RESEARCH ARTICLE

In vivo negative regulation of SARS-CoV-2 receptor, ACE2, by interferons and its genetic control [version 1; peer review: 2 approved with reservations]

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Abstract

Background: Angiotensin I converting enzyme 2 (ACE2) is a receptor for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and differences in its expression may affect susceptibility to infection.

Methods: We performed a genome-wide expression quantitative trait loci (eQTL) analysis using hepatitis C virus-infected liver tissue from 190 individuals.

Results: We discovered that polymorphism in a type III interferon

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gene (*IFNL4*), which eliminates IFN- λ 4 production, is associated with a two-fold increase in *ACE2* RNA expression. Conversely, among genes negatively correlated with *ACE2* expression, IFN-signalling pathways were highly enriched and *ACE2* was downregulated after IFN- α treatment. Negative correlation was also found in the gastrointestinal tract where inflammation driven IFN-stimulated genes were negatively correlated with *ACE2* expression and in lung tissue from a murine model of SARS-CoV-1 infection suggesting conserved regulation of *ACE2* across tissue and species.

Conclusions: We conclude that *ACE2* is likely a negatively-regulated interferon-stimulated gene (ISG) and carriage of *IFNL4* gene alleles which modulates ISGs expression in viral infection may play a role in SARS-CoV-2 pathogenesis with implications for therapeutic interventions.

Keywords

ACE2, IFNL4, HCV, ISG

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Introduction

Entry of coronaviruses into susceptible cells depends on the binding of the spike (S) protein to a specific cell-surface protein and subsequent S protein priming by cellular proteases. Similar to severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1), infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; coronavirus disease 2019 (COVID-19) virus) employs Angiotensin I converting enzyme 2 (ACE2) as a receptor for cellular entry¹.

Epidemiological studies have indicated that the risk for serious disease and death from COVID-19 is higher in males, in older individuals and those with co-morbidities^{2–4} and it varies across ethnic groups⁵. Host genetic variation is important in determining susceptibility and disease outcome for many infectious diseases⁶ and it is likely to be important in determining SARS-CoV-2 susceptibility and outcome. Polymorphisms in the host genome could drive differences in *ACE2* expression, which may affect susceptibility to SARS-CoV-2 infection and its consequences. Thus a better understanding of *ACE2* expression, its regulatory mechanisms *in vivo* and its association with host genetics, especially during viral infection will provide insights on SARS-CoV-2 pathogenesis and help in repurposing antiviral drugs and development of vaccine strategies.

Additionally host genetic variation could impact on differential immune responses to the infection^{7–9}. The earliest immune defence mechanism activated upon virus invasion is the innate immune system¹⁰. Virus-induced signalling through innate immune receptors prompts extensive changes in gene expression¹¹ and it has been shown that host genetics contribute to transcriptional heterogeneity in response to infections^{7,8,12}. Therefore, in the context of infectious diseases, it is important to investigate infected tissue to observe infection-triggered immune response heterogeneity and to understand the role of host genetics. For instance in the context of hepatitis C virus (HCV) infection, hepatic interferon (IFN)-stimulated genes (ISGs) induction varies considerably between individuals with some patients showing constant ISGs expression at high levels while others show almost no detectable induction of innate immune system^{7,8}. This differential innate immune activation is strongly associated with the genetic variation in the *IFNL* locus^{7,8} on chromosome 19q13.2. The causal variant is likely to be the dinucleotide exonic variant rs368234815 in the *IFNL4* gene¹³. This variant [$\Delta G > TT$] results in a frameshift, abrogating production of functional IFN- $\lambda 4$ protein. Lack of production of IFN- $\lambda 4$ in individuals carrying rs368234815 TT/TT genotype is associated with no or low levels of expression of liver ISGs, higher viral load and paradoxically with higher rates of spontaneous clearance and treatment response to IFN- α and direct-acting antivirals^{13,14}. Lack of production of IFN- $\lambda 4$ is also associated with better outcome of RNA virus respiratory tract infections (including coronaviruses) in children¹⁵. This locus has also been associated with viral evolution^{16,17}. It has been shown that IFN- $\lambda 4$ is highly conserved in mammals and therefore functionally relevant, but in humans, the dinucleotide insertion (rs368234815 TT allele) has a gradient in frequency that rises from Africa (0.29–0.44) to Europe

(0.58–0.77) and reaches near fixation in East Asia (0.94–0.97) indicating positive selection has favoured the elimination of IFN- $\lambda 4$ in humans¹⁸. The molecular link between the genotype and the phenotype has not been fully defined, in part due to limitations in detection of IFNL proteins and mRNAs in the liver biopsies of patients with chronic HCV infection^{19,20}.

To understand the impact of *IFNL* locus and other host genetic factors on *ACE2* expression in the presence of RNA virus infection, we performed a genome-wide eQTL analysis for *ACE2* expression in 190 HCV-infected liver biopsies. We observed that host genetic polymorphism of the *IFNL* region tagging a variant that eliminates IFN- $\lambda 4$ production was significantly associated with increase in *ACE2* RNA expression. We also observed that increase in age and presence of liver cirrhosis were associated with increased *ACE2* expression. Additionally, we identified negative correlation of *ACE2* with ISGs in virus infected liver biopsies. We discovered the same pattern in gastrointestinal tract where inflammation driven ISGs were negatively correlated with *ACE2* expression in two independent cohorts. The interferon-associated down-regulation of *ACE2* was also identified in lung tissue in a murine model of SARS-CoV-1 infection. Furthermore, we detected downregulation of *ACE2* transcripts after IFN- α treatment in virus-infected liver biopsies. Due to the conserved pattern of down-regulation of *ACE2* in presence of up-regulation of ISGs across tissues, inflammatory responses, infections and species, we conclude that *ACE2* is likely a negatively-regulated ISG and the genetic variation in the *IFNL* locus which modulates ISGs expression in viral infection may potentially play a role in SARS-CoV-2 pathogenesis.

