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Research Article

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Research Article

Type 1 interferon auto-antibodies are elevated in patients with decompensated liver cirrhosis

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Abstract

Patients with decompensated liver cirrhosis, in particular those classified as Childs-Pugh class C, are at increased risk of severe coronavirus disease-2019 (COVID-19) upon infection with severe acute respiratory coronavirus 2 (SARS-CoV-2). The biological mechanisms underlying this are unknown. We aimed to examine the levels of serum intrinsic antiviral proteins as well as alterations in the innate antiviral immune response in patients with decompensated liver cirrhosis. Serum from 53 SARS-CoV-2 unexposed and unvaccinated individuals, with decompensated liver cirrhosis undergoing assessment for liver transplantation, were screened using SARS-CoV-2 pseudoparticle and SARS-CoV-2 virus assays. The ability of serum to inhibit interferon (IFN) signalling was assessed using a cell-based reporter assay. Severity of liver disease was assessed using two clinical scoring systems, the Child-Pugh class and the MELD-Na score. In the presence of serum from SARS-CoV-2 unexposed patients with decompensated liver cirrhosis there was no association between SARS-CoV-2 pseudoparticle infection or live SARS-CoV-2 virus infection and severity of liver disease. Type I IFNs are a key component of the innate antiviral response. Serum from patients with decompensated liver cirrhosis contained elevated levels of auto-antibodies capable of binding IFN-α2b compared to healthy controls. High MELD-Na scores were associated with the ability of these auto-antibodies to neutralize type I IFN signalling by IFN-α2b but not IFN-β1a. Our results demonstrate that neutralizing auto-antibodies targeting IFN-α2b are increased in patients with high MELD-Na scores. The presence of neutralizing type I IFN-specific auto-antibodies may increase the likelihood of viral infections, including severe COVID-19, in patients with decompensated liver cirrhosis.

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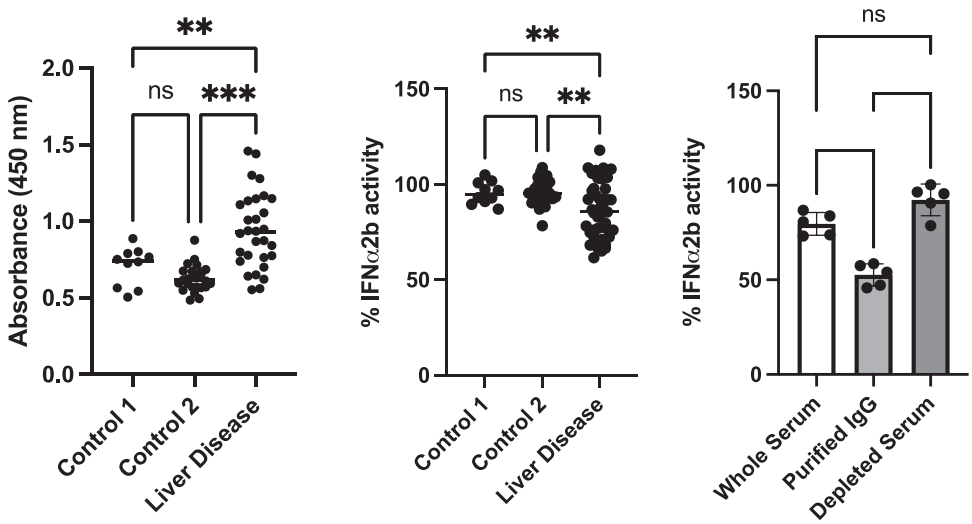
Graphical Abstract



Keywords: decompensated liver cirrhosis, type I interferon, auto-antibodies, SARS-CoV-2, COVID-19

Abbreviations: AAb: auto-antibodies; AIH: autoimmune hepatitis; BMI: body mass index; COVID-19: coronavirus disease-2019; HCC: hepatocellular carcinoma; HE: hepatic encephalopathy; IFN: interferon; MELD-Na: model for end-stage liver disease Na; NASH: Non-alcoholic steatohepatitis; PBC: primary biliary cholangitis; PSC: primary sclerosing cholangitis; SARS-CoV-2: severe acute respiratory coronavirus 2; SHBG: sex hormone binding globulin.

Introduction

Pre-existing chronic liver disease is an important risk factor for coronavirus disease-2019 (COVID-19)-associated mortality upon infection with severe acute respiratory coronavirus 2 (SARS-CoV-2) [1–3]. Early retrospective studies of patients with liver cirrhosis and SARS-CoV-2 infection identified high rates of mortality [4, 5]. The patients most at risk were those with decompensated liver cirrhosis, which refers to the occurrence of an acute deterioration of liver function. Liver transplantation is the sole curative option for patients with decompensated liver cirrhosis and patients are prioritized for transplantation based on two clinical scoring systems, the Childs-Pugh score and the model for end-stage liver disease (MELD)-Na score. Data from international registries of COVID-19 in chronic liver disease patients identified increased mortality in patients with Child-Pugh class B and C [6, 7]. Deaths occurred in 8% of SARS-CoV-2 infected non-cirrhotic patients but 32% of infected cirrhotic patients, and this increased to 51% in Child-Pugh class C patients [7].

The biological mechanisms underlying the described risk factors for severe COVID-19 are complex and remain only partially resolved. In patients with chronic liver disease, a number of mechanisms have been postulated as possible causes of the increased risk for severe COVID-19 [2]. These include hypercoagulation driven by systemic inflammation, alterations in the gut-liver axis, acute hepatic decompensation due to infection of hepatocytes, altered innate immunity enhancing viral replication, and reduced adaptive immune responses due to cirrhosis-associated immune dysfunction.

One described biological mechanism in elderly individuals is the presence of auto-antibodies (AAb) capable of neutralizing type I interferons (IFN) [8, 9]. The presence of IFN AAb increases with age in the general population—in individuals over the age of 75 between 2% and 8% possess auto-antibodies capable of neutralizing 100 pg/ml of IFN-α2 in human embryonic kidney293T type I IFN reporter assays [8]. Neutralizing AAb against type I IFNs are enriched in individuals with severe COVID-19, being present in 18% of individuals who die from SARS-CoV-2 infection [8], and are also present in ~5% of life-threatening influenza pneumonia cases in individuals younger than 70 years old [10].

We hypothesized that cirrhosis may influence SARS-CoV-2 infection in two ways: firstly, by altering innate antiviral proteins in the serum and enhancing viral infectivity; and secondly, by dysregulating antiviral signalling pathways. In the present study, we demonstrate that viral infectivity is not altered in the presence of serum from patients with decompensated liver cirrhosis, but these patients do have elevated levels of auto-antibodies capable of neutralizing type I IFN.

