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ORIGINAL ARTICLE

Basic Study

Hepatic angiotensin-converting enzyme 2 expression in metabolic dysfunction-associated steatotic liver disease and in patients with fatal COVID-19

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Abstract

BACKGROUND

Metabolic dysfunction-associated steatotic liver disease (MASLD), characterised by hepatic lipid accumulation, causes inflammation and oxidative stress accompanied by cell damage and fibrosis. Liver injury (LI) is also frequently reported in patients hospitalised with coronavirus disease 2019 (COVID-19), while pre-existing MASLD increases the risk of LI and the development of COVID-19-associated cholangiopathy. Mechanisms of injury at the cellular level remain unclear, but it may be significant that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which causes COVID-19, uses angiotensin-converting expression enzyme 2 (ACE2), a key regulator of the 'anti-inflammatory' arm of the renin-angiotensin system, for viral attachment and host cell invasion.

AIM

To determine if hepatic ACE2 levels are altered during progression of MASLD and in patients who died with severe COVID-19.

METHODS

ACE2 protein levels and localisation, and histological fibrosis and lipid droplet

accumulation as markers of MASLD were determined in formalin-fixed liver tissue sections across the MASLD pathological spectrum (isolated hepatocellular steatosis, metabolic dysfunction-associated steatohepatitis (MASH) +/- fibrosis, end-stage cirrhosis) and in post-mortem tissues from patients who had died with severe COVID-19, using ACE2 immunohistochemistry and haematoxylin and eosin and picrosirius red staining of total collagen and lipid droplet areas, followed by quantification using machine learning-based image pixel classifiers.

RESULTS

ACE2 staining is primarily intracellular and concentrated in the cytoplasm of centrilobular hepatocytes and apical membranes of bile duct cholangiocytes. Strikingly, ACE2 protein levels are elevated in non-fibrotic MASH compared to healthy controls but not in the progression to MASH with fibrosis and in cirrhosis. ACE2 protein levels and histological fibrosis are not associated, but ACE2 and liver lipid droplet content are significantly correlated across the MASLD spectrum. Hepatic ACE2 levels are also increased in COVID-19 patients, especially those showing evidence of LI, but are not correlated with the presence of SARS-CoV-2 virus in the liver. However, there is a clear association between the hepatic lipid droplet content and the presence of the virus, suggesting a possible functional link.

CONCLUSION

Hepatic ACE2 levels were elevated in nonfibrotic MASH and COVID-19 patients with LI, while lipid accumulation may promote intra-hepatic SARS-CoV-2 replication, accelerating MASLD progression and COVID-19-mediated liver damage.

Key Words: Metabolic dysfunction-associated steatotic liver disease; Angiotensin-converting enzyme 2; Immunohistochemistry; COVID-19; COVID-19-associated cholangiopathy

Core Tip: There has been much recent interest in angiotensin-converting expression enzyme 2 (ACE2) as the fulcrum of the 'anti-inflammatory' renin-angiotensin system pathway because severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), targets ACE2 for viral attachment and host cell invasion. Previously ACE2 mRNA has been measured in metabolic dysfunction-associated steatotic liver disease (MASLD) and COVID-19 infection but, uniquely, we used immunohistochemistry, alongside measurement of fibrosis and lipid, to show that ACE2 protein levels and hepatic lipid content are correlated across the MASLD pathophysiological spectrum and in COVID-19 patients showing evidence of liver injury. Hepatic lipids are also associated with the presence of SARS-CoV-2 virus in the liver suggesting a possible functional link.

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INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously defined as non-alcoholic fatty liver disease (NAFLD)[1], is globally the most prevalent chronic liver disease, comprising a spectrum of liver pathology progressing through isolated hepatocellular steatosis, metabolic dysfunction-associated steatohepatitis (MASH) both without and with fibrosis, to end-stage cirrhosis[2,3]. Progressive liver injury (LI) at the cellular level due to lipid infiltration in MASLD is characterised by inflammation leading to aberrant tissue repair and accumulation of extracellular matrix proteins, particularly collagens, through activation of hepatic stellate cells, portal fibroblasts and bone marrow myofibroblasts by fibrogenic cytokines including interleukin 6, transforming growth factor-β1 and angiotensin II[4]. Angiotensin II, the precursor for which (angiotensinogen) is made in the liver, is the principal peptide hormone product of the renin-angiotensin system (RAS) that plays classical endocrine roles in systemic regulation of blood pressure and electrolyte balance. More recently the RAS has also been recognised in regulating the response to local tissue injury in systems as diverse as heart, vasculature and liver, including a role in the progression of liver fibrosis; indeed, it may be a general regulator of local tissue inflammatory responses[5].

The RAS has both 'classical' and 'protective' arms, together forming a homeostatic feedback system in which angiotensin-converting enzyme 1 converts angiotensin I to the 'pro-inflammatory' angiotensin II, while angiotensin-converting expression enzyme 2 (ACE2) converts angiotensin I into 'anti-inflammatory' angiotensin 1-9, and angiotensin II into angiotensin 1-7, with ACE2 expression determining the balance between the classical and protective RAS pathways. It

may therefore be significant that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), uses ACE2 for viral attachment and as a host cell invasion entry point, raising the possibility that it might influence the degree of inflammation in infected tissues[5-7].

SARS-CoV-2 was initially considered to be a respiratory infection due to predominant pulmonary pathology. However, extrapulmonary manifestations of COVID-19 include systemic inflammation, thrombotic complications, gastrointestinal symptoms, and liver as well as kidney injury[8,9]. There is also evidence that pre-existing MASLD is a risk factor for the development of LI in COVID-19, including COVID-19-associated cholangiopathy[10], while metabolic conditions such as obesity, diabetes and cardiovascular disease have also been shown to be key risk factors for COVID-19-associated mortality[11,12].

