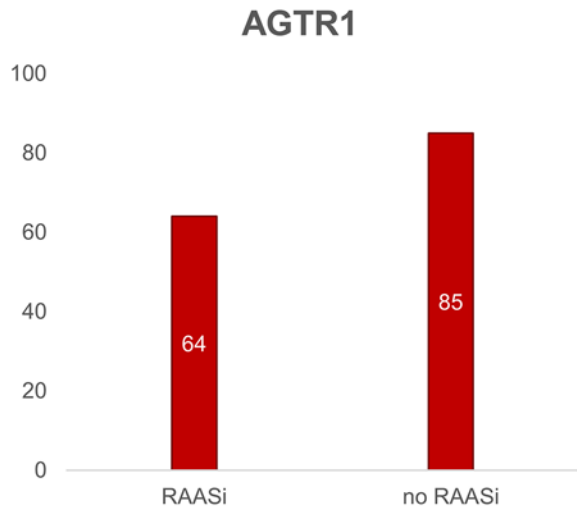


Fig. 1. Histomorphological characteristics of RAAS components and SARS-CoV-2 entry factors in lethal COVID-19 cases versus controls. Graphs display % positivity in varying cellular populations. * indicates p values <0.05 . Exemplary annotations: green = positive, red = negative. Arrows: DESs/macrophages; asterisks: END; circles: ALVs. Error bars denote 100 μm . ALV, alveolar

pneumocyte; DES, desquamated cell; END, endothelium; RAAS, renin-angiotensin-aldosterone system; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; AGTR1, angiotensin receptor 1; AGTR2, angiotensin receptor 2; MasR, Mas-receptor; ACE2, angiotensin converting enzyme-2; TMPRSS2, transmembrane protease serine subtype 2.



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amongst patients with RAASi-intake for AGTR2 (57 vs. 85%, $p = n.s.$), ACE2 (43 vs. 62%, $p = n.s.$), and, significantly, MasR (29 vs. 69%, $p = 0.04$). Positivity of ALVs behaved inversely, with a more prevalent incidence of positive staining for ACE2 and MasR in cases with RAASi-intake (ACE2: 71 vs. 62%, $p = n.s.$; MasR: 93 vs. 69%, $p = n.s.$). SARS-CoV-2 viral load was significantly higher in patients with RAASi-intake ($57,517$ vs. $15,980/10^6$ RPPH1 copies, $p = 0.0041$).

Detection of SARS-CoV-2 in COVID-19 Lung Tissue

Figure 4 displays the *in-situ* detection of SARS-CoV-2. SARS-CoV-2 was detected in various cellular populations (1/27, 4% in ALVs; 9/27, 33% in DESs; and 3/27, 11% in the END). Double staining with ERG (ETS-related gene) revealed viral nucleocapsid protein in close association with endothelial structures (ERG = brown and SARS-CoV-2 N = red) (Fig. 4). SARS-CoV-2 viral load mea-

sured by quantitative reverse transcriptase polymerase chain reaction was significantly higher in cases staining positive ($115,910$ vs. $19,701/10^6$ RPPH1 copies; $p = 0.001$). Most notably, all cases with positive detection of SARS-CoV-2 had a history of arterial hypertension with RAASi therapy.

ACE/ACE2 Ratio and TMPRSS2 Gene Expression in COVID-19 Patients versus Controls Grouped by RAASi-Intake

Gene expression of ACE, ACE2 and TMPRSS2 according to RAASi-intake is summarized in Figure 5. ACE/ACE2 ratio was slightly higher amongst COVID-19 patients than controls (6.47 vs. 5.16 ; $p = n.s.$). However, when grouped according to RAASi-intake, COVID-19 patients displayed significantly lower ACE/ACE2 ratios than those without RAASi (4.58 vs. 11.07 ; $p = 0.04$), while there was no significant difference in controls (4.73 vs.