Methods and materials

Participant recruitment and processing

Study participants were approached after being referred for transplant assessment at the Irish National Liver Transplant Centre at St Vincent's Hospital from December 2019 to 20. This was early in the SARS-CoV-2 pandemic, prior to the development of SARS-CoV-2 vaccines. Patients eligible for inclusion were those over 18 years who were undergoing liver transplant assessment regardless of disease aetiology. The only exclusion criteria was an inability to provide written informed consent. Consecutive patients were recruited at outpatient clinic appointments or as inpatients during their clinical assessment for consideration for listing on the liver transplant waitlist. Prior to taking part in the study, they were fully informed of the procedures and gave written consent. Blood samples were taken at the time of clinical assessment for clinical laboratory analysis and research. Blood for serum isolation was collected in serum blood collection tubes with no anticoagulant or preservative. Blood was allowed to clot and then centrifuged at 1000 rcf for 10 minutes. Serum was collected and centrifuged a second time before being stored in aliquots at −80°C. Control serum from two independent cohorts were utilized. Control cohort 1 included serum from 10 healthy donors (StemCell Technologies), which had a similar BMI and gender breakdown but were younger than the full

Table 1. Clinical demographics of patient and control cohorts

	Full patient cohort*	Child-Pugh B	Child-Pugh C	Control cohort 1	Control cohort 2
n	53	27	23	10	25
Mean age (SD)	55 (11)	54 (12)	55 (10)	42 (6)	60 (5)
Sex	24	11	12	5	11
Female	29	16	11	5	14
Male					
Median MELD-Na score (range)	17 (6-43)	14 (6-27)	20 (9-43)	N/A	N/A
Aetiology	4	2	2	N/A	N/A
AIH	19	9	9		
ALD	7	1	6		
NASH	8	4	4		
PBC	11	8	2		
PSC	4	3	0		
Other					
HCC diagnosis	10	7	2	N/A	N/A
HE stage	11	5	5	N/A	N/A
0	23	14	8		
1	11	5	6		
2	6	2	4		
3	1	1	0		
4					
Ascites	11	8	1	N/A	N/A
None	22	9	12		
Mild/moderate	14	7	7		
Severe	6	3	3		
Complicated/refractory					
Portal hypertension	8	7	0	N/A	N/A
None	18	9	8		
Ascites	15	5	9		
Varices	12	6	6		
UGI bleed					
Mean BMI (SD)	28.0 (6.4)	26.0 (5.0)	30.9 (7.2)	30.3 (6.2)	ND

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; BMI, body mass index; HCC, hepatocellular carcinoma; HE, hepatic encephalopathy; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis. N/A, not applicable; ND, not done. * Two individuals were classified as Childs-Pugh A and one individual was not assigned a Childs-Pugh class.

patient cohort (average age of 55 years in the patient cohort and 42 years in the control cohort; Table 1). Control cohort 2 included serum from 25 healthy donors, which had a similar gender breakdown and age compared to the full patient cohort (average age of 60 years; Table 1).

SARS-CoV-2 serological screening

Serum from all participants were screened for the presence of antibodies against SARS-CoV-2 S and N proteins. Serum IgG and IgM responses against the spike protein of SARS-CoV-2 were determined as previously described [11]. Serum IgG responses against the nucleocapsid protein of SARS-CoV-2 were determined as previously described [12]. Absorption at 450 nm was measured using a CLARIOstar® Plus plate reader. Sero-reactivity was subsequently confirmed in a SARS-CoV-2 pseudoparticle assay, as detailed below.

Pseudoparticle assays

The effect of serum on SARS-CoV-2 entry was assessed using a lentivirus-based pseudotype system, using the reference sequence of Wuhan-Hu-1 spike, as described elsewhere [13]. An expression vector was constructed in pcDNA3.1(–) to produce spike lacking the C-terminal 18 amino acids of the cytoplasmic tail [14], based on spike plasmids kindly provided by Prof. Nigel Temperton (Medway School of Pharmacy). To produce pseudoparticles, the spike plasmid was co-transfected into near-confluent wild-type human embryonic kidney-293T cells along with packaging (p8.91) and firefly luciferase reporter-expressing lentivirus genome (pCSFLW) plasmids at a ratio of 1:5:5. Plasmid were mixed with lipofectamine 2000 reagent, as per the manufacturer's instructions, and incubated with cells overnight in OptiMEM before switching to standard growth media (high glucose DMEM with 10% FCS and 1% pen/strep). At 2 days post-transfection, conditioned medium containing pseudoparticles was harvested, clarified by centrifugation and filtered using a 0.45-μm filter. Pseudoparticles (25 μl) were incubated with serum (50 μl at a final dilution of 1:3 dilution) at 37°C for 1 hour before addition of target cells (50 000 per well of a 96-well plate, in an equal volume) to assess infectivity. Target cells used were lentiviral transduced human embryonic kidney-293T cells expressing an EGFP fusion protein linked to human ACE2 (plasmids kindly provided by Dr Jacob Yount, Ohio State

University) and human TMPRSS2 [15]. Firefly luciferase activity was measured using luciferase assay system detection kit (Promega), as per manufacturer's instructions, and read using a Mithras LB940 (Berthold) plate reader 2 days post-transduction. Infectivity was quantified as a percentage using an infected well treated with DMEM alone as 100% infection. Data presented represent two independent experimental repeats.

Live virus assay

For all SARS-CoV-2 work using infectious viruses, experiments were carried out in a dedicated BSL3 facility at Queen's University Belfast. The virus strain used was "BT20.1", which is pre-variant of concern isolate carrying the D614G mutation in spike and retaining an intact polybasic cleavage site [16]. Modified human airway epithelial A549 cells expressing human ACE2 and TMPRSS2 (kindly provided by Dr Suzannah Rihn, MRC-University of Glasgow Centre for Virus Research) were used in well clearance infection assays exploiting the cytopathic nature of this isolate [17]. This assay measures cell lysis, so a higher clearance of the modified A549 monolayer indicates higher viral replication. Cell monolayers (~90% confluent) were infected with virus at an MOI of 0.1 plaque-forming units per cell, and 2 hours post-infection patient serum was added to the cell monolayer at a final dilution of 1:3. At 2 days post-infection cells were fixed before staining with crystal violet. Stained wells were visualized using a CELIGO imaging cytometer (Nexelom) and images were analysed in Fiji [18], using white pixel intensity quantification. Clearance was quantified as a percentage using an uninfected control sample as the baseline for no clearance (0%) and an infected well treated with DMEM alone as the maximum clearance (100%). Data presented represent two independent experimental repeats.

Mass spectrometry analysis

Serum samples were immunodepleted of albumin and IgG using Proteome Purify 2 Human Serum Protein Immunodepletion Resin (R&D). The removal of these high-abundance serum proteins enhanced the proteomic detection of less abundant proteins present in the sample. After reduction with dithiothreitol and iodoacetic acid-mediated alkylation, digestion was performed using trypsin overnight at 37°C. Digested immunodepleted samples were loaded onto a Q-Exactive high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system (ThermoFisher Scientific, Hemel Hempstead, UK). Sample loading was conducted by an auto-sampler onto a C18 trap column (C18 PepMap, 300 µm id × 5 mm, 5 µm particle size, 100 Å pore size; ThermoFisher Scientific). The trap column was switched on-line with an analytical Biobasic C18 Picofrit column (C18 PepMap, 75 µm i.d. × 50 cm, 2 µm particle size, 100 Å pore size: Dionex). Data were acquired with Xcalibur software (Thermo Fisher Scientific). Data analysis, processing, and visualization were performed using MaxQuant v1.5.2.8 (http://www.maxquant.org) and Perseus v.2.0.7.0 (www.maxquant.org/) software. The Andromeda search engine was used to explore the detected features against the UniProtKB/SwissProt database for *Homo sapiens*. The false discovery rate was set to 1% for both peptides and proteins using a target-decoy approach. The intensities were log2 transformed, with proteins filtered based on detection in > 85% of samples and data imputation was performed to replace missing values.