Given the occurrence of LI in patients hospitalised with COVID-19, the increased frequency and severity of LI predicted in COVID-19 patients by pre-existing MASLD, and the possible involvement of the RAS in progression of liver fibrosis[5,13], it is surprising that to date there have been only a few investigations of ACE2 expression in MASLD or COVID-19 patients. Also, most studies on hepatic ACE2 levels have been at the mRNA level, which may not be a true surrogate for enzyme activity[14-16]. Therefore, to determine the localisation of ACE2 protein in the liver and whether or not hepatic ACE2 levels are altered in the progression of MASLD, we carried out an immunohistochemical analysis of ACE2 protein in archival human liver tissues scored for MASLD pathology. Then, to determine if increased susceptibility to viral-mediated liver damage is associated with pre-existing MASLD, we assessed hepatic lipid droplet accumulation and fibrosis as markers of MASLD in patients who had died from COVID-19 and correlated these with ACE2 protein levels and the presence of SARS-CoV-2.

MATERIALS AND METHODS

Human tissue acquisition

Anonymised unstained formalin-fixed, paraffin-embedded (FFPE) liver sections from six independent patients for each MASLD stage (steatosis, MASH without fibrosis, MASH with fibrosis, cirrhosis) and eight healthy patient controls were obtained from the Lothian NRS Human Annotated Bioresource under generic ethical approval from the East of Scotland Research Ethics Service REC 1 (Reference: 20/ES/0061 IRAS 281531). Anonymised unstained post-mortem FFPE liver tissue sections from patients who died from COVID-19 were obtained from the 'Inflammation in COVID-19: Exploration of Critical Aspects of Pathogenesis' (ICECAP) study established in 2020 in Edinburgh as a rapid response to the COVID-19 pandemic (https://www.ed.ac.uk/inflammation-research/research/icecap), under the authority of the East of Scotland Research Ethics Service REC 1 (Reference: 16/ES/0084). The ICECAP patient cohort (n = 11) was collated from consecutive independent patients with fatal COVID-19 referred for hospital post-mortem during the first wave of the COVID-19 pandemic during 2020. Sample numbers were therefore constrained by COVID-19 pandemic control measures and the number of patients referred to the post-mortem service, as described in Dorward *et al*[17]. The study was conducted according to the guidelines of the Declaration of Helsinki and in compliance with the Human Tissue Act 2004.

Tissue histology and immunohistochemical staining

5 μm tissue sections were stained for histology, using haematoxylin and eosin (HE) and picrosirius red (PSR) staining of total collagen, according to standard protocols[18,19]. Diaminobenzidine (DAB) immunohistochemistry for ACE2 was carried out using the Leica Bond™ III automated staining system using the bond Polymer Refine Detection Kit (Leica Biosystems, DS9800, United Kingdom). Briefly, slides were dewaxed in xylene, then rehydrated in graded ethanols and washed in phosphate buffered saline. Following this, slides were incubated in hydrogen peroxide for 5 min and then with either the primary anti-ACE2 antibody (Abcam, ab15348; 1:1200 dilution), a primary isotype control antibody as negative control (Thermofisher, AB_2532938), or a second primary anti-ACE2 antibody (Bioss, bs-1004R-A488, 1:200) for 1 hour, followed by incubation with the Refine Detection Kit Polymer for 15 minutes, DAB refine reagent for 10 minutes, and finally haematoxylin counterstain for 5 minutes. Two 5-minute washes in TBST were carried out between each step. All staining was undertaken by trained staff at the SuRF immunodetection and histological imaging facility (Queen's Medical Research Institute, Edinburgh, United Kingdom).

Image acquisition and processing

Entire liver tissue sections stained for ACE2 or total collagen and counterstained with HE were scanned using a Zeiss Axioscan.Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) and images captured at × 20 magnification. Whole slide.czi image files were imported into QuPath open-source software version 3.0[20] for quantitative analysis. The QuPath open-source software machine learning-based Pixel Classifier tool was trained to detect ACE2 DAB staining, PSR collagen staining and lipid droplet vacuoles in merged 2000 μm^2 regions from multiple whole-slide scanned images of ACE2 DAB-stained and PSR-stained liver sections selected randomly across each of the different MASLD disease states and from the ICECAP patient cohort to avoid a training bias, as described in Kendall *et al*[21]. 'Gaussian', 'Laplacian of gaussian', and 'weighted deviation' filters in QuPath 3.0 were selected for these analyses.

Briefly, a machine learning-based image pixel classifier was trained to identify the tissue section in raw.czi whole-slide scanned images and to annotate 'whole tissue' regions for future measurement. During annotation, small artifacts or marks on individual section images were removed automatically based on size, while any larger artifacts and liver capsule tissue were removed during subsequent manual review of images. A second set of pixel classifiers were then trained to classify pixels in annotated ACE2-stained sections as 'DAB-positive' or 'DAB-negative', or pixels in annotated PSR-stained sections as 'PSR-positive' or 'Lipid droplet-positive'. Positive ACE2-DAB staining was defined by comparing

staining patterns in liver sections obtained using two different primary anti-ACE2 antibodies generated against non-overlapping ACE2 antigenic peptide determinants with primary non-specific tissue 'isotype' antibody and 'omission of primary antibody' negative controls. Positive collagen-PSR staining was defined by comparing red-stained collagen fibrils with surrounding, yellow-stained tissues within single liver sections[19], while lipid droplet vacuoles were recognised within hepatocytes by holes left in tissue sections following tissue processing. Pixel classifiers were reviewed by an expert liver pathologist, blinded to clinical metadata, to confirm their validity and to ensure that they were selecting positive features correctly, with data being presented as the total percentage of positive pixels for each class within the annotated 'whole tissue' region.