Results

To understand the link between host genetics and *ACE2* expression in presence of RNA virus infection, we used genotyped autosomal SNPs in the host genome to undertake a genome-wide eQTL analysis for the expression of *ACE2* in 190 virus-infected livers. Due to a dominant effect of *IFNL4* locus on hepatic gene expression, we used both additive and dominant genetic models using linear regression and adjusted for population structure by including the first five host genetic principal components (PCs) as covariates. We also added age, sex and liver cirrhosis status as covariates to account for possible confounding. The outcome variable in the linear regression analysis was the expression of *ACE2* RNA in $\log_{10}$ -transformed transcripts per million ($\log_{10}(\text{TPM}+1)$). There was no inflation in the association test statistics (Supplementary Figure 1²¹). We used a P-value threshold of 5×10^{-8} to declare a convincing finding. Across the human genome, in both dominant and additive analysis, the most associated signals were observed for three SNPs in the *IFNL* locus (Figure 1a, Supplementary Figures 2, 3, 4 and Supplementary Table 1²¹). For all three SNPs the dominant model had lower P-values (rs12980275, $P_{\text{dom}} = 9.9 \times 10^{-11}$, $P_{\text{add}} = 1.6 \times 10^{-8}$; rs8103142, $P_{\text{dom}} = 7.8 \times 10^{-10}$, $P_{\text{add}} = 2.5 \times 10^{-8}$; rs12979860, $P_{\text{dom}} = 3.9 \times 10^{-9}$, $P_{\text{add}} = 6.5 \times 10^{-8}$). In European populations these SNPs are in high linkage disequilibrium (LD) with each other and with the likely causal variant rs368234815 SNP (not typed in

our genotyping array; Supplementary Figure 5²¹; LD between the three associated SNPs and rs368234815 from 1000 genomes study, CEU population, $r^2_{rs12980275} = 0.90$, $r^2_{rs8103142} = 1.0$, $r^2_{rs12979860} = 0.98$).

To understand the impact of polymorphisms in the *IFNL4* gene and other host factors on *ACE2* expression in presence of viral infection, we focused on the impact of *IFNL4* SNP

rs12979860 on *ACE2* RNA expression (Figure 1b). This SNP is an *IFNL4* intronic SNP and closest to rs368234815 SNP among the three associated SNPs (Supplementary Figure 5²¹), where rs12979860 C allele is in linkage disequilibrium with rs368234815 TT allele ($r^2 = 0.98$). Using the dominant genetic model: C/C versus C/T and T/T genotypes i.e. those that do not produce IFN- $\lambda 4$ protein and those that do produce IFN- $\lambda 4$ protein, we

Figure 1. Impact of host genetics and other factors on *ACE2* expression in HCV infected liver. (a) Manhattan plot of association tests between host genetic variation and *ACE2* expression (in $\log_{10}$ -transformed transcripts per million, $\log_{10}(TPM+1)$) in virus infected liver biopsies using a dominant genetic model. The dashed line indicates 5×10^{-8} significance threshold. Significant SNPs are indicated and their IDs are shown. (b) Forest plot of the effect sizes of SNP rs12979860 (dominant model C/C versus C/T and T/T genotypes), cirrhosis status, age and sex on *ACE2* expression. The black circles indicate the point estimate of the effect sizes and the black lines indicate their 95% confidence interval. (c) Distribution of *ACE2* expression stratified by SNP rs12979860 genotypes (dominant model). Black circle shows the mean and the lines indicate its 95% confidence interval. (d) The relationship between *ACE2* expression and age. The blue and red lines show the linear regression fit (for C/C and non-C/C genotypes respectively) and the grey area indicates their 95% confidence interval. (e) Distribution of *ACE2* expression stratified by host liver cirrhosis status. The black dot and lines indicate the mean and its 95% confidence interval. *ACE2* = Angiotensin I converting enzyme 2; TPM = transcripts per million; SNP = single nucleotide polymorphism;

observed a two-fold higher expression of *ACE2* ($P = 3.9 \times 10^{-9}$, mean expression for C/C = 4.34 TPM, mean expression for non-C/C = 2.03 TPM) in individuals with C/C versus non-C/C genotypes (Figure 1c).

Additionally, we obtained weak evidence that *ACE2* expression increased with age ($P = 0.04$) in both C/C and non-C/C patients (Figure 1d) and patients with cirrhosis had a 1.5 fold higher *ACE2* expression relative to non-cirrhotic patients ($P = 0.02$, mean expression for cirrhotic = 3.86 TPM and mean expression for non-cirrhotic = 2.52 TPM, Figure 1e).