Type I IFN reporter assays

Highly sensitive commercially available human monocytic THP-1 dual reporter cells (InvivoGen), which express a secreted luciferase (Lucia) under the control of five IFN-stimulated response elements, were cultured in RPMI 1640 (Gibco, ThermoFisher), 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated foetal bovine serum, 100 µg/ml normocin, and penicillin-streptomycin at 37°C in 5% CO_2. Selection pressure was maintained with 10 µg/ml of blasticidin and 100 µg/ml of zeocin added every other passage. Patient serum, at a final dilution of 1 in 40, was combined with either IFN-α2b (InvivoGen; 500 pg/ml), IFN-α8 (InvivoGen; 50 pg/ml), or IFN-β1a (InvivoGen; 100 pg/ml), and incubated with THP-1 dual reporter cells for 18 hours at 37°C. These type I IFNs were selected as they show either intermediate (IFN-α2b) or high (IFN-α8) antiviral activity against SARS-CoV-2 and IFN-β1a is expressed by a wide range of cell types, compared to the restricted expression of IFN-α [19]. The concentrations of the type I IFNs were selected to be approximately half the [IC_{50}] in the THP-1 dual reporter cell system. Luminescence intensity was measured with a CLARIOstar® Plus microplate reader (BMG Labtech) and luciferase activity values were normalized against IFN-only positive and media-only negative controls and expressed as a percentage. Data presented represent two independent experimental repeats.

IgG depletion

IgG was purified from serum using the NAb™ Protein G Spin kit (ThermoFisher Scientific), as per manufacturer's instructions. The IgG-depleted flow-through was collected along with the purified IgG. The concentration of purified IgG was quantified using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific). Purified IgG and IgG-depleted serum were assessed for IFN-α2b inhibition as described above. A final concentration of 200 µg/ml/well was selected for purified IgG, representative of the concentration of IgG in diluted serum. The IgG-depleted serum was analysed at a 1 in 40 dilutions alongside the original serum sample.

IFN inhibitory IgG enzyme-linked immunosorbent assay (ELISA)

To assess the binding capacity of serum purified IgG to IFN-α2b, Nunc 96-well Maxisorp plates (ThermoFisher Scientific) were coated with IFN-α2b overnight at 4°C. Plates were blocked and incubated for 1 hour at room temperature with 20 µg of serum-purified IgG or a 1:40 dilution of healthy plasma. Plates were washed with 0.05% PBS Tween20 then incubated for 1 hour with 500 ng/ml horse radish peroxidase-conjugated goat anti-human IgG (ThermoFisher Scientific). TMB (BioLegend) was added to each well and once colour developed the reaction was stopped by the addition of 1 M sulphuric acid. Absorbance at 450 nm was read on CLARIOstar® Plus microplate reader (BMG Labtech).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 9.3.0), using either parametric or non-parametric analysis as indicated, depending on the data distributions and

as detailed in figure legends. Statistical analysis of mass spectrometry data was done using Perseus, utilizing *t*-test to compare between two groups and a significance cut-off of $q < 0.1$.

Results

Identification of SARS-CoV-2 naïve individuals undergoing liver transplant assessment

A cohort of 56 individuals undergoing transplant assessment at the Irish National Liver Transplant Centre at St Vincent's Hospital from December 2019 to 20 were assessed for serological responses against SARS-CoV-2 spike (S) and nucleocapsid (N) antigens (Supplementary Fig. S1). S-antigen IgG or IgM reactivity was evident in three individuals, suggesting previous asymptomatic/unreported infection with SARS-CoV-2 (Supplementary Fig. S1A and B). Two of these individuals also demonstrated N-antigen IgG reactivity (Supplementary Fig. S1C), and all three demonstrated viral neutralization using SARS-CoV-2 pseudoparticle assays (Supplementary Fig. S1D). Upon exclusion of the three seropositive individuals, a total of 53 SARS-CoV-2 unexposed and unvaccinated individuals with varying degrees of chronic liver disease were included in the study as detailed in Table 1.

These 53 individuals presented for liver transplant assessment with a range of underlying aetiologies, including alcoholic liver disease ($n = 19$), non-alcoholic steatohepatitis (NASH; $n = 7$), autoimmune hepatitis (AIH; $n = 4$), primary biliary cholangitis ($n = 8$), and primary sclerosing cholangitis ($n = 11$). The total cohort included 45% females and had a mean age of 55 years, in line with national Irish trends for liver transplantation [20]. The cohort included 27 individuals classified as Child-Pugh B and 23 individuals classed as Child-Pugh C (Table 1). Two individuals in the cohort were Child-Pugh A and one individual refused assessment for Child-Pugh classification. The distribution of aetiology was largely similar between patients classified as Child-Pugh B or Child-Pugh C, with slightly fewer NASH patients and slightly more primary sclerosing cholangitis patients in the Child-Pugh B group. An HCC diagnosis was present in 26% of Child-Pugh B patients but only 9% of Child-Pugh C patients. Child-Pugh C patients were more likely to have complications associated with hepatic encephalopathy, ascites and portal hypertension (Table 1).

Serum from patients with decompensated liver disease does not alter viral replication or infectivity

Serum from patients within the cohort were screened using a SARS-CoV-2 pseudoparticle assay (Fig. 1A). The addition of serum from different individuals resulted in wide variation in pseudoparticle infectivity, with serum from some individuals inhibiting infection while others enhanced infection. Pre-incubation of diluted serum from patients classified as Child-Pugh B with SARS-CoV-2 pseudoparticles did not result in significantly different pseudoparticle infectivity compared to patients classified as Child-Pugh C (Spearman $r = 0.1166$, $P = 0.4056$; Fig. 1B). There was no significant correlation between pseudoparticle infectivity and MELD-Na scores across the cohort (Fig. 1C). No large differences in pseudoparticle infectivity were evident between patients with different disease aetiologies, although the overall size of the cohort limits the ability to distinguish more subtle differences between disease aetiologies (Supplementary Fig. 2A). Due to the size of the cohort additional clinical parameters that may influence viral infectivity or replication, such as ascites, HCC, portal hypertension and BMI, could not be incorporated into the analysis.

To confirm the findings from the pseudoparticle assay, a live virus SARS-CoV-2 (strain BT20.1) assay was established using modified A549 cells expressing ACE2 and TMPRSS2 (Fig. 1D). Similar to the pseudoparticle assay, addition of diluted serum from patients classified as Child-Pugh B did not result in significant differences in clearance of modified A549 cells compared to serum from patients classified as Child-Pugh C (Fig. 1E). There was also no significant correlation between clearance of modified A549 cells and MELD-Na scores across the cohort (Spearman $r = 0.1364$, $P = 0.3302$; Fig. 1E). Again no large differences in clearance of modified A549 cells were evident between patients with different disease aetiologies (Supplementary Fig. 2B).

Neither age nor sex affected pseudoparticle infectivity (Fig. 2A and B). Likewise, neither age nor sex affected SARS-CoV-2-induced clearance of modified A549 cells (Fig. 2C and D). Despite the lack of association between viral infectivity and clinical parameters, it was evident that some individual serum samples were capable of influencing either pseudoparticle infectivity or clearance of modified A549 cells. When directly comparing the two viral assays utilized, we observed no correlation between infectivity in the SARS-CoV-2 pseudoparticle assay and clearance of modified A549 cells upon SARS-CoV-2 infection (Spearman $r = -0.001048$, $P = 0.9941$; Fig. 2E), indicating the two assays are influenced by distinct intrinsic antiviral serum proteins.

