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Intestinal protein characterisation of SARS-CoV-2 entry molecules ACE2 and TMPRSS2 in Inflammatory Bowel Disease (IBD) and fatal COVID-19 infection

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Abstract: The coronavirus SARS-CoV-2 contributes to morbidity and mortality mainly as a result of immune-pathology in the lungs. Recent data has shown multi-system involvement with widespread viral tropism. Here we present a detailed intestinal protein characterisation of SARS-Cov-2 entry molecules ACE2 and TMPRSS2 in patients with Inflammatory Bowel Disease ([IBD]; Ulcerative Colitis [UC] and Crohn's disease [CD]) with age- and sex-match non-IBD controls; and in those with fatal COVID19 infection. Our study showed that IBD gut inflammation did not influence ACE2 and TRPMSS2 expression. Of interest, colonic protein expression of ACE2 and TRPMSS2 are cytoplasmic distinct to the membranous pattern in the ileum. We observed a significant increase in immune cells within the lamina propria in UC and CD that expressed ACE2 and TRMPSS2 when compared to non-IBD controls. These were identified as plasma cells with multiple myeloma oncogene 1/interferon regulatory factor 4 (MUM1/IRF4) expression. In six fatal COVID19 cases, there was no gut inflammation despite evidence of viral tropism within the enterocytes. Our data provides evidence for tissue expression of entry molecules ACE2 and TMPRSS2 including a close apposition to plasma cells – both pointing towards a role of the gut in the immune response to SARS-CoV-2 infection.

Key words: COVID19; SARS-CoV-2; IBD; UC; Crohn's disease; inflammation; Gut biology

The pathogenic mechanisms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in mediating the clinical syndrome of COVID19 are increasingly better understood. The SARS-Cov-2 virus gains entry to host cells upon binding of its spike (S) proteins to the angiotensin I converting enzyme 2 (ACE2) receptor where the transmembrane protease (TMPRSS2) primes the S proteins to facilitate this process¹. Although mortality and morbidity associated with COVID19 are driven largely by immunopathology in the lung, viral protein and RNA are detected throughout the body². ACE2 and TRMPSS2 for examples, are highly expressed within the gastrointestinal tract, where transcriptomics analysis show highest expressions in the ileal and colonic enterocytes³. Diarrhoea is the most common GI symptoms and there is considerable debate whether this is linked to more severe COVID19 (reviewed in⁴). Pertinently, two on-going questions arise on the importance of the gastrointestinal tract and its role in faecal-oral transmission and as a site of host immune response to SARS-CoV-2. Of immediate relevance is the potential importance of local tissue factors such as the presence of chronic inflammation and in common chronic immune-mediated gastrointestinal conditions such as Inflammatory Bowel Diseases (IBD) comprising of Ulcerative Colitis (UC) and Crohn's disease (CD) that affect more than 10 million people globally. In this context, we characterized the gastrointestinal protein expression of ACE2 and TRMPSS2 in individuals with UC and CD; and also in those with fatal COVID19 (n=11, 10 and 6 respectively; East of Scotland Ethical Review No 20/ES/0061 and 16/ES/0084 for IBD and COVID19-ICECAP post-mortem studies respectively).

In UC, ACE2 and TMPRSS2 display cytoplasmic expression; whereas in CD, they have a membranous pattern that is more pronounced in the ileum likely due to presence of the brush border in the small bowel (**Fig 1A**). In UC and CD, ACE2 and TMPRSS2 protein expression levels are similar in actively inflamed and unaffected parts of the colon (**Fig 1B and 1C**) suggesting that IBD gut inflammation does not lead to an increase of ACE2 and TMPRSS2. *In silico* analysis of our previously published colonic gene microarray dataset (99 CD, 129 UC and 50 non-IBD controls; Data set available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) (accessed September 2020) accession: GSE11223 and GSE20881)⁵ support this, by showing no differences in ileo-colonic ACE2 and TMPRSS2 across these groups; and no correlation with inflammation (**Fig 1D and 1E**). Secondly, we observed a significant increase in immune cells within the lamina propria in UC and CD that expressed ACE2 and TRMPSS2 when compared to non-IBD controls (**Fig 1F and 1G**). On histopathological review (GI-pathologist, KK), these were identified as plasma cells together with the finding of protein co-localisation with multiple myeloma oncogene 1/interferon regulatory factor 4 (MUM1/IRF4) expression, a commonly used plasma cell marker (**Fig 1H and II**). Thirdly, in six fatal COVID19 cases (using tissue generated from the ICECAP post-mortem study), there was no gut inflammation despite evidence of Sars-CoV-2 viral tropism within the enterocytes². Subsequent analysis demonstrates that