To detect common regulatory mechanisms, biological function and the context of *ACE2* expression, we performed correlation

analysis accounting for multiple testing to identify genes correlated with *ACE2* expression in virus-infected livers. We observed large correlation coefficients (maximum of 0.6 and minimum of -0.5) and detected 1530 genes correlated with *ACE2* expression at 1% false discovery rate (FDR) and with correlation coefficients of > 0.3 or < -0.3 . Considering separately the genes that were positively correlated ($N=1362$) and those that were negatively correlated ($N=168$) with *ACE2* expression (Supplementary Tables 2 and 3²¹), we performed a gene set enrichment analysis, observing that genes involved in type I IFN signalling pathways were enriched among genes negatively correlated with *ACE2* expression (Figures 2a and 2b, Supplementary Table 4²¹). We also observed that genes involved in extracellular structure organisation were enriched among

Figure 2. Negative correlation of transcript levels of *ACE2* with interferon-stimulated genes in HCV infected liver biopsies. (a) Expression of four representative interferon-stimulated genes (ISG) and their observed negative correlation with *ACE2* expression. Pearson's correlation coefficient is shown for each gene. (b) Gene Ontology (GO) gene set enrichment analysis among genes with significant negative correlation with *ACE2* expression (at false discovery rate of 1% and correlation coefficient of < -0.3). Only the top ten enriched gene sets are shown (from a total of 22 gene sets), which are all ISGs related pathways. (c) Expression of the four representative ISGs and their observed negative correlation with *ACE2* in an independent HCV infected liver biopsy data set. Pearson's correlation coefficient is shown for each gene. (d) *ACE2* expression in paired liver biopsy samples from before and after pegylated IFN- α treatment taken at different time points post treatment. (e) Log fold change ($\log_{10}$ (post-treatment / pretreatment)) of *ACE2* expression after pegylated IFN- α treatment. At all time points we observe a reduction in mean *ACE2* expression with the biggest median drop at 16 hours. The reported P-values are from paired t-tests for each time point. Combining all time points, downregulation of *ACE2* after IFN- α treatment is highly significant ($P = 6.5 \times 10^{-5}$, paired t-test).

genes positively correlated with *ACE2* expression (Supplementary Figure 6 and Supplementary Table 5²¹). We used an independent data set of liver biopsies⁹ from 28 patients (6 non-HCV infected controls and 22 HCV-infected cases, GSE84346) and replicated concordant correlation signs for 162 of the 168 genes negatively correlated with *ACE2* (Figure 2c and Supplementary Table 6²¹). This represents a significant enrichment of concordant correlation signs relative to the null hypothesis of no association between correlation coefficients in the two datasets ($P = 2.2 \times 10^{-16}$, binomial test). In this replication cohort 18 patients had two biopsies taken, one before and another after treatment with pegylated IFN- α at different time points ($N_{4\text{hrs}} = 4$, $N_{16\text{hrs}} = 3$, $N_{48\text{hrs}} = 3$, $N_{96\text{hrs}} = 3$, $N_{144\text{hrs}} = 5$). At all time points we observed a down-regulation of *ACE2* expression (Figure 2d), with the biggest median drop at 16 hours post treatment. These reductions were nominally

significant at 48 and 144 hours after IFN- α treatment (paired t-test, Figure 2e), but across all time points represent a highly significant down-regulation of *ACE2* after IFN- α treatment ($P = 6.5 \times 10^{-5}$, paired t-test).

To further explore the negative correlation of ISGs with *ACE2* expression in a known site of SARS-CoV-2 replication, we explored the relationship between *ACE2* and ISGs expression in the gastrointestinal (GI) tract in a gene expression study of terminal ileum biopsies in inflammatory bowel disease (IBD) in treatment-naïve young donors (RISK cohort²², GSE57945). In intestinal biopsies, there was a striking decrease of *ACE2* expression with increasing severity of inflammation that was independent of the abundance of transcriptional markers of epithelial identity²³ (Figure 3a and Supplementary Figure 7a²¹)

Figure 3. Conservation of negative correlation of *ACE2* expression with ISGs across tissues, conditions and species. (a) *ACE2* expression in terminal ileum biopsies of IBD patients grouped based on disease severity and histologic assessment of inflammation. The black dots and lines indicate the mean and its 95% confidence interval in each group (Ulcerative Colitis without ileal involvement; iCD = ileal Crohn's Disease; Micro Infl. = Microscopic Inflammation, Macro Infl. = Macroscopic Inflammation; DU = Deep Ulcers). Data are shown as $\log_{10}$ -transformed RPKM relative to the epithelial cell identity metagene (see methods) (b) Expression of genes which are part of the Gene Ontology category "response to type I interferon" (GO ID: 0034340) and which in the liver are negatively correlated with *ACE2* (FDR of 1% and correlation coefficient of < -0.3) in the IBD cohort stratified by disease severity. The black dots and lines show the mean and its 95% confidence interval for each group. (c) Negative correlation of representative ISGs with *ACE2* in the terminal ileum biopsies of IBD patients. Pearson's correlation coefficients are shown. (d) Volcano plot of lung tissue differential gene expression pattern induced by SARS-CoV-1 infection in mouse model vs. mock. *ACE2* and representative ISGs which are part of the Gene Ontology category "response to type I interferon" (GO ID: 0034340) and which in the liver are negatively correlated with *ACE2* are indicated by red dots.

and ISGs had increasing expression with rise in disease activity and were negatively correlated with *ACE2* expression (Figures 3b, 3c and Supplementary Figure 7b²¹). Genes associated with epithelial cell structure and function were enriched among genes that were positively correlated with *ACE2* in both liver and intestine, while genes associated with type I interferon signalling pathways were enriched among genes that negatively correlated with *ACE2* expression in both tissues (Supplementary Figure 8²¹). These data were supported by analysis of a second independent IBD cohort²⁴ (GSE137344, Supplementary Figure 9²¹).

Since the pattern of gene expression incorporating down-regulation of *ACE2* in presence of ISGs was consistent in two models of viral chronic infection and/or inflammation in different tissue, we addressed whether a similar pattern of gene regulation was observed in lung tissue using data from mouse models of SARS-CoV-1 infection²⁵ (GSE59185). Indeed, we observed in SARS-CoV-1 infected lung the same associated down-regulation of *ACE2* in the presence of up-regulation of classical ISGs (Figure 3d).

