Humoral response to SARS-CoV-2 infection among liver transplant recipients

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Original research

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ABSTRACT

Objective Immunosuppressive agents are known to interfere with T and/or B lymphocytes, which are required to mount an adequate serologic response. Therefore, we aim to investigate the antibody response to SARS-CoV-2 in liver transplant (LT) recipients after COVID-19.

Design Prospective multicentre case–control study, analysing antibodies against the nucleocapsid protein, spike (S) protein of SARS-CoV-2 and their neutralising activity in LT recipients with confirmed SARS-CoV-2 infection (COVID-19-LT) compared with immunocompetent patients (COVID-19-immunocompetent) and LT recipients without COVID-19 symptoms (non-COVID-19-LT).

Results Overall, 35 LT recipients were included in the COVID-19-LT cohort. 35 and 70 subjects fulfilling the matching criteria were assigned to the COVID-19-immunocompetent and non-COVID-19-LT cohorts, respectively. We showed that LT recipients, despite immunosuppression and less symptoms, mounted a detectable antinucleocapsid antibody titre in 80% of the cases, although significantly lower compared with the COVID-19-immunocompetent cohort (3.73 vs 7.36 index level, p<0.001). When analysing anti-S antibody response, no difference in positivity rate was found between the COVID-19-LT and COVID-19-immunocompetent cohorts (97.1% vs 100%, p=0.314). Functional antibody testing showed neutralising activity in 82.9% of LT recipients (vs 100% in COVID-19-immunocompetent cohort, p=0.024).

Conclusions Our findings suggest that the humoral response of LT recipients is only slightly lower than expected, compared with COVID-19 immunocompetent controls. Testing for anti-S antibodies alone can lead to an overestimation of the neutralising ability in LT recipients. Altogether, routine antibody testing against separate SARS-CoV-2 antigens and functional testing show that the far majority of LT patients are capable of mounting an adequate antibody response with neutralising ability.

Significance of this study

What is already known about this subject?

- Immunosuppressive agents interfere with T and/or B lymphocytes, which are required to mount an adequate humoral response.
- Some reports suggest that humoral response after SARS-CoV-2 infection may be impaired in liver transplant (LT) recipients.

What are the new findings?

- Presence and level of antinucleocapsid antibodies after confirmed SARS-CoV2 infection are significantly lower in LT recipients when compared with immunocompetent controls. However, presence and levels of anti-spike (S) antibodies are similar between these groups.
- The majority of LT recipients is able to produce functional antibodies against SARS-CoV-2 after a confirmed SARS-CoV2 infection. This neutralising ability is associated with the presence of antinucleocapsid antibodies.
- The findings in the non-COVID-19-LT group confirm that both assays show specificity for detection of SARS-CoV-2 antibodies in the LT population.

How might it impact on clinical practice in the foreseeable future?

- Antinucleocapsid antibodies, when tested alone, may be a suboptimal tool to confirm previous contact with SARS-CoV-2 in immunosuppressed (LT) recipients.
- LT patients have a less severe impairment of the immune response to SARS-CoV-2 than previously thought and antinucleocapsid antibodies may indirectly indicate which patients are able to mount functional antibodies to neutralise the virus.
- Caution must be taken when interpreting the results of testing for anti-S antibodies, since those results can overestimate the ability of neutralising the virus on subsequent exposure.

INTRODUCTION

COVID-19 is caused by SARS-CoV-2. It has become a global pandemic since its first identification in Wuhan, Hubei province, China, in December 2019.[1] Due to its sudden spread and novelty, there is still a lack of knowledge, and several issues remain unsolved. In this phase of the pandemic, available data on clinical disease course and optimal management of COVID-19 are progressively increasing. However, knowledge on host immune response,

reinfection rates and duration of antibody response after infection remains incomplete.[2–4]

To gain immunity after an infection, an adequate serologic response is required. Several studies have shown that the majority of patients in the general population can develop antibodies against SARS-CoV-2.[5–7] Nonetheless, insights gained in the general population are difficult to extrapolate to specific categories of patients such as liver transplant (LT) recipients using immunosuppressive therapy. Since immunosuppressive agents interfere with T and/or B lymphocytes, which are required to mount an adequate serologic response, we hypothesised that the serologic response after SARS-CoV-2 infection may be impaired in LT recipients. Indeed, it has been demonstrated for other viruses (eg, H1N1) that the ability to develop an induced immune response in individuals on immunosuppressive therapy is suboptimal.[8] Recently, in a large cohort of patients with chronic inflammatory bowel disease on biological therapy seroconversion was observed in fewer infliximab-treated than vedolizumab-treated patients.[9]

There are currently limited data available concerning seroconversion after SARS-CoV-2 infection in LT recipients or solid organ transplant (SOT) recipients in general. The few studies performed show that the majority of SOT recipients seroconverted after SARS-CoV-2 infection, however major drawbacks of those studies are low sample size, cross-sectional design, use of different antibody tests and antibody testing at different time points.[10–14] Only one prospective case–control study is currently published. The authors showed a lower incidence of IgG antibodies against the nucleocapsid protein of the SARS-CoV-2 in LT recipients compared with immunocompetent controls after COVID-19, with a faster decrease of the IgG titre over 6 months.[15] Nonetheless, whether this finding reflects a complete and protective humoral immune response against SARS-CoV-2 still needs to be demonstrated, since nucleocapsid-protein antibodies represent a marker of previous infection, rather than neutralising ability.

Therefore, we aimed to prospectively investigate the antibody response to SARS-CoV-2 in LT recipients after COVID-19, testing both the antibodies directed against nucleocapsid protein as well as against the spike (S) protein of SARS-CoV-2. We included a negative and a positive control group to assess the specificity of the findings. Additionally, we aimed to analyse the neutralising ability of these antibodies against SARS-CoV-2 and investigate the possible risk factors of an altered humoral response in the LT population.

METHODS

Study design

We conducted a multicentre, prospective case–control study in LT recipients after confirmed SARS-CoV-2 infection. LT recipients were consecutively recruited from 1 October 2020 to 28 February 2021 in five European secondary and tertiary LT centres from Switzerland, The Netherlands, Belgium and Italy. Two control cohorts were included: the first comprising immunocompetent patients with confirmed SARS-CoV-2 infection, without a history of LT, and the second including LT recipients without SARS-CoV-2 infection.