To explore factors contributing to the consistent individual variation, mass spectrometry was performed comparing samples that inhibited and enhanced either pseudoparticle infectivity or A549 clearance (Supplementary Fig. 3). No significant differences in the serum proteome were observed between samples that inhibited or enhanced pseudoparticle infectivity (data not shown). Two proteins were significantly different between samples that inhibited or enhanced viral clearance of modified A549 cells—alpha-2-macroglobulin (A2M) and sex hormone binding globulin (SHBG) (Supplementary Fig. 3A). Linear regression analysis demonstrated that elevated A2M and SHBG levels were associated with reduced viral replication, as assessed via clearance of modified A549 cells (Supplementary Fig. 3B and C). Neither of these proteins showed a relationship with SARS-CoV-2 pseudoparticle infectivity (Supplementary Fig. 3D and E).

An increased MELD-Na score is associated with inhibition of type I IFN signalling

The results from the SARS-CoV-2 pseudoparticle and live virus experiments suggested that there was no difference in intrinsic serum antiviral proteins between patients classified as either Child-Pugh class B or C. In addition to intrinsic serum antiviral proteins, type I IFNs expressed by epithelial cells and innate immune cells play an important role in suppressing viral infection. To assess whether serum from decompensated patients influenced type I IFN signalling, recombinant IFNs were pre-incubated with serum and signalling activity was assessed using a cell-based reporter (Fig. 3). Pre-incubation of recombinant IFN-α2b (500 pg/ml) with serum from patients classified as either Child-Pugh class B or C did not result in

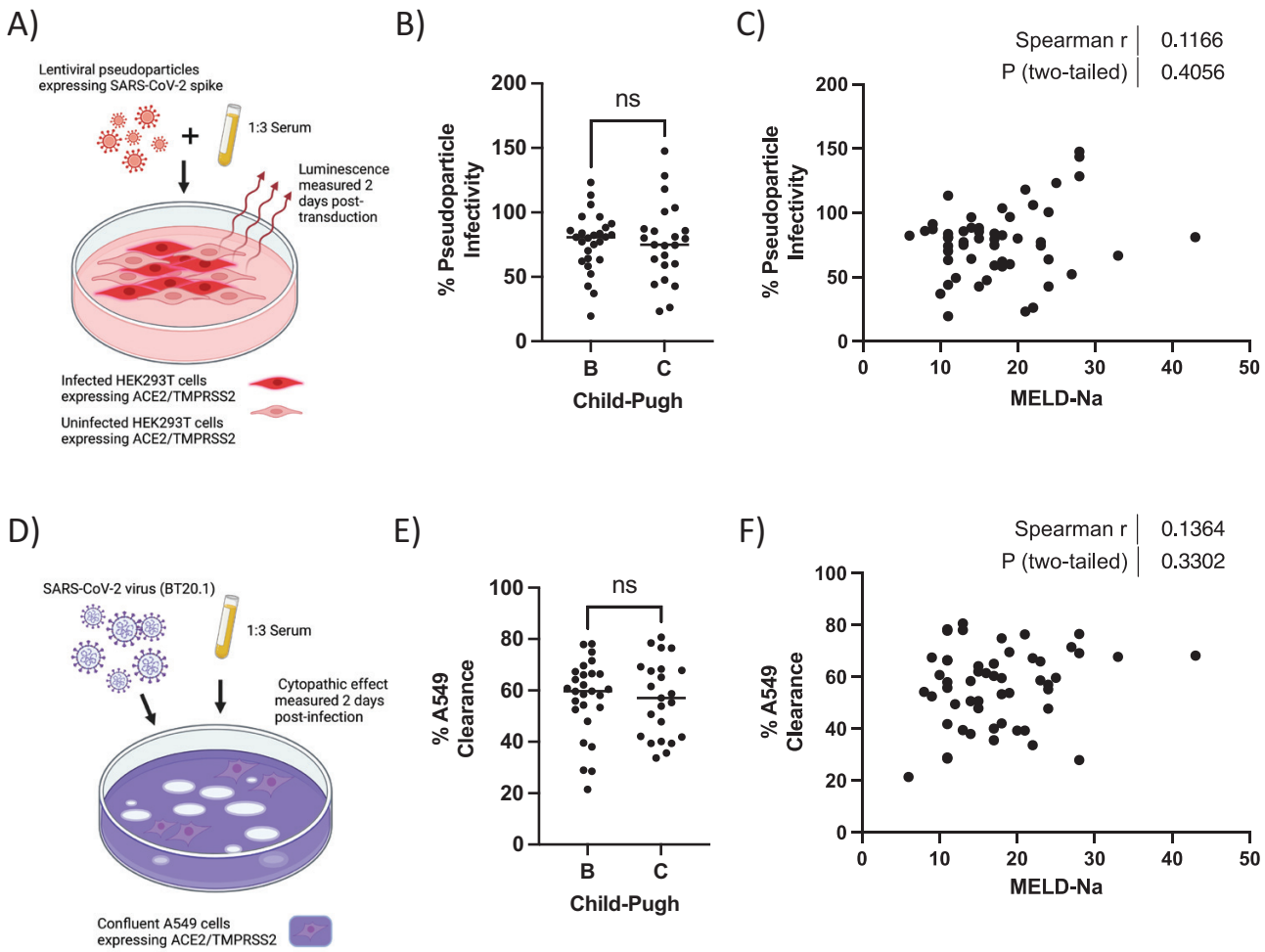


Figure 1. Serum from patients with varying severity of decompensated liver disease does not alter viral infectivity *in vitro*. (A) Schematic representation of the viral pseudoparticle (PP) assay. (B) Infectivity of SARS-CoV-2 PPs in the presence of serum from patients grouped by Child-Pugh class and analysed using the Mann–Whitney test. (C) Spearman correlation analysis of MELD-Na score and SARS-CoV-2 PP infectivity. (D) Schematic representation of the live virus assay using modified A549 lung epithelial cells. (E) Infectivity of the SARS-CoV-2 BT20.1 strain in the presence of serum from patients grouped by Child-Pugh class and analysed using the Mann–Whitney test. (F) Spearman correlation analysis of MELD-Na score and infectivity of the SARS-CoV-2 BT20.1 strain. Data for the SARS-CoV-2 PP infectivity assay and the A549 clearance assay represent the average from two independent experimental repeats and are expressed as a % of infectivity/clearance observed in the presence of DMEM only. Horizontal lines indicate the group median. ns, not significant. Created with BioRender.com.

a significant difference between groups (Fig. 3A). In contrast, recombinant IFN-α2b signalling showed a highly significant relationship with MELD-Na score (Fig. 3B). Increasing MELD-Na score was associated with a decrease in IFN signalling, indicating that the patient serum was capable of blocking the activity of recombinant IFN-α2b. There was no relationship observed between signalling induced by recombinant IFN-α8 (50 pg/ml) and either Child-Pugh class (Fig. 3C) or MELD-Na score (Fig. 3D). Likewise, there was no relationship observed between signalling induced by recombinant IFN-β1a (100 pg/ml) and either Child-Pugh class (Fig. 3E) or MELD-Na score (Fig. 3F).

The inhibition of recombinant IFN-α2b signalling observed in patients with decompensated liver cirrhosis was significantly greater than that observed in healthy control cohorts (control cohort 1 vs. liver disease patients, adjusted $P = 0.008$; control cohort 2 versus liver disease patients, adjusted $P = 0.001$; Fig. 4A). Amongst the patients with decompensated liver cirrhosis neither patient sex (Fig. 4B) or chronological age (Fig. 4C) were associated with the inhibition of recombinant IFN-α2b signalling. There was no association with a particular disease aetiology (Supplementary Fig. 4A), although this observation is limited by the small cohort size. There was also no correlation between IFNα2b activity and viral infectivity in either the pseudoparticle assay (Supplementary Fig. 4B) or the live virus assay (Supplementary Fig. 4C), likely due to the lack of cell types capable of producing IFNα in these model systems.