Discussion

To understand the impact of host genetic factors on *ACE2* expression in the presence of RNA virus infection, we performed a genome-wide eQTL analysis for *ACE2* expression in 190 HCV-infected liver biopsies. Using infected tissue is important, since genetically driven differences in innate immune responses are only likely to be observed when innate immune responses are triggered. We observed that genome-wide host genetic polymorphisms in the *IFNL* region were significantly associated with *ACE2* expression in the presence of viral infection. The likely causal mechanism is the variant rs368234815 [$\Delta G > TT$], which results in a frameshift and abrogates production of IFN- $\lambda 4$ ¹³. In the context of HCV infection, production of IFN- $\lambda 4$ is associated with high hepatic ISG expression (low *ACE2* expression) and low viral load, but paradoxically with lower rates of spontaneous clearance in acute phase of infection and lower rates of response to treatment in the chronic phase of infection. Production of IFN- $\lambda 4$ is also associated with worse outcome of RNA virus respiratory tract infection in children¹⁵.

Interferon lambda receptor (*IFNLRI*) is largely restricted to tissues of epithelial origin^{26,27}, therefore, IFN- λ proteins (type III IFN) may have evolved specifically to protect the epithelium. Overall, *IFNL* genes lead to a pattern of gene expression which is similar to type I IFN genes, but the time course and pattern of expression may vary¹⁰. This has been explored in HCV, where a slower, but sustained impact of *IFNL* signalling is seen²⁸. *In vitro* studies have revealed that ISG expression and anti-viral activity induced by recombinant *IFNL4* are comparable to that induced by *IFNL3*²⁹, although the tight regulation of *IFNL4*³⁰ may impact on its ability to induce a rapid antiviral state^{14,31}. However, once established, the *IFNL4* transcriptional module may also be highly sustained (as seen here and in other HCV cohorts³²) and also noted elsewhere, e.g. after childbirth³³.

In mice, the type III IFN response is restricted largely to mucosal epithelial tissues, with the lung epithelium responding to both type I and III IFNs³⁴ and intestinal epithelial cells responding exclusively to type III IFNs. Among nonhematopoietic cells, epithelial cells are potent producers of type III IFNs. In mouse models, type III IFNs seem to be the primary type of IFN found in the bronchoalveolar lavage in response to influenza A virus infection and play a critical role in host defence³⁵. The data from the GI tract indicate that this gene expression pattern is conserved amongst tissues, consistent with emerging data³⁶.

To understand the context of *ACE2* expression *in vivo* and detect common regulatory mechanisms in presence of RNA virus infection, we measured the correlation between *ACE2* expression and approximately 15000 other genes expressed in HCV-infected liver biopsies. We observed a down-regulation of *ACE2* in the presence of up-regulation of classical ISGs. This was replicated in a second cohort of HCV-infected liver biopsies. The same observation was confirmed in the gastrointestinal tract in an inflammatory condition and in lung in a murine model of SARS-CoV-1 infection. This indicates a robust maintenance of the transcriptional downregulation of *ACE2* in presence of ISGs up-regulation across infections, inflammatory conditions, tissues and species. We also detected down-regulation of *ACE2* in virus-infected liver biopsies after pegylated-IFN- α treatment with the biggest drop at 16 hours post treatment.

We have presented evidence that *ACE2* may be negatively regulated by IFNs *in vivo*. We have also demonstrated that *ACE2* expression in presence of RNA virus infection is modulated by genetic variation in the *IFNL* region. This regulation is likely due to confirmed differential activation of innate immune system in the liver in response to RNA virus infection associated with this region⁷⁻⁹. Therefore, given the prominent role of type III IFNs in defence of epithelial surfaces such as that in the lung from viral infections, we hypothesise that the genetic variation in the *IFNL* region may also play a role in modulating innate immune responses to SARS-CoV-2 infection. Genetic variation resulting in production of IFN- $\lambda 4$ is associated with high ISGs level and downregulation of *ACE2* which may limit the ability of SARS-CoV-2 and other related coronaviruses to enter cells, but may, if sustained, also have impacts on inflammation and interfere with lung tissue repair^{37,38}. Indeed *ACE2*^{-/-} mice suffer from enhanced disease following virus infection of the lung through an angiotensin-driven mechanism³⁹.

These data are derived from an *in vivo* assessment and the downregulation of *ACE2* is consistent across conditions, tissues and species. The data are also potentially consistent with up-regulation of *ACE2* seen in early time points by IFN- α *in vitro*⁴⁰. However, we note that measuring *ACE2* expression using cell cultures stimulated by type I and III IFNs we observed large amounts of variability between cell lines and also between donors when using primary bronchial epithelial cells (Supplementary Figures 10, 11 and 12²¹). The likely explanation for the difference is that the regulation of this physiologic receptor in an *in vivo* setting is distinct from

studies *in vitro*, but the full kinetics of this need further study during natural infection.

This study is relevant to the expression of *ACE2* during SARS-CoV-2 infection. Although we did not study this directly in the respiratory tract, such studies should be performed to confirm these data. Furthermore the overall impact of *IFNL4* polymorphism on the clinical course should be assessed, especially given the very variable distribution of *IFNL4* alleles in different ethnic groups^{18,41}. Finally, the genetic data add weight to the idea of a careful exploration of IFN- λ pathways in therapy for SARS-CoV-2⁴².