Study population

Consecutive adult LT recipients with confirmed SARS-CoV-2 infection (COVID-19-LT cohort) were included in this study. Confirmed SARS-CoV-2 infection was based on a positive real-time reverse transcription-PCR assay, performed on oropharyngeal and nasopharyngeal swabs at the local treatment centres according to WHO guidelines.[16] Information regarding date of SARS-CoV-2 infection, COVID-19-related symptoms, indication for LT, time since LT, comorbidities and immunosuppressive therapy was collected. Control subjects with confirmed SARS-CoV-2 infection and who did not use immunosuppressive drugs, matched for time interval between SARS-CoV-2 infection to antibody testing, age and gender (ratio 1:1) were used as a first control cohort (COVID-19-immunocompetent control cohort). Information regarding the date of SARS-CoV-2 infection, COVID-19-related symptoms and comorbidities was collected. In addition, LT recipients without any signs or symptoms suggestive of SARS-CoV-2 infection were matched 2:1 for age, gender and time from LT to sampling and were included as a second control cohort (non-COVID-19-immunosuppressed control cohort). To ascertain that there was no prior infection with SARS-CoV-2, these patients were requested to complete a questionnaire (online supplemental appendix A) regarding symptoms history and exposure risk levels to COVID-19 since February 2020 (when the pandemic extended to Europe). Demographic and clinical information was also recorded.

Patient and public involvement statement

Patients or the public were not involved in the design, or conduct, or reporting of our research. Results of the present study will be disseminated by the investigators in collaboration with the (inter)national patient associations.

Serological analysis

Serum samples were collected from all the participants included in the study. In the COVID-19-LT cohort, as well as in the COVID-19-immunocompetent cohort, the samples were drawn between 4 and 8 weeks after the detection of the SARS-CoV-2 infection. In order to maximise the standardisation of the measurement, all serum samples were sent to and centralised at the Clinical Microbiology Laboratory of the LUMC for SARS-CoV-2 antibody analysis, according to standard procedures. Two different assays for detecting SARS-CoV-2-specific antibodies were performed. First, an assay detecting SARS-CoV-2 IgG targeting the nucleocapsid protein was performed using the IgG quantitative assay (Abbott Diagnostics). At a predefined index value threshold of 1.4 for positivity, this assay has a documented sensitivity of 100% and a specificity of 99.6% in immunocompetent subjects.[17] Additionally, an assay detecting SARS-CoV-2 total-Ig antibodies targeting the receptor-binding domain (RBD) of the S protein was performed using the Wantai SARS-CoV-2 total antibody assay (Wantai Diagnostics). At a predefined ratio value threshold of 1.1 for positivity, this assay has a sensitivity of 94.5% and a specificity of 100%.[18]

Neutralising activity assay

In order to test antibody efficacy, serum samples from the COVID-19-LT and COVID-19-immunocompetent groups were analysed for their neutralising ability. Serum samples of patients from the non-COVID-19-LT group with positive antibodies against SARS-CoV-2 were also included in this analysis. Neutralisation assays against live SARS-CoV-2 wild-type virus were performed using the microneutralisation assay, previously described by Algaissi and Hashem.[19] The virus used for this assay was the clinical isolate SARS-CoV-2/human/NLD/Leiden-0008/2020 (GenBank accession number: MT705206.1). Neutralisation titre was calculated by dividing

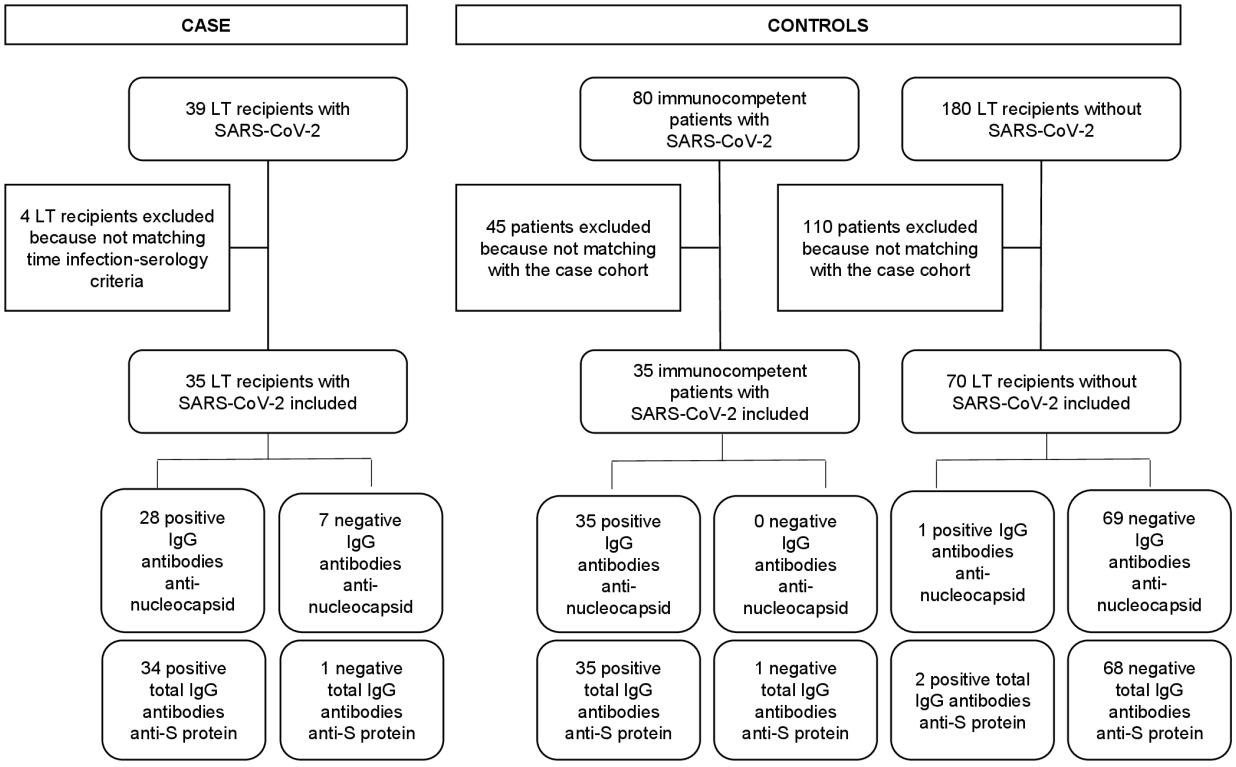

Figure 1 Flowchart of the study. LT, liver transplant; S, spike.

the number of positive wells with complete inhibition of the virus-induced cytopathogenic effect, by the number of replicates, and adding 2.5 to stabilise the calculated ratio. The neutralising antibody titre was defined as the log2 reciprocal of this value. All neutralisation titers above five were considered as positive.

