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**Study of B-Cell Immune Memory in COVID-19 and
Post-Vaccinal Immunity**

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AUTHOR’S SUMMARY

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The dissertation consists of 160 pages and includes 7 tables and 29 figures. It is structured into 12 chapters. The reference list comprises 361 literature sources, the majority of which were published after 2020.

All investigations were carried out by the PhD student at the Department of Medical Microbiology and Immunology “Prof. Dr. Elisei Yanev”, Faculty of Medicine, Medical University – Plovdiv. The statistical analyses were performed at the Department of Social Medicine and Public Health, Faculty of Public Health, Medical University – Plovdiv, with the active participation of the PhD student.

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ABBREVIATIONS

| | |
|---|--|
| ADCC — Antibody-Dependent Cellular Cytotoxicity | MBC — Memory B Cells |
| AID — Activation-Induced Deaminase | MFI — Mean Fluorescence Intensity |
| BAU/ml — Binding Antibody Units per millilitre | MZB — Marginal Zone B Cells |
| BCR — B-Cell Receptor | NK — Natural Killer Cells |
| Breg — Regulatory B-lymphocytes | NCSMB — Non-Class-Switched Memory B Cells |
| CD — Cluster of Differentiation | PB — Plasmablasts |
| COVID — Coronavirus Disease | PBMCs — Peripheral Blood Mononuclear Cells |
| CSMB — Class-Switched Memory B Cells | PBS — Phosphate-Buffered Saline |
| CSR — Class-Switch Recombination | RBD — Receptor-Binding Domain |
| CVID — Common Variable Immune Deficiency | SARS — Severe Acute Respiratory Syndrome |
| ELFA — Enzyme-Linked Fluorescent Assay | SARS-CoV-2 — Severe Acute Respiratory Syndrome Coronavirus 2 |
| ELISPOT — Enzyme-Linked ImmunoSpot Assay | SFU — Spot-Forming Units |
| FACS — Fluorescence-Activated Cell Sorting | SHM — Somatic Hypermutation |
| GC — Germinal Center | TCR — T-Cell Receptor |
| IFN — Interferon | TLR — Toll-Like Receptor |
| IgA — Immunoglobulin A | TNF — Tumor Necrosis Factor |
| IgG — Immunoglobulin G | Treg — Regulatory T-lymphocytes |
| IgM — Immunoglobulin M | mRNA — Messenger Ribonucleic Acid |
| IL — Interleukin | |
| IQR — Interquartile Range | |
| LLPC — Long-Lived Plasma Cells | |

I. INTRODUCTION

Understanding and defining the concept of immune (immunological) memory is of central importance for modern immunology. In the early days of immunology as a distinct science, this concept referred to the phenomenon of resistance to re-infection with the same disease after recovery or vaccination. In the second half of the 20th century, immune memory came to denote the capacity for a faster, stronger and more specific immune response upon re-encounter with an antigen, reflecting the existence of clonally expanded antigen-specific lymphocytes. The latter part of this definition essentially mirrors the dogmatic view in immunology at that time that immune memory is a property solely of the acquired (adaptive) immune system, since only its cells can undergo somatic rearrangements of the genes encoding antigen receptors. For this reason, for decades it was assumed that immune memory is characteristic only of vertebrates and represents an evolutionary advantage most clearly demonstrated by the maternal-fetal transfer of immunoglobulins in mammals.

It is clear that immune memory preserves information on the type of antigen precisely through clonal proliferation of antigen-specific memory lymphocytes. Information regarding the route and portal of entry of infection is encoded in the degree of differentiation of memory lymphocytes and their tissue localization. It remains unclear whether and how other types of information about the pathogen (for example, the presence of particular molecular structures or the repetitiveness of epitopes) may also be retained. Not least, the question regarding the duration of persistence of memory lymphocytes in the organism remains open.

The two main paradigms embedded in the classic definition of immune memory – namely its restriction to the adaptive immune system and to vertebrate animals – have been challenged and refuted over the past two decades. It has been unequivocally demonstrated that features of immune memory are also characteristic of cellular elements of the innate immune system, although in this case they are epigenetically rather than genetically determined. Even more strikingly, forms of immune memory have been shown to exist in invertebrate animals as well as in other kingdoms of living organisms, albeit not always using mechanisms analogous to the adaptive immunity of vertebrates. In this sense, some immunologists even propose that immune memory should be considered not only at the level of the individual, but also in an evolutionary context.