Methods

Boson patient cohort

For this study, we used patient data from the BOSON cohort that has been described elsewhere in details⁴³. In summary, The BOSON study is a phase 3 randomized open-label trial to determine the efficacy and safety of treatment with sofosbuvir, with and without pegylated IFN- α , in treatment-experienced patients with cirrhosis and HCV genotype 2 infection and treatment-naïve or treatment-experienced patients with HCV genotype 3 infection. All patients provided written informed consent before undertaking any study-related procedures. The BOSON study protocol was approved by each institution's review board or ethics committee before study initiation. The study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines and the Declaration of Helsinki (clinical trial registration number: [NCT01962441](#)).

RNA extraction, library prep, sequencing and mapping for the BOSON cohort

Liver biopsy samples were available for 198 patients. Total RNA was extracted from patient liver biopsies at baseline (pre-treatment) using RNeasy mini kits (Qiagen, 74104). Briefly, liver biopsy samples were mechanically disrupted in the presence of lysis buffer and homogenized using a QIAshredder (Qiagen, 79654). Tissue lysates were then centrifuged (8000 g for 1 minute) and clarified supernatants were transferred into new microcentrifuge tubes (pellets were discarded). Next, 350 μ L volume of 70% ethanol was added to the lysates and samples were mixed by gentle vortexing. 700 μ L of sample was then transferred into RNeasy spin columns (Qiagen, 74104; with 2 mL collection tubes) and centrifuged at 10000 rpm for 15 seconds. Column flow-through was discarded. DNase (Qiagen, 79254) digestion was subsequently performed to eliminate any contamination from genomic DNA. 80 μ L of DNase I solution (10 μ L DNase I stock + 70 μ L Buffer RDD) was added directly to RNeasy spin columns and incubated at room temperature for 15 minutes. Following DNase incubation, the columns were washed with 350 μ L of Buffer RW1 and centrifuged at 10000 rpm for 15 seconds. Flow-through was discarded and 500 μ L of Buffer RPE was added to the spin columns. Columns were then centrifuged again at 10000 rpm for 15 seconds and flow-through was discarded. An additional 500 μ L of Buffer RPE was added to the spin columns and columns were centrifuged at 10000 rpm for 2 minutes. Finally, spin columns

were transferred into new microcentrifuge tubes and 30 mL of RNase-free water was added directly to the column membrane. Columns were then centrifuged at 10000 rpm for 1 minute to elute the RNA.

RNA yield was quantified using a NanoDrop spectrophotometer. Selected samples were also run on an Agilent TapeStation system to assess RNA quality and purity. Library preparation from purified RNA samples was performed using the Smart-Seq2 protocol⁴⁴, used along with previously described indexing primers during amplification (see Additional file 5 of [45](#)).

High-throughput RNA sequencing of prepared libraries was performed on the Illumina HiSeq 4000 platform to 75bp PE at the Wellcome Center for Human Genetics (Oxford, UK). Reads were trimmed for Nextera, Smart-seq2 and Illumina adapter sequences using [skewer-v0.1.125](#)⁴⁶. Trimmed read pairs were mapped to human genome GRCh37 using [HISAT2](#) version 2.0.0-beta⁴⁷. Uniquely mapped read pairs were counted using [featureCounts](#)⁴⁸, [subread-1.5.0](#)⁴⁹, using exons annotated in ENSEMBL annotations, release 75. Mapping QC metrics were obtained using [picard-tools-1.92](#) [CollectRnaSeqMetrics.jar](#). Three samples were excluded after QC checks due to low sequencing depth which left 195 samples for analysis. Genes were filtered using the criteria of having a count per million (CPM) of 1.25 in at least 10 samples to remove low expressed genes. Function cpm from [edgeR](#)⁵⁰ version 3.20.9 was used to calculate the CPM values. After removing low expressed genes we were left with 14882 genes. To normalise for library size and gene length, transcripts per million (TPM) values were calculated from unique mapped read counts and $\log_{10}(\text{TPM}+1)$ was used in the analysis.

For the replication cohort⁹ (GSE84346), the read counts were downloaded from GEO. Gene expression data for 46 liver biopsy samples from 28 individuals were available for this data set. 22 individuals had chronic HCV infection while 6 individuals did not have HCV infection and were enrolled as controls. Among the 22 individuals with chronic HCV infection, 18 individuals were treated with pegylated interferon-alpha and a second biopsy was taken post-treatment. Genes were filtered using the criteria of having a count per million (CPM) of 1.25 in at least 10 pre-treatment samples to remove low expressed genes. After removing low expressed genes we were left with 14661 genes. To normalise for library size and gene length, transcripts per million (TPM) values were calculated from unique mapped read counts and $\log_{10}(\text{TPM}+1)$ was used in the analysis.

Host genotyping

Host genome-wide genotyping was performed on 567 patients from the BOSON cohort as described previously¹⁶. Briefly, genomic DNA was extracted from buffy coat using Maxwell RSC Buffy Coat DNA Kit (Promega, AS1540) as per the manufacturer's protocol and quantified using Qubit (ThermoFisher). DNA samples from patients were genotyped using the Affymetrix UK Biobank array¹⁶. After quality control and filtering of the human genotype data, approximately 330,000 common SNPs with minor allele frequency greater than 5%

were available for analysis. Both liver RNA transcriptomic and human genome-wide SNP data were obtained on a total of 190 patients of mainly White self-reported ancestry infected with HCV subtype 3a.

Statistical analysis

To test for association between autosomal human SNPs and *ACE2* expression in $\text{Log}_{10}(\text{TPM}+1)$ unit, we performed linear regression using [PLINK](#)⁵¹ version 1.9 using additive and dominant genetic models adjusted for the human population structure by adding the first five genetic principal components as covariates. We also added host cirrhosis status, age and sex as covariates to the analysis. To assess the impact of age per 10 years increase, we divided the age by 10 before adding it as a covariate. For 190 patients both host genome-wide genotyping data and hepatic *ACE2* expression data were available. We used a significance threshold of 5×10^{-8} . Investigating the impact of rs12979860 on *ACE2* expression (in $\text{log}_{10}(\text{TPM}+1)$ unit) we used linear regression with a dominant genetic model (C/C versus C/T and T/T genotypes) with the same exact covariates as in the GWAS model described above.