Statistical analysis

Qualitative data are described using frequencies and percentages. Normality of the distribution was preassessed according to Kolmogorov-Smirnov test. Quantitative variables are described using mean (SD) or median (IQR) when appropriate. Comparisons between independent groups were performed using the Mann-Whitney U test and t-test for continuous variables and χ^2 test or Fisher's exact test for categorical variables. Missing data were not imputed. To assess factors associated with positive IgG antibody response against the nucleocapsid protein, a univariate logistic regression analysis was performed. The same analysis was performed for factors associated with presence of neutralising antibodies. Correlation between IgG levels against the nucleocapsid protein and neutralisation titers was calculated using a Spearmen rank correlation test.

All tests are two sided, and a p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS, V.25.0 (IBM SPSS) and R environment.

RESULTS

COVID-19-LT cohort characteristics

Overall, 39 liver recipients with confirmed SARS-CoV-2 infection were recruited. Of those, 35 recipients were included in the analysis fulfilling the required inclusion criteria (figure 1). Infection dates were between the 41st week of 2020 and the 2nd week of 2021, therefore we can assume that the results of our study were related mostly to wild-type lineage. Male gender was most prevalent (25 recipients, 71.4%) and mean age was 56.7±13.9 years. Demographic characteristics are summarised in table 1. The majority of patients were transplanted for viral aetiology (8, 22.9%), whereas 12 (34.3%) patients had a hepatocellular carcinoma at the time of LT. Two (5.7%) patients had undergone a combined liver–kidney transplantation. Regarding immunosuppression (IS), almost half of the patients were on a dual regimen (15, 42.9%), with calcineurin inhibitors (32, 91.4%) as the most frequently represented drug (table 2).

Regarding COVID-19-related symptoms, 21 patients (60.0%) experienced fatigue, followed by cough (18 patients, 51.4%), fever (15 patients, 42.9%) and dyspnoea (11 patients, 31.4%). Only two patients needed hospitalisation longer than 5 days and one patient (2.9%) required intensive care unit (ICU) management and mechanical ventilation. IS was reduced in four patients, particularly when a mycophenolate mofetil (MMF) containing regimen was present.

COVID-19-immunocompetent cohort characteristics

An equal number of matched controls were selected from a group of immunocompetent patients with confirmed PCR-swab positivity to SARS-CoV-2. Serology was evaluated at the same laboratory with the same techniques. Overall, of 80 patients recruited, 35 were included in the analysis (figure 1), fulfilling the matching characteristics. Demographic characteristics are reported in table 1.

Diabetes mellitus and chronic kidney disease were more frequently present in the LT cohort in comparison to the immunocompetent cohort (31.4% vs 8.6%, p=0.017% and 25.7% vs 2.9%, p=0.006, respectively).

Regarding COVID-19 symptoms, almost all patients had fever (33 patients, 94.3%), followed by fatigue, dyspnoea and cough. In comparison to the LT cohort, fever and dyspnoea were more frequently present (p<0.001 and p=0.003, respectively).

Table 1 General characteristics of patients included in the COVID-19-LT cohort versus patients in the COVID-19-immunocompetent cohort

	COVID-19-LT group (n=35)	COVID-19-immunocompetent group (n=35)	P value
General information			
Age in years, mean±SD	56.7±13.6	60.7±13.9	0.223
Male gender, n (%)	25 (71.4)	22 (62.9)	0.445
Ethnicity, n (%)			0.208
Caucasian	31 (88.6)	34 (97.1)	
African	3 (8.6)	0 (0)	
Asian	1 (2.9)	1 (2.9)	
History of smoking, n (%)	6 (17.1)	1 (2.9)	**0.023**
BMI in kg/m^2, n (%)			**0.028**
18–24.9	21 (60)	10 (28.6)	
24.9–29.9	8 (22.9)	16 (45.7)	
>30	6 (17.1)	9 (25.7)	
Comorbidities, n (%)			
Hypertension	14 (40.0)	14 (40.0)	1.000
Diabetes mellitus	11 (31.4)	3 (8.6)	**0.017**
Cardiovascular disease	4 (11.4)	4 (11.4)	1.000
Chronic kidney disease	9 (25.7)	1 (2.9)	**0.006**
Chronic lung disease	2 (5.7)	3 (8.6)	0.643
Present malignancy	2 (5.7)	1 (2.9)	0.555
Chronic liver disease	35 (100)	0 (0)	
COVID-19-related data			
COVID-19-related symptoms, n (%)			
Fever	15 (42.9)	33 (94.3)	**<0.001**
Cough	18 (51.4)	22 (62.9)	0.404
Dyspnoea	11 (31.4)	24 (68.6)	**0.003**
Fatigue	21 (60.0)	30 (85.7)	**0.024**
Anosmia/dysgeusia	5 (14.3)	7 (20.0)	0.600
Gastrointestinal	8 (22.9)	14 (40.0)	0.142
Other	7 (20.0)	14 (40.0)	0.094
COVID-19 therapy, n (%)			**<0.001**
Antiviral	1 (2.9)	16 (45.7)	
Chloroquine	0 (0.0)	13 (37.1)	
Steroids	2 (5.7)	0 (0.0)	
Antibiotics	3 (8.6)	12 (34.3)	
Bacterial superinfection, n (%)	2 (5.7)	0 (0.0)	
Highest level of medical support, n (%)			**<0.001**
Hospitalisation<5 days	30 (85.7)	11 (31.4)	
Hospitalisation>5 days	4 (11.4)	24 (68.6)	
Intensive care unit	1 (2.9)	0 (0.0)	
Concomitant medication			
ACE inhibitors	4 (11.4)	3 (8.6)	0.690
Angiotensin receptor blockers	7 (20)	6 (17.1)	
NSAIDs	4 (11.4)	0 (0)	
Serology-related data			
Positive IgG antibodies against nucleocapsid protein of SARS-CoV-2, n (%)	28 (80)	35 (100)	**0.005**
IgG index level, median (IQR)	3.73 (1.67–5.1)	7.36 (6.58–8.19)	**<0.001**
Positive total-Ig antibodies against spike protein of SARS-CoV-2, n (%)	34 (97.1)	35 (100)	0.314
Presence of neutralising antibodies against SARS-CoV-2, n (%)	29 (82.9)	29 (100)*	**0.024**
Neutralising antibody titre, median (IQR)	60 (12.5–120)	80 (50–120)*	0.135
Weeks from COVID-19 diagnosis to sampling, median (IQR)	6.3 (5.6–9.35)	6.9 (5.35–7.55)	0.495

*Number of analysed patients=29.
ACE, angiotensin converting enzyme; BMI, body mass index; LT, liver transplant; NSAIDs, non-steroidal anti-inflammatory drugs .