Fig. 4. Detection of SARS-CoV-2 N-protein by immunohistochemistry and RNA by ISH. Top left: positivity for viral N-protein in hyaline membranes and in the endothelial compartment; top right: positivity for viral RNA in ALVs, endothelia and hyaline membranes. Scale bars denote 100 μ m. Bottom: double staining with N-protein (red) and ERG (brown) reveals close association of viral proteins and the END (black arrows: endothelial cell surface coated with N-protein). SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; N-protein, nucleocapsid protein; ISH, *in-situ* hybridization; END, endothelium; ALV, alveolar pneumocyte; ERG, ETS-related gene.

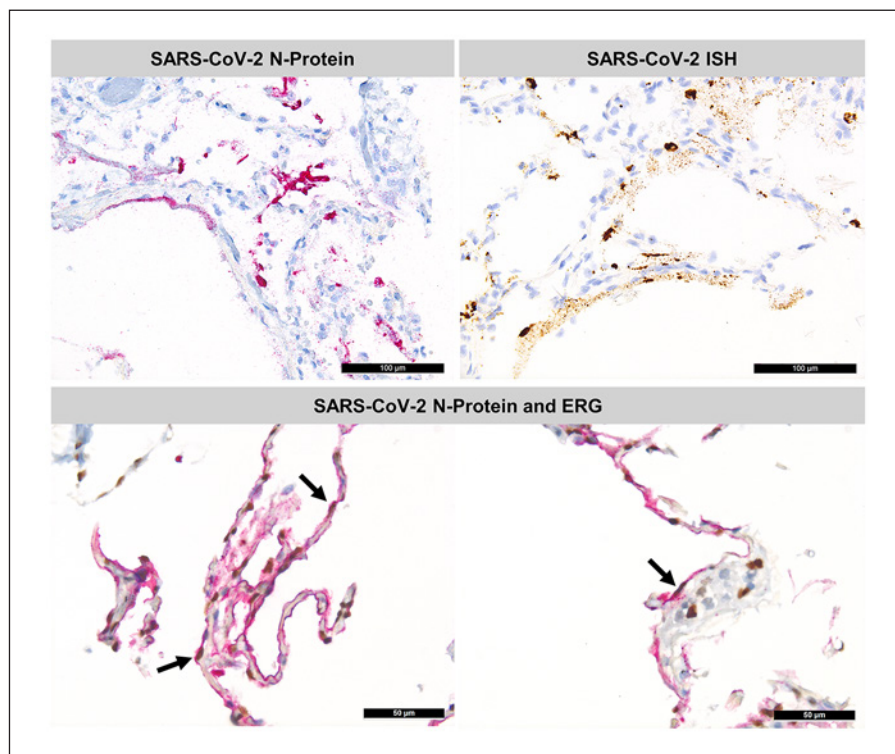
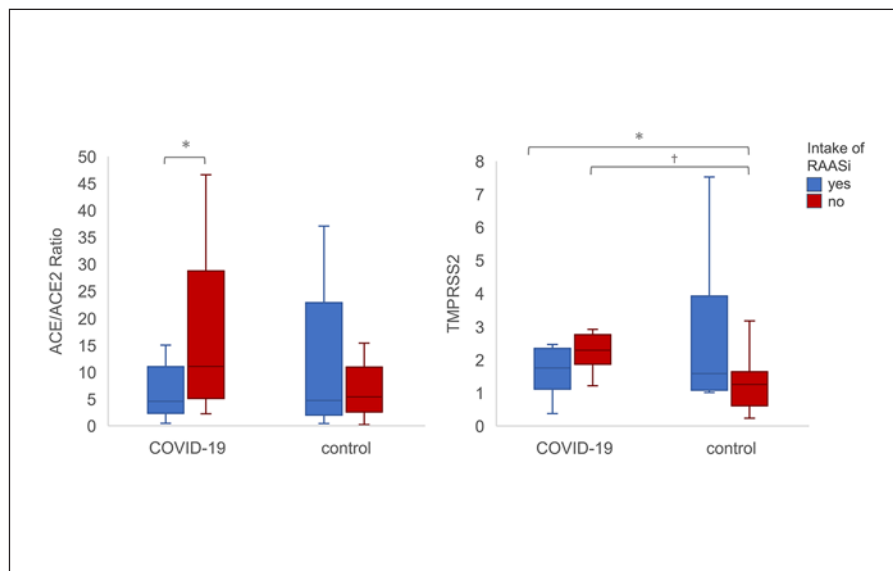


