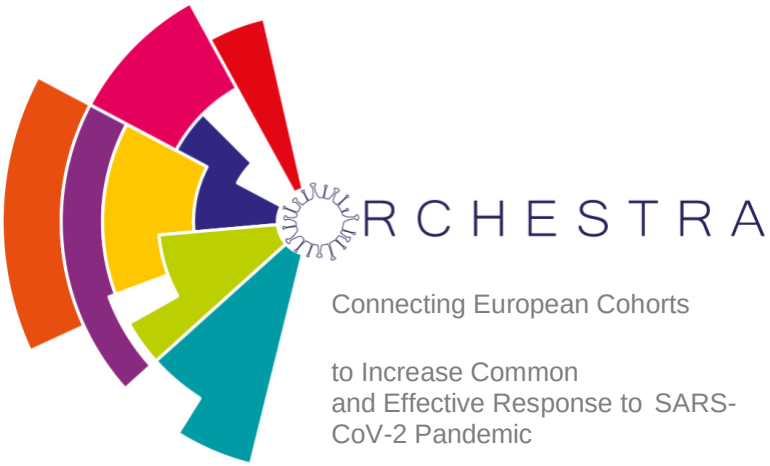

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First report on immunity analysis from prospective cohorts (COVID-19, vaccinated and not vaccinated)

Samir Kumar-Singh, Benoit Visseaux, Surbhi Malhotra, Maddalena Giannella

Universiteit Antwerpen (UANTWERPEN)

University of Bologna (UNIBO)

Institut National de la Sante et de la Recherche Medicale (INSERM)

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Executive summary

WP and deliverable context

The present report is part of ORCHESTRA project, a three-year international research project aimed at tackling the coronavirus pandemic. ORCHESTRA provides an innovative approach to learn from the pandemic SARS-CoV-2 crisis, derive recommendations to further management of COVID-19 and be prepared for the possible future pandemic waves. The ORCHESTRA project aims at delivering sound scientific evidence for the prevention and treatment of the infections caused by SARS-CoV-2 assessing epidemiological, clinical, microbiological, and genotypic aspects of population, environment, and socio-economic features. The project builds upon existing, and new large scale population cohorts in Europe (France, Germany, Spain, Italy, Belgium, Romania, Netherlands, Luxemburg, and Slovakia) and non-European countries (India, Perú, Ecuador, Colombia, Venezuela, Argentina, Brazil, Democratic Republic of Congo, and Gabon) including SARS-CoV-2 infected and non-infected individuals of all ages and conditions. The primary aim of ORCHESTRA is the creation of a new pan-European cohort applying homogenous protocols for data collection, data sharing, sampling, and follow-up, which can rapidly advance the knowledge on the control and management of the COVID-19. Within ORCHESTRA project, the Work Package 6 (WP6) aims at providing innovative laboratory capabilities combining serology, immunology, viral and human genomes, microbiota and epigenetic analysis. It aims to describe markers and physiopathology of various COVID-19 outcomes including severe cases, long COVID and vaccine efficiency across various patient populations gathered within ORCHESTRA cohorts.

The objectives of WP6 are distributed in two parts: (1) a retrospective part on frozen samples obtained during 2020 and (2) a prospective part starting in 2021. The goal for the serological markers part is to allow the description of the SARS-CoV-2 variants (both known variants and novel mutations) according to epidemics waves, stages of SARS-CoV-2 infection (mild vs severe) and setting (outpatients vs hospitalized). Given the evidence that anti-SARS-CoV-2 antibodies, CD4+ T cells, and CD8+ T cells can all participate in protective immunity to SARS-CoV-2, WP6 further intends to assess T cell responses, especially CD4+ T cells in vaccinated individuals as well as individuals recovering from natural infection by recent SARS-CoV-2 variants.

Content of the document

The present report describes the response to SARS-CoV-2 exposure performed in the context of the prospective studies in mixed populations with number of tested serological timepoints, serological markers, and T cell immunity. This report conforms with the objective to allow the description of serological and T cell markers across patients included in the prospective part of the Orchestra study along with viral variant, genomic, microbiota, and clinical data analyses for all included patients where such data is available. In the original study plan, immunity analysis of unvaccinated fragile populations was also planned. However, due to country legislation on mandatory vaccination, the fragile population could not be tested for non-vaccinated individuals. Serology analysis of non-vaccinated and immunocompetent individuals has been performed by WP3 and had been reported in D3.5. In addition, serology results for non-vaccinated hospitalized cohorts have been reported earlier as D6.4 mostly covering French cohorts. Furthermore, immunological responses in vaccinated and non-vaccinated healthcare workers as part of WP5 has been partially reported as D5.5 and would be further updated as part of D5.6. While the current document contains the analysis of different cohorts from different centres in Italy as well as of Spain, additional fragile and hospitalized cohorts are currently being planned for Brazil (pregnant cohort), Luxembourg (Parkinson cohort) and South America (Zika). These results will be provided in the final report on immunity analysis for prospective cohorts (D6.9).

Dissemination level: Public

Core content

Detailed description of the conducted tests

Sample collection

All serum and PBMC samples from various cohorts including fragile populations, 1 month after receiving their 3rd dose booster vaccine, patients with breakthrough infections, patients receiving monoclonal antibody therapy and community based COVID-19 infections collected in Verona (UNIVR, Italy), Vicenza (Italy), Treviso (Italy) and Padova (Italy), as described in **Table 1** were analysed by WP6 (UAntwerpen) for this deliverable. In addition, cellular immunity analysis by T-spot conducted at UNIBO, Italy has also been included.

The fragile cohorts consist of patients who received a solid organ transplant (SOT) and cancer patients. Since these patient populations are known to be less likely to develop adequate immune responses, we aimed to evaluate the antibody titres and cellular immunity at different timepoints after different vaccination doses. In addition, we included patients who were fully vaccinated but still developed a breakthrough infection and determined the antibody concentration and T cell response in breakthrough infection with the omicron and other viral variants.

Table 1: The number of samples included per cohort. V3M1: 1 month after the 3rd vaccination.

Cohort	Work Package	Population/ time point	Serology	Sero neutralisation	T cell Analysis
UNIVR SOT Cohort	WP4	V3M1	81	0	0
Vicenza SOT Cohort	WP4	V3M1	110	0	25
Treviso SOT Cohort	WP4	V3M1	323	0	0
Padova SOT Cohort	WP4	V1M6, V3M1	264	0	0
SAS SOT Cohort	WP4	V3M1	16	0	0
UNIVR Oncology Cohort	WP4	V3M1	73	73	0
SAS Oncology Cohort	WP4	V1M6, V3M1	31	0	0
UNIVR Breakthrough Cohort	WP2	Breakthrough	208	208	98
UNIBO Hospitalised Cohort	WP2	Omicron	17	17	13
UNIVR monoclonal study	WP2	Bamlanivimab; Bamlanivimab/ Etesevimab; Casirivimab/ Imdevimab	539	539	106
Total			**1631**	**1615**	**242**

IgG measurements in serum

IgG titres were measured in serum samples using V-PLEX SARS-CoV-2 Panel 6 Kit (IgG) from Meso Scale Discovery (MSD, MD, USA) according to the manufacturer instructions. IgG titres to the following antigens were measured: Nucleocapsid, RBD, Spike, Spike (D614G), Spike (B.1.1.7), Spike (B.1.351) and Spike (P.1).