ACE2 and TRMPSS2 enterocyte expressions are not different compared to age- and sex-matched non-IBD controls (**Fig 2A and 2B**). In line with IBD, we identified MUM1+ plasma cells co-localised with ACE2 within the lamina propria in Covid19 (**Fig 2C and 2D**).

Gene expression datasets have shown either no upregulation or a trend towards a reduction in ACE2 expression in IBD⁶⁻⁹. To date, clinical databases such as SECURE-IBD¹⁰ have not shown an increase in susceptibility or severity of COVID19 in patients with IBD. Our report which mainly focused on protein (rather than gene) expression data support this. In addition to this, we did not find any evidence of gut inflammation in our cohort of patients with fatal COVID19. Our finding of plasma cells expressing ACE2 in inflamed IBD gut is noteworthy. Recent studies point to the importance of the humoral immune response, particularly IgA neutralizing antibodies to SARS-CoV-2, which is more potent, occur rapidly and remains more persistent. Gaebler et al. show that SARS-CoV-2 antigen persistence in the gut may be the key factor that drives antibody evolution and potency¹¹. COVID19 patients have been shown to develop metabolically hyperactive plasmablasts during inflammatory states¹². IgA antibodies dominated the early SARS-CoV-2-specific antibody response compared with IgG and IgM and was associated with expansion of IgA plasmablasts with mucosal homing characteristics¹³. Although our primary data does not show gut inflammation as a specific factor for SARS-CoV-2 entry mechanism, the high expression of ACE2 and TMPRSS2 along with the apposition of plasma cells in the gut lamina propria suggest that the gastrointestinal tract may play a role in SARS-CoV-2 entry and host humoral immune response in COVID19.

Contributions: All authors contributed to the study conception and design led by GTH. MJM performed all immunohistochemistry experiments and carried out primary analyses. KK carried out histopathological interpretation and curated IBD gut specimens. SCC and JAC contributed to manuscript preparation and data analyses. CDR, CDL and DAD provided Covid19 gut section and all relevant clinical data and interpretation. EJT carried out microarray data analyses. K All authors read and approved the final manuscript. GTH and MJM wrote the manuscript.

Competing interest: None.

Ethical approval: East of Scotland Research Ethical Approval No 20/ES/0061 and No 16/ES/0084

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Consent for Publication: All human subjects have consented for publication of research data via ethical approvals above.

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Availability of data and materials: Full data presented in this data is available on request to corresponding author.

Supplementary:

Methodology

Patient cohorts

Intestinal resection specimens from 21 patients with Inflammatory Bowel Disease (Crohn's Disease n=11 [ileum n=3, colon n=9], Ulcerative Colitis n=10 [colon n=10]) and 10 non-IBD control patients (colon resection for colorectal cancer) were studied. All surgical resections were carried out between 1st Jan 2016 and 31st December 2020 in the Western General Hospital, Edinburgh (East of Scotland Research Ethical Approval No 20/ES/0061). The groups were matched for age- and gender; mean age 66, 64 and 68 years old for UC, CD and non-IBD controls at time of resection respectively. Histological analysis of inflamed vs. non-inflamed areas in IBD resection specimens were carried out by GI pathologist (KK).

Additionally, enteric tissue samples were obtained from post-mortem examinations conducted on six patients with pre-mortem PCR-confirmed SARS-CoV-2 infection a median of 19.6 hours (IQR 13.6-47.2) after death, as previously reported² (East of Scotland Research Ethical Approval No 16/ES/0084). Formalin-fixed paraffin-embedded (FFPE) tissue blocks were prepared following standard processes. Patients with Covid-19 were all male and had a mean age of 71 years. None of the patients had pre-existing intestinal disease. All patients had hypoxic respiratory failure with radiological evidence of viral pneumonitis. SARS-CoV-2 was present in colon tissue from all six patients (detected using multiplex PCR using ARTIC Network protocol, confirmed by sequencing, as described with subgenomic messenger RNA detected in 5/6, indicative of viral RNA synthesis).