To determine the correlation between ACE2 levels, fibrosis, lipid infiltration and the presence of SARS-CoV-2 virus in ICECAP study cohort liver tissues, data on hepatic ACE2 protein expression, PSR-positive area, lipid droplet-positive area and NAFLD activity score (NAS) and non-alcoholic steatohepatitis-clinical research network (NASH-CRN) scoring obtained in this study were then compared with existing spatial information in liver sections from the ICECAP patient cohort for the presence of SARS-CoV-2 RNA and spike protein reported in Dorward *et al*[17].

Manual scoring of MASLD spectrum and ICECAP study post-mortem liver tissue

HE and PSR-stained MASLD spectrum liver tissue sections and post-mortem liver tissue sections from the ICECAP study were scored for histological features of MASLD activity and scarring using the ordinal NAS and NASH-CRN fibrosis scale: Steatosis, MASH without fibrosis (- fibrosis), MASH with fibrosis (+ fibrosis), and MASLD cirrhosis[22]. One patient (Patient G) in the ICECAP study cohort had received a diagnosis of primary biliary cholangitis, following admission with severe COVID-19[17], but none of the other patients had previously been diagnosed with any pre-existing liver disease, although several had metabolic risk factors for MASLD, including obesity and type 2 diabetes mellitus[17]. Scoring was carried out by an expert liver pathologist who was blinded to all clinical information. Data reported in this study was collected and analysed using established NAFLD histopathological scoring systems; however, recent studies have shown that 99% of patients with NAFLD meet MASLD criteria and that data collected on NAFLD patients can be used under the new MASLD definition[23,24].

Statistical analysis

GraphPad Prism version 6 (GraphPad Soft-ware Inc., United States) was used for statistical analysis and graphical figure production. Normality tests were carried out on all individual datasets prior to performing statistical analyses. Normally distributed data with three or more groups were analysed by one-way Analysis of Variance (ANOVA) with Bonferroni's post-hoc test. Data without normal distribution was analysed with Mann-Whitney *U* test for two groups or the Kruskal-Wallis with Dunn's post-hoc test for groups of three or more. Spearman's rank correlations were used to assess the strength and direction of associations between variables. A P value of < 0.05 was considered statistically significant. Data are presented as scatter plots with boxplot overlay showing the median and interquartile range unless otherwise stated in the figure legend.

RESULTS

Hepatic ACE2 protein expression in patients with MASLD

Immunohistochemical staining for ACE2 protein in liver biopsy tissue sections from normal liver controls and patients across the MASLD spectrum, showed that in healthy liver tissue ACE2 immunostaining was primarily intracellular in nature and confined to the cytoplasm of centrilobular hepatocytes and the apical membrane of cholangiocytes (Figures 1 and 2). In MASLD patients, there was no change in ACE2 staining intensity in cholangiocytes across the MASLD spectrum; rather, expression was consistently high across all stages and localised to cholangiocyte apical (luminal) bile duct membranes (Figure 2A and B). In contrast, ACE2 displayed a punctuate granular intracellular cytoplasmic staining pattern in centrilobular hepatocytes, with staining localised close to the plasma membrane in some steatotic hepatocytes (Figure 2C and D).

ACE2 protein levels were significantly elevated in MASH without fibrosis ($P = 0.02$; Figure 3A) and were also increased in simple steatosis although this did not achieve significance compared to normal healthy liver controls ($P = 0.33$; Figure 3A). In contrast, ACE2 protein levels in MASH with fibrosis and cirrhosis levels did not show any increase in comparison to normal healthy liver controls ($P = 0.99$ and $P = 0.65$; Figure 3A), suggesting that the elevation in ACE2 protein levels associated with the early stages of MASLD (simple steatosis, MASH without fibrosis) is reversed during progression to MASH with fibrosis and in cirrhosis. There was no statistically significant correlation between ACE2 protein levels and histological fibrosis (r_s 0.3, $P = 0.09$; Figure 3B), however, hepatic ACE2 protein levels and liver lipid droplet content were significantly correlated across the MASLD spectrum (Spearman's rank correlation r_s 0.5, $P = 0.01$; Figure 3C), suggesting a possible functional link.

Hepatic SARS-CoV-2 virus association with ACE2 protein levels in patients with fatal COVID-19

Computational quantification in patients who died with severe COVID-19 showed a non-statistically significant trend towards increased hepatic ACE2 protein levels in patients without any histopathological evidence of LI, compared to normal liver controls ($P = 0.31$; Figure 4A). Hepatic ACE2 protein levels were, however, significantly increased in COVID-19 patients showing evidence of LI ($P = 0.04$; Figure 4A), although no correlation was found between hepatic ACE2 protein levels and histological fibrosis content (Spearman's rank correlation r_s 0.29, $P = 0.39$; Figure 4B) or hepatic ACE2