Another key concept in immunology is that of protective immunity. It is not limited to quantitative measures of the immune response, such as specificity, speed of development or magnitude, but rather refers to its quality – namely the ability to provide immediate protection of the organism upon re-exposure to a pathogen. Protective immunity may be mediated by the presence of neutralizing antibodies, active effector cells or other components of the immune system that ensure rapid defense. It may wane over time, even if immunological memory is preserved.

Distinguishing between the mechanisms of persistent protective immunity and those mediating secondary memory immune responses is clearly of crucial importance both for the adequate assessment of an individual's immune status and for the development of effective vaccines and immunization schedules against infectious diseases, tailored to the dynamics of the epidemic process and the demographic and clinical characteristics of the population.

The COVID-19 pandemic, which erupted in 2020 and led to the rapid clinical deployment of various vaccines against SARS-CoV-2, against the backdrop of relatively widely accessible immunological analytical techniques, has enabled investigation of the development and dynamics of protective immunity and immune memory to an unprecedented degree and on a new methodological level.

II. AIM

To assess the dynamics and duration of the humoral and memory B-cell immune response after recovery from SARS-CoV-2 infection and after vaccination with mRNA vaccines against SARS-CoV-2.

III. OBJECTIVES

Objective 1: To evaluate the dynamics and duration of the humoral immune response by determining serological markers – SARS-CoV-2-specific RBD-IgG antibodies (BAU/ml) and virus-neutralizing antibodies (% inhibition).

Objective 2: To characterize the memory immune response by quantifying antibody-secreting cells using B-ELISpot.

Objective 3: To assess the T-cell immune response using an interferon-gamma-based test (T-Spot).

Objective 4: To characterize B-lymphocyte subpopulations by multiparameter flow cytometry.

Objective 5: To identify S1-specific memory B-lymphocytes by multiparameter flow cytometry.

IV. MATERIALS AND METHODS

1. Materials

We conducted a longitudinal, prospective study at the Department of Medical Microbiology and Immunology – "Prof. Dr. Elisey Yanev," Medical University of Plovdiv, during the period 2021-2023. Participants were enrolled before or at the onset of the relevant immunological events (SARS-CoV-2 infection or first vaccine dose), which allowed for baseline assessment and longitudinal follow-up. Whole peripheral blood was collected in 3x6 ml tubes with sodium heparin and 1x5 ml tubes for serum separation at four time points for vaccinated participants: T0 (baseline – at the time of administration of the second dose of the respective vaccine), T1 (1 month after the second dose), T2 (6 months after the second dose), and T3 (12 months after the second dose). For the group of previously infected individuals, biological material was collected at three time points: T1 (1 month after the onset of symptoms), T2 (6 months after the onset of symptoms), and T3 (12 months after the onset of symptoms). Peripheral blood mononuclear cells (PBMCs) and serum were isolated from all samples and stored at -80°C or transferred to a liquid nitrogen tank (-196°C) for long-term storage (until the relevant immunological tests were performed). The samples in the group of recovered patients were collected from hospitalized patients at the Infectious Diseases Clinic of St. George University Hospital in Plovdiv, while blood samples from vaccinated individuals were taken at the Department of Medical Microbiology and Immunology. The group of healthy controls included individuals who had not been infected and had not been vaccinated against SARS-CoV-2. Samples from this group were collected and stored in the period 2019-2020 and tested once. All participants completed an informed consent form, and the study was conducted in accordance with the ethical standards and principles of the Declaration of Helsinki.