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissues biopsies were processed into 4µm sections and stained for haematoxylin and eosin. Sections were de-paraffinised in xylene, rehydrated through graded alcohol, and heated for 5 minutes under pressure for antigen retrieval in Citrate buffer pH6 (2mM Sodium Citrate and 8mM Citric Acid). Endogenous peroxidase activity was blocked using 3% H₂O₂ for 30 minutes and non-specific background staining was blocked for 30 minutes in 10% horse serum in Tris-buffered saline (TBS). Primary antibodies for ACE2 (Cat Ab15348; Abcam) and TMPRSS2 (Cat Ab92323; Abcam) were incubated overnight at 4°C diluted at 1:1000. Sections were incubated in ImmPRESS detection kit (Vector Laboratories, UK) and visualised using 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako, UK). Samples were then counterstained, dehydrated, and mounted. Antibody specificity for ACE2 and TMPRSS2 was confirmed using an IgG isotype control (Cat Ab172730; Abcam). Slides were scanned using Zeiss AxioScanner (Zeiss, Germany) and analysed using Carl Zeiss

Zen 2.6 Blue edition programme (Zeiss, Germany). Staining intensity for ACE2 and TMPRSS2 was performed using a weighted histo-score method for cytoplasmic and membrane expression in the epithelia and lamina propria. In brief, the score was calculated by sum of (1 X % cells staining weakly positive) + (2 X % cells staining moderately positive) + (3 X % cells staining strongly positive) with a maximum of 300 (100% strongly stained) and a minimum of 0 (100% with no staining). The percentage of ACE2 or TMPRSS2 positive immune cells within the lamina propria was calculated by counting the percentage of positive cells within three representative 0.6mm x 0.6mm areas. ACE2 and TMPRSS2 staining intensity and positive immune cell counts were scored by two independent observers.

Immunofluorescence

Tissue sections were de-paraffinised and rehydrated before undergoing antigen retrieval in Citrate Buffer pH6. Samples were blocked for 1 hour using 2% fetal calf serum and incubated in primary antibodies ACE2 (1:1000, Cat Ab15348; Abcam) and Anti-MUM1 (1:100, Cat Ab247079; Abcam) overnight at 4°C and for 1 hour at room temperature respectively. Sections were incubated in Alexa Fluor® 488 (Cat Ab150077; Abcam) and Alexa Fluor® 555 (Cat Ab150078; Abcam) respectively and visualised using Zeiss LSM 780 Confocal followed by analysis on Carl Zeiss ZEN 2 blue edition software (Zeiss, Germany). Staining was categorised based on absence and/ or presence of ACE2 and/ or MUM1, analysed in three representative 0.6mm X 0.6mm areas within the lamina propria, and scored by two independent observers.

Gene microarray

Gene expression for ACE2 and TRMPSS2 were analysed from gut biopsies taken from 99 Crohn's Disease, 129 Ulcerative Colitis and 50 non-IBD control patients. Full details of tissue acquisition and processing are previously detailed⁵. The whole data set is available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/> (accessed September 2020) accession: GSE11223 and GSE20881.

Statistics

Statistical analysis was performed using SPSS Version 25.0 (IBM, USA) and GraphPad Prism Version 5 (GraphPad Software, USA) for Mann-Whitney U-Test. Interclass correlation coefficients were employed to confirm two independent scores of tissues staining intensity.

