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Single cell RNA sequencing analysis did not predict hepatocyte infection by SARS-CoV-2

To the Editor:

It was with great interest that we read the research article by Wang *et al.* In their manuscript, the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) particles in hepatocytes is shown with additional arguments for viral replication and cytopathy in infected hepatocytes, which provides a partial explanation for the high prevalence of abnormal liver laboratory tests in patients with COVID-19.[1]

It has already been proposed that SARS-CoV-2 might directly infect liver cells.[2] It certainly seemed that a risk stratification for direct liver cell infection could be predicted based on single cell transcriptomic data for the SARS-CoV-2 entry factors ACE2 (SARS-CoV-2 cellular entry) and TMPRSS2 (spike protein priming).[3] Many researchers have analyzed single cell RNA sequencing (scRNASeq) datasets. The general conclusion was that the liver is a low-risk organ for SARS-CoV-2 infection,[4] though cholangiocytes proved to be the cells with the highest ACE2 expression.[5,6] This led to the hypothesis that cholangiocytes are the most likely target of a direct SARS-CoV-2 infection in the liver.[2]

We did note, however, that these scRNASeq analysis manuscripts analyzed only 1 liver dataset, did not focus on liver tissue specifically or reported only on ACE2 expression without considering TMPRSS2 expression. Additionally, since substantial clinical data on COVID-19 infection and chronic liver disease (CLD) is currently limited, we thought it would be informative to evaluate the potential vulnerability of individual cell types in patients with CLD. Enhanced ACE2 and TMPRSS2 expression in cirrhotic livers would leave these patients potentially more susceptible to liver infection with possible worse disease outcomes.

To address these issues, we analyzed 3 publicly available human liver datasets published by Aizarani *et al.*,[7] Macparland *et al.*[8] and Ramachandran *et al.*[9] The latter includes cirrhotic livers from patients undergoing orthotopic liver transplantation, caused by non-alcoholic liver disease, alcohol-related liver disease and primary biliary cholangitis. Using these 3 datasets, we verified ACE2 and TMPRSS2 expression in healthy and diseased human livers (Fig. 1A-B).

Cholangiocytes are among the highest expressors of ACE2 in all datasets, which is in line with previous scRNAseq reports. However, only a low percentage of cholangiocytes express *ACE2* RNA, except for the MacParland dataset (14.29% *vs.* 0.99 and 0.82 %). ACE2 expression in hepatocytes from the dataset by Ramachandran *et al.* shows a higher frequency of ACE2 positive cells (10.2%) compared to the other datasets. However, seeing as they are KRT7, EPCAM and ALB positive, these hepatocytes presumably represent cells undergoing a ductular reaction (Fig. S1-2). Hepatocytes from the datasets by Aizarani (0.73% ACE2+) and Macparland (0.26% ACE2+) do not show KRT7 or EPCAM expression. TMPRSS2 is expressed by a higher percentage of hepatocytes and cholangiocytes in all datasets, suggesting that this is not the limiting factor for cellular entry, as is also the case for other tissues.[6] Other cell types such as immune, endothelial and mesenchymal cells express limited to no ACE2 or TMPRSS2. Analysis of double positive cells of healthy individuals shows that only 0.04% and 0.03% of the hepatocytes co-express ACE2 and TMPRSS in the Aizarani and Macparland studies. Respectively, 0.45% and 2.52% of cholangiocytes co-express ACE2 and TRMPRSS2 in the Ramachandran and Macparland studies (no co-expression in Aizarani study). Furthermore, when comparing cells from healthy and diseased livers, we do not see any increase in ACE2 expression nor in ACE2-TMPRSS2 co-expression in cholangiocytes.

In conclusion, scRNAseq analyis does not point towards hepatocytes as a likely point of entry for SARS-CoV-2 infection. The low expression of ACE2 seen in this data presumably represents technical limitations of the scRNAseq technique, rather than an absolute absence of ACE2 in these cells, leading to an underestimation of ACE2 expressing hepatocytes. Indeed, even in alveolar epithelial type II cells, the cell type playing a crucial role in SARS-CoV pathogenesis, ACE2 expression levels were reported to be low in single cell analysis.[6] Interestingly, while the percentage of ACE-TMPRSS2 co-expressing hepatocytes is extremely low in the 2 datasets containing representative hepatocytes,[7,8] it is not zero. Since the human liver is estimated to contain tens of billions of hepatocytes,[10] this very low percentage could still leave millions of hepatocytes at risk. However, this does not explain the absence of SARS-CoV-2 viral particles in cholangiocytes, which leaves the possibility of alternate cellular entry receptors or requirements for co-receptors, as hypothesized by Wang *et al.*, to explain the seemingly hepatocyte-specific tropism of SARS-CoV-2 in the liver. Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) using antibodies against ACE2 and TMPRSS2 could help to gain more insight into the identity of cell types at risk of SARS-Cov-2 infection. Finally, despite great insights into cellular identities across the entire human body in health and disease, the findings by Wang *et al.*[1] highlight a need for caution when interpreting analyses of scRNAseq data for cell susceptibility to SARS-CoV-2 viral infection.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