1.1. Inclusion criteria and distribution by groups

The study included four well-defined groups of individuals to enable comparative analysis of immune responses elicited by natural infection and mRNA vaccination. Group 1 (n=157) included individuals who received the BNT162b2 mRNA vaccine (Pfizer-BioNTech) according to the standard two-dose schedule with a 21-day interval between doses. Group 2 (n=18) included individuals vaccinated with mRNA-1273 (Moderna), also administered in two doses according to the manufacturer's instructions and with a 28-day interval between doses. Participants in both vaccine groups had no documented history of previous SARS-CoV-2 infection, confirmed by medical records and serological screening prior to vaccination. Group 3 (n=110) consists of individuals who have recovered from SARS-CoV-2 infection, confirmed by positive RT-PCR. These participants were not vaccinated against SARS-CoV-2 before or during the study period and served as a reference group representing immunity acquired through natural exposure to the virus. Group 4 (n=18) consisted of individuals who had not previously been infected and had not been vaccinated against SARS-CoV-2 (healthy controls). The number of participants in each group for the specified time periods is as follows: for those vaccinated with BNT162b2: T0 (before vaccination, n=42), T1 (1 month after full immunization, n=128), T2 (6 months, n=78) and T3 (12 months, n=22), with new participants being monitored at each stage. The mRNA-1273 cohort is smaller, with T0 (n=6), T1 (n=13), T2 (n=9), and T3 (n=3). In the group of COVID-19 survivors, follow-up begins at T1 (n=54), followed by T2 (n=41) and T3 (n=33).

2. Methods

2.1. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral venous blood was collected into three 6-ml Vacutainer tubes containing sodium heparin as anticoagulant.

Samples were kept at room temperature (18–22°C) and processed within 24 hours of collection to ensure optimal cell viability. PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque™ PLUS ($\rho = 1.077$ g/ml; Cytiva, UK). Six milliliters of blood were carefully layered over 3 ml of Ficoll in a sterile 15-ml conical tube, taking care not to disrupt the interface. Centrifugation was performed at $400 \times g$ for 30 minutes at room temperature without brake to allow separation of blood components according to density. Four distinct layers were observed after centrifugation: plasma (top), a mononuclear cell ring, Ficoll, and erythrocytes (bottom). The mononuclear cell layer was collected and washed twice with PBS (without Ca^{2+} and Mg^{2+}) by centrifugation at 400 rpm for 15 minutes to remove residual proteins and Ficoll. Cell concentration and viability were assessed by Trypan Blue (0.4% in PBS) exclusion using an automatic cell counter LUNA™ III (Logos Biosystems, South Korea).

2.2. Cryopreservation of peripheral blood mononuclear cells (PBMCs)

Isolated and purified PBMCs that were not used immediately were subjected to cryopreservation to maintain their viability and functionality for subsequent analyses.

Cells were resuspended in 1 ml of chilled freezing medium consisting of 90% RPMI 1640 and 10% dimethyl sulfoxide (DMSO, $\geq 99.7\%$). DMSO was used as an intracellular cryoprotectant to prevent ice crystal formation. The suspension was aliquoted as 2-ml per cryovial (CryoTube™, Nunc), labeled with sample ID and date. Cryovials were placed in a controlled rate freezing

container (Mr. Frosty™, Thermo Fisher Scientific, MA, USA) filled with isopropanol to ensure a gradual temperature decrease of 1°C/min. After 24 hours at –80°C, some vials were transferred into a liquid nitrogen tank (–196°C) for long-term storage. To prevent thermal shock, the cryoprotective medium was added slowly by dropwise addition with continuous gentle mixing. For subsequent use, cells were rapidly thawed at 37°C, and DMSO was removed by dilution with pre-warmed PBS or RPMI 1640 followed by two washes (400 × g, 10 min). All procedures were performed under aseptic conditions in a Class II laminar flow hood, and handling of liquid nitrogen was carried out with appropriate protective gloves, goggles and lab coat.

2.3. Thawing of peripheral blood mononuclear cells (PBMCs)

Frozen cryovials containing PBMCs were thawed rapidly in a 37°C water bath for approximately 1 minute, until almost fully liquefied. After removal from the bath, 1 ml of pre-warmed (37°C) RPMI 1640 medium was added to each vial slowly along the tube wall to minimize osmotic and mechanical stress on the cells. The cell suspension was transferred to a sterile 15-ml conical tube, avoiding direct aspiration of the cell pellet to prevent membrane damage. Cryovials were rinsed with an additional 2 ml of pre-warmed RPMI 1640, which was also added to the tube. The resulting mixture was incubated for 5 minutes at 37°C in a water bath to facilitate complete resuspension and adaptation of the cells after cryostress.