Five serum samples that demonstrated inhibition of IFNα2b activity were selected for IgG purification, generating matched IgG-depleted serum and purified IgG. In comparison to matched whole serum, the purified IgG showed significantly stronger inhibition of IFNα2b activity (mean IFNα2b activity with whole serum is 79.6%rr vs. 52.6% with purified IgG; $P = 0.0001$; Fig. 4D). Removal of the IgG fraction restored IFNα2b activity, with the IgG-depleted serum showing a mean IFNα2b activity of 92.3% (Fig. 4D). The neutralizing effect of AAbs against type I IFNs was dependent on the dilution of serum, with the neutralizing effect lost at 1 in 360 dilution (Fig. 4E).

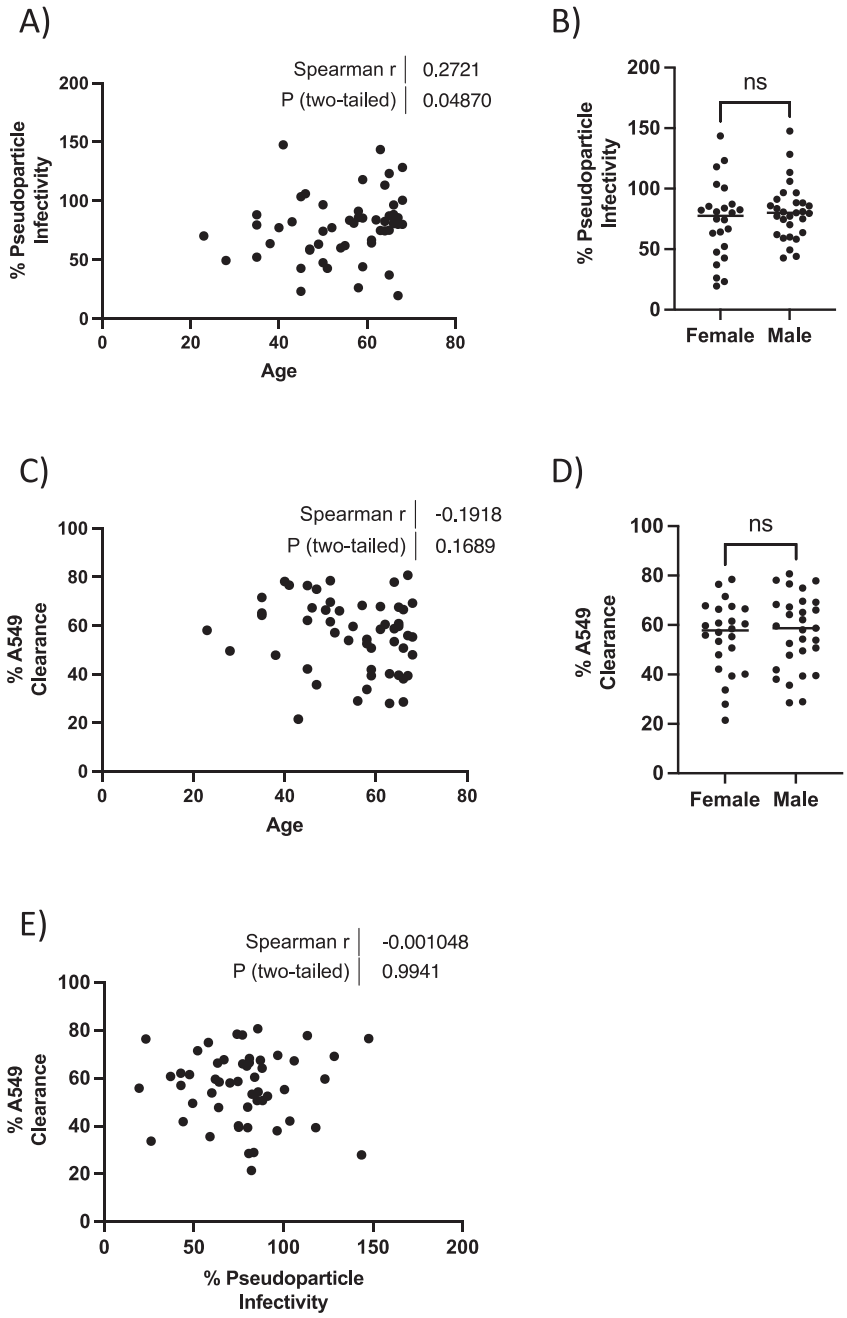


Figure 2. Viral infectivity *in vitro* in the presence of patient serum is not associated with age or gender. (A) Spearman correlation analysis of chronological age and infectivity of SARS-CoV-2 PP in the presence of serum from patients with decompensated CLD. (B) Infectivity of SARS-CoV-2 PP in the presence of serum from patients grouped by sex and analysed using the Mann–Whitney test. (C) Spearman correlation analysis of chronological age and infectivity of the SARS-CoV-2 BT20.1 strain (measured as clearance of modified A549 cells) in the presence of serum from patients with decompensated CLD. (D) Infectivity of the SARS-CoV-2 BT20.1 strain in the presence of serum from patients grouped by sex and analysed using the Mann–Whitney test. (E) Spearman correlation analysis of infectivity of SARS-CoV-2 PP versus infectivity of the SARS-CoV-2 BT20.1 strain in the presence of serum from patients with decompensated CLD. Data for the SARS-CoV-2 PP infectivity assay and the A549 clearance assay represent the average from two independent experimental repeats and are expressed as a % of infectivity/clearance observed in the presence of DMEM only. Horizontal lines indicate the group median. ns, not significant.

Patients with more severe decompensated liver disease possess auto-antibodies capable of inhibiting type I IFN signalling

As neutralizing AAb against type I IFNs have been implicated in severe COVID-19 [8], we explored whether the inhibition of IFNα2b activity was mediated by auto-antibodies capable of directly binding IFNα2b. IgG reactivity against recombinant IFNα2b was evident in a large number of serum samples from decompensated patients (Fig. 5A). This result highlights that the direct binding IgG is able to inhibit IFNα2b signalling in patients, as opposed to auto-antibodies targeting the type I IFN receptor. The level of IgG reactivity observed in patients with decompensated liver cirrhosis was significantly greater than that observed in healthy control cohorts (control cohort

