APENDIX 1

1) MATERIALS AND METHODS

Patients

The cohort comprised 129 adult LTRs follow-up at the Liver Transplant Unit of University Hospital Marqués de Valdecilla, Santander (Spain) with a negative history for COVID-19 who had previously received two doses of mRNA-1273 SARS-CoV-2 and were subsequently vaccinated 4 moths after the second dose (IQR= 133-139 days) with a homologous third dose of the mRNA-1273 SARS-CoV-2 vaccine between September 27, 2021, and October 10, 2021. Neither severe side-effect nor rejection event was documented after third dose. Mild local pain and self-limited fever were reported as the main side-effects. No adjustment of immunosuppression was performed within vaccination procedure. Clinical data including comorbidities and immunosuppressive regimen were obtained from patient´s medical records and routine blood test up to one month after the third vaccine. This study was approved by the Institutional Ethics Committee of Cantabria (internal code: 2021.391) and complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki.

Measurement of SARS-CoV-2 spike antibodies

The IgG antibodies directed against the receptor binding domain (RBD) of the spike glycoprotein [IgG(S-RBD)] of the SARS-CoV-2 was assessed using the ARCHITECT IgG II Quant test (Abbot), measured a median of 43 days (IQR, 37-49 days) after the second vaccine dose and a median of 30 days (IQR, 30-33.7 days) after the third vaccine injection (the sensitivity with the test is near 100% in this range of analysis (1). Values higher than 50 AU/mL were considered positive as specified by the manufacturer. The results of this assay have been shown to correlate with in vitro neutralization of SARS-CoV-2 (2). This interpretation is limited by its lack of association with vaccine effectiveness and its lack of comparability with other assays, using different cut-offs (3). Thus, for surrogate measures of antibody neutralization, we examined IgG(S-RBD) levels at or

above 4,160 AU/mL given that this threshold is recommended by the manufacturer and is used a surrogate measure of vaccine protection because it corresponds to a 0.95 probability of obtaining an in-vitro plaque reduction neutralization test. In addition, such cut-off was chosen according to a recent study that reported that lower anti-S1 titers were associated with significantly more breakthrough infection in vaccinated recipients (4).

Definitions

Groups were defined based on the level of anti-S1 IgG antibodies measured after the second vaccine dose: seronegative; low responders and high responders. Seronegatives were defined as levels <50 AU/mL, low responders with levels between 50 and 4,159 AU/mL and high responders with levels ≥4160 AU/mL as estimate neutralizing antibody.

The primary composite endpoint was defined as the cumulative percentage of patients switching from no-to low-responder status, from low-to high responders status or doubling their anti-S1 IgG level.

Assessment of SARS-CoV-2 T-Specific Response by Flow Cytometry

The assay was validated by the Spanish Society of Immunology and based on the surface expression of activation-induced markers (AIM) after exposure with specific SARS-CoV-2 peptide pools (5). This procedure has been previously published by our group (6). Briefly, peripheral blood mononuclear cells (PBMCs) from heparinized blood were isolated by Ficoll gradient and cultured at 10^6/mL in TexMACS medium (Miltenyi Biotec, Bergisch Gladbach, Germany) during 24 h at 37 °C in a flat-bottom 96-well plate in 0.1% DMSO, PepTivator SARS-CoV-2 Prot S, Prot M and Prot N (Miltenyi Biotec) at final concentration of 1 ug/mL and Dynabeads Human T activator CD3/CD28 (Gibco Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) as a positive control. After cell culture, the PBMCs were washed and stained with the following monoclonal antibodies: anti-CD3 (FITC) clone UCHT 1 (Inmunotech SAS Beckman Coulter, Marseille, France),

anti-CD4 (APC-Vio 770) clone VIT4 (MiltenyiBiotec, Bergisch Gladbach, Germany), anti-CD8 (ECD) clone SFCI21Thy2D3 (Beckman Coulter, 737659, Brea, CA, USA), anti-CD134 (PE) clone 134-1 (Cytognos, Salamanca, Spain), and anti-CD25 (PE-Cy7) clone 2A3 (Beckman Coulter). The stained PBMCs samples were washed with 150 µL of phosphate buffer saline (PBS) and centrifuged during 5 min at 1800 rpm. Finally, 2 µL of 7-Aminoactinomycin D staining solution (Tonbo Biosciences, San Diego, CA, USA) and 90 µL of PBS were added before the samples were acquired on the CytoFLEX flow cytometer (Beckman Coulter). The results were expressed as the frequency in the AIM ($CD25^{+}CD134^{+}$) ratio obtained after specific activation to negative non-stimulated control. A ratio >3 in one of the specific SARS-CoV-2 peptide pools was considered positive.

Statistical analysis

Statistically analysis was performed with IBM SPSS Statistics v22.0 for Mac (IBM Corp, Armonk, NY). The distribution of continuous variables was assessed using Kolmogorov–Smirnov/Shapiro–Wilk tests. The results were expressed as mean (standard deviation) or median (range or interquartile range-IQR), according to data distribution, and qualitative variables as absolute value and proportions. Comparisons between groups were performed with the unpaired Student´s t-test, the Mann-Whitney test or Fisher's exact test as appropriate. A two-sided *p*-value < 0.05 was considered statistically significant.