Cells were then centrifuged for 10 minutes at 400 × g at room temperature to remove DMSO and other remnants of the cryoprotective medium. After discarding the supernatant, the cell pellet was resuspended in 1 ml of pre-warmed RPMI 1640. Cell concentration and viability were determined on a LUNA III cell counter after Trypan Blue staining (0.4% in PBS). Before immunophenotyping or culture, cells were allowed to rest at room temperature for approximately 1 hour to restore metabolic activity and stabilize the cell membrane.

2.4. Detection of anti-SARS-CoV-2-specific RBD-IgG by ELFA (Enzyme-Linked Fluorescent Assay)

SARS-CoV-2-specific RBD-IgG antibodies were measured using the VIDAS® SARS-CoV-2 IgG kit (bioMérieux, France), catalog no. 424114 (LOT: 1008826440), based on an enzyme-linked fluorescent immunoassay (ELFA). Serum samples were pre-centrifuged and stored at –80°C until analysis.

The solid phase receptacle (SPR) was pre-coated with recombinant SARS-CoV-2 RBD antigen. In the first step, IgG antibodies against SARS-CoV-2 in the sample bound to the immobilized antigen. This was followed by automatic washing of unbound components and addition of a conjugate – anti-human IgG antibodies linked to alkaline phosphatase. After a second wash, a specific fluorogenic substrate was added, which was hydrolyzed by the enzyme to a fluorescent product. Fluorescence was measured automatically at 450 nm by the VIDAS® PC instrument.

Results were interpreted by VIDAS® PC software and expressed as a fluorescence index (Relative Fluorescence Value, RFV).

Result interpretation:

- Index (i) < 1.00 – negative result; anti-SARS-CoV-2 RBD IgG not detected.
- Index (i) ≥ 1.00 – positive result; presence of specific IgG antibodies.

For quantitative assessment, RFV values were converted to BAU/ml (binding antibody units per milliliter) using the formula:

$$\text{BAU/ml} = \text{RFV index} \times 20.33$$

2.5. Determination of the degree of viral inhibition by ELISA

Neutralizing antibodies against SARS-CoV-2 were measured using the GenScript cPass™ SARS-CoV-2 Surrogate Virus Neutralization Test Kit, catalog no. L00847-C (GenScript Biotech, USA) (LOT: A210608). This is a blocking ELISA (sVNT ELISA) that mimics the specific interaction between viral RBD and the human ACE2 receptor without using live virus or cell culture. Serum samples were pre-centrifuged and stored at -80°C until testing.

Samples and controls were diluted 1:10 in the supplied sample dilution buffer. The diluted samples were incubated with HRP-conjugated RBD antigen (HRP-RBD) at room temperature for 30 minutes. The mixture was then transferred to a 96-well ELISA plate pre-coated with recombinant human ACE2 receptor and incubated for 15 minutes at 37°C . Unbound conjugate was washed away with PBST (PBS + 0.05% Tween-20) three times. TMB substrate was added to each well; it reacts with HRP to produce a blue color inversely proportional to the concentration of neutralizing antibodies in the sample. After 15 minutes of incubation in the dark, the reaction was stopped with stop solution (0.2 M H_2SO_4), and the color changed to yellow. Optical density at 450 nm was measured with a BioTek 800 TS (Agilent Technologies, USA) plate reader.

The percentage of inhibition was calculated as:

$$\% \text{ Inhibition} = (1 - (\text{OD sample} / \text{OD negative control})) \times 100\%.$$

Internal validation of the test required inclusion of a positive control ($\geq 50\%$ inhibition) and a negative control ($< 20\%$ inhibition) as a condition for assay validity.

2.6. ELISpot

2.6.1. B-SPOT

B-cell ELISpot was performed using the ELISpot Path: Human IgG (SARS-CoV-2, RBD) kit, catalog no. 3850-2H (Mabtech AB, Sweden) (LOT: 3850-4HPW-R1-1), according to the manufacturer's instructions. Previously isolated PBMCs were thawed at 37°C and resuspended in R10 culture medium containing RPMI-1640, 10% FBS, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) (Capricorn Scientific, Ebsdorfergrund, Germany). The concentration of viable cells was determined using an automatic cell counter (Luna II, Logos Biosystems, Anyang, South Korea), with viability $> 90\%$.