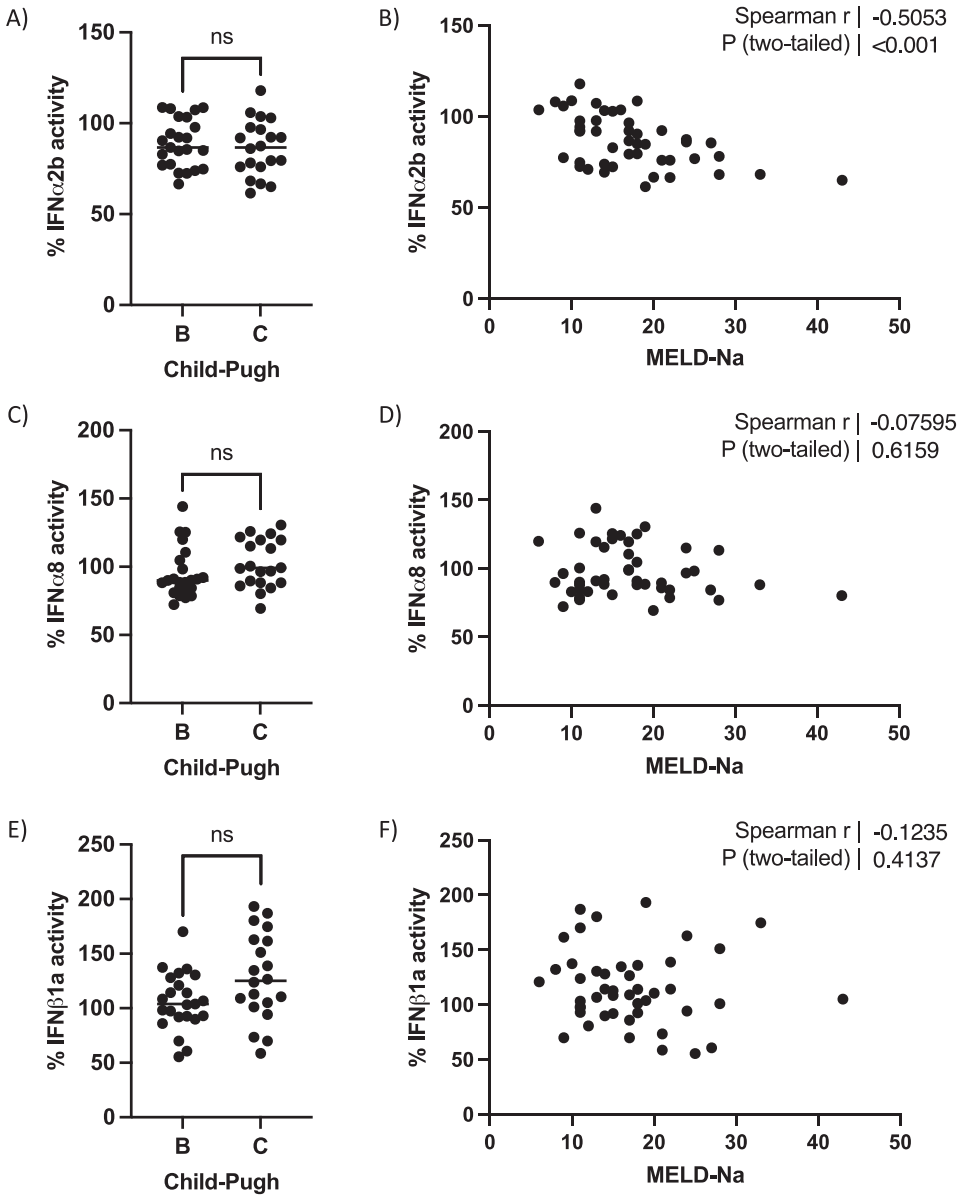


Figure 3. Inhibition of IFNα signalling is correlated with MELD-Na score but not Child-Pugh class. (A) Type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum grouped by Child-Pugh class, analysed using the Mann–Whitney test. (B) Spearman correlation analysis of type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum with MELD-Na scores. (C) Type I IFN activity following pre-incubation of IFN-α8 (50 pg/ml) in the presence of a 1:40 dilution of serum grouped by Child-Pugh class, analysed using the Mann–Whitney test. (D) Spearman correlation analysis of type I IFN activity following pre-incubation of IFN-α8 (50 pg/ml) in the presence of a 1:40 dilution of serum with MELD-Na scores. (E) Type I IFN activity following pre-incubation of IFN-β1a (100 pg/ml) in the presence of a 1:40 dilution of serum grouped by Child-Pugh class, analysed using the Mann–Whitney test. (F) Spearman correlation analysis of type I IFN activity following pre-incubation of IFN-β1a (100 pg/ml) in the presence of a 1:40 dilution of serum with MELD-Na scores. Horizontal lines indicate median. ns = not significant.

1 vs. liver disease patients, adjusted $P = 0.002$; control cohort 2 vs. liver disease patients, adjusted $P \leq 0.001$; Fig. 5A).

Amongst the patients with decompensated liver cirrhosis the level of IFNα2b binding activity did not differ between Child-Pugh class (Fig. 5B), with chronological age (Fig. 5C) or between sexes (Fig. 5D). This IFNα2b binding activity was present in both samples that inhibited IFNα2b signalling ('inhibitory', reduced IFNα2b activity to < 80% of the positive control) and samples that did not ('non-inhibitory,' IFNα2b activity to > 85% of the positive control) (Fig. 5E). Furthermore, there was no significant correlation between the level of binding of IFNα2b and the reduction in IFNα2b signalling activity (Fig. 5F). This lack of significant difference in the level of IFNα2b binding between the 'inhibitory' versus 'non-inhibitory' samples, in combination with the lack of correlation between IFNα2b activity and ELISA binding, demonstrates that the type I IFN AAbs induced in patients with decompensated liver disease includes both neutralizing and non-neutralizing B cell clones.