Twenty-four patients (68.6%) needed to be hospitalised for longer than 5 days.

Non-COVID-19-LT cohort characteristics

Overall, of 180 LT recipients recruited, 70 were included in the final analysis (figure 1), fulfilling the matching criteria. Difference in underlying liver aetiology as indication for LT was neither found between the two LT groups nor in the IS regimen used. Demographic characteristics are reported in table 2.

Immunological response against SARS-CoV-2 nucleocapsid protein

In the COVID-19-LT cohort, 28 out of 35 (80%) developed a positive IgG antibody titre to the SARS-CoV-2 nucleocapsid protein, with a median IgG index level of 3.73 (IQR 1.67–5.10). In the COVID-19-immunocompetent control cohort, all included patients developed antibodies, with a median index level of 7.36 (6.58–8.19) (p<0.001) (table 1). In the LT recipient control group, only one patient had a detectable positive

Table 2 General characteristics of patients included in the COVID-19-liver transplant (LT) cohort versus patients in the non-COVID-19-LT cohort

	COVID-19-LT group (n=35)	Non-COVID-19-LT group (n=70)	P value
General information			
Age, mean±SD	56.7±13.9	57.1±12.9	0.879
Male gender, n (%)	25 (71.4)	52 (74.3)	0.755
Ethnicity, n (%)			0.412
Caucasian	31 (88.6)	65 (92.9)	
African	3 (8.6)	2 (2.9)	
Asian	1 (2.9)	3 (4.3)	
History of smoking, n (%)	6 (17.1)	30 (42.9)	**0.033**
BMI, n (%)			0.251
18–24.9	21 (60)	30 (42.9)	
24.9–29.9	8 (22.9)	22 (31.4)	
>30	6 (17.1)	18 (25.7)	
Comorbidities, n (%)			
Hypertension	14 (40.0)	35 (50)	0.333
Diabetes mellitus	11 (31.4)	21 (30)	0.881
Cardiovascular disease	4 (11.4)	11 (15.7)	0.554
Chronic kidney disease	9 (25.7)	35 (50)	**0.017**
Chronic lung disease	2 (5.7)	7 (10)	0.460
Present tumour	2 (5.7)	5 (7.1)	0.782
Chronic liver disease	35 (100)	66 (94.3)	
LT-related data			
LT, n (%)	35 (100)	70 (100)	1.000
Re-LT, n (%)	6 (17.1)	7 (10)	
Underlying liver aetiology, n (%)			0.200
Alcoholic liver disease	4 (11.4)	13 (18.6)	
Viral hepatitis	8 (22.9)	17 (24.3)	
NAFLD/NASH	5 (14.3)	5 (7.1)	
PSC/PBC/AIH	8 (22.9)	20 (28.6)	
Other	10 (28.6)	5 (7.1)	
Immunosuppressive therapy, n (%)			
Calcineurin inhibitors	32 (91.4)	59 (84.3)	0.310
Tacrolimus	30 (93.8)	55 (93.2)	
Ciclosporin	2 (6.2)	4 (4.8)	
Mycophenolate mofetil (MMF)	12 (34.3)	29 (41.4)	0.479
mTOR inhibitors	2 (5.7)	12 (17.1)	0.104
Sirolimus	2 (100)	3 (25.0)	
Everolimus	0 (0)	9 (75.0)	
Steroids	7 (20.0)	21 (30.0)	0.275
Azathioprine	1 (2.9)	1 (1.4)	
Dose immunosuppressive therapy, mg/day, median (IQR)			
Tacrolimus	3 (2–4.3)	3 (1.5–5)	0.869
MMF	1000 (937.5–1625)	1500 (1000–2000_	0.150
Steroids	6.25 (5–9.4)	5 (5–10)	0.951
≥2 immunosuppressant agents	15 (42.9)	42 (60.0)	0.096
Serology-related data			
Positive IgG antibodies against nucleocapsid protein of SARS-CoV-2, n (%)	28 (80)	1 (1.4)	**<0.001**
IgG index level, median (IQR)	3.73 (1.67–5.1)	0.03 (0.02–0.06)	**<0.001**
Positive total-Ig antibodies against spike protein of SARS-CoV-2, n (%)	34 (97.1)	2 (2.9)	**<0.001**
Years from transplant to sampling, median (IQR)	5.95 (1.38–9.33)	6.00 (1.73–9.99)	0.838
Within 1 year, n (%)	5 (14.3)	12 (17.1)	0.583

AIH, autoimmune hepatitis; BMI, body mass index; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

response (table 2, figure 2). This patient neither reported any COVID-19-related symptoms since February 2020 nor had there been any contact with someone who had tested positive for SARS-CoV-2.

Comparing the two COVID-19-positive cohorts regarding factors associated with the development of positive antinucleocapsid antibody response, neither time from COVID-19 diagnosis to sampling (online supplemental figure S1A) nor age (online supplemental figure S2) seemed to affect IgG levels. However, when considering only the COVID-19-LT cohort, we found higher age to be positively associated with presence of SARS-CoV2 IgG antibodies (OR 1.073, 95% CI 1.008 to 1.143), whereas an underlying liver aetiology of primary sclerosing cholangitis/primary biliary cholangitis/autoimmune hepatitis prior to LT was negatively associated with presence of antibodies (OR 0.048, 95% CI 0.006 to 0.366) (online supplemental table S1).

In table 3 and online supplemental table S3, the characteristics of the LT patients who did not develop any detectable positive IgG antibody response against nucleocapsid protein are summarised.

Immunological response against SARS-CoV-2 S protein

All but one COVID-19-LT patient (97.1%) developed positive total-Ig antibody response against the S protein of SARS-CoV-2, with total-Ig ratio levels ranging from 5 to 18, the upper limit of quantification (figure 3). In the COVID-19-immunocompetent control cohort, all 35 included patients developed strong antibody responses, except one patient with a weaker response who had tested negative in the antinucleocapsid antibody assay. In the non-COVID-19-LT group, only two (2.9%) patients showed anti-S seroreactivity (table 2), of which one was strong (figure 3). These two patients neither reported any COVID-19-related symptoms since February 2020 nor did they report contact with people testing positive for SARS-CoV-2.