Measurements were performed in randomized batches. Briefly, 96-well plates were blocked with MSD blocking buffer A for 30 minutes. All plates were then washed three times with PBS-Tween20 (0.05%). Samples were diluted 1:5,000 in Diluent 100 (MSD), loaded on the plates and incubated for two hours, after which the plates were washed three times again. Detection antibody with a sulfo-tag was added and after another one-hour incubation step plates were washed and read with MSD Gold Read Buffer B on the QuickPlex SQ 120 (MSD). Quantitative IgG results were measured in Antibody Units (AU)/mL, converted to WHO Binding Antibody Units (BAU)/mL using a conversion factor provided by MSD. The detection range is described in **Table 2**.

Stratification of IgG responses

Antibody responses were stratified into negative, inconclusive, low titre, medium titre or high titre based on the quantitative IgG measurements as described in **Table 2**.

Table 2: Stratification of quantitative IgG results.

	Negative	Inconclusive	Low	Medium	High	Units
anti-Spike	<4.76	4.76 - <53	53 - <241	241 - <832	>832	BAU/mL
anti-RBD	<5.58	5.58 - <45	45 - <205	205 - <817	>817	BAU/mL
anti-N	<8.20	8.20 - <12	12 - <295	295 - <713	>713	BAU/mL

The upper limit for “Negative” was determined as the average plus one standard deviation of anti-Spike IgG measurements in 50 serum samples collected before 2019. The lower limits for “Low”, “Medium” and “High” were based on the BAU/mL concentrations of “Low” (NIBSC code 20/140), “Mid” (NIBSC code 20/148) and “High” (NIBSC code 20/150) WHO International Standards for anti-SARS-CoV-2 immunoglobulins.

ACE2 neutralisation measurements in serum

ACE2 neutralisation was measured in serum samples using V-PLEX SARS-CoV-2 Panel 6, 13 and 23 Kit (ACE2) from Meso Scale Discovery (MSD, MD, USA) according to the manufacturer instructions. The antibodies capable of blocking the binding of ACE2 to the following antigens were measured: RBD, Spike, Spike (D614G), Spike (B.1.1.7), Spike (B.1.351), Spike (P.1), Spike (P.2), Spike (B.1.526.1), Spike (B.1.617), Spike (B.1.617.1), Spike (B.1.617.2), Spike (B.1.617.3), Spike (AY.4.2), Spike (B.1.1.529; BA.1), Spike (B.1.617.2; AY.4)

Measurements were performed in randomized batches. Briefly. 96-well plates were blocked with MSD blocking buffer A for 30 minutes. All plates were then washed three times with PBS-Tween20 (0.05%). All samples were diluted 1:50; loaded on the plates and incubated for one hour. ACE2 with a sulfo-tag was added and after another one-hour incubation step plates were washed and read with MSD Gold Read Buffer B on the QuickPlex SQ 120 (MSD).

The calibration curve was used to calculate neutralizing antibody concentrations in samples, by backfitting the measured signals for samples to the calibration curve. Neutralising antibody concentrations were measured in Units (U)/mL, which corresponds to neutralizing activity of 1 µg/mL monoclonal antibody to SARS CoV-2 Spike protein and reported as such. ULQ for the neutralizing antibody concentrations was 1,050 U/mL and LLQ was 0.26 U/mL.

Additionally, the results were reported as percent inhibition (% inhibition), calculated using the equation below:

$$\%\ Inhibition = \left(1 - \frac{Sample\ Signal}{Average\ Signal\ of\ the\ Blanc}\right) \times\ 100\%$$

All results have been submitted to ORCHESTRA REDCap and all corresponding viral variant results have been provided to the WP6 microbiological database.

Description of PBMC isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated according to ORCHESTRA protocol (WP6 prospective sample management Manual dated 04/11/2021) and frozen at -80°C in fetal bovine serum (FBS) with 10% DMSO before being transported on dry ice to University of Antwerp. On the day of procedure, PBMCs were thawed and rested overnight in RPMI 1640 medium (Gibco, Thermofisher Scientific, MA, USA) supplemented with 5% heat-inactivated AB serum (Sigma Aldrich), 100 U/ml penicillin (Biochrom, Berlin, Germany) and 0.1 mg/ml streptomycin (Biochrom). The cells were counted and 100,000 – 150,000 PBMCs per treatment condition were plated for stimulation. For stimulation, 2 pools of lyophilized peptides were utilized. The first pool was of 15-mer sequences with 11 amino acids (aa) overlap, covering the immunodominant sequence domains of the surface glycoprotein ("Protein S") of SARS-CoV-2 (Miltenyi Biotech, Leiden, Netherlands). The second pool was a 102 peptide sequence of the nucleocapsid phosphoprotein ("NCAP") of SARS-CoV-2 (JPT peptide technologies, Berlin, Germany). Both peptide pools were utilized at 1 µg/ml per peptide. Stimulation controls were performed with equal volumes of sterile water and 10% DMSO as unstimulated negative control. Cell Activation Cocktail, composed of PMA (Phorbol-12-myristate 13- acetate) and ionomycin (Bio Legend, Amsterdam, Netherlands) in DMSO was used as positive control. Incubation was performed at 37 °C, 5% CO2 for 2 hours after which 2 µg/ml brefeldin A (Bio Legend, Amsterdam, Netherlands) was added and further incubated for 4 hours before being collected for flow cytometry analysis.

Flow cytometry

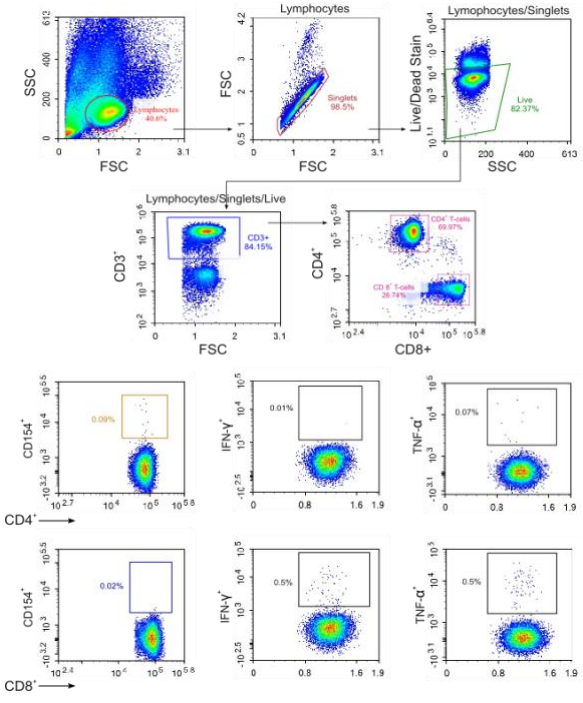

Figure 1. Gating strategy for analysis of flow cytometry.