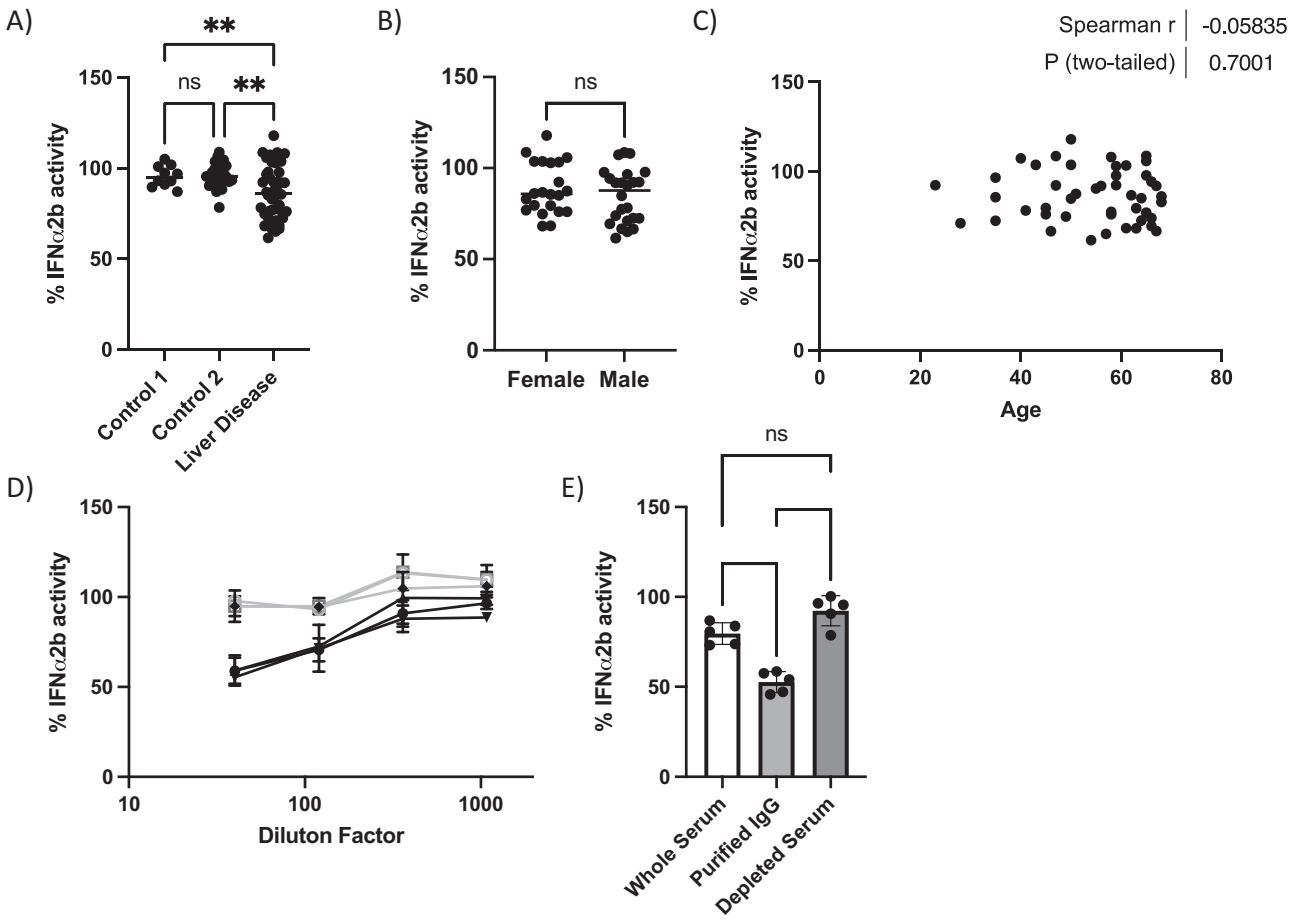


Figure 4. Inhibition of IFNα signalling is due to IgG auto-antibodies present in patients with decompensation liver disease but is not associated with sex or age. (A) Type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum comparing two cohorts of healthy controls (Table 1) and the liver disease patient cohort, analysed using a Welch ANOVA test. (B) Type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum from liver disease patients grouped by sex and analysed using the Mann–Whitney test. (C) Spearman correlation analysis of type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum from liver disease patients with age. (D) Type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) with varying dilutions of serum. Serum samples with evidence of inhibition of Type I IFN activity are black ($n = 3$) and unmatched health controls are grey ($n = 3$). (E) Comparison of Type I IFN activity following pre-incubation of IFNα2b (500 pg/ml) in paired samples of whole serum, purified IgG and IgG-depleted serum, analysed using the repeated measures one-way ANOVA test. For (A) and (B) the horizontal lines indicate the group median. For (D) and (E) the error bars denoted the standard deviation. **, P-value < 0.01; ***, P-value < 0.001; ns = not significant.

Discussion

Pre-existing chronic liver disease is an important risk factor for severe COVID-19, especially in individuals with decompensated cirrhosis where the mortality rate in certain sub-groups is above 50% [7]. While vaccination is an important public health measure to reduce severe COVID-19, cirrhotic patients have poor responses to a variety of vaccines [21], and demonstrate reduced T-cell responses and rapid loss of antibody responses to SARS-CoV-2 vaccines [22, 23]. Understanding the immunological basis for susceptibility in this patient population is essential. In this study, we examined intrinsic and innate antiviral responses to SARS-CoV-2 infection in unexposed decompensated cirrhotic patients, to understand alterations in viral immune responses in the absence of adaptive immune responses, induced by either natural exposure or vaccination. Three individuals with strongly neutralizing Ab responses against SARS-CoV-2 were identified and excluded from further analysis. Our results demonstrate that viral infectivity is not altered in the presence of serum from patients with decompensated liver cirrhosis, indicating that there is no defect in intrinsic antiviral protein expression within the serum. In contrast, these patients display defects in key antiviral signalling pathways caused by neutralizing AAb against type I interferon capable of inhibiting antiviral IFN signalling.

Neutralizing AAb to type I IFNs have been identified as an important cause of severe COVID-19 in elderly individuals [8, 9]. We report that serum from patients with higher MELD-Na significantly reduced the signalling activity of 500 pg/ml IFNα2b. Removal of IgG from these serum samples restored IFNα2b activity, confirming the presence of AAb with IFN-neutralizing activity. Direct comparison of the level of neutralization observed in our study with previous reports [8, 9, 24–27], is hampered by the lack of standardized methodologies for detecting and quantifying neutralizing type I IFN AAb. A limitation of our study is the relatively small patient cohort size, which excludes the possibility of investigating the effect of aetiology or other clinically relevant conditions, such as the presence of HCC. Together these limitations