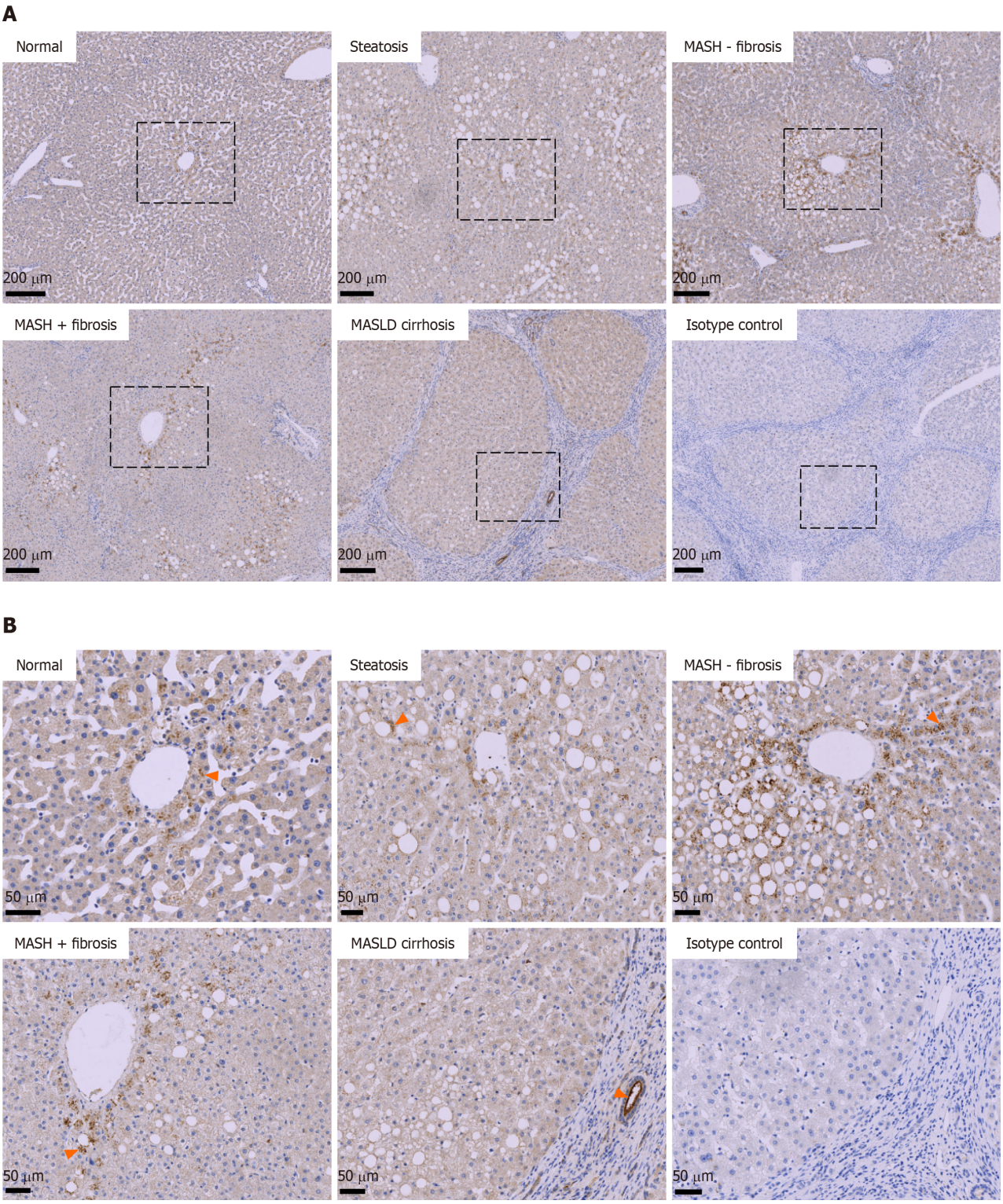

Figure 1 Angiotensin-converting enzyme 2 protein expression pattern in normal and metabolic dysfunction-associated steatotic liver disease livers. A: Representative images of angiotensin-converting enzyme 2 immunostaining across the metabolic dysfunction-associated steatotic liver disease histological spectrum. Scale bars: 200 μm; B: Representative views of boxed areas in panel A (positive and specific staining is indicated with arrowheads). Scale bars: 50 μm. MASH: Metabolic dysfunction-associated steatohepatitis; MASLD: Metabolic dysfunction-associated steatotic liver disease.

protein levels and liver lipid droplet content (Spearman's rank correlation r_s 0.04, P = 0.89; Figure 4C). The pattern of ACE2 immunostaining in post-mortem liver tissue from patients with fatal COVID-19 was similar to that seen in uninfected MASLD spectrum liver sections, also showing cytoplasmic granular staining in centrilobular hepatocytes (Figure 4D), and strong staining in apical membranes of bile duct cholangiocytes (Figure 4E). Interestingly, ACE2 staining in cholangiocytes colocalises with the previously-reported presence of SARS-CoV-2 spike protein in this patient cohort [17].