Neutralising activity of antibodies against SARS-CoV-2

In 29 out of 35 (82.9%) patients in the COVID-19-LT cohort, activity of neutralising antibodies against SARS-CoV-2 was detected. The median neutralisation titre at 120 TCID/60 μL was 60 (IQR 12.5–120) (figure 4). From the COVID-19-immunocompetent cohort, we were able to analyse 29 samples out of 35, and in all patients activity of neutralising antibodies against SARS-CoV-2 was detected (p=0.024). In this group, the median neutralisation titre at 120 TCID/60 μL was 80 (IQR 50–120). The three patients from the non-COVID-19-LT group who had positive antibodies against either nucleocapsid protein or S protein were also analysed for their neutralising activity. In none of these patients activity of neutralising antibodies against SARS-Cov-2 was detected.

In table 4, the characteristics of the COVID-19-LT patients who did not have neutralising antibodies against SARS-CoV-2 are summarised. Interestingly, all patients who had no detectable activity of neutralising antibodies also had negative IgG

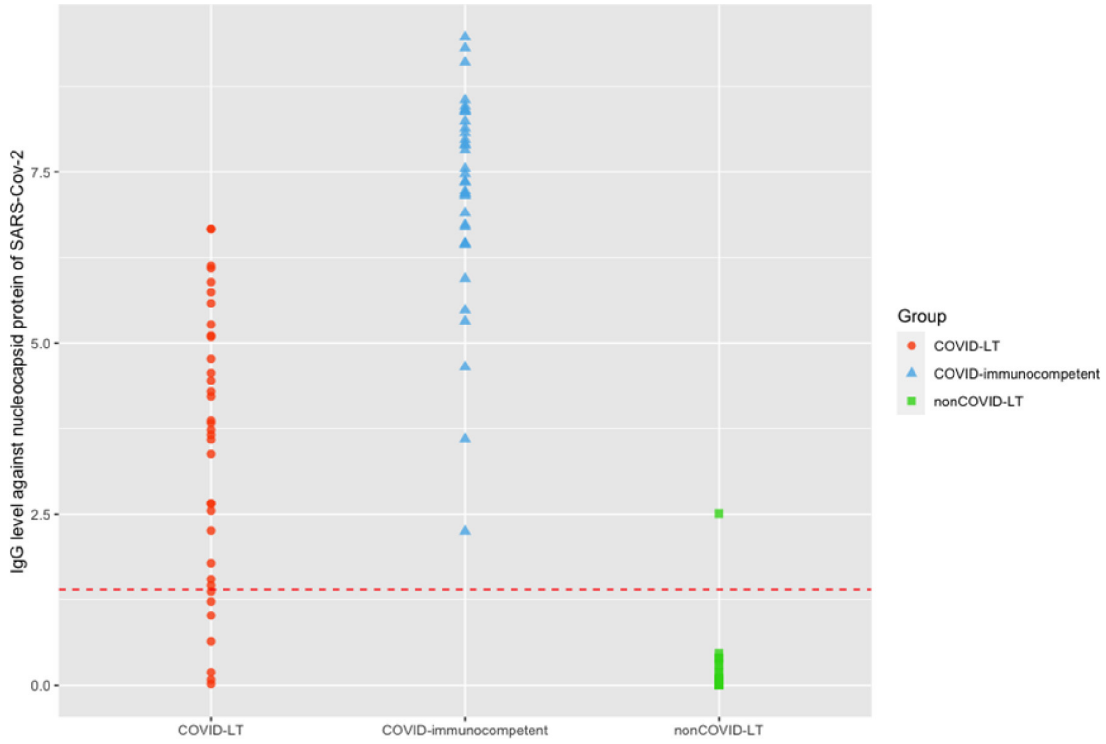

Figure 2 IgG levels against nucelocapsid protein of SARS-CoV-2 in the different groups. LT, liver transplant.

antibodies against the nucleocapsid protein, except for one patient. Positive IgG antibodies against nucleocapsid protein were positively associated with presence of neutralising activity of antibodies (OR 67.5, 95% CI 5.1 to 893.6) (online supplemental table S2). In addition, we found a moderate correlation between levels of IgG antibodies against nucleocapsid protein and neutralisation titre (correlation coefficient 0.393, p=0.02).

DISCUSSION

In this prospective, multicentre study, we aimed to evaluate the humoral immune response against both the nucleocapsid protein and the S protein of LT recipients after confirmed SARS-CoV-2 infection. We adopted a strategy including two consecutive control groups: the COVID-19-immunocompetent group with confirmed SARS-CoV-2 infection and the non-COVID-19-LT group without reported SARS-CoV-2 infection, in order to enhance the specificity of our findings (online supplemental graphical abstract). We showed that, despite the use of immunosuppressive drugs and less symptoms indicative of severe disease, LT recipients were able to mount a detectable antinucleocapsid antibody titre in 80% of the cases. This was significantly lower in comparison to the frequency and median values observed in the immunocompetent COVID-19 cohort. Only one patient in the non-COVID-19-LT cohort showed antinucleocapsid IgG antibodies just above the threshold of positivity, suggesting high specificity of the chosen method. Interestingly, when analysing the anti-S protein antibody response, no difference in positivity rates were found between the COVID-19-LT cohort and the COVID-19-immunocompetent cohort (97.1% vs 100%). This can be explained by the fact that the anti-S antibody test that was used is more sensitive than the antinucleocapsid test and also detects patients with COVID-19 with weak, immature or waning immunity. Regarding the negative control group of LT patients without reported COVID-19, only two patients developed detectable anti-S responses. Since the relatively strong anti-S serum reactivity measured in one of them could be related to subclinical COVID-19 infection in the past, misclassification cannot be excluded in this case. Regarding neutralising activity, this was found to be lower in the COVID-19-LT cohort. In addition, neutralising activity was significantly associated with the antinucleocapsid antibody titre.

Serological assays represent the test of choice to determine prior exposure to SARS-CoV-2. However, whether the detected antibodies are capable of neutralising the virus and providing protection on subsequent exposure still remains a point of debate. Antibodies against RBD of the S protein have been shown to be the primary source of neutralising antibodies against the virus,[20 21] while the nucleocapsid protein, remaining folded and not being exposed at the virus particle surface, is usually not the primary target of circulating antibodies. Nevertheless, effector functions of antinucleocapsid antibodies might still mediate protection, although they are unlikely to mediate an active neutralising effect.[22] In this regard, our finding of the presence of antibodies against the S protein in almost all LT recipients is very promising. However, when testing the actual neutralising ability of the antibodies in these patients, we found neutralising activity in 82.9% of the patients, which significantly differed from the COVID-19-immunocompetent group where neutralising activity was found in all patients. This result suggests that not all LT recipients develop functional antibodies. Interestingly, the presence of antinucleocapsid antibodies was associated with neutralising activity. It can be speculated that antinucleocapsid antibodies may reflect the extent of protection conferred by SARS-CoV-2 exposure in LT recipients. Since the assessment of neutralising ability of antibodies is laborious and relatively time consuming, measurement of antinucleocapsid antibody titre might be useful as a surrogate marker for lack of neutralising activity, despite the presence of anti-S antibodies. However, this observation needs to be confirmed. Neutralising ability was tested on a strain isolated from a Dutch patient at the beginning