After stimulation, cells were further stained with Zombie Aqua Fixable Viability Kit (BioLegend) for 15 min in the dark at room temperature. Cells were washed with cell staining buffer (PBS 1% bovine serum albumin, 2mM EDTA) unless stated otherwise. Cells were stained with surface antibody mixture including anti-CD3-APC Fire750, anti-CD4-FITC, anti-CD8a-BV570 and anti-CD154-APC (BioLegend) for 15 min at room temperature. Cells were washed with cell staining buffer and fixed/permeabilized using inside stain kit (Miltenyi Biotec) for 20 min in the dark at room temperature. Cells were washed with permeabilization buffer and stained with antibodies directed towards intracellular cytokines (anti-IFN-**γ**-PE and anti-TNF-α-PE/Cyanine7 (BioLegend) for 15 min in the dark at room temperature. Finally, cells were washed in cell staining buffer before analysis on NovoCyte Quanteon 4025 flow cytometer (Agilent, CA, USA), and analysed using FlowJo v10.8.1 (BD Biosciences, CA, SA). The gating strategy is shown in **Figure 1**.

T-SPOT.COVID assay

This commercially available EliSpot (enzyme-linked immunospot) assay (Oxford Immunotec Ltd) enumerates IFN-γ secreting SARS-CoV-2–specific T cells (both CD4+ and CD8+ cells), at single cell level, upon in vitro stimulation with viral peptides. In particular, two antigen peptide pools are used; one pool contains peptides from the spike S1 protein, including the receptor-binding domain, and the other contains peptides from the nucleocapsid protein.

Blood samples were collected in lithium heparin tubes. Peripheral blood mononuclear cells (PBMCs) were isolated according to ORCHESTRA protocol (WP6 prospective sample management Manual dated 04/11/2021) and then investigated according to the T-SPOT.COVID protocol. An aliquot of 2.5x105 T cells was incubated in the anti–IFN-γ antibody coated wells together with the viral peptides for 16-19 hours in a 5% CO2 incubator. In response to SARS-CoV-2 stimulation, T cells specifically release IFN-γ, which is then bound by the antibody coated to the well. Cytokine release is visualized by an enzyme-labeled detection antibody. The end results are blue spots, each of which represents the area of one cell secreting several molecules of IFN-γ. Coloured spots were counted using an automated EliSpot plate reader (AID EliSpot Reader System, Germany). A mitogen stimulation and a negative control were included to determine general T-cell responsiveness and background, respectively. Cells incubated with phytohemagglutinin (PHA-P) mitogen and with medium alone were used as positive and negative controls, respectively.

An EliSpot result was considered negative when the number of spot-forming cells (SFCs) in the wells stimulated with peptides (using the formula “sample − negative control”) was < 4, borderline when the number of SFCs was between 5 and 7, low positive for 8 - 20 SFCs and positive for > 20 SFCs. A negative result identified a patient without SARS-CoV-2–specific cell-mediated immunity (CMI) but with global T-cell responsiveness and a positive result identified a patient with detectable SARS-CoV-2 - specific CMI.

Results

Anti-Spike and anti-RBD titres consistently increased in SOT patients upon administration of COVID-19 vaccine and further increased after 3rd dose booster

Despite an immunocompromised status, 59% (95% CI [55, 62]) and 63% (95% CI [59, 66]) of SOT patients developed anti-Spike and anti-RBD titres, respectively, upon the administration of the 3rd dose of a COVID-19 vaccine. This number is higher than the initial 20% (95% CI [18, 22]) and 24% (95% CI [22, 26]) of patients who developed high anti-spike and anti-RBD titres after the 2nd dose. (**Figure 2, Table 3**).

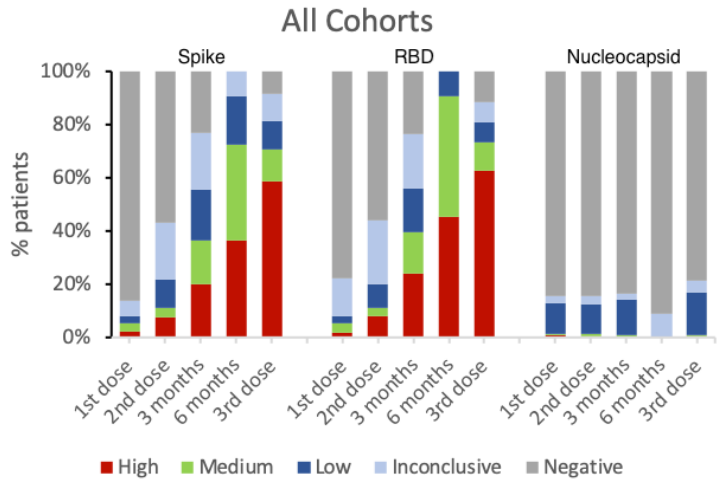

Figure 2. Combined serology results from all SOT cohorts

Table 3: Combined serology results from all SOT cohorts

Anti-Spike	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
1st dose*	938 (86%) [84, 88%]	65 (6%) [5, 8%]	31 (3%) [2, 4%]	30 (3%) [2, 4%]	27 (2%) [2, 4%]	1091
2nd dose*	600 (57%) [54, 60%]	229 (22%) [19, 24%]	113 (11%) [9, 13%]	35 (3%) [2, 5%]	82 (8%) [6, 10%]	1059
3 months*	272 (23%) [20, 25%]	256 (21%) [19, 24%]	230 (19%) [17, 22%]	195 (16%) [14, 19%]	238 (20%) [18, 22%]	1191
6M after 1st dose	0 (0%) [0, 0%]	1 (9%) [0, 41%]	2 (18%) [2, 52%]	4 (36%) [11, 69%]	4 (36%) [11, 69%]	11
1M after 3rd dose	67 (8%) [7, 11%]	79 (10%) [8, 12%]	85 (11%) [9, 13%]	94 (12%) [10, 14%]	467 (59%) [55, 62%]	792