Fig. 3. Trends in % positivity of RAAS components and SARS-CoV-2 entry factors in lethal COVID-19 cases, grouped by intake of RAASi. A lower incidence of endothelial positivity of AGTR2, ACE2, and MasR is noted in cases with RAASi-intake. Conversely, ALV positivity is higher for ACE2 and MasR amongst cases with RAASi-intake. *indicates p values <0.05 . Viral load is mapped as per 10^6 RPPH1 copies. ALV, alveolar pneumocyte; DES, desqua-

mated cell; END, endothelium; RAASi, renin-angiotensin-aldosterone system inhibitor; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; AGTR1, angiotensin receptor 1; AGTR2, angiotensin receptor 2; ACE2, angiotensin converting enzyme-2; MasR, Mas-receptor; TMPRSS2, transmembrane protease serine subtype 2; RPPH1, RNase P-gene.

Fig. 5. Gene expression analysis of ACE/ACE2 ratio and TMPRSS2 in COVID-19 versus controls. Left: patients with RAASi-intake have a significantly lower ACE/ACE2 ratio amongst COVID-19 patients ($*p < 0.05$), while there was no significant difference in the control arm. Right: TMPRSS2 gene expression did not significantly differ with RAASi-intake. An overall significantly lower TMPRSS2 expression was observed in COVID-19 versus controls, irrespective of RAASi-intake ($*p < 0.05$). Amongst patients without RAASi-intake, TMPRSS2 expression was higher in COVID-19 ($^{\dagger}p < 0.01$). ACE/ACE2, angiotensin converting enzyme/angiotensin converting enzyme-2; TMPRSS2, transmembraneproteaserinesubtype2; COVID-19, coronavirus disease 2019; RAASi, renin-angiotensin-aldosterone system inhibitor.



5.38, $p = \text{n.s.}$). ACE and ACE2 were decreased in COVID-19 patients versus controls, and even more decreased in cases with RAASi-intake, not achieving significance (Table 1). Overall TMPRSS2 expression was higher in COVID-19 patients than controls (1.76-fold; $p = 0.04$), in particular amongst patients without RAASi-intake (1.83-fold; $p = 0.003$). Amongst COVID-19 cases, TMPRSS2- ($\rho = -0.629$, $p = 0.001$) and ACE-gene expression ($\rho = -0.629$, $p = 0.001$) strongly negatively correlated with viral load, while no significant correlation was observed for ACE2 (online suppl. Results); these correlations were not observed in controls.

Discussion

This analysis of SARS-CoV-2 entry factors and RAAS components revealed subtle differences between COVID-19 patients and controls, but more characteristic, significant RAASi-dependent differences amongst COVID-19 patients, both at the IHC and transcriptional level, which are systematically discussed below. ACE2 exhibited subtle nonsignificant lower positivity in ALVs and higher positivity in the END in COVID-19 patients versus controls. These findings, together with an overall downregulation of ACE2 gene expression, may indicate a redistribution of ACE2 in different cellular populations in COVID-19. ACE2 expression amongst sub-anatomical locations of the respiratory tract and cellular populations has previously been reported as heterogeneous [23–

26], with the most abundant expression in type 1 and, in particular, type 2 pneumocytes [26]. Our results support the premise of SARS-CoV-2-dependent ACE2 depletion, which may be the pathophysiological hallmark of COVID-19-associated DAD.

As described above, an overall paucity of ACE2 leads to accumulation of Ang-II and, by extension, an increased activity of AGTR1/AGTR2. Here, a slight, albeit nonsignificant increase of AGTR2 positivity amongst COVID-19 patients was observed, while AGTR1 staining showed weak staining; this is expected as AGTR2 has higher pulmonary tissue specificity [27, 28]. In line with these observations, MasR, which is activated by ACE2 and Ang1-7, correspondingly displayed marginally lower positivity in ALVs and END of COVID-19 patients. The MasR/ACE2/Ang1-7-axis has a known protective role against ALI and chemokine production [29, 30]; its downregulation thus contributes to a proinflammatory microenvironment, which may facilitate COVID-19-associated DAD [31].