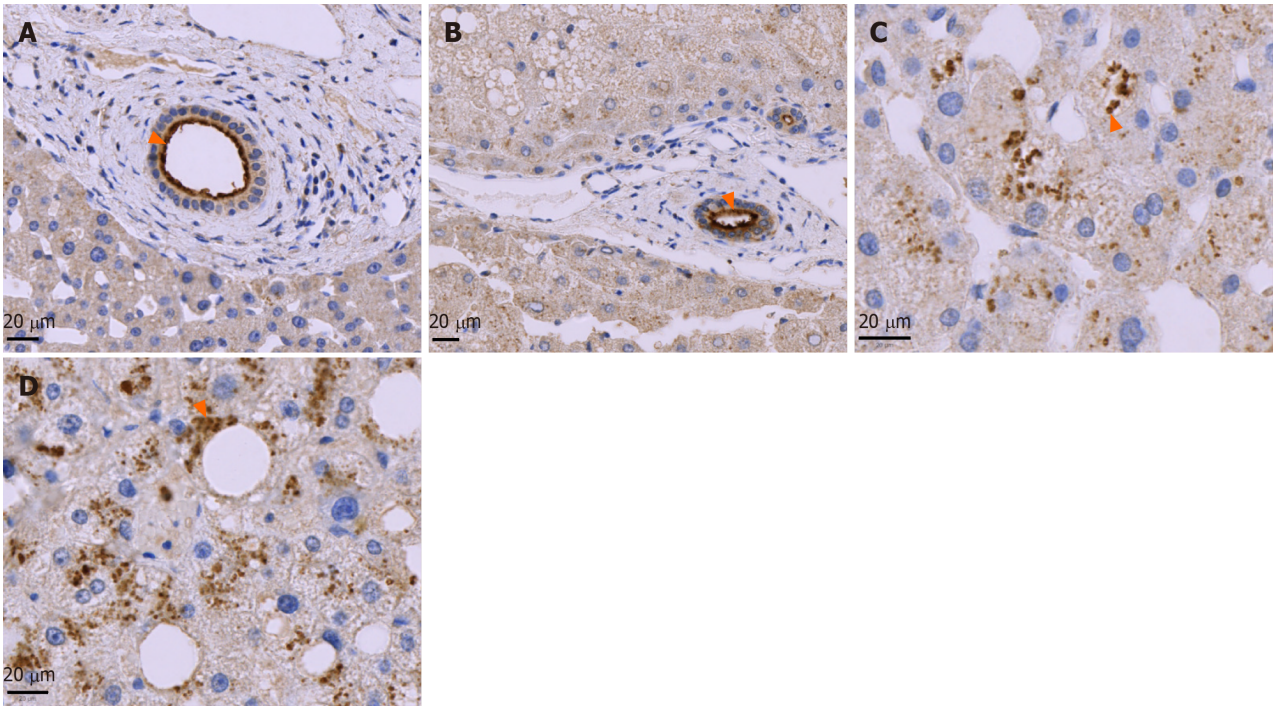

Figure 2 Hepatic angiotensin-converting enzyme 2 immunolocalization in cholangiocytes and hepatocytes in normal and metabolic dysfunction-associated steatotic liver disease. Representative images of liver sections showing strong and precise immunostaining for angiotensin-converting enzyme 2 (ACE2) on the apical membrane of cholangiocytes. A: Normal liver; B: Metabolic dysfunction-associated steatotic liver disease (MASLD)-related cirrhosis (arrowheads); C: Normal liver showing punctuate granular intracellular ACE2 immunostaining in hepatocytes (arrowhead); D: Metabolic dysfunction-associated steatohepatitis showing ACE2 immunostaining localised close to the plasma membrane in some steatotic hepatocytes (arrowhead). Scale bars: 20 μm.

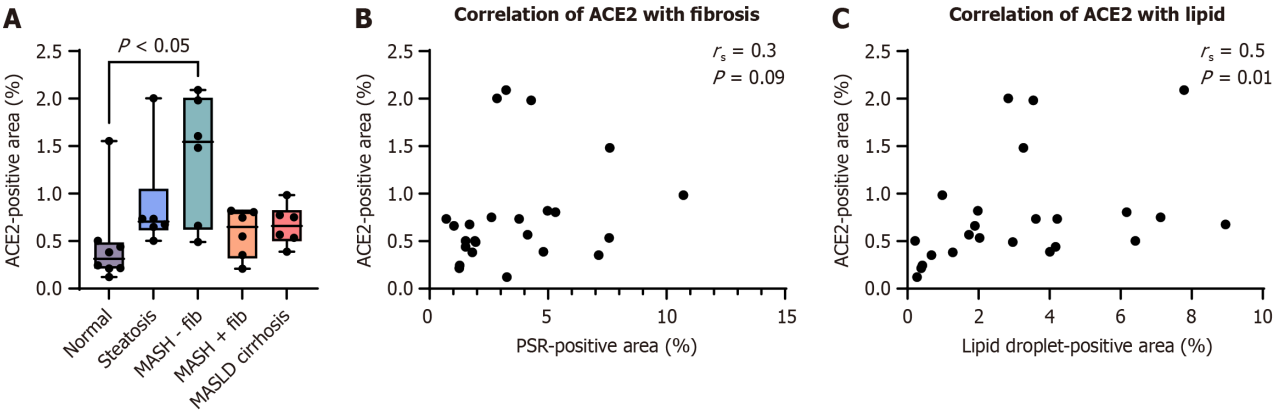

Figure 3 Hepatic angiotensin-converting enzyme 2 protein expression is elevated in metabolic dysfunction-associated steatohepatitis without fibrosis and correlates with hepatocyte lipid droplet content. A: Computational quantification of hepatic angiotensin-converting enzyme 2 (ACE2) immunostaining in tissue sections across the histopathological metabolic dysfunction-associated steatotic liver disease (MASLD) spectrum. Metabolic dysfunction-associated steatohepatitis (MASH) - fib = MASH without fibrosis, MASH + fib = MASH with fibrosis. Data was analysed using the Kruskal-Wallis test and Dunn's post-hoc multiple comparisons (Normal *vs* MASH - fib: $P < 0.05$; other comparisons not statistically significant); B: Correlation between histological fibrosis (picrosirius red-positive area) and hepatic ACE2 protein levels (ACE2-positive area) across the MASLD spectrum assessed by Spearman's rank correlation coefficient: $r_s = 0.3$, $I = 0.09$, not statistically significant); C: Correlation between lipid droplet content (lipid droplet-positive area) and hepatic ACE2 protein levels (ACE2-positive area) across the MASLD spectrum assessed by Spearman's rank correlation coefficient: $r_s = 0.5$, $P = 0.01$). ACE2: Angiotensin-converting enzyme 2.

Increased hepatic lipid droplet content correlates with the presence of hepatic SARS-CoV-2 RNA

Quantification of the total lipid droplet-positive area (Figure 5A) on whole-slide images of PSR-stained liver sections showed a statistically significant correlation between the total lipid droplet-positive area and the presence of hepatic SARS-CoV-2 RNA ($P = 0.01$; Figure 5B). In contrast, there was no correlation between hepatic SARS-CoV-2 RNA and ACE2 protein levels ($P = 0.22$; Figure 5C), or liver fibrosis as measured by the % PSR-positive area ($P = 0.93$; Figure 5D).