For the BOSON Cohort, $\text{Log}_{10}(\text{TPM}+1)$ values were calculated and used to estimate Pearson's correlation coefficient between *ACE2* and all other genes. The [qvalue](#) (version 2.10.0) package in R was used to calculate false discovery rate. We used FDR of 1% and correlation coefficient of >0.3 or <-0.3 to decide on genes significantly correlated with *ACE2*. To test for enrichment we used [enrichGO](#) function from the [clusterProfiler](#) (version 3.15.2) package⁵², limiting the analysis to GO "biological process" class and maximum gene set size of 500.

An independent liver biopsy dataset⁹ (GSE84346) was used to replicate the negative correlation of *ACE2* with 168 genes found in the BOSON cohort. We used the $\text{Log}_{10}(\text{TPM}+1)$ values from the 28 baseline liver biopsy samples and calculated Pearson's correlation coefficient between *ACE2* and the 168 genes. Assuming a null hypothesis of no association between correlation coefficients signs in the two data sets, one can use a binomial test to assess this null hypothesis where the probability of a negative correlation sign was estimated from the BOSON cohort by dividing the number of genes with negative correlation with *ACE2* (3558) divided by total number of genes (14881) tested against *ACE2*. To test the hypothesis of down-regulation of hepatic *ACE2* expression when treated with pegylated interferon-alpha, we used the 18 individuals with liver biopsy samples taken before and after the treatment. We used one-sided paired t-test to perform hypothesis testing for downregulation of *ACE2* for each of the time points and across all time points.

RISK cohort

The RISK study is an observational prospective cohort study with the aim to identify risk factors that predict complicated course in pediatric patients with Crohn's disease as previously described⁵³. The RISK study recruited treatment-naïve patients with a suspected diagnosis of Crohn's disease. The Paris modification of the Montreal classification were used to classify patients

according to disease behaviour (non-complicated B1 disease (non-stricturing, non-penetrating disease); complicated disease, composed of B2 (stricturing) and/or B3 (penetrating) behaviour) as well as disease location (L1, ileal only, L2, colonic only, L3, ileocolonic and L4, upper gastrointestinal tract). 322 samples were investigated with ileal RNA-seq. Individuals without ileal inflammation were classified as non-IBD controls. Patients with Crohn's disease were followed over a period of 3 years. Patients were largely of European (85.7%) and African (4.1%) ancestry. RPKM expression values for the RISK cohort⁵³ were retrieved from GEO (GSE57945). The dataset was filtered to (n=19,556) genes that had an expression value ≥ 0.1 in $>10\%$ of the patients.

Statistical analysis of RISK cohort

To account for the potential loss of epithelial cells contribution to gene expression a metagene score was generated based on the average expression of epithelial identity genes²³. RPKM data were transformed and presented as: $\text{RPKM}+1/\text{epithelial cell metagene}$. For the ISG metagene score, we used 17 genes that were significantly negatively correlated with *ACE2* in the liver and were part of the GO term "response to type I interferon" (GO ID: 0034340). For the intersection of *ACE2* correlated gene expression, genes were ranked based on their Pearson's correlation coefficient with *ACE2* for each patient subgroup. Intersected lists of *ACE2* expression positively (Pearson's correlation coefficient > 0.5) and negatively (Pearson's correlation coefficient < -0.5) correlated genes were extracted (positive correlation: n = 2067; negative correlation: n = 2264). BOSON liver *ACE2* expression and RISK *ACE2* expression positively and negatively correlated gene sets were intersected based on Entrez gene identifiers using [Cytoscape](#) (version 3.7.1) and visualized using the [Cytoscape Venn and Euler Diagrams](#) (Version 1.0.3) plugin. Functionally grouped networks of terms and pathways were analysed using the [Cytoscape](#) (version 3.7.1) [ClueGO](#) (version 2.5.6) and [CluePedia](#) (version 1.5.6) plug-in⁵⁴. The analysis was performed by accessing the Gene Ontology Annotation (GOA) Database for Biologic processes, Cellular components, Immune system processes and Molecular function, the [Reactome pathways database](#) and the [KEGG database](#). Only pathways with an adjusted enrichment p-value ≤ 0.05 were considered (Two-sided hypergeometric test, Bonferroni step down p-value correction). GO terms were grouped based on the highest significance when more than 50% of genes or terms were shared. The filtered RISK gene expression data (n=19,556; expression value ≥ 0.1 in $>10\%$ of the patients) served as reference gene set.