Table 3 General characteristics of patients with and without IgG antibodies against nucleocapsid protein of SARS-CoV-2 from the COVID-19-LT cohort

	Positive IgG antibodies (n=28)	Negative IgG antibodies (n=7)
General information		
Age, mean±SD	59.5±10.9	45.6±17.6
Male gender, n (%)	22 (78.6)	3 (42.9)
BMI>24.9, n (%)	14 (50.0)	0 (0)
Comorbidities, n (%)		
Hypertension	12 (42.9)	2 (28.6)
Diabetes mellitus	11 (39.3)	0 (0)
Cardiovascular disease	4 (14.3)	0 (0)
Chronic kidney disease	7 (25.0)	2 (28.6)
Chronic lung disease	2 (7.1)	0 (0)
COVID-19-related data		
COVID-19-related symptoms, n (%)		
Fever	14 (50.0)	1 (14.3)
Cough	16 (57.1)	2 (28.6)
Dyspnoea	11 (39.3)	0 (0)
Fatigue	18 (64.3)	3 (42.9)
Anosmia/dysgeusia	3 (10.7)	2 (28.6)
Gastrointestinal	7 (25.0)	1 (14.3)
Other	5 (17.9)	2 (28.6)
COVID-19 therapy, n (%)		
Antiviral	1 (3.6)	0 (0)
Chloroquine	0 (0)	0 (0)
Steroids	2 (7.1)	0 (0)
Antibiotics	3 (10.7)	0 (0)
Highest level of medical support, n (%)		
Domiciliary	19 (67.9)	7 (100)
Hospitalisation<5 days	2 (7.1)	0 (0)
Hospitalisation≥5 days	6 (21.4)	0 (0)
Intensive care unit	1 (3.6)	0 (0)
Concomitant medication		
ACE inhibitors	3 (10.7)	1 (14.3)
Angiotensin receptor blockers	6 (21.4)	1 (14.3)
NSAIDs	4 (14.3)	0 (0)
LT-related data		
Underlying liver aetiology, n (%)		
Alcoholic liver disease	4 (14.3)	0 (0)
Viral hepatitis	8 (28.6)	0 (0)
NAFLD/NASH	5 (17.9)	0 (0)
PSC/PBC/AIH	3 (10.7)	5 (71.4)
Other	8 (28.6)	2 (28.6)
Transplant indication		
End-stage liver disease	11 (39.3)	1 (14.3)
Hepatocellular carcinoma	12 (42.9)	0 (0)
Acute liver failure	1 (3.6)	2 (28.6)
Other	4 (14.3)	4 (57.1)
Immunosuppressive therapy, n (%)		
Calcineurin inhibitors	25 (89.3)	7 (100)
Tacrolimus	23 (82.1)	7 (100)
Ciclosporin	2 (7.1)	0 (0)
Mycophenolate mofetil	8 (28.6)	4 (57.1)
mTOR inhibitors	2 (7.14)	0 (0)
Sirolimus	2 (7.14)	0 (0)
Everolimus	0 (0)	0 (0)
Steroids	4 (14.3)	3 (42.9)
≥2 immunosuppressant agents	12 (42.9)	3 (42.9)
Dose immunosuppressive therapy, mg/day, median (IQR)		
Tacrolimus	3.5 (2.25–4.75)	2 (1.25–2.38)
Mycophenolate mofetil	1000 (937.5–1625)	1000 (875–1250)
Steroids	6.25 (5–8.13)	7 (5.5–8.5)
Serology-related data		
Positive total-Ig antibodies against spike protein of SARS-CoV-2, n (%)	28 (100)	6 (85.7)
Total-Ig index level, median (IQR)	18.3 (18.3–18.3)	18.3 (17.0–18.3)
Weeks from diagnosis to sampling, median (IQR)	6.5 (5.6–8.7)	6.0 (5.4–10.0)
Years from LTx to sampling, median (IQR)	6.2 (1.7–10.2)	5.6 (0.8–6.9)

ACE, angiotensin converting enzyme; AIH, autoimmune hepatitis; BMI, body mass index; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NSAIDs, non-steroidal anti-inflammatory drugs; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

of the pandemic, which comprises the Wuhan sequence with the D614G mutation, which had become dominant at that time. It is therefore unlikely that SARS-CoV-2 mutations have had influence on our results, since all patients in our cohort were infected at the time when this first variant was dominant.

In the general population, seroconversion rates after SARS-CoV-2 infection are reported to be higher than 90%.[23 24] The decline of the antibodies titre is variable and not always related with the rate of neutralisation.[25] In our COVID-19-LT cohort, 80% of patients developed antinucleocapsid antibodies with a significantly lower median level when compared with the COVID-19-immunocompetent control group. These data are in line with those from a recent Spanish study in which 77.4% of LT patients had antinucleocapsid IgG at 3 months versus the immunocompetent group in which 100% seroconverted.[15] Another study underlined that only 51% of SOT recipients (10 were LT recipients) developed antinucleocapsid antibodies, with lower rates in kidney transplant recipients.[14] Therefore, our data further corroborate the idea that the use of antinucleocapsid IgG testing, contrary to what international guidelines suggest,[16 26] is probably a suboptimal tool to confirm previous contact with SARS-CoV-2 in immunosuppressed (LT) recipients. On the other hand, thanks to the introduction of LT negative controls, our study emphasises that cross-reactivity with other CoV species is irrelevant for the proposed assay, while still ensuring adequate specificity when the test is adopted in LT patients.[27]

In the general population, factors associated to humoral response are age, sex and severity of the disease.[28] In our study, older COVID-19-LT patients displayed a higher rate of antinucleocapsid seroconversion. This finding should be interpreted in light of the fact that older patients are often more symptomatic and severely ill. In this regard, it should also be noted that in a context of consecutive patients included prospectively, the COVID-19-LT cohort had less COVID-19 symptoms than the COVID-19-immunocompetent control group. Nevertheless, there was a presence of antinucleocapsid seroconversion of 80% and anti-S seroconversion of 97.1%. Previous studies had suggested that the COVID-19 severity in LT patients was not higher than that of the general population[29] and the use of tacrolimus could even prevent a severe disease course.[30] Therefore, to what extent the severity of the clinical presentation is attenuated in LT patients due to use of IS and whether this affects humoral immunity still remains unsolved.