2) Patients and Results

Baseline characteristics of the 129 LTRs included in this study are summarized in Table S1. Briefly, all patients were Caucasian, and most patients were man (n=99, 76.7%) with median age of 60,6 (IQR, 56-28). The most frequent etiology was alcoholic cirrhosis (n= 56 43.4%), followed by hepatitis C virus-induced liver cirrhosis (n=24, 18.6%), being the decompensation of cirrhosis (includes refractory ascites and spontaneous bacterial peritonitis, bleeding from gastroesophageal varices and encephalopathy; n=61, 47.3%) the main indication for liver

transplantation, followed by hepatocellular carcinoma (n=52, 40.3%). The median time between transplantation and the initiation of vaccination was 7.0 years (IQR, 4 to 12 years). Comorbidities were common in our LTRs, with 94.9% diagnosed with at least 1 co-morbidity, including arterial hypertension (n=78, 60.5%), diabetes mellitus (n=47, 36.4%), and CKD (n=46, 35.9%). Regarding immunosuppressive therapy prior to vaccination, calcineurin inhibitors (CNIs) were used as the backbone of the immunosuppressive regimen in 115 LTR (89.1%). Everolimus was used in 10 patients (7.8%). Thirty two patients (24.8%) received mycophenolate mofetil (MMF).

Antibody response and levels after SAS-Cov2

The overall an antibody response developed in 113 (87.6%) of patients after two doses of mRNA-1273 SARS-CoV-2 vaccine, and a striking increase in antibody level occurred after third vaccine dose administration. Their antibody levels increased significantly from a median 4,774.4 [IQR, 1,394.5-14,090.5] AU/mL to 30,319 [IQR, 18,829-41,062.5] AU/mL (p=0.01).

The primary composite endpoint was reached in 117 patients (90.7%). In detail, 12/16 (75%) changed from no-to low-responders status, 43/51 (95.2%) from low-to high responder status and 62/62 (100%) at least doubled their anti-S1 IgG level. Therefore, only 8 of 51 low responder remained with a weak humoral response after third dose; 1,417 [IQR, 629-2,446] AU/mL to 26,077 [12,156-38,155] AU/mL (p=0.01). In contrast, all LTRs with high response after 2 dose vaccination showed a strong response after third vaccine dose; and their antibody levels increased significantly from a median of 12,413 [IQR, 5,761.7-23,494.9] AU/mL to 39,371 [IQR, 28,066.2-43,625] AU/mL, p=0.01

Specific T-Cell Immune Response

To examine the specific T-cell response, PBMCs were exposed to anti-SARS-CoV-2 peptides using an unstimulated negative control and CD3/CD28 as positive control, as showed in the Material and Methods section. The assessment of vaccine-specific T-cell response has been proposed to

fully evaluate the immune response against the vaccine. The assay was addressed in those 16 seronegative to 2 dose vaccination.

3. Table S1. Baseline characteristics of Liver Transplant Recipients (LTRs) and comparison of LTRs with positive and negative antibody response after third dose of mRNA-1273 SARS-CoV-2 Vaccine

Variables	All LTRs (n=129)	Seropositive (n=125)	Seronegative (n=4)	p
Age (years), median (IQR)	63 (56-68)	63 (56-68)	67.5 (66.25-69.5)	0.162
Sex (male)	99 (76.7)	97 (77.6)	2 (50)	0.198
Race (caucasian)	129 (100)	125 (100)	4 (100)	-
Aetiology of liver disease				0.605
Alcohol	56 (43.4)	54 (43.2)	2 (50)	
HCV	24 (18.6)	24 (19.2)	0 (0)	
Alcohol + HCV	12 (9.3)	12 (18.4)	0 (0)	
Other	37 (28.7)	35 (28)	2 (50)	
Transplant indication				0.491
Hepatocelullar carcinoma	52 (40.3)	51 (40.8)	1 (25)	
Decompensated cirrosis(*)	61 (47.3)	58 (46.4)	3 (75)	
Other (**)	16 (12.4)	16 (12.8)	0 (0)	
Interval since transplantation (years), n (%)				0.279
<1	8 (6.2)	7 (5.6)	1 (25)	
1-3	15 (11.6)	14 (11.2)	1 (25)	
3-6	30 (23.3)	30 (24)	0 (0)	