Evidence of undiagnosed liver disease in patients who died with severe COVID-19

NAS and NASH-CRN scoring on post-mortem HE-stained and PSR-stained liver sections from patients who died with

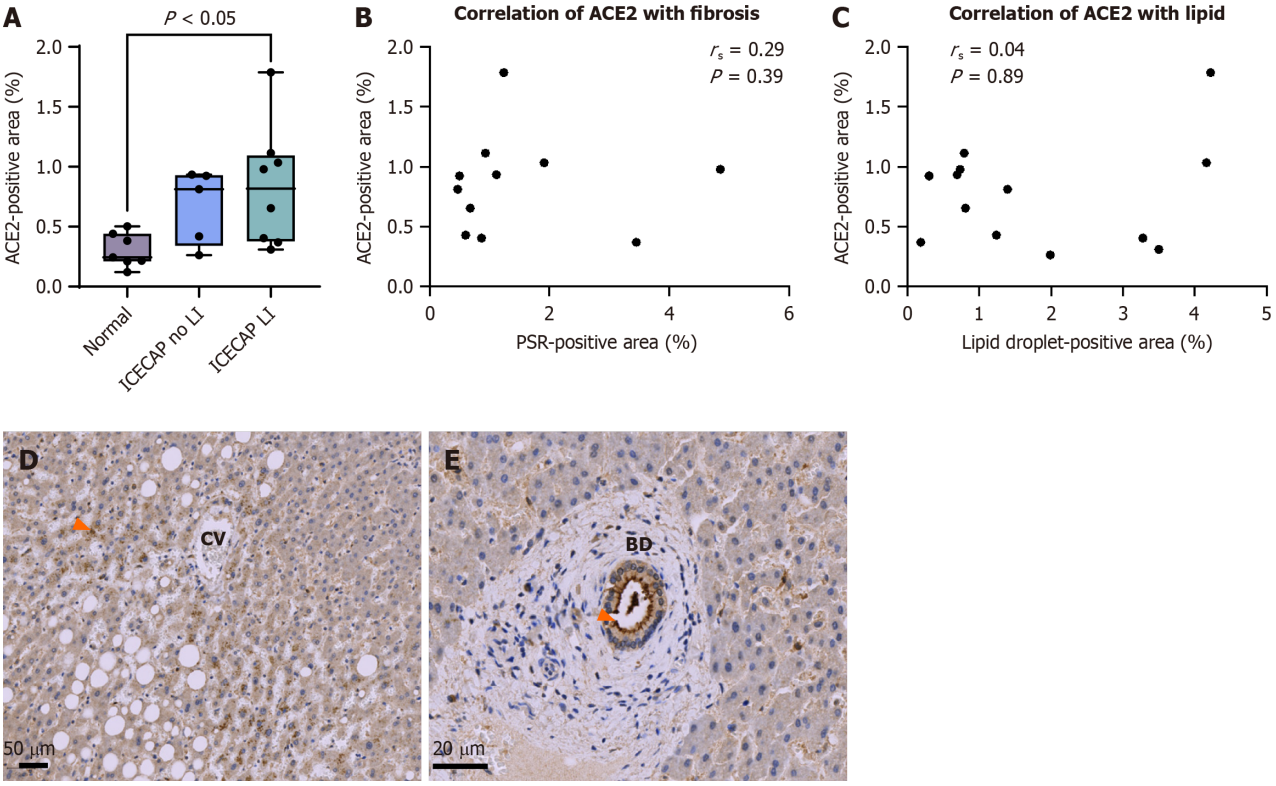

Figure 4 Angiotensin-converting enzyme 2 protein levels are increased in post-mortem livers of patients who died with severe coronavirus disease 2019. A: Computational quantification of hepatic angiotensin-converting enzyme 2 (ACE2) protein immunostaining in post-mortem tissue sections from the coronavirus disease-19 'Exploration of Critical Aspects of Pathogenesis' (ICECAP) patient cohort with and without histopathological evidence of liver injury (LI) (ICECAP + LI and ICECAP no LI), including steatosis and/or fibrosis. Groups were compared by one-way ANOVA with Bonferroni's post-hoc test (Normal *vs* ICECAP + LI: $P < 0.05$, comparison of Normal *vs* ICECAP no LI not statistically significant); B: Correlation between histological fibrosis (picrosirius red-positive area) and hepatic ACE2 protein levels (ACE2-positive area) assessed by Spearman's correlation coefficient: $r_s = 0.29$, $P = 0.39$, not statistically significant; C: Correlation between lipid droplet content (lipid droplet positive-area) and hepatic ACE2 protein levels (ACE2-positive area) assessed by Spearman's correlation coefficient: $r_s = 0.04$, $P = 0.89$, not statistically significant; D: Representative image showing granular ACE2 immunostaining pattern (arrowhead) in the cytoplasm of hepatocytes in pericentral areas of the liver lobule (central vein) in a steatotic post-mortem liver. Scale bar: 50 µm; E: Representative image showing a bile duct is shown with strong and specific staining localised to the apical membrane of cholangiocytes (arrowhead). Scale bar: 20 µm. ACE2: Angiotensin-converting enzyme 2; LI: Liver injury. CV: Central vein; BD: Bile duct.

severe COVID-19 showed that 3 out of 4 patients who were positive for the SARS-CoV-2 virus also displayed evidence of pre-existing chronic liver disease (Table 1). However, none of the patients who died with severe COVID-19 showed any evidence of lobular inflammation or hepatocyte ballooning, regardless of hepatic SARS-CoV-2 status.

DISCUSSION

In this study we sought to better understand the association between pre-existing MASLD and the increased frequency and severity of LI in COVID-19. We approached this by investigating the level and localisation of ACE2 protein, a potential regulator of local tissue inflammation, alongside the histological fibrosis and lipid droplet accumulation across the MASLD histological spectrum and in livers from patients who had died with severe COVID-19.