Specifically in SOT recipients, it is known from previous respiratory viral infections that they may have an impaired humoral response,[31] mainly imputed to the use of IS.[32] Reported risk factors of a reduced humoral response in SOT recipients included transplant-infection interval, use of angiotensin converting

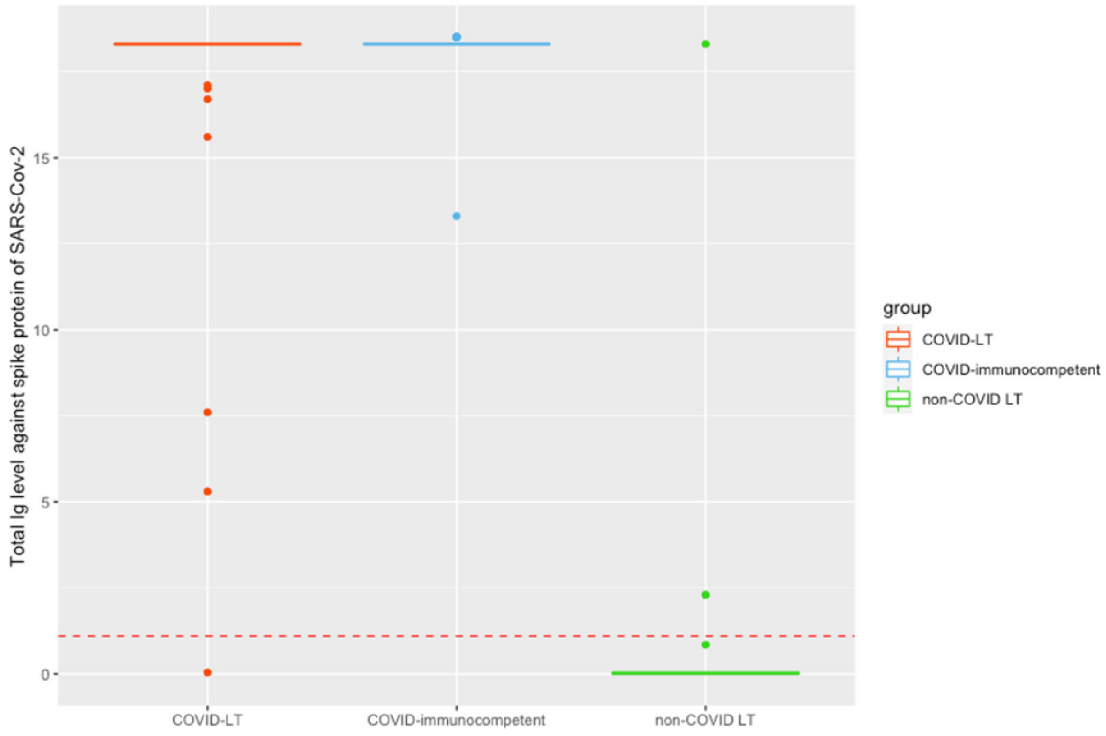

Figure 3 Total-Ig levels against spike protein of SARS-CoV-2 in the different groups. LT, liver transplant.

enzyme inhibitors and kidney transplantation compared with other SOTs.[14 15] Although it may be hypothesised that higher dosages of IS therapy may contribute to these findings, we did not observe any association with IS dose. Indeed, several reports failed to demonstrate a role for IS in reducing antiviral responses, although most studies were underpowered for this objective.[15 33]

Only in one report it was suggested that patients who did not develop antibodies against SARS-CoV-2, more frequently had dual IS.[14] In our cohort, despite the low numbers of patients with negative antinucleocapsid antibody response, five out of seven were transplanted for cholestatic or autoimmune causes, implying a multidrug IS regimen, often including MMF or

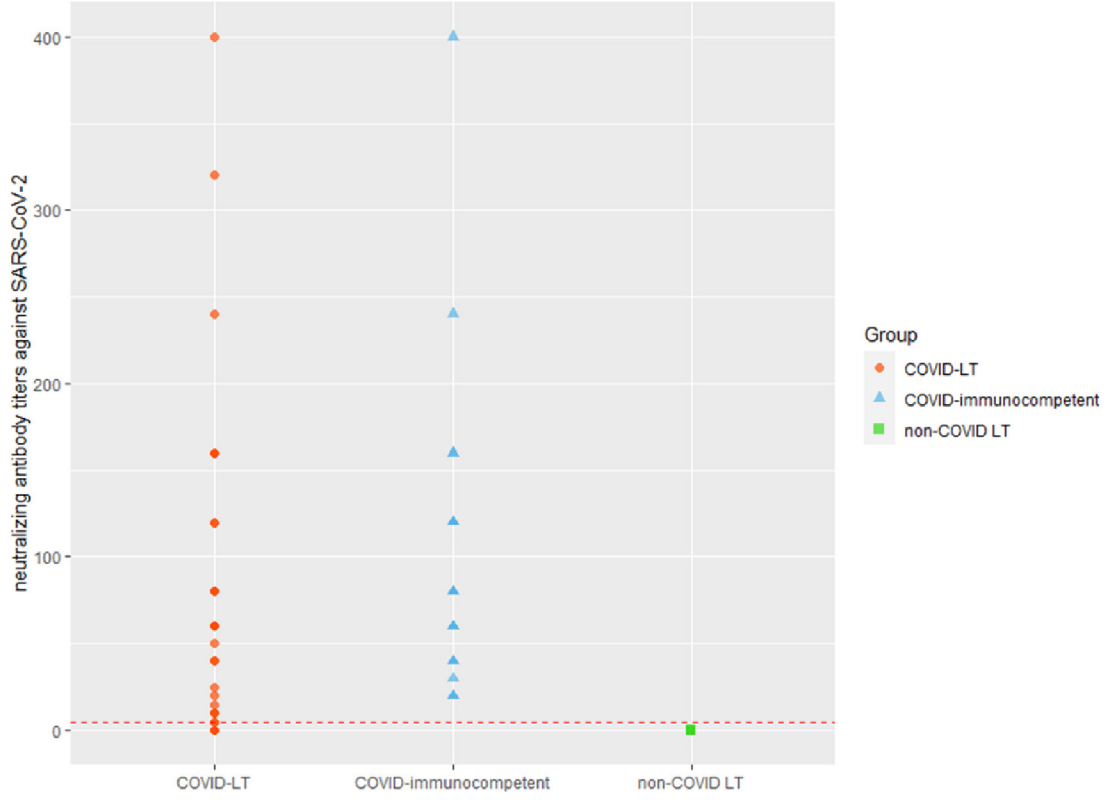

Figure 4 Virus-neutralising titers against SARS-CoV-2 in different groups. LT, liver transplant.