Anti-RBD	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
1st dose*	849 (78%) [75, 80%]	155 (14%) [12, 16%]	28 (3%) [2, 37%]	37 (3%) [2, 5%]	22 (2%) [1, 3%]	1091
2nd dose*	590 (56%) [53, 59%]	258 (24%) [22, 27%]	94 (9%) [7, 11%]	32 (3%) [2, 4%]	85 (8%) [6, 10%]	1059
3 months*	278 (23%) [21, 26%]	244 (20%) [18, 23%]	197 (17%) [14, 19%]	186 (16%) [14, 18%]	286 (24%) [22, 27%]	1191
6M after 1st dose	0 (0%) [0, 0%]	0 (0%) [0, 0%]	1 (9%) [0, 41%]	5 (45%) [17, 77%]	5 (45%) [17, 77%]	11
1M after 3rd dose	91 (11%) [9, 14%]	59 (7%) [6, 10%]	60 (8%) [6, 10%]	85 (11%) [9, 13%]	497 (63%) [59, 66%]	792

Anti-Nucleocapsid	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
1st dose*	921 (84%) [82, 87%]	30 (3%) [2, 4%]	124 (11%) [10, 13%]	8 (1%) [0, 1%]	8 (1%) [0, 1%]	1091
2nd dose*	892 (84%) [82, 86%]	36 (3%) [2, 5%]	115 (11%) [9, 13%]	10 (1%) [0, 2%]	6 (1%) [0, 1%]	1059
3 months*	994 (83%) [81, 86%]	26 (2%) [1, 3%]	159 (13%) [11, 15%]	10 (1%) [0, 2%]	2 (0%) [0, 1%]	1191
6M after 1st dose	10 (91%) [59, 100%]	1 (9%) [0, 41%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	11

1M after 3rd dose	622 (79%) [76, 81%]	35 (4%) [3, 6%]	128 (16%) [14, 19%]	3 (0%) [0, 1%]	4 (1%) [0, 1%]	792

**Previously reported in Deliverable 6.6*

Cellular immunity in VICENZA SOT cohort 1 month after 3rd dose

In total 25 patients were included. Relative frequencies of CD3+, CD4+ and CD8+ cells are highlighted in **Table 4**.

Table 4. Relative frequencies of lymphocytes in SOT cohort after third dose.

Cell type	Relative Frequency (mean % ± SD)
CD3	84.6 ± 9.7%
CD4	45.9 ± 13.4%
CD8	39.4 ± 11.1%

CD4+ CD154+ activation following stimulation with nucleocapsid and spike peptides are graphically represented in **Figure 3**, data is presented as box and whisker plots, with individual data point overlay. The mean CD4+ CD154+ activation after stimulation with nucleocapsid and spike peptides, was 0.6% and 1.13%, respectively. IFN-γ+ activation after stimulation with nucleocapsid and spike peptides, was 0.10% and 0.32%. TNF-α+ activation after stimulation with nucleocapsid and spike peptides, was 0.21% and 0.67%. This data suggests that IFN-γ and TNF-α secreting CD4+ T cells in solid organ transplanted patients display responses 1 month after 3rd dose vaccination against SARS-CoV-2. However, longitudinal analysis over different timepoints in this group is needed for data comparison and interpretation.

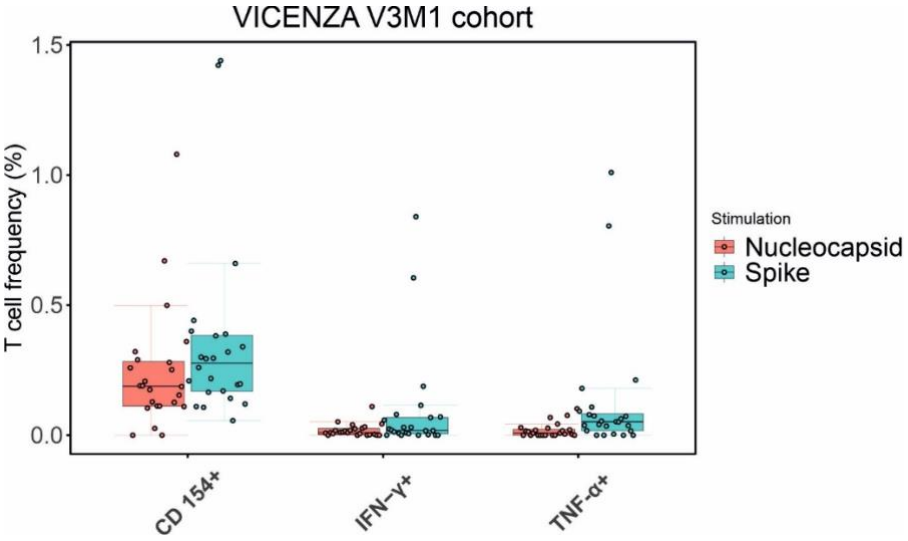

Figure 3. CD4+ T cell responses at 1 month after 3rd dose in SOT patients.

High Anti-Spike and anti-RBD titres in oncology patients upon administration of COVID-19 3rd dose booster

The majority of oncology patients developed high titres against spike (90%, 95% CI [81, 96]) and RBD (93%, 95% CI [85, 98]) 1 month after administration of 3rd dose booster.

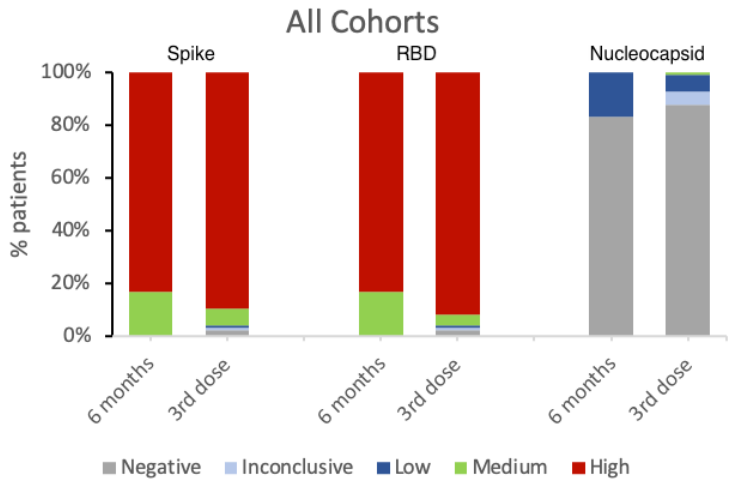

Figure 4. Combined serology results from all Oncology cohorts.