Immunohistochemical analysis in tissue samples from patients diagnosed with MASLD revealed a non-statistically significant trend towards increased ACE2 protein levels in simple steatosis compared to normal controls; however, ACE2 protein levels were significantly increased in patients at the next stage of MASLD, namely MASH without fibrosis. This is consistent with previous observations that hepatic ACE2 mRNA levels are significantly upregulated in MASH patients compared to normal controls[25]. Intriguingly, increased ACE2 protein levels were not seen in the progression to MASH with fibrosis and in cirrhosis, suggesting that ACE2 may only be involved in regulating responses in earlier stages of chronic liver disease. There was also a correlation between ACE2 protein levels and lipid droplet content throughout the MASLD spectrum, indicating that elevated ACE2 may be a response to metabolic stress resulting from increased lipid load.

Ex vivo evidence for ACE2 expression in cholangiocytes has been convincing but so far ACE2 expression in hepatocytes is less well characterised. While most previous *in vivo* studies of ACE2 in liver and other tissues have looked at ACE2 mRNA levels[25], the current study is one of the few to monitor the presence of ACE2 in liver sections using immunohistochemistry, allowing protein distribution to be visualised at the cellular level. This revealed consistently high ACE2 staining in cholangiocytes across the MASLD histological spectrum, localised to the cholangiocyte apical (luminal)

Table 1 Non-alcoholic steatohepatitis activity score and non-alcoholic steatohepatitis-clinical research network histological feature scores in livers positive or negative for the presence of severe acute respiratory syndrome coronavirus 2 mRNA in the 'Inflammation in coronavirus disease 2019: Exploration of critical aspects of pathogenesis' study of patients who died of fatal coronavirus disease 2019

Case	Ballooning	Inflammation	Fat	Fibrosis	Hepatic SARS-CoV-2 PCR result
A	0	0	0	0	-
B	0	0	1	3	+
C	0	0	1	2	+
D	0	0	1	0	-
E	0	0	0	0	+
F	0	0	0	1c	-
G	0	0	1	3*	-
H	0	0	0	0	-
I	0	0	2	1b	+
J	0	0	0	3	-
K	0	0	0	0	-

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

membrane (Figures 1 and 2), which accords well with previous *ex vivo* data[26]. However, ACE2 staining primarily in the cytoplasm of centrilobular hepatocytes displayed a punctuate granular intracellular immunostaining pattern, with staining localised close to the plasma membrane in some steatotic hepatocytes (Figure 2C and D). This could support the notion that ACE2 is expressed and trafficked between multiple cellular compartments in liver, as reported previously for other tissues[27-29].

A cytoplasmic location for an enzyme that processes blood-borne peptide hormones and is used by SARS-CoV-2 and other coronaviruses for viral attachment and host cell invasion, might seem paradoxical. However, ACE2 is a transmembrane protein with the catalytic moiety positioned extracellularly and thus exposed to the vascular capillary lumen, with a cytoplasmic moiety within the cell[30,31]. The primary anti-ACE2 antibodies used in this study are raised against independent cytoplasmic peptide antigens; but this does not seem to be a sufficient explanation for the granular nature of hepatocyte cytoplasmic staining. Instead, following processing of its peptide hormone substrate, ACE2 can be internalised and cycle through several intracellular compartments before being reinserted into the cell membrane. Also, under certain circumstances the transmembrane domain can be cleaved causing the extracellular catalytic moiety to be released into the systemic circulation while the rest of the protein cycles through the cytoplasm[32]. Interestingly, a similar SARS-CoV-2-mediated cleavage of the ACE2 transmembrane domain has been proposed as part of viral attachment and invasion process following spike protein binding[33,34]. A similar granular cytoplasmic staining pattern has also been reported in several other epithelial cell types, including the basal layer epithelium of the oral mucosa and in pancreatic β-cells[35-37], which suggests that ACE2 trafficking between multiple cellular compartments may be a general feature of cells in which ACE2 is expressed.

Post-mortem liver tissue sections from patients with fatal COVID-19 also showed cytoplasmic granular staining for ACE2 in pericentral hepatocytes and strong apical staining in bile duct cholangiocytes where the presence of SARS-CoV-2 spike protein has previously been reported[17]. This suggests that cholangiocytes may also be susceptible to direct SARS-CoV-2-mediated damage which may contribute to the COVID-19-associated cholangiopathies seen in long COVID. However, the similarity of the hepatic ACE2 staining pattern in the COVID-19 samples to that seen across the uninfected MASLD spectrum suggests this might not be dependent on the presence of SARS-CoV-2 virus in these patients. The relatively limited number of patients in the ICECAP study group only allows us to draw preliminary conclusions, but the fact that the hepatic ACE2 levels, though increased, did not positively correlate with lipid droplet accumulation in these patients suggests that this increase was not just related to pre-existing chronic liver disease but might also be influenced by severe COVID-19, as has been postulated in post-mortem lung tissue[38]. Thus, the increased hepatic expression of ACE2 in these patients could be multifactorial-relating to acute systemic inflammation, oxidative stress, and drug-induced LI associated with hospitalisation for severe COVID-19, as well as pre-existing MASLD[8,9]. Indeed, *in vitro* studies have suggested that cell energy stress, including hypoxia and inflammation, could be important regulators of ACE2 activity[39,40]. Our study of hepatic ACE2 expression may therefore highlight the importance of the local counter-regulatory RAS in the human liver in addition to other metabolic, antioxidant, and anti-inflammatory mechanisms in *in vivo* MASLD models[41].