Resources for statistical analysis and data visualization:

[Prism](#) version 8.0 (GraphPad Software)

Excel for Mac Version 15.32 (Microsoft)

[Cytoscape](#) 3.7.1 (<https://cytoscape.org/>)

[Cytoscape](#) 3.7.1 plugin [ClueGO](#) (Version 2.5.6)

[Cytoscape](#) 3.7.1 plugin [CluePedia](#) (version 1.5.6)

[Cytoscape](#) 3.7.1 plugin [Venn and Euler Diagrams](#) (Version 1.0.3)

[Morpheus](#) (<https://software.broadinstitute.org/morpheus/>)

R (Version 3.6.1)

RStudio (Version 1.2.5001)

GENESIS cohort

GENESIS is funded by the National Institute of Diabetes and Digestive and Kidney Diseases and managed by Emory University for the recruitment of self-identified African American subjects with IBD⁵⁵. We used a subset of 195 GENESIS cohort subjects with ileal transcriptomic profiles as an additional replication cohort to test for negative correlation of ACE2 expression with interferon-stimulated genes expression. Pearson correlation tests between normalized expression values for *ACE2* and four ISGs confirmed that this pattern of negative correlation is also observable in a cohort enriched for African American ancestry. This dataset includes 158 IBD patients along with 37 controls. Subjects with ileal inflammation were included as IBD, while non-IBD controls did not have ileal inflammation. This dataset is enriched for African American ancestry (70%), and gender was equally distributed. Full descriptions of age, sex, race, disease status and other phenotypic information are available in a prior publication²⁴. Additionally, ileal transcriptomic profiles sequenced on the NextSeq 550 platform are available in the GEO repository (GSE57945) for all subjects.

Data analysis for SARS-CoV-1 mouse model

The mouse lung tissue microarray data were downloaded from GEO using the accession number GSE59185²⁵. In case of multiple probes per gene, they were collapsed into a single feature, which resulted in 21217 features. Three lung tissue samples were from mice infected with wild type virus and three lung tissue samples were from mock infection. We then used LIMMA (version 3.34.9)⁵⁶ to perform differential gene expression analysis between these two conditions. The log fold change and the p-values were used to make a volcano plot. The genes highlighted as red on the volcano plot are 17 genes which were significantly negatively correlated with *ACE2* in the human liver and were part of the GO term “response to type I interferon” (GO ID: 0034340).

Cell line and human primary bronchial epithelial cell culture

Cell lines used in this study were hepatocyte cell lines Huh7, Huh7.5 (Gift from Charlie Rice), HepaRG, HepG2, human kidney cell line HEK 293T, Human lung fibroblast cell line MRC5, human colorectal tumor cells CaCO2 and Vero E6. All cell lines except the HepaRG cell line were cultured in DMEM media at 37°C, 5% CO₂. HepaRG cell line was cultured at 37°C, 5% CO₂ in Williams' E medium (with Glutamine) (Gibco-Invitrogen) supplemented with glutamine (2 mM), penicillin/streptomycin (50 U/mL), gentamycin (20 µg/mL), insulin bovine (5 µg/mL, Roche-Boehringer-Manheim, France), hydrocortisone hemisuccinate (7x10⁻⁵ M, Roche-Boehringer-Manheim) and FCS (10% selected, non-decomplemented, Fetaclone II-Hyclone-PERBIO France). The cells were allowed to differentiate in the presence of 2% (v/v) DMSO to the medium. Cells were stimulated with 100ng of IFNα2 (Cat # NBP2-34971-20ug), IFNL3 (Cat # 5259-IL-025) or IFNL4 (Cat # 9165-IF-025) all from Biotechnie-R&D systems, Abingdon, UK.

Human primary bronchial epithelial cells were obtained using flexible fibreoptic bronchoscopy under light sedation with

fentanyl and midazolam from healthy control volunteers. Participants provided written informed consent. The study was reviewed by the Oxford Research Ethics Committee B (18/SC/0361). Airway epithelial cells were taken by 2mm diameter cytology brushes from 3rd to 5th order bronchi and cultured in Airway Epithelial Cell medium (Promocell, Heidelberg, Germany) in submerged culture. They were expanded in submerged culture on collagen-coated PureCol (Advanced BioMatrix) plasticware at 37°C 5% CO₂ using Pneumacult-Ex (Stemcell) supplemented with g/mL gentamicin and 15ng/mL amphotericin, and 100 IU/mL penicillin and 100 µg/mL streptomycin. At passage 1, cells were seeded onto collagen-coated transwells, once confluent cultures were airlifted to air-liquid interface (ALI) by removal of the media on the apical side. Basal media was replaced with Pneumacult-ALI maintenance media (Stemcell) and changed every 2 days. Following differentiation (approximately 4 weeks at ALI) cells were stimulated on the basolateral side with (IFNL3 and IFNL4 at 100ng/ml). At 4, 8, 12 and 24 hours post-stimulation cells were lysed with RLT buffer (Qiagen) and stored at -80.

RNA isolation and quantification

RNA was extracted from cell lysates using the RNEasy Mini Kit (Qiagen, 74104). RNA concentration was determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) at 260 nm.

cDNA synthesis and RT-qPCR analysis

cDNA was reverse-transcribed from template RNA either using a two-step reverse transcription using AppScript cDNA synthesis kit (Appleton Woods, ARP601). Samples were incubated at 42°C for 30 mins, followed by 85°C for 10 mins to inactivate reverse transcriptase. All RT-qPCR reactions were either performed using the Roche Light Cycler 480 instrument using AppProbe reagents (Appleton Woods, ARP305). Cycling conditions are as follows: 95°C 5 minutes; amplification 94°C 10 seconds, 58°C 30 seconds, 72°C 10 seconds, for 50 cycles followed by final cooling to 40°C for 10 seconds. Primers were designed using the Roche Universal Probe library system. Relative gene expression was calculated using the comparative cycle threshold method⁵⁷ normalised to expression of the housekeeping gene GAPDH and expressed relative to a mock treated sample.

Data availability

Underlying data

The liver gene expression read counts are submitted to Gene Expression Omnibus (GEO) under accession number GSE149601. The raw FASTQ files are deposited in the European Genome-phenome Archive under the accession code EGAS00001004996. Human genotype data underlying this manuscript are deposited in the European Genome-phenome Archive under accession code EGAS00001002324.