Table 5: Combined serology results from all Oncology cohorts

Anti-Spike	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
6M after 1st dose	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	1 (17%) [0, 64%]	5 (83%) [36, 99%]	6
1M after 3rd dose	2 (2%) [0, 0%]	1 (1%) [0, 7%]	1 (1%) [0, 7%]	6 (6%) [2, 15%]	88 (90%) [81, 96%]	98

Anti-RBD	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
6M after 1st dose	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	1 (17%) [0, 64%]	5 (83%) [36, 99%]	6
1M after 3rd dose	2 (2%) [0, 0%]	1 (1%) [0, 7%]	1 (1%) [0, 7%]	4 (4%) [1, 12%]	90 (92%) [85, 98%]	98

Anti-Nucleocapsid	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
6M after 1st dose	5 (83%) [36, 99%]	0 (0%) [0, 0%]	1 (17%) [0, 64%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	6
1M after 3rd dose	86 (88%) [85, 98%]	5 (5%) [0, 10%]	6 (6%) [1, 12%]	1 (1%) [0, 0%]	0 (0%) [0, 0%]	98

Anti-Spike and anti-RBD and titres in patients with breakthrough infection

Globally large-scale vaccination campaigns targeted at decreasing COVID-19 severity have been successful, however with new circulating variants of SARS-CoV-2, breakthrough infections, defined as SARS-CoV-2 infection 2 weeks after full vaccination, are a matter of concern. We observed

breakthrough infections (n=208) with the omicron viral variant in 15 (7.2%) patients. High anti-spike (67%, 95% CI [38, 88]) and anti-RBD (80%, 95% CI [52, 96]) titres were seen in patients identified with breakthrough infections with omicron variant whereas only 44% (95% CI [37, 51]) and 48% (95% CI [40, 55]) of patients with breakthrough infections with non-omicron variants show high anti-spike and anti-RBD titres, respectively. (**Figure 5**)

Table 6A: Serology results from UNIVR Breakthrough infections with the omicron viral variant.

Target	Timepoint	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
Anti-Spike	T0 breakthrough	1 (7%) [0, 3%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	4 (27%) [8, 55%]	10 (67%) [38, 88%]	15
Anti-RBD	T0 breakthrough	1 (7%) [0, 3%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	2 (13%) [2, 49%]	12 (80%) [52, 96%]	15
Anti-Nucleocapsid	T0 breakthrough	9 (60%) [32, 84%]	0 (0%) [0, 0%]	5 (33%) [12, 62%]	1 (7%) [0, 3%]	0 (0%) [0, 0%]	15

Table 6B: Serology results from UNIVR Breakthrough infections with other, non-omicron, viral variants.

Target	Timepoint	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
Anti-Spike	T0 breakthrough	10 (5%) [3, 9%]	11 (6%) [3, 10%]	29 (15%) [10, 21%]	58 (30%) [24, 37%]	85 (44%) [37, 51%]	193
Anti-RBD	T0 breakthrough	12 (6%) [3, 11%]	9 (5%) [2, 9%]	20 (10%) [6, 16%]	60 (31%) [25, 38%]	92 (48%) [40, 55%]	193
Anti-Nucleocapsid	T0 breakthrough	157 (81%) [75, 87%]	7 (4%) [1, 7%]	26 (13%) [9, 19%]	0 (0%) [0, 0%]	3 (2%) [0, 4%]	193

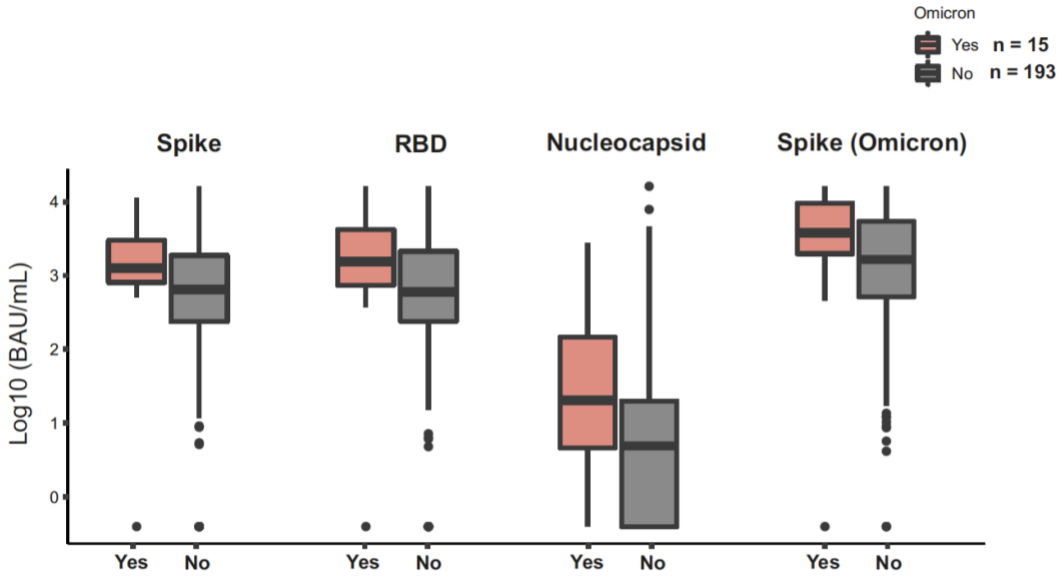

Figure 5. Serology results from UNIVR breakthrough infections with the omicron (pink) or other (grey) viral variant.

Cellular immunity in UNIVR Breakthrough Cohort

The presence of T cells reactive against SARS-CoV-2 was assessed by measuring the proportion of CD4+ and CD8+ T cells that express the activation markers CD154 (CD40L) and upregulation of cytokines IFN-γ along with TNF-α, after in vitro stimulation with the immunodominant region of spike and nucleocapsid proteins. In total 60 patients were included. The overall PBMC counts of the study group were low. Relative frequencies of CD3+, CD4+ and CD8+ cells are listed in **Table 7**.

Table 7. Relative frequencies of lymphocytes after breakthrough infection in study group.

Cell type	Relative Frequency (mean % ± SD)
CD3	76.1 ± 1.3%
CD4	62.0 ± 1.5%
CD8	26.3 ± 1.2%

CD4+ CD154+ activation following stimulation with nucleocapsid and spike peptides are graphically represented in **Figure 6**, data is presented as box and whisker plots (whiskers indicating SD), with individual data point overlay. The median CD154+ activation after stimulation with nucleocapsid and spike peptides, was 0.07% and 0.08%, respectively. IFN-γ+ activation after stimulation with nucleocapsid and spike peptides, was 0% for both. TNF-α+ activation after stimulation with nucleocapsid and spike peptides, was 0.03% and 0%.