Although a recent SARS-CoV-2 RNA *in situ* hybridisation study provided robust evidence that hepatocytes are susceptible to infection with SARS-CoV-2 *in vivo*[42], we did not observe any clear association between the level of hepatic ACE2 expression or the degree of fibrosis and the presence of SARS-CoV-2 in patients who died with severe

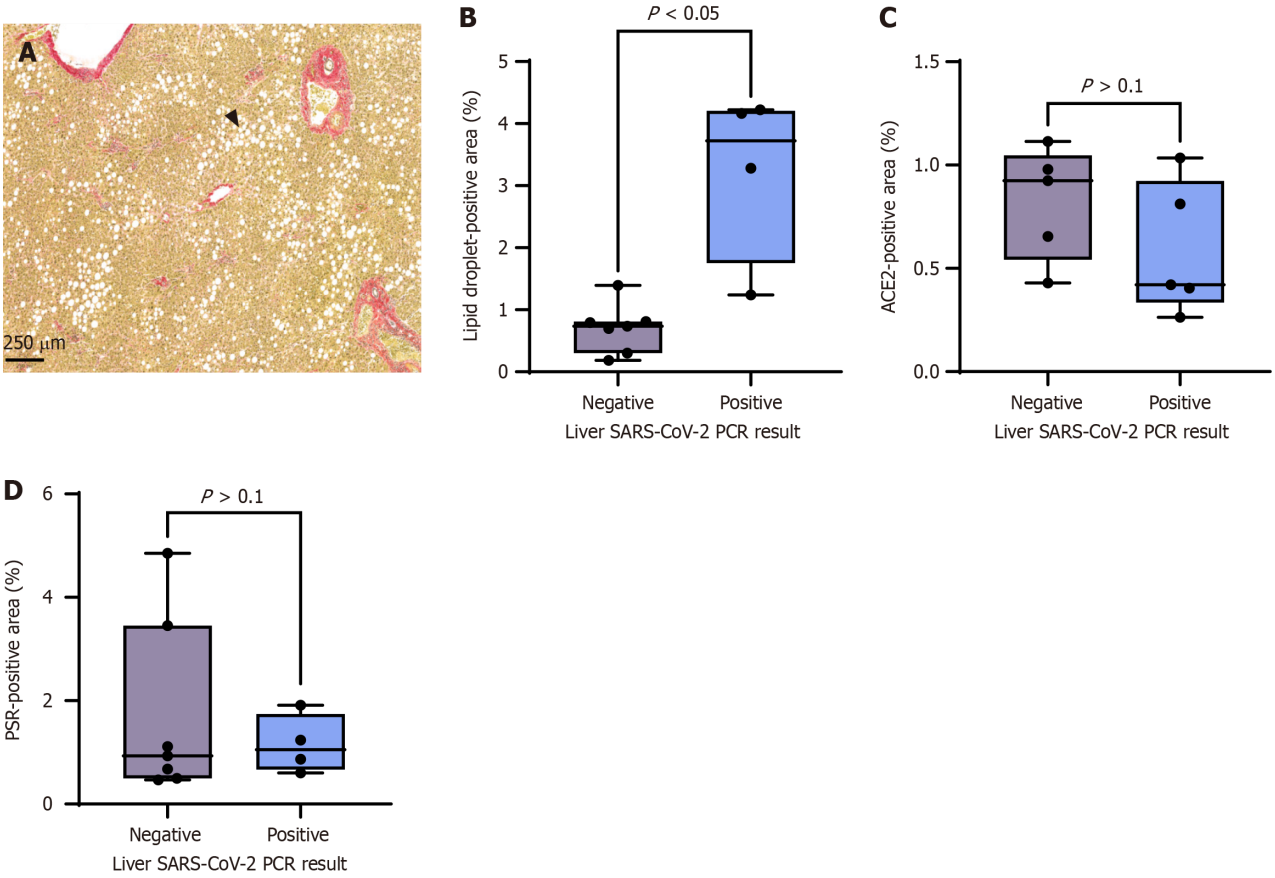

Figure 5 Hepatic lipid droplet content correlates with detectable severe acute respiratory syndrome coronavirus 2 RNA. A: Representative image showing extensive hepatocellular steatosis (arrowhead) in a post-mortem liver section from an ICECAP study patient who died with severe coronavirus disease-19. Scale bar: 250 μm; B: Hepatic lipid droplet content (lipid droplet-positive area); C: Hepatic angiotensin-converting enzyme 2 (ACE2) protein levels (ACE2-positive area); D: Histological fibrosis (picrosirius red-positive area) in ICECAP patient post-mortem liver sections were compared with the presence or absence of detectable liver severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral RNA (liver SARS-Cov-2 PCR result), in the ICECAP study patient cohort[17], using the Mann-Whitney test for unpaired group comparisons (hepatic lipid droplet content *vs* detectable liver SARS-CoV-2 viral RNA $P < 0.05$; other comparisons not statistically significant). PSR: Picrosirius red; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

COVID-19, suggesting that these may not be predictors of direct viral-mediated damage (Figure 5C and D). However, there was a clear association between increased hepatic lipid droplet accumulation and the presence of SARS-CoV-2 virus in the liver. This observation is consistent with previous studies that found a high proportion of patients had evidence of hepatocellular steatosis at post-mortem[43]. A similar association between SARS-CoV-2 and lipid droplets was also seen in lung pneumocytes[44]. Lipid droplets have been shown to promote SARS-CoV-2 replication in human cells/cell lines *in vitro*[45], while it has also been suggested that coronaviruses can hijack host factors to collectively orchestrate a unique lipid microenvironment optimal for viral replication[46,47], suggesting that something similar might be happening in liver.

CONCLUSION

In conclusion, our data showed that hepatic ACE2 levels are elevated in nonfibrotic MASH patients, but not in fibrotic MASH or cirrhosis, while hepatic ACE2 levels are also elevated in COVID-19 patients with LI. Moreover, our results raise the possibility that the virus may have used the lipid droplets as a template for intra-hepatic viral replication either to promote direct viral-mediated liver damage or by impairment of mitochondrial function[48], which could accelerate MASLD progression and/or promote COVID-19-associated cholangiopathy[10]. Larger patient groups and further mechanistic analyses will be needed to distinguish between these possibilities.

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FOOTNOTES

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