Vincent De Smet: Conceptualization, Writing – original draft, Writing – review & editing. Stefaan Verhulst: Formal analysis,

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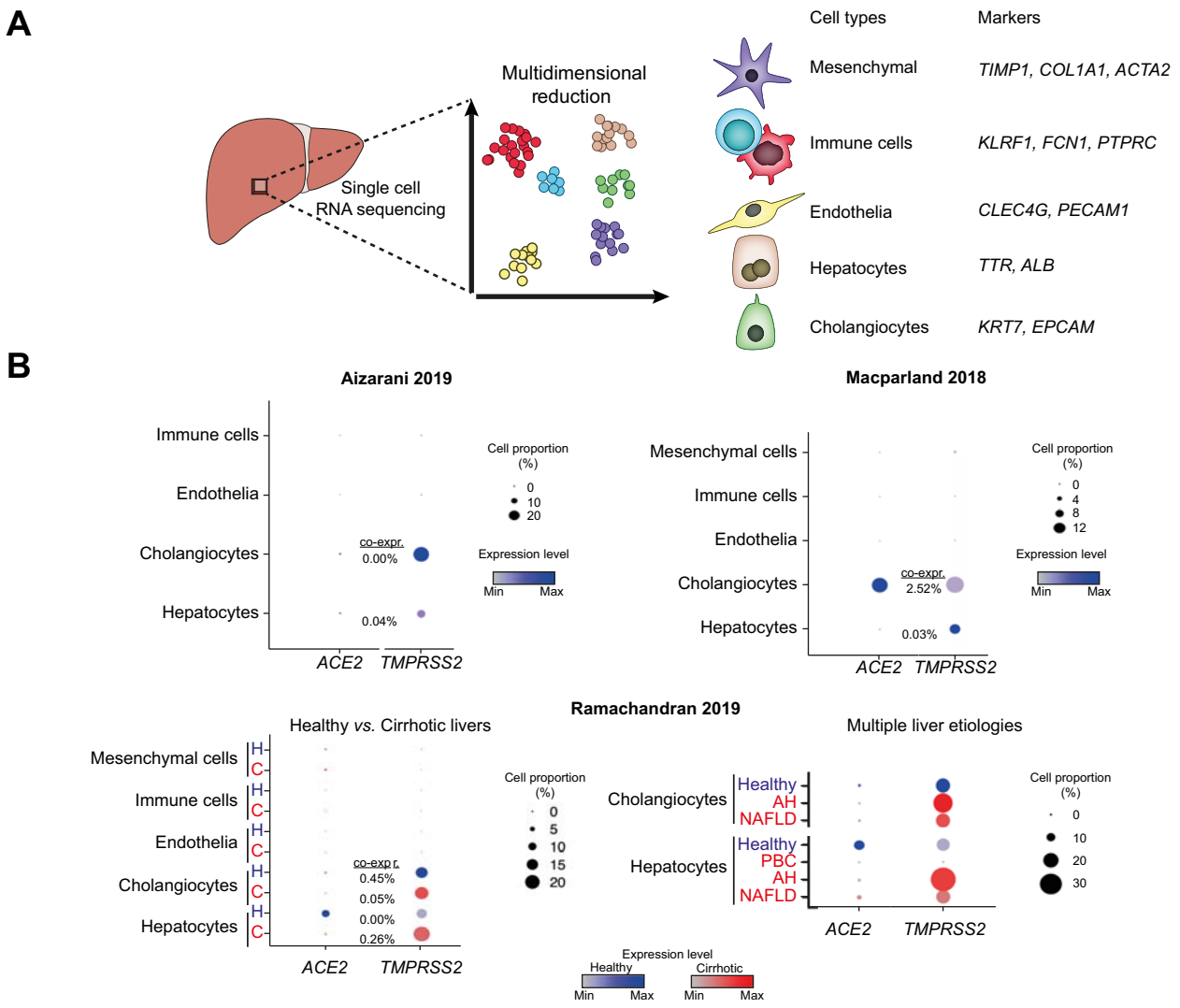

Fig. 1. scRNAseq analysis of ACE2 and TMPRSS2 expression in livers. (A) Schematic of liver cell types represented in the 3 publicly available scRNAseq data sets of human liver tissue. Cellular markers used for cluster identification are shown. (B) *ACE2* and *TMPRSS2* RNA (co)expression in liver cells. All raw cell counts were normalized, scaled and clustered using principle component analysis. Cell types were identified using the same markers as in the original papers (Fig. S1). Surface of dots represents percentage of cells with greater-than-zero RNA expression (per cell type). Color intensity represents expression value of genes.