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Figure Legends:

Figure 1: ACE2 and TMPRSS2 protein and gene expression in Ulcerative Colitis (UC), and Crohn's Disease (CD) patients and non-IBD controls. **A:** Immunohistochemistry of ACE2 and TMPRSS2 protein expression within the cytoplasm (C) and membrane (M) of non-IBD (n=10), UC (colon) (n=9), and CD (ileum and colon) (n=11) patients. **B:** ACE2 protein weighted histoscore expression within the cytoplasm (C) or membrane (M) of uninfamed (n=8) or inflamed (n=7) colonic tissue. **C:** TMPRSS2 protein weighted histoscore expression within the cytoplasm (C) or membrane (M) of uninfamed (n=8) or inflamed (n=7) colonic tissue. **D:** ACE2 gene expression in uninfamed (n=57) vs. inflamed (n=67) colonic UC vs non-IBD controls (n=50). **E:** TMPRSS2 gene expression in uninfamed (n=57) vs. inflamed (n=67) colonic UC vs non-IBD controls (n=50). **F:** ACE2 positive cells (%) within lamina propria in inflamed and uninfamed colon of IBD vs. non-IBD controls (** $p < 0.001$ and *** $p < 0.0001$). **G:** TMPRSS2 positive cells (%) within lamina propria in inflamed and uninfamed colon of IBD vs. non-IBD controls (p=NS). **H:** ACE2 expressing cells within the lamina propria of uninfamed and inflamed IBD colon. **I:** Immunofluorescence co-staining of ACE2 and MUM1 (plasma cell marker) within the lamina propria of colonic IBD. Error bars represent standard error of mean (SEM). DAPI was included as a nuclear stain and scale bars represent 20 μm .

Figure 2: ACE2, TMPRSS2 and MUM1 protein expression in fatal COVID19 (SARS-CoV-2 PCR positive) ileum and colon sections; and non-IBD controls. **A:** ACE2 protein weighted histoscore expression of cytoplasm (C) or membrane (M) of COVID19 ileum or colon versus non-IBD controls. **B:** TMPRSS2 protein weighted histoscore expression of cytoplasm (C) or membrane (M) of COVID19 ileum or colon versus non-IBD controls. **C:** Immunohistochemistry of ACE2 and TMPRSS2 protein expression within the ileum and colon of COVID-19. **D:** Immunofluorescence co-staining of ACE2 and MUM1 (plasma cell marker) within the lamina propria of COVID-19 positive ileal tissue. Error bars represent standard error of mean (SEM). DAPI was included as a nuclear stain and scale bars represent 20 μm .