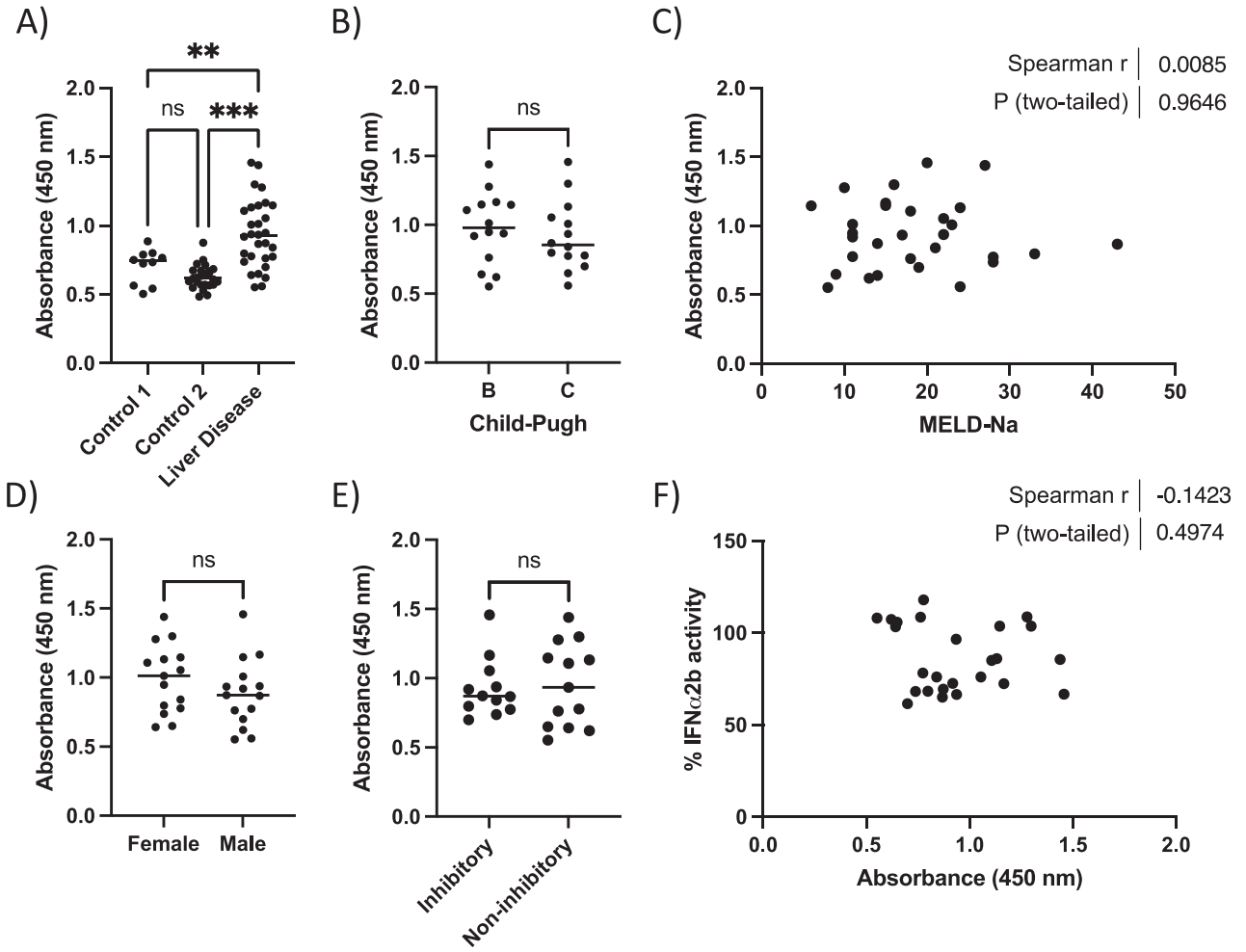


Figure 5. IgG autoantibodies from patient serum directly bind recombinant IFNα2b. (A) Comparison of IFNα2b ELISA absorbances in serum comparing two cohorts of healthy controls (Table 1) and the liver disease patient cohort, analysed using a Welch ANOVA test. (B) Comparison of IFNα2b ELISA absorbances in serum from patients with liver disease grouped by Child-Pugh class, analysed using the Mann–Whitney test. (C) Spearman correlation analysis of the IFNα2b ELISA absorbance readings in serum from liver disease patients and age. (D) Comparison of IFNα2b ELISA absorbances in serum from patients with liver disease grouped by sex and analysed using the Mann–Whitney test. (E) Comparison of IFNα2b ELISA absorbances in serum samples with evidence of inhibition of Type I IFN activity ('inhibitory') versus serum samples lacking inhibition of Type I IFN activity ('non-inhibitory'). Data were analysed using the Kruskal–Wallis test. (F) Spearman correlation analysis of the type I IFN activity in samples following pre-incubation of IFNα2b (500 pg/ml) in the presence of a 1:40 dilution of serum and IFNα2b ELISA absorbance readings. **, *P*-value < 0.01; ***, *P*-value < 0.001; ns = not significant.

highlight the need to explore the relative frequency of type I IFN AAb systematically in a wider range of chronic liver disease patients and age-matched health controls. Our study did not include longitudinal follow-up of the patient cohort, and as such, we lacked data on subsequent infections with SARS-CoV-2. This excluded the possibility of stratifying infection outcomes based on the antiviral activities evident in sera and is an important limitation of our results.

While IFNα2b neutralization was more frequent in decompensated patients with high MELD-Na scores, there was statistical correlation observed for either IFNα8 or IFNβ1a activity. A non-significant trend was observed when using IFNα8, with the serum samples from patients with the highest MELD-Na scores showing a slight reduction in signalling (Fig. 3D). This may relate to the fact that IFNα8 is a potent inducer of IFN signalling and IFN-stimulated genes (ISGs), and as such the quantity used in our *in vitro* assays may have been too high to efficiently neutralize. IFNβ neutralizing AAb have been rare or completely lacking in critical COVID-19 patients possessing other neutralizing type I IFN AAb [8, 28, 29]. IFNβ is a potent inducer of ISGs and the lack of neutralizing AAb in this cohort could make it a potential treatment for COVID-19 in patients with decompensated liver disease. IFNβ has been used clinically in COVID-19 with positive results [29, 30]. The reason for the relative lack of AAb is unknown but may stem from IFNβ being a more potent inducer of ISGs (therefore it is expressed at lower concentrations).

Type I IFNs play an important role in the initial response of the host to a wide variety of viral infections, including respiratory tract infections [31, 32]. In SARS-CoV-2 infection, rapid induction of IFN responses early in infection is required to limit SARS-CoV-2 replication [33–35]. Studies of the various IFNα subtypes have identified significant differences in the antiviral effects against SARS-CoV-2 [19]. This differential antiviral effect of different IFNα subtypes is thought to be due to differences in the induction of subsets of ISGs in responding cells [19, 36]. Subtypes with high antiviral activity tend to induce robust expression of antiviral mediators such

as *MX2*, *IFIT1*, *OAS2*, and *RSAD2*. In contrast, subtypes with low antiviral activity preferentially induce gene signatures associated with cellular proliferation. The neutralization of specific IFNα subtypes likely alters the dynamics between IFNα subtypes with high and low antiviral activity.

IFN neutralizing AAb have been reported in a number of conditions with elevated IFN expression including autoimmune polyendocrinopathy syndrome [37], and system lupus erythematosus [38, 39], as well as conditions such as myeloproliferative neoplasms where IFN therapeutics are used [40]. Repeated activation of type 1 IFNs has been reported in chronic liver diseases [41]. This chronic activation of type I IFNs may explain why neutralizing AAb are present in cirrhotic patients. The presence of neutralizing AAb against type I IFNs in patients with cirrhosis may act to reduce their initial response to infection and contribute to a more severe disease course. They are also of potential relevance for other viral infections. Bacterial infection is a risk factor for mortality in patients with liver cirrhosis [42]. However, data on the frequency of respiratory viral infections in this patient population is sparse. Respiratory viruses have been detected in ~20% of cirrhotic patients, often with bacterial co-infection, and are associated with poor clinical outcomes [43, 44]. The inhibition of IFN signalling may be an unrecognized component of the systemic immune dysregulation in chronic liver disease that could increase susceptibility to respiratory viral infection [45].

While serum from patients with high MELD-Na scores did not alter SARS-CoV-2 cell entry or viral replication in *in vitro* models, a high degree of inter-individual variation was observed in these assays. The lack of a healthy control comparator for these viral assays is a limitation of our study, which means we are unable to determine whether this high degree of inter-individual variation is also a characteristic of serum from healthy individuals. Human serum contains a wide variety of proteins with both pro-viral and antiviral effects. We identified two serum proteins associated with reduced clearance of modified A549 cells upon infection with SARS-CoV-2. A2M is a highly abundant serum protein and has been identified as a possible inhibitor of SARS-CoV-2 entry, with an [IC_{50}] of 54.2 μg/ml [46]. In human plasma, A2M has a concentration of ~1 mg/ml, indicating it may play an important role in inhibiting SARS-CoV-2 entry. SHBG has been associated with mortality during SARS-CoV-2 infection in both men and women [47], although other studies have failed to identify this association with SHBG and clinical outcomes [48].

Our data highlight that an intrinsic defect in antiviral protein expression is not present in the serum of patients with decompensated liver cirrhosis. However, AAb targeting type I IFNs are highly prevalent in these decompensated patients, and high MELD-Na scores are associated with the presence of neutralizing AAb. Our results suggest that the presence of neutralizing AAb targeting type I IFN may explain the increased likelihood of severe COVID-19 in chronic liver disease patients upon SARS-CoV-2 infection and are of relevance to other viral infections in this patient population.

Supplementary Data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

This study received ethics approval from St Vincent's Hospital Group Ethics Board as per the 1975 Declaration of Helsinki guidelines. All participants provided written informed consent.

Conflict of interests

None declared.

Author contributions

G.G., T.K.G., C.G.G.B., and M.R. designed the study. S.C., S.O.N., S.A., G.B., W.M., A.H.D., N.M.B., O.T., D.C., U.F.P., P.D., T.K.G., C.G.G.B., and M.W.R. collected the data. G.G., C.G.G.B., and M.R. analysed the data and drafted the original manuscript. All authors revised and edited the manuscript. All authors approved the final version of the manuscript.

Data availability

The data that support the findings of this study are available on request from the corresponding author. Mass spectrometry data are available at https://doi.org/10.17605/OSF.IO/SEQV5.

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