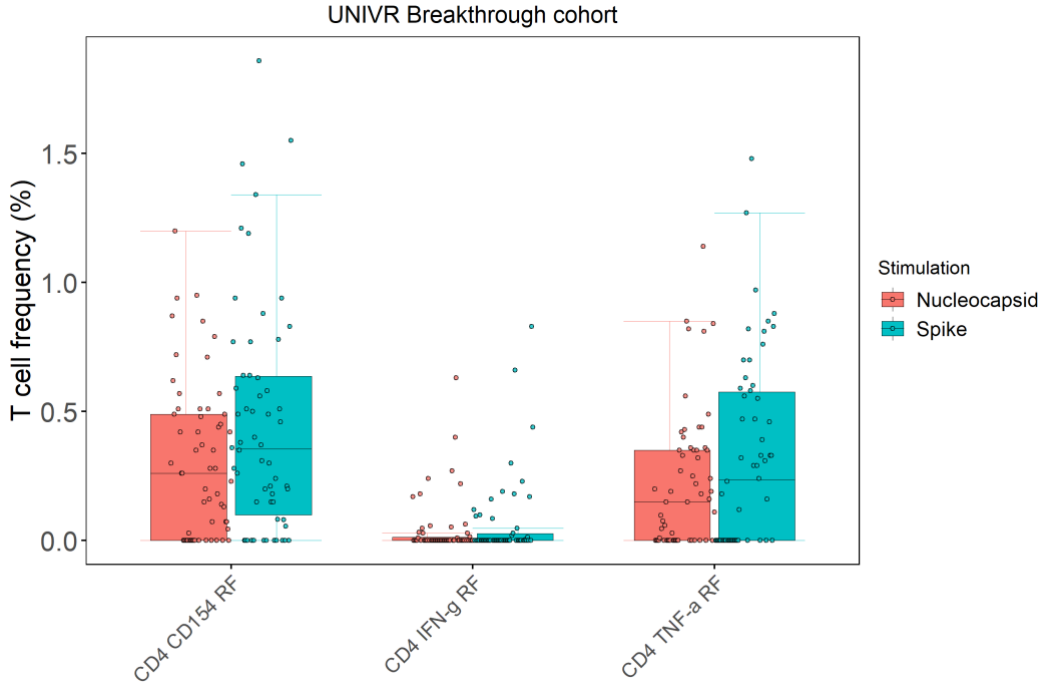

Figure 6. CD4 T-cell responses in UNIVR breakthrough cohort.

High Anti-Spike and anti-RBD and titres in patients infected with UNIBO omicron variant of SARS-CoV-2

Despite high anti-spike and anti-RBD titres, patients are still susceptible breakthrough infections with omicron.

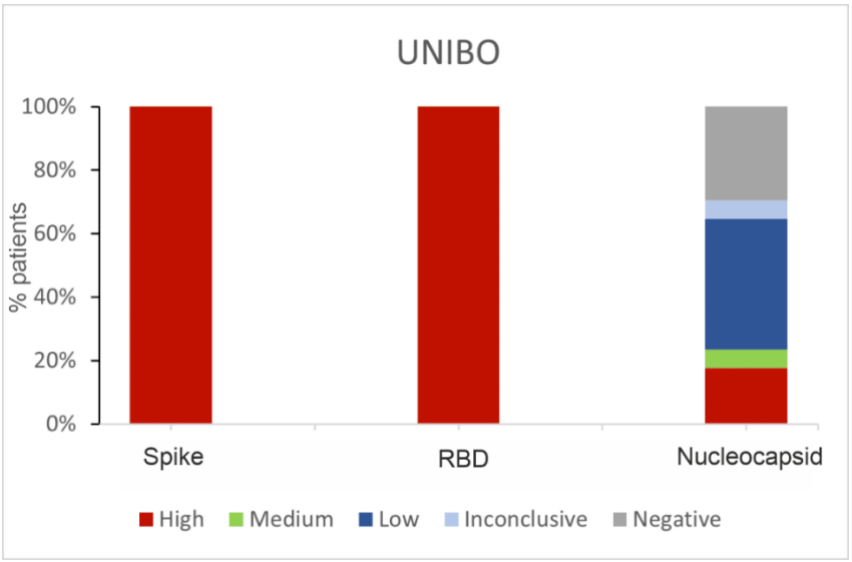

Figure 7. Serology results from UNIBO omicron cohorts.

Table 8: Serology results from UNIBO omicron cohorts

	Timepoint	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	High n (%) [95% CI]	Total number of patients
Anti-Spike	T0 Omicron	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	17 (100%) [80, 100%]	17
Anti-RBD	T0 Omicron	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	17 (100%) [80, 100%]	17
Anti-Nucleocapsid	T0 Omicron	5 (29%) [10, 56%]	1 (6%) [0, 29%]	7 (41%) [18, 67%]	1 (6%) [0, 29%]	3 (18%) [4, 43%]	17

Cellular immunity in UNIBO Omicron Cohort

In total 13 patients were included. Relative frequencies of CD3+, CD4+ and CD8+ cells are highlighted in **Table 9**. CD4 T-cells showed increased expression of CD154+ activation marker and expression of inflammatory markers - IFN-γ & TNF-α after stimulation with spike and nucleocapsid peptides. CD4+ activation following stimulation with nucleocapsid and spike peptides are graphically represented in **Figure 8**, data is presented as box and whisker plots (whiskers indicating SD), with individual data point overlay. The median CD154+ activation after stimulation with nucleocapsid and spike peptides, was 1.64% and 1.34% respectively. IFN-γ+ activation after stimulation with nucleocapsid and spike peptides, was 0.67% and 0.45% respectively. TNF-α+ activation after stimulation with nucleocapsid and spike peptides, was 0.87% and 0.97% respectively.

Table 9. Relative frequencies of lymphocytes in UNIBO omicron cohort

Cell type	Relative Frequency (mean % ± SD)

CD3	84.6 ± 33.4%
CD4	64.8 ± 27.2%
CD8	20.5 ± 13.9%

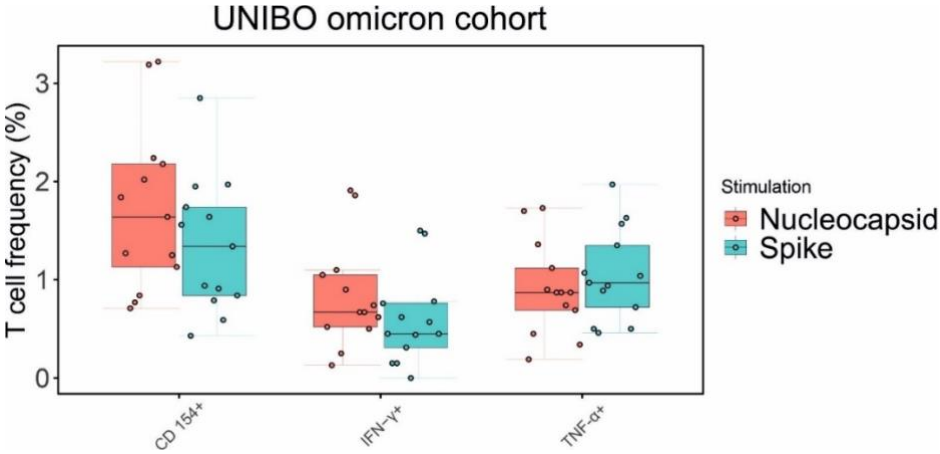

Figure 8. CD4+ T cell responses in UNIBO omicron cohort.