Due to patient privacy concerns the gene expression FASTQ files and the human genotype data can only be accessed by making an application to the data access committee. The Information on access to the study data is available at <http://www.stop-hcv.ox.ac.uk/data-access>.

Extended data

Figshare: Extended data for the paper “In vivo negative regulation of SARS-CoV-2 receptor, ACE2, by interferons and its genetic control”. <https://doi.org/10.6084/m9.figshare.c.5284667.v1>²¹

This project contains the following extended data:

- Supplementary tables in CSV and xlsx formats
- Supplementary Figures

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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? **Christopher J. McCormick**

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The paper by Ansari *et al.* reports that ACE2 is negatively regulated by a type I/type III interferon response. Initial work takes advantage of the known link between an IFNL4 polymorphism (delta G>TT) that disrupts this gene's open reading frame, and the paradoxical stimulatory effect that this has on interferon-stimulated gene (ISG) expression in HCV chronically infected livers. By undertaking an expression quantitative trait loci (eQTL) analysis, the authors convincingly show that high ACE2 transcript levels are significantly associated with single nucleotide polymorphisms (SNPs) linked to the G>TT IFNL4 SNP in HCV chronically infected livers. Additional analysis confirms that there is a negative correlation between ISG expression and ACE2 expression. This conclusion is further supported by the finding that there is a significant drop in ACE2 expression in HCV-infected livers after patients undergo treatment with pegylated IFN-alpha. Analysis is then extended to other tissues that include human small intestine biopsies from patients with varying levels of inflammatory bowel disease and the lungs of BALB/c mice infected with a mouse-adapted strain of SARS-CoV-1. Findings made confirm that ACE-2 expression negatively correlates with ISG expression.

Overall the work is clearly presented and described in such a way that it could be reproduced. All datasets used are either directly available or available on request; the latter being for legitimate reasons. The work has academic merit as few studies have looked at ACE2 expression *in vivo* in response to interferon combined with viral infection. The literature is appropriately cited but does fail to mention certain key studies that are of relevance. Firstly, two very distinctive mRNA transcripts are produced from the human ACE2 gene locus; one that encodes canonical ACE2 and another that encodes for a truncated form of ACE2 that lacks the capacity to bind to spike or act as a carboxypeptidase^{1,2}, [ref-3]. It is this truncated form of ACE2 that has been suggested to account for earlier reports that ACE2 expression is upregulated in response to IFN. Given the study's finding that ACE2 expression increased with cirrhosis, it would also be appropriate to acknowledge earlier studies looking at ACE2 expression in response to liver damage⁴.

Most of the analysis that has been undertaken is appropriate and robust. However, there are one or two points that need attending to.

- The principle one relates to whether the authors have considered that they could be seeing changes to 'short' ACE2 transcript levels rather than the transcript encoding the canonical form? Were transcript reads used to assess ACE2 levels restricted to sequences derived from the first 9 exons, and if not does the depth of sequence reads allow reanalysis? Failing that, can the sequence data be used in some way to rule out short ACE2 expression in the HCV-infected liver? The same question applies to the analysis of ACE2 expression in the terminal ileum. Also, given the existence of the truncated ACE2 transcript, it is important that the authors make clear what primers were used to detect ACE2 in their qPCR assays.
- Another aspect of the analysis that could introduce confounding effects is the inclusion of both uninfected and HCV-infected patients when looking at whether a correlation exists between ACE2 and ISG expression (Fig 2c). It would be preferable for the analysis to be restricted to just the HCV-infected group. Related to this point, it appears that while the methods and results section suggest that the analysis involves 22 HCV patients and 6 uninfected controls, only 27 data points appear to be plotted on the Fig 2c graphs.
- The conclusions drawn in the discussion are broadly appropriate, assuming reanalysis of the data to deal with the points raised above does not invalidate any of the key findings. The authors suggest changes to ACE2 expression might either affect SARS-CoV-2 cell entry or have consequences for tissue repair. I agree with Reviewer 1 (Stuart Neil) that one has to be cautious about whether modulation of ACE2 expression would ultimately have an impact on cell entry, but feel that all that is required here is that this be brought out in the discussion a bit more. Similarly, there could be a benefit to acknowledging in the discussion that much of the analysis has been undertaken in tissues experiencing a chronic inflammatory response rather than a more acute response, as would be encountered from a respiratory infection of the lung. Have the authors looked at the multitude of existing GWAS studies on COVID (<https://www.covid19hg.org/results/>) to see if the G>T IFNL4 SNP or ones in linkage disequilibrium with this SNP have been surveyed? Certainly, these SNPs have not yet been reported as having an association with severe disease. If the SNPs have been surveyed and there is no clear association, it would be valuable to hear any thoughts the authors have on why this might be.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Positive strand RNA virologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 28 June 2021

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Stuart J.D. Neil

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The authors study the expression of the SARS CoV2 receptor in the liver of HCV patients. They find that a risk allele in IFNL4 negatively correlates with ACE2 expression, suggesting IFNs downregulate ACE2. This is replicated in gut epithelial biopsies and in historical analysis of GE in mouse lungs from a SARS CoV1 infection model. In all, this study makes a strong case that ACE2 is a negatively regulated ISG, suggesting the quality and quantity of IFN induction early after infection may limit viral spread.

This is a valuable study, but is essentially observational. The major limitation is that the authors have not sought to confirm these data in ex vivo cultured human lung epithelium, the correlation with protein level, or the impact that reduction of ACE2 has on viral entry. The latter is an important caveat because SARS2 doesn't need much ACE2 on the cell surface to enter, and the extant data in the literature indicates that the VOCs need less. Any further information and discussion on these issues would be important to include in a revised manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: virology and virus host interactions

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.