Figures

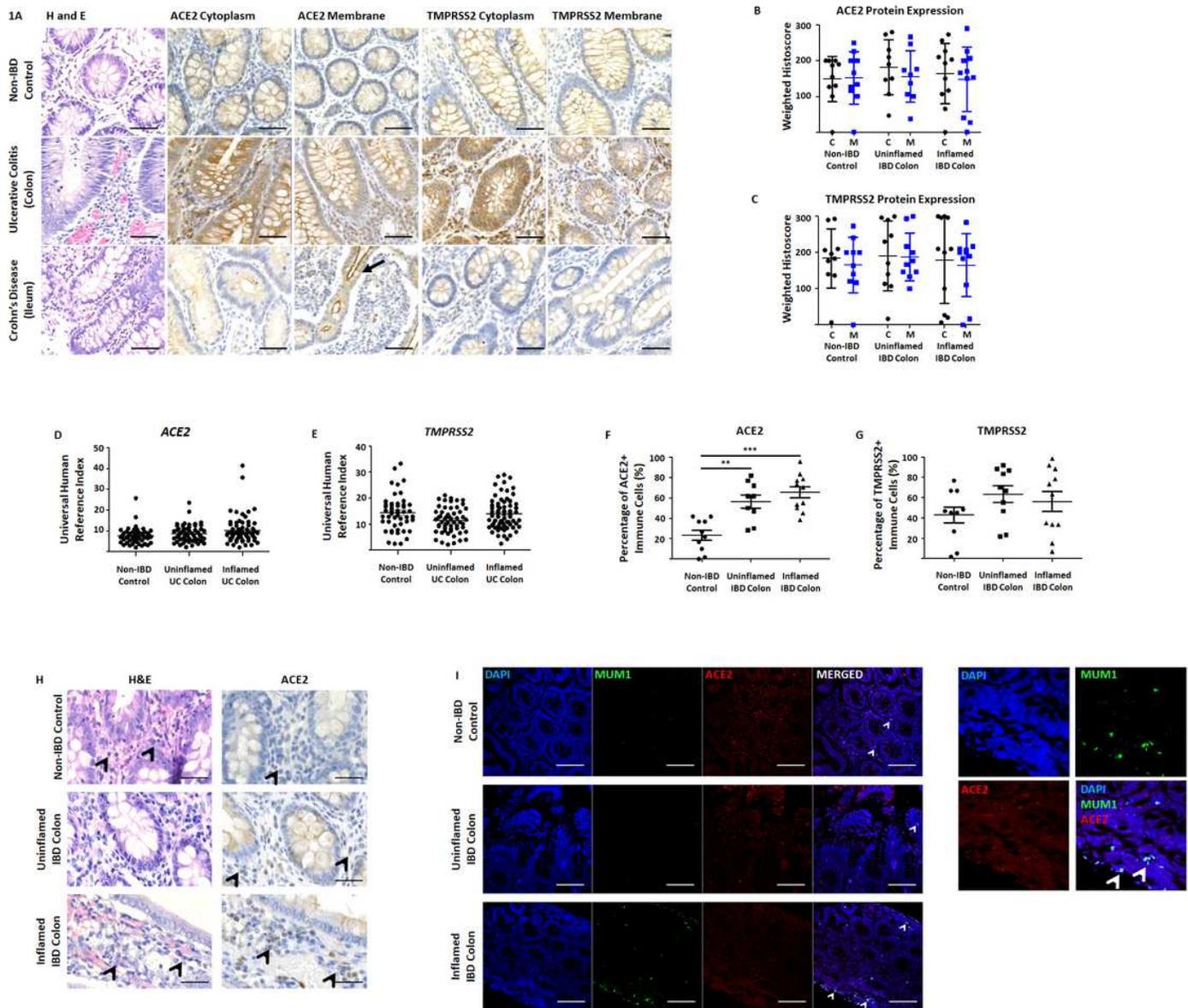


Figure 1

ACE2 and TMPRSS2 protein and gene expression in Ulcerative Colitis (UC), and Crohn's Disease (CD) patients and non-IBD controls. A: Immunohistochemistry of ACE2 and TMPRSS2 protein expression within the cytoplasm (C) and membrane (M) of non-IBD (n=10), UC (colon) (n=9), and CD (ileum and colon) (n=11) patients. B: ACE2 protein weighted histoscore expression within the cytoplasm (C) or membrane (M) of uninflamed (n=8) or inflamed (n=7) colonic tissue. C: TMPRSS2 protein weighted histoscore expression within the cytoplasm (C) or membrane (M) of uninflamed (n=8) or inflamed (n=7) colonic tissue. D: ACE2 gene expression in uninflamed (n=57) vs. inflamed (n=67) colonic UC vs non-IBD controls (n=50). E: TMPRSS2 gene expression in uninflamed (n=57) vs. inflamed (n=67) colonic UC vs non-IBD controls (n=50). F: ACE2 positive cells (%) within lamina propria in inflamed and uninflamed

colon of IBD vs. non-IBD controls (** $p < 0.001$ and *** $p < 0.0001$). G: TMPRSS2 positive cells (%) within lamina propria in inflamed and uninflamed colon of IBD vs. non-IBD controls ($p=NS$). H: ACE2 expressing cells within the lamina propria of uninflamed and inflamed IBD colon. I: Immunofluorescence co-staining of ACE2 and MUM1 (plasma cell marker) within the lamina propria of colonic IBD. Error bars represent standard error of mean (SEM). DAPI was included as a nuclear stain and scale bars represent 20 μm .