To activate and differentiate B-lymphocytes into antibody-secreting cells (ASC), cells were stimulated with a cocktail containing:

- R848 (1 $\mu\text{g/ml}$), a synthetic TLR7/8 agonist;
- IL-2 (10 ng/ml), a cytokine supporting B-cell proliferation, both supplied with the ELISpot kit.

Stimulated cells were incubated for 5 days at 37°C , 5% CO_2 in 24-well plates. At the end of the stimulation period, cells were centrifuged and resuspended in fresh R10 medium (Capricorn Scientific, Germany) and prepared for ELISpot analysis.

The cell suspension was seeded into pre-activated ELISpot plates coated with SARS-CoV-2 RBD antigen. Between 50,000 and 200,000 cells were added per well. Plates were incubated for 18–24 hours at 37°C and 5% CO_2 . After incubation, plates were washed five times with PBS to

remove non-adherent cells and components. For detection of secreted RBD-specific IgG, biotinylated anti-IgG antibodies were added, followed by streptavidin-conjugated alkaline phosphatase and a chromogenic substrate (BCIP/NBT) provided by the manufacturer. After the final incubation and washing, plates were air-dried at room temperature.

Spots corresponding to individual antibody-secreting B-lymphocytes were visualized and counted using an automated ELISpot reader BIOREADER® 7000-F (BIO-SYS GmbH, Germany). Results were expressed as the number of IgG-secreting cells per 10⁶ PBMCs, with background signals from control wells considered.

2.6.2. T-SPOT

SARS-CoV-2-specific T-cell responses were assessed using the commercial T-SPOT®.COVID kit (Oxford Immunotec Ltd., UK) (LOT: VEC7000000), based on the ELISpot technique for detection of interferon-gamma secreted by effector T cells. The concentration of previously isolated PBMCs was measured using an automated counter, and 250,000 viable cells were seeded per well.

Cells were plated onto ELISpot plates pre-coated with anti-IFN- γ antibody and stimulated with the following antigen panels supplied by the manufacturer:

- **Panel A:** containing SARS-CoV-2 spike protein, used to assess immune responses following vaccination or infection;
- **Panel B:** containing nucleocapsid protein, indicative of natural infection;
- **Positive control:** phytohemagglutinin (PHA), a non-specific mitogen to verify T-cell viability and functionality;
- **Negative control:** RPMI 1640 medium, to determine background reactivity.

Samples were incubated at 37°C and 5% CO₂ for 16–20 hours to allow specific T-lymphocytes to secrete IFN- γ upon antigen encounter. After incubation, plates were washed three times with PBS and a secondary anti-IFN- γ antibody conjugated to alkaline phosphatase was added. Following washing, a chromogenic substrate was added, resulting in dark blue spots at sites of IFN- γ secretion. Spots were counted using the automated ELISpot reader BIOREADER 7000-F (BIO-SYS GmbH, Germany). Results were expressed as spot-forming units (SFU), and control wells (PHA and negative) were used to validate the test.

2.7. Preparation of S1-specific tetramers for detection of specific memory B-lymphocytes

Two different S1-specific tetramers were prepared by conjugating biotinylated SARS-CoV-2 S1 protein to streptavidin labeled with fluorochromes BUV395 (BD Biosciences, catalog no. 564176, LOT 2069860) and BV421 (BD Biosciences, catalog no. 563259, LOT 2258281), following a modified protocol for tetramer formation used in B-cell immunophenotyping.

Initially, 100 μ g of biotinylated S1 protein were dissolved in 1 ml PBS. For long-term storage, 0.5 ml of this solution were mixed with 0.5 ml glycerol and stored at –20°C at a final concentration of 50 μ g/ml. For short-term storage, another 0.5 ml were mixed with 0.5 ml PBS, aliquoted into 20 tubes (50 μ l each) and stored at –80°C, also at 50 μ g/ml.

To prepare a working streptavidin solution, 5 μ l of a 100 μ g/ml stock were diluted in 45 μ l PBS to a final concentration of 10 ng/ μ l. Tetramer complexes were formed by mixing 3 μ l of S1 protein (50 μ g/ml) with 47 μ l PBS and, in a separate tube, 3.3 