Table 4 General characteristics of patients with and without neutralising antibodies against SARS-CoV-2 from the COVID-19-LT cohort

	Presence of neutralising antibodies (n=29)	Absence of neutralising antibodies (n=6)
General information		
Age, mean±SD	58 (11.5)	50.3 (20.8)
Male gender, n (%)	23 (79.3)	2 (33.3)
BMI>24.9, n (%)	14 (48.3)	0 (0)
Comorbidities, n (%)		
Hypertension	13 (44.8)	1 (16.7)
Diabetes mellitus	11 (37.9)	0 (0)
Cardiovascular disease	4 (13.8)	0 (0)
Chronic kidney disease	8 (27.6)	1 (16.7)
Chronic lung disease	2 (6.9)	0 (0)
COVID-19-related data		
COVID-19-related symptoms, n (%)		
Fever	14 (48.3)	1 (16.7)
Cough	15 (51.7)	3 (50)
Dyspnoea	10 (30.5)	1 (16.7)
Fatigue	18 (62.1)	3 (50)
Anosmia/dysgeusia	4 (13.8)	1 (16.7)
Gastrointestinal	7 (24.1)	1 (16.7)
Other	6 (20.7)	1 (16.7)
COVID-19 therapy, n (%)		
Antiviral	1 (3.5)	0 (0)
Chloroquine	0 (0)	0 (0)
Steroids	2 (6.9)	0 (0)
Antibiotics	3 (0.3)	0 (0)
Highest level of medical support, n (%)		
Domiciliary	20 (69)	6 (100)
Hospitalisation<5 days	5 (17.2)	0 (0)
Hospitalisation≥5 days	3 (10.3)	0 (0)
Intensive care unit	1 (3.45)	0 (0)
Concomitant medication, n (%)		
ACE-inhibitors	4 (13.8)	0 (0)
Angiotensin receptor blockers	6 (20.7)	1 (16.7)
NSAIDs	4 (13.8)	0 (0)
Liver transplant-related data		
Underlying liver aetiology, n (%)		
Alcoholic liver disease	4 (13.8)	0 (0)
Viral hepatitis	8 (27.6)	0 (0)
NAFLD/NASH	5 (17.2)	0 (0)
PSC/PBC/AIH	4 (13.8)	4 (66.7)
Other	8 (27.6)	2 (33.3)
Transplant indication, n (%)		
End-stage liver disease	11 (37.9)	1 (16.7)
Hepatocellular carcinoma	12 (41.4)	0 (0)
Acute liver failure	2 (6.9)	1 (16.7)
Other	4 (13.8)	4 (66.7)
Immunosuppressive therapy, n (%)		
Calcineurin inhibitors	26 (89.6)	6 (100)
Tacrolimus	24 (82.7)	6 (100)
Ciclosporin	2 (6.9)	0 (0)
Mycophenolate mofetil	10 (34.5)	2 (33.3)
mTOR inhibitors	2 (6.9)	0 (0)
Sirolimus	2 (6.9)	0 (0)
Everolimus	0 (0)	0 (0)
Steroids	5 (17.2)	2 (33.3)
≥2 immunosuppressant agents, n (%)	11 (37.9)	4 (66.7)
Dose immunosuppressive therapy, mg/day, median (IQR)		
Tacrolimus	3 (2.25–5)	2 (1–2)
Mycophenolate mofetil	1000 (812.5–1375)	1500 (1250–1750)
Steroids	7.5 (5–10)	5 (5–5)
Serology-related data		
Positive IgG antibodies against nucleocapsid protein of SARS-CoV-2, n (%)	27 (93.1)	1 (16.67)
IgG index level, median (IQR)	4.22 (2.65–5.27)	0.42 (0.11–0.92)
Positive total-Ig antibodies against spike protein of SARS-CoV-2, n (%)	29 (100)	5 (83.33)
Total-Ig index level, median (IQR)	18.26 (18.26–18.31)	17.62 (8.27–18.26)
Weeks from diagnosis to sampling, median (IQR)	6.7 (5.9–10.7)	5.6 (4.95–6.82)
Years from LTx to sampling, median (IQR)	6.0 (1.42–9.12)	6.5 (2.1–11.1)

ACE, angiotensin converting enzyme; AIH, autoimmune hepatitis; BMI, body mass index; LT, liver transplant; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NSAIDs, non-steroidal anti-inflammatory drugs; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

steroids. Additionally, four out of five cholestatic/autoimmune patients with negative antinucleocapsid antibodies had negative neutralising antibodies.

Recently, much more attention has been given to the interplay between innate and adaptive immunity in maintaining immunological memory from both B cells and T cells, despite the decay of antibodies detectability.[34] This has implications not only in preventing reinfections but also in reducing disease severity. Further studies with this trajectory are necessary to increase the understanding and strengthen our findings, which seem to suggest less severe impairment of the immune response of LT patients to SARS-CoV-2 than previously thought concerning the presence and titers of antibodies. However, the complexity of immune response is probably not completely depicted by the antibodies' level. One of the limitations we acknowledge in our study is not having analysed the T-cell activity, which is more recently recognised as playing a complementary role in the immune response.[35] Other limitations of our study include the relatively small sample size and the lack of a longitudinal assessment of the serological response over time. Additionally, the severity of SARS-CoV-2 infections in the two cohorts (COVID-19-LT and COVID-19-immunocompetent) is probably relatively mild compared with other studies where the hospitalisation and ICU admission rates were higher.[14 15] Nevertheless, our study introduces several elements of novelty with respect to the previously published literature, such as the introduction of a more comprehensive testing of the humoral response and the presence of a negative control group, aiming to corroborate the specificity of our findings.

In conclusion, our findings suggest that the humoral response of LT recipients is only slightly lower than expected compared with that of COVID-19 immunocompetent controls. Additionally, we showed that the majority of LT recipients is capable of mounting an adequate neutralising activity against SARS-CoV-2 and that neutralising ability was associated with the presence of antinucleocapsid antibodies. However, caution must be taken when interpreting the presence of antibody levels against the S protein of SARS-CoV-2, since those results can overestimate the ability of neutralising the virus. In addition, we showed that antinucleocapsid antibodies, although specific for SARS-CoV-2 when tested alone, might not be suitable as a single tool for testing past exposure to SARS-CoV-2 in this population, while testing for anti-S antibodies can add sensitivity. Our results confer major insight into the natural immunity to SARS-CoV-2

in LT recipients and may have potential implications for vaccine-related immunity and better interpretation of the serological assays adopted in the clinical setting.

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