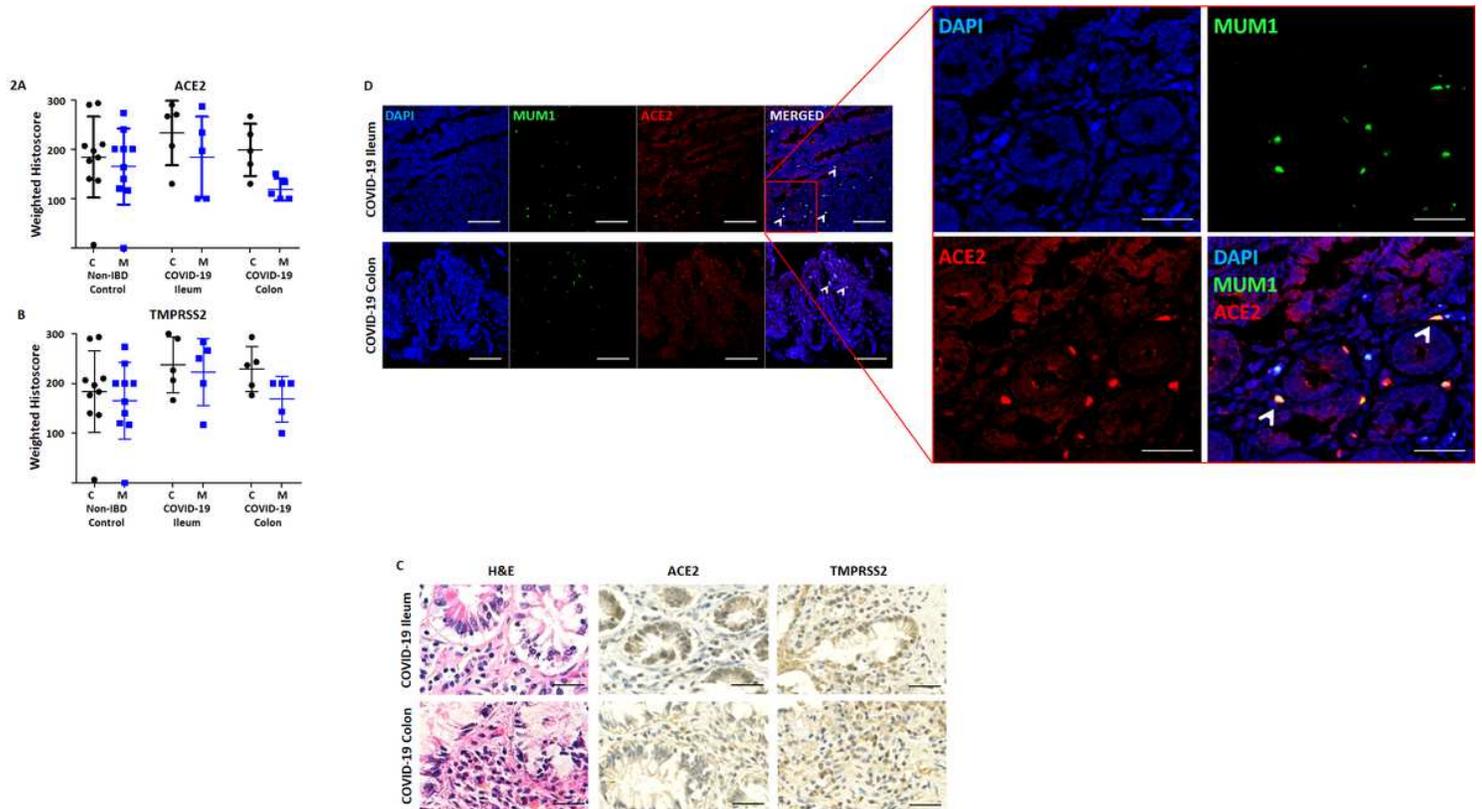


Figure 2

ACE2, TMPRSS2 and MUM1 protein expression in fatal COVID19 (SARS-CoV-2 PCR positive) ileum and colon sections; and non-IBD controls. A: ACE2 protein weighted histoscore expression of cytoplasm (C) or membrane (M) of COVID19 ileum or colon versus non-IBD controls. B: TMPRSS2 protein weighted histoscore expression of cytoplasm (C) or membrane (M) of COVID19 ileum or colon versus non-IBD controls. C: Immunohistochemistry of ACE2 and TMPRSS2 protein expression within the ileum and colon of COVID-19. D Immunofluorescence co-staining of ACE2 and MUM1 (plasma cell marker) within the lamina propria of COVID-19 positive ileal tissue. Error bars represent standard error of mean (SEM). DAPI was included as a nuclear stain and scale bars represent 20 μm .