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Supplementary data

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Author names in bold designate shared co-first authorship

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Vincent De Smet[#]
Stefaan Verhulst[#]
Leo A. van Grunsven[*]
Liver Cell Biology Research Group, Vrije Universiteit Brussel, Jette, Belgium

[*]Corresponding author. Address: Liver Cell Biology Research Group, Vrije Universiteit Brussel (VUB) Laarbeeklaan 103, D022, 1090 Jette, Belgium. Tel.: +32 2 02 477 4407.
E-mail address: lvgrunsv@vub.be (L.A. van Grunsven)

[#] VDS and SV contributed equally.

SARS-CoV-2: Is the liver merely a bystander to severe disease?

To the Editor:

We read the recent article from Wang *et al.* with great interest.[1] They report that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-positive patients with ≥1-week history of increased aminotransferases have worse acute pulmonary disease (radiological and physiological) than those without. They also report higher ferritin levels, higher proportions of patients with a low albumin and raised direct bilirubin, and histological features (albeit in only 2 patients) possibly in keeping with viral-mediated liver injury. Considering that interleukin-6 (IL-6) and C-reactive protein (CRP) are similar between patients with normal and prolonged abnormal liver aminotransferases, the authors speculate that liver injury is a direct effect of SARS-CoV-2 viral hepatitis rather than the result of indirect immune-mediated injury. The fact that increases in liver aminotransferases occur and tend to parallel the severity of pulmonary disease remains unquestioned,[2] however, whether the liver injury is a true viral hepatitis rather than a bystander to the multi-organ pathophysiology of critical illness requires further discussion.

Wang *et al.* provide evidence for direct viral infection based on electron microscopy where they identified multiple intra-hepatocyte microvesicular structures with "crowns" as SARS-CoV-2 virions. However, normally occurring clathrin-coated vesicles have a similar appearance. Additionally, the tissue is undergoing autolysis, as is usual for post-mortem tissue, and autolysed multi-vesicular bodies (MVBs) are seen in the images. It is therefore possible that the observed cytosolic microvesicles are the intraluminal vesicles of autolysed MVBs. In the context of systemic inflammation, hepatocytes are known to produce MVBs which release the contained vesicles as extracellular vesicles by exocytosis during non-apoptotic cell death (*e.g.* pyroptosis).[3] Indeed, the authors demonstrate TUNEL-positive hepatocytes (not specific for apoptosis, but also positive in non-apoptotic cell death and autolysis[4]) and elevated lactate dehydrogenase levels (a marker of non-apoptotic cell death), supporting pyroptosis and autolysis as alternate explanations for these clinical and tissue findings, respectively. Moreover, as the authors acknowledge, hepatocytes express little to no angiotensin converting enzyme-2 (ACE2) receptors, the cellular entry point for SARS-CoV-2. Taken together, and in the absence of SAR-CoV-2 *in situ* hybridisation, immunohistochemistry/immunoelectron microscopy or demonstration of SARS-CoV-2 RNA or proteins within the liver, we believe the authors, as others, have mislabelled these electron microscopic structures as SARS-CoV-2 virions.[5]

Regarding the blood parameters in the study, aminotransferases (in particularly aspartate aminotransferase) are not specific for liver injury and are also released after acute muscle injury. The authors identify higher levels of creatinine kinase in patients with raised aminotransferases raising the possibility of a predominantly muscle rather than hepatic source. Acute and chronic infective illnesses drive catabolic processes that involve muscle (protein) breakdown.[6] In keeping with this, patients with severe pulmonary SARS-CoV-2 infection lose weight and we have found them to have a high incidence of critical illness neuromyopathy on recovery from their respiratory failure. Notwithstanding this, the real elephant in the room is the greater degree of respiratory compromise that associates with only modest liver aminotransferase derangement and the complete lack of clinical correlation with clinically significant liver disease. Parameters disturbed in severe acute liver failure are lactate, glucose and international normalized ratio – these were all well preserved in the data presented by the authors. The patterns of direct bilirubin and albumin are therefore unlikely due to poor synthetic liver function. Reductions in albumin more likely reflect increased systemic endothelial permeability and albumin loss from the circulation, something which commonly and rapidly occurs in acute systemic illnesses in patients without liver disease.[7]

Despite IL-6 and CRP being similar between patient groups, lymphocyte subset depletion, neutrophil counts, ferritin and markers of fibrinolysis are all significantly increased in patients with prolonged abnormal aminotransferases, clearly suggesting increased immune activation, as we have previously highlighted.[2] Furthermore recent studies have confirmed increased NETosis, a form of non-apoptotic and highly immunogenic cell death causing bystander damage and coagulation changes, accompanies disease severity.[8] Immune-mediated bystander damage then remains a credible mechanism for liver enzyme release and has already been shown to be involved in chimeric antigen receptor T cell-mediated cytokine release syndrome.[9]

In conclusion, we do not believe that the findings of Wang *et al.* conclusively demonstrate a direct cytotoxic effect of SARS-CoV-2 on the liver. Based on the above perspectives, we feel that raised liver aminotransferases associated with SARS-CoV-2 positivity are more likely attributable to illness severity, in which host response and iatrogenic harm (*i.e.* drugs, ventilation) drive bystander liver injury, thus explaining its association with

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