TMPRSS2, which showed ubiquitous IHC positivity without significant morphological differences between COVID-19 patients and controls, strikingly presented with significantly higher gene expression in COVID-19. This may be explained by a higher enzymatic activity due to SARS-CoV-2-dependent internalization of ACE2 and thus supports our findings of decreased ACE2 in COVID-19. However, male gender-specific co-expression between TMPRSS2 and other genes related to SARS-CoV-2 entry has been reported [32, 33],

highlighting a possible explanation for the male predominance of COVID-19. This is further supported by the role of *TMPRSS2-ERG* fusions in prostate cancer [34] and the strong regulation of *TMPRSS2* by androgens [35]. Thus, the male predominance amongst COVID-19 cases in our cohort (70.4% vs. 56.5%) may similarly serve as an explanation for the increased *TM-PRSS2* gene expression.

Comparison of RAAS component-staining as well as *TMPRSS2* and *ACE2* gene expression yielded characteristic findings dependent on RAASi-intake, summarized in Figure 6. Interestingly, COVID-19 patients with RAASi-intake had shorter hospitalization times, were more likely to present with exudative DAD and presented with higher pulmonary viral loads. Furthermore, all COVID-19 patients with positive viral detection suffered from arterial hypertension treated with RAASi. Therefore, RAASi may predispose to more severe disease dynamics, outcome and extensive viral replication, leading to earlier death. This may conceivably be due to RAASi-dependent increase of ACE2, which was previously described in the literature [3, 36, 37], possibly also reflected by observations on ALVs in this study as well as the significantly decreased *ACE/ACE2* ratio in COVID-19 patients taking RAASi. Indeed, a decreased *ACE/ACE2* ratio has previously been observed in ARDS [38]. Additionally, the findings of this study mirror analyses performed on the same cohort, linking differing immunopathological [22] and cardiopathological [39] characteristics to hospitalization time and viral load; whether this is connected to changes in RAAS remains to be elucidated. In contrast to our observations however, a series of population-based studies have refuted any associations between RAASi and unfavorable prognosis of COVID-19 [40–43], although one study reported a higher risk of hospitalization among COVID-19 patients taking RAASi (OR 1.84) or ARB (OR 1.61) [40]. Another prospective randomized trial reported no significant difference of disease severity upon discontinuation of RAASi but suggests that discontinuation may lead to faster recovery [44]. Our data should thus be interpreted with caution, especially because of several potential unmeasured confounding factors and the limited statistical power of a smaller cohort.

Mounting evidence suggests that the endothelium plays an instrumental role in COVID-19 [31], characterized by an upregulation of thromboinflammatory genes [17, 45] leading to increased incidence of (micro-)thrombotic events [46]. Endothelial tropism for SARS-CoV-2 was observed in this study, further underlining these findings. Interestingly, endothelial cells expressed less