Monoclonal antibody therapy provides extended passive immunity and does not compromise natural immunity

Anti-spike and anti-RBD IgG levels reached up to 7 million BAU/mL after mAb treatments, and while the levels dropped slightly 7 days after administration (D7) and more so after D28, the levels were still on average >2.4 million BAU/mL for both anti-RBD and anti-spike at D28, suggesting that mAb therapy could potentially provide patients with sustained passive immunity for up to 1 month (**Figure 9**). Compared to the bamlanivimab alone, bamlanivimab/etesevimab combination performed better in neutralising B.1.526.1, B.1.617.1, B.1.617.2, B.1.617.3 and P.2 variants, although its ability to neutralize B.1.351 and P.1 variants remained low. The overall best neutralization for delta (B1.617.1, B.1.617.2, B.1.617.3) variants was observed in casirivimab/imdevimab treatment group (**Figure 10**).

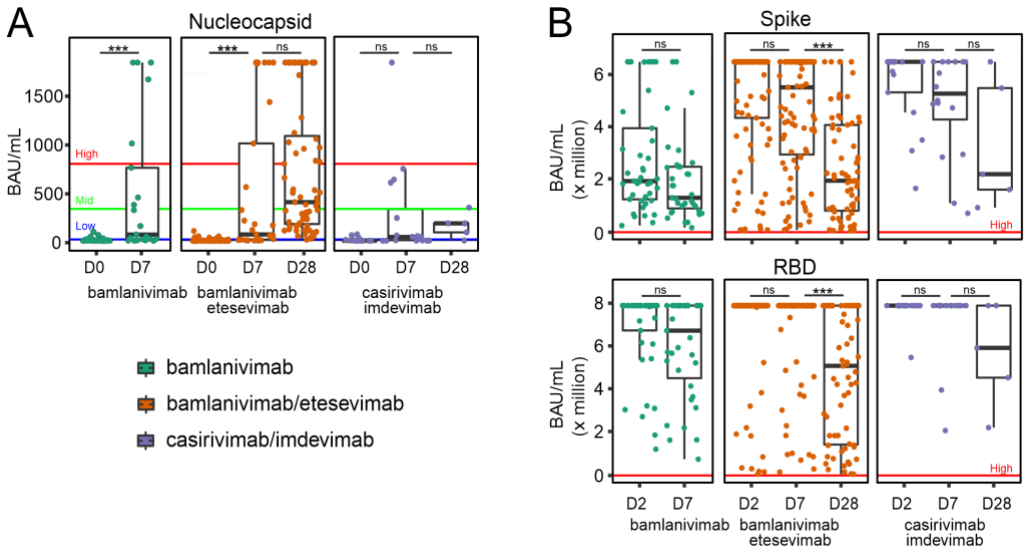

Figure 9. Serology of patients receiving mAb therapy. (A) Natural immunity assessment after day 7 and day 28 of mAb treatment. Anti-nucleocapsid measurements at day 7 and day 28 were compared with anti-nucleocapsid titres at time point before infusion. Coloured lines marked with Low, Mid, High indicate the SARS-CoV-2 WHO standards. (B) Synthetic mAb stability studied with antispike and anti -RBD measurements at day 2, day 7 and day 28 after mAb treatment on samples enumerated in A.