6-11	29 (22.5)	29 (23.2)	0 (0)	
>11	47 (36.4)	45 (36)	2 (50)	
ABO group				0.93
A	53 (51)	51 (40.8)	2 (50)	
B	8 (7.7)	7 (5.6)	1 (25)	
AB	2 (1.9)	2 (1.6)	0 (0)	
0	41 (39.4)	40 (32)	1 (25)	
Previous medical history				
Hypertension	78 (60.5)	75 (60)	3 (75)	0.546
Diabetes	47 (36.4)	44 (35.2)	3 (75)	0.103
Chronic Kidney Disease	46 (35.9)	42 (33.9)	4 (100)	0.007
Cardiovascular Disease	34 (26.4)	33 (26.4)	1 (25)	0.95
Chronic Lung Disease	12 (9.3)	12 (9.6)	0 (0)	0.515
Inmunosupressive régimen				<0.001
Without Mycophenolate	97 (75.2)	96 (76.8)	1 (25)	
Monotherapy [CNI/imTOR]	91 [85/6] (70.5)	91 (72.8)	0 (0)	
Association with CNI*	6 (4.7)	5 (4)	1 (25)	
With Mycohpenolate	32 (24.8)	29 (23.2)	3 (75)	
Monotherapy	8 (6.2)	6 (4.8)	2 (50)	
Association with CNI	24 (18.6)	23 (18.4)	1 (25)	
Inmunosupression (dose – mg) mean (SD)				

Mycophenolate (n=32)	1,093.8 (482.6)	1,068.9 (394.7)	1,666.7 (577.6)	0.022
Prednisone (n=3)	4.2 (2.9)	4.2 (±2.9)	-	-
Inmunosupression (through concentration -*µg/L-) mean (SD)*				
Cyclosporine	n=12 66.9 (33.9)	n=12 66.9 (33.9)	-	-
Tacrolimus	n=103 4.9 (1.4)	n=100 4.9 (1.4)	n=3 5.2 (0.6)	0.773
Everolimus	n=10 4.8 (1.6)	n=9 5.1 (1.4)	n=1 2.1 (0)	0.069
Laboratory parameters, mean (SD)				
Haemoglobin (g/dL)	14.8 (9.1)	14.9 (9.3)	12.7 (1.5)	0.635
Platelets (G/L)	169.3 (60.5)	169.9 (61.3)	150.0 (25.2)	0.518
Leukocytes (G/L)	5.9 (1.6)	5.9 (1.6)	6.5 (1.4)	0.474
Lymphocytes (G/L)	3.4 (1.8)	3.4 (1.8)	1.5 (0.8)	0.832
eGFR (ml/min/1.73m2)	68.2 (19.9)	69.4 (18.9)	32.3 (17.3)	<0.001
Serum albumin (g/dL)	4.4 (0.3)	4.4 (0.3)	4.3 (0.1)	0.632

(*) Descompensation of cirrhosis: including ascites, bleeding from gastroesophageal varices and encephalopathy.

(**) Other transplant indications: Fulminant hepatic failure and acute chronic liver failure.

Quantitative variables are expressed as n (%). Abbreviations: IQR: interquartile range; SD: Standard Deviation; HCV: Hepatitis C Virus; CNI: Calcineurin inhibitors; imTOR: Mammalian

target of rapamycin inhibitors; Ratio Le/Ly: Ratio Leukocytes/Lymphocytes; eGFR: estimated Glomerular Filtration Rate. *CNI was associated with prednisone, imTOR or both.

4. REFERENCES

(1) Chapuy-Regaud S, Miédougé M, Abravanel F, Da Silva I, Porcheron M, Fillaux J, et al. Evaluation of Three Quantitative Anti-SARS-CoV-2 Antibody Immunoassays. Microbiol Spectr 2021;9(3):e0137621. doi: 10.1128/spectrum.01376-21

(2) Perkmann T, Perkmann-Nagele N, Koller T, Mucher P, Radakovics A, Marculescu R, et al. Anti-Spike Protein Assays to Determine SARS-CoV-2 Antibody Levels: a Head-to-Head Comparison of Five Quantitative Assays. Microbiol Spectr. 2021 Jun 30:e0024721. doi: 10.1128/Spectrum.00247-21.

(3) Criscuolo E, Diotti RA, Strollo M, et al. Weak correlation between antibody titers and neutralizing activity in sera from SARS-CoV-2 infected subjects. J Med Virol 2021;93:2160-7.

(4) Ebinger JE, Fert-Bober J, Printsev I, et al. . Antibody responses to the BNT162b2 mRNA vaccine in individuals previously infected with SARS-CoV-2. Nat Med 2021;27:981–4. 10.1038/s41591-021-01325-6.

(5) Grifoni A., Weiskopf D., Ramirez S.I. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell. 2020;181:1489–1501.e15. doi: 10.1016/j.cell.2020.05.015

(6) San Segundo D, Comins-Boo A, Irure-Ventura J, Renuncio-García M, Roa-Bautista A, González-López E, Merino-Fernández D, Lamadrid-Perojo P, Alonso-Peña M, Ocejo-Vinyals JG, Gutiérrez-Larrañaga M, Guiral-Foz S, López-Hoyos M. Immune Assessment of BNT162b2 m-RNA-

Spike Based Vaccine Response in Adults. Biomedicines. 2021;9(8):868. doi: 10.3390/biomedicines9080868.