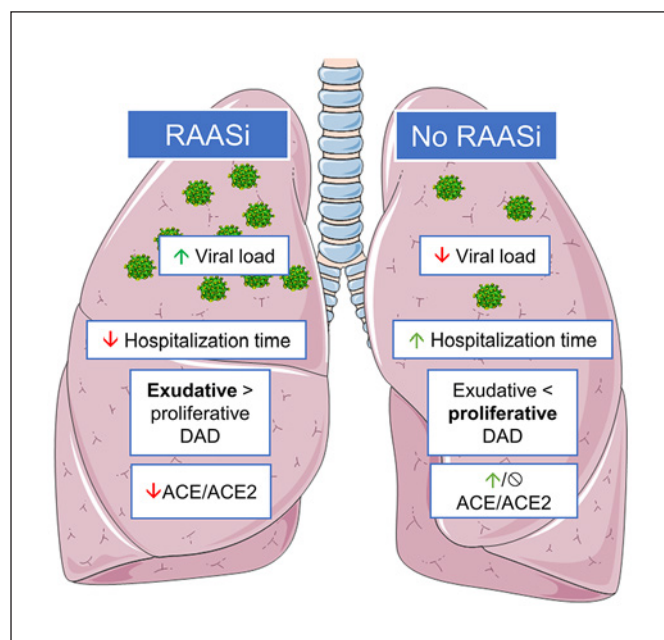


Fig. 6. Summary of clinical and pathological characteristics in lethal COVID-19, dependent on RAASi-intake. Patients with RAASi-intake were observed to have higher viral loads, shorter hospitalization times (time till death) and significantly lower ACE/ACE2 ratios. A higher proportion of patients with RAASi-intake presented with exudative DAD, in line with the shorter hospitalization times. Original lung artwork by Servier Medical Art®, licensed under a Creative Commons Attribution 3.0 Unported License. COVID-19, coronavirus disease 2019; RAASi, renin-angiotensin-aldosterone system inhibitor; ACE/ACE2, angiotensin converting enzyme/angiotensin converting enzyme-2; DAD, diffuse alveolar damage.

ACE2 and MasR under RAASi, while ALVs expressed more. These findings may be significant, taking into consideration the pathophysiological ties between RAAS and vascular inflammation and remodeling [47]. Previously performed *in-vitro* studies demonstrated an upregulated ACE2/MasR axis and increased ADAM17-mediated shedding of ACE2 in CD34+ hematopoietic stem/progenitor cells under hypoxic stimulation. These effects were mirrored by increase of vascular endothelial growth factor and stromal-derived factor 1 α , implying a crucial role of ACE2/MasR in vascular repair [48]. Thus, a decreased activity of ACE/MasR in COVID-19 cases of our cohort may signify a dysregulation in vascular signaling and remodeling in addition to thromboinflammation. Further studies of RAAS expression patterns within the endothelium in particular and the associated expression patterns in other cellular populations in the pulmonary microenvironment are required.

The complex interaction between the Kallikrein-Kinin system and RAAS is well established. ACEi have previously been found to increase bradykinin synthesis, thus stimulating BDKRB1/2, which, in turn, results in an up-regulation of endothelial nitric oxide synthase, angiogenesis-related genes and prostaglandins, while simultaneously downregulating NF- κ B [19, 49]. Diffuse positivity of inflammatory cells (mostly granulocytes, lymphocytes, and macrophages) for ACE2 and BDKRB1/2 in our cohort potentially supports this. However, staining patterns for BDKRB1/2 were diffuse in all samples, which rendered difficulties in statistical analysis between COVID-19 patients and controls. Thus, although this study does not offer any histological evidence of a “bradykinin storm,” its inherent role in severe COVID-19 needs to be further elucidated.

This study has several limitations. Firstly, its retrospective design may give rise to confounding factors which may have influenced the expression of ACE and ACE2. Its limited sample size ($n = 50$) suggests that results may not be representative enough and could explain why some p values did not reach significance. Secondly, controls were pooled from a wide variety of histological subgroups, all of which likely possess extensive variations in RAAS component expression. Thirdly, several COVID-19 patients without RAASi therapy (69%) suffered from arterial hypertension; these are patients who were treated with other antihypertensive drugs; the extent of which these impact RAAS is currently unclear. Fourthly, the heterogeneity of lung tissue is likely not sufficiently captured with the TMA. Lastly, IHC positivity was only measured by a binary scale as staining intensity of diaminobenzidine does not reflect antigen quantity. In summary, our findings underscore the heterogeneity of the expression of RAAS components described in the literature and identify a potential link between RAASi-intake and disease dynamics in COVID-19, which urgently requires further investigation.

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Statement of Ethics

This study has received approval by the Ethics Committee of Northwestern and Central Switzerland (ID 2020-00629). Samples used in this study were obtained as part of routine medical care. Written informed consent was obtained from participants prior to the study (or next of kin).

Conflict of Interest Statement

The authors have no financial interests to disclose. A.T. is a co-editor of *Pathobiology* but withdrew in all respective functions in connection to the present manuscript.

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Author Contributions

Study design was contributed by A.T. and J.D.H. J.D.H. and A.T. completed the manuscript. Clinical data acquisition was contributed by J.D.H. and A.S. Histology was contributed by J.D.H., M.M., and A.T. Statistics was contributed by J.D.H. Critical revision of the manuscript was contributed by A.S., S.B., K.M., C.Z., and M.M. All authors read and approved of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary Material files. Further enquiries can be directed to the corresponding author.

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