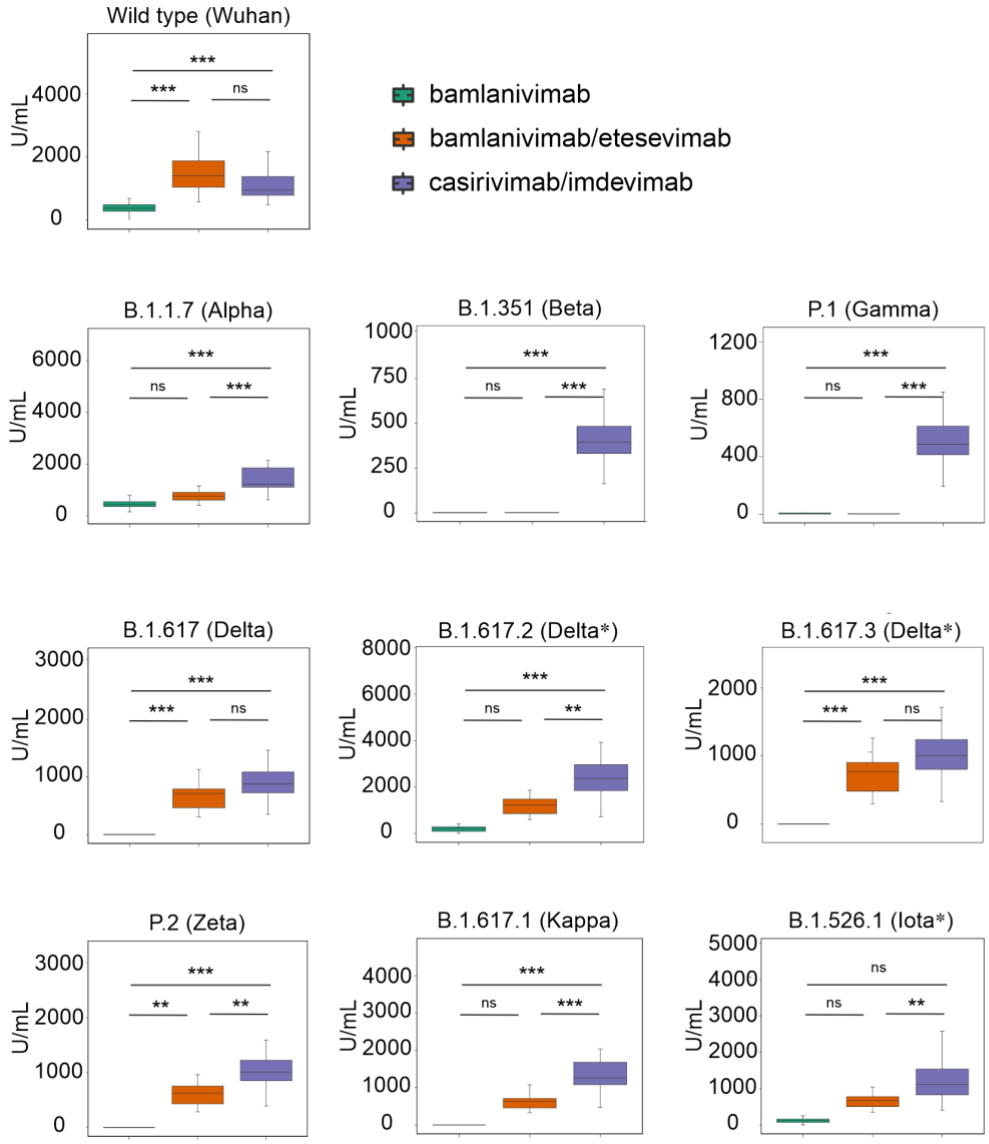

Figure 10. Seroneutralization in a sub-selection of patients receiving mAb therapy. Anti-RBD, Ant-Spike (WT, Wuhan) and Anti-Spike (B.1.1.7/alpha) measurements in patients treated with bamlanivimab (n=41 (D2) and n=35 (T2), bamlanivimab-etesevimab (n=97 (D2), n=91 (D7), n=67 (D28) and casirivimab-imdevimab (n=17 (D2), n=16 (D7), n=5 (D28). (D) Neutralizing antibody measurements against 10 different SARS-CoV-2 variants of concern (VOCs). Box plots indicate median (middle line), 25th, 75th percentile (box), and 5th and 95th percentile (whiskers). ***: P < 0.001. **: P < 0.010. *: P < 0.050. ns: non-significant.

CD4 T cell responses in patients receiving monoclonal antibody therapy were not different between the therapy groups

CD4+ T cells stimulated with SARS-CoV-2 nucleocapsid and spike peptides showed increased expression of CD154+, IFN-γ and TNF-α at D28 after administration of mAb therapy suggesting development of natural cellular immunity that was not different between the groups. CD4+ activation following stimulation with nucleocapsid and spike peptides are graphically represented in **Figure 11**, data is presented as box and whisker plots (whiskers indicating SD), with individual data point overlay.

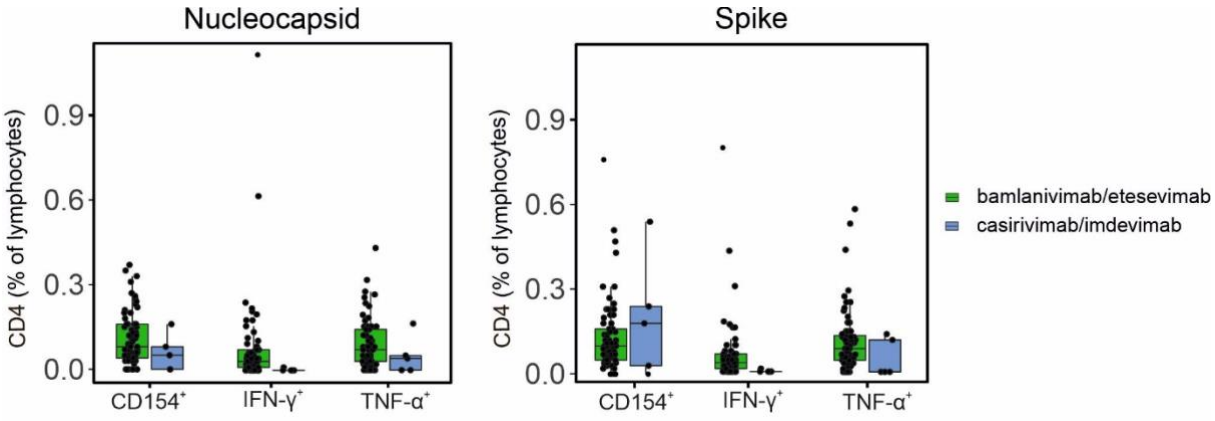

Figure 11. CD4 T cell responses of patients receiving bamlanivimab/etesevimab and casirivimab/imdevimab therapy

SARS-CoV-2–specific cell-mediated immunity in UNIBO WP4 SOT cohort

A total number of 118 patients was included in order to analyse SARS-CoV-2–specific CMI at 3 different time-points (**Table 10**). Some of these patients were analysed at more than one time-point.

Table 10. Number of samples included per types of SOT and time-points analysed

Time-point	Heart	Liver	Kidney	Lung	Total number of patients
2nd dose	4	8	6	3	21
3M after 1st dose	19	37	35	19	110
1M after 3rd dose	3	7	3	3	16

At 3 months after the 1st dose of vaccination 10% of patients (11/110) showed detectable SARS-CoV-2 Spike–specific T cell responses (i.e., low positive [8.2%] and positive [1.8%] EliSpot results); 3.6% had a borderline EliSpot result. Higher percentages of patients with positive (18.8%; 3/16) and borderline (12.5%; 2/16) EliSpot results were observed after the administration of 3rd dose booster vaccine compared to the previous time-points **(Table 11).**

Table 11. SARS-CoV-2–EliSpot results from UNIBO WP4 SOT cohort. All SARS-CoV-2 EliSpot results obtained at the 3 time-points are shown in **Figure 11**.

Time-point	Negative n (%) [95% CI]	Inconclusive n (%) [95% CI]	Low n (%) [95% CI]	Medium n (%) [95% CI]	Total number of patients
2nd dose	21 (100%) [84, 100%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	0 (0%) [0, 0%]	21
3M after 1st dose	95 (86%) [79, 92%]	4 (4%) [1, 9%]	9 (8%) [4, 15%]	2 (2%) [0, 6%]	110
1M after 3rd dose	11 (69%) [41, 89%]	2 (13%) [2, 38%]	3 (19%) [4, 46%]	0 (0%) [0, 0%]	16

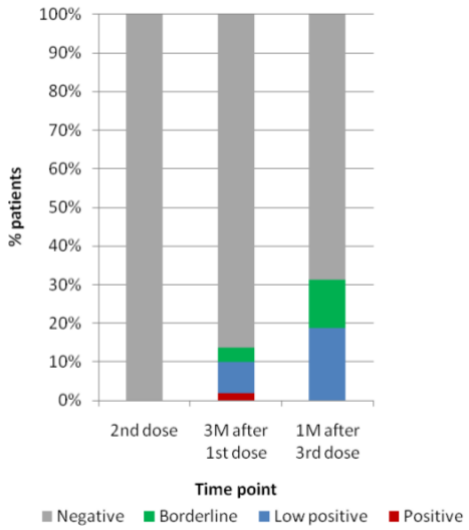

Figure 11. Graphic representation of the EliSpot results from UNIBO WP4 SOT cohort

Acknowledgments

The WP6 want to acknowledge the cohorts that provided the samples and associated clinical data:

UNIVR SOT Cohort (WP4)

UNIVR Oncology Cohort (WP4)

UNIVR Breakthrough Cohort (WP4)

Vicenza SOT Cohort (WP4)

Treviso SOT Cohort (WP4)

Padova SOT Cohort (WP4)

UNIBO Hospitalized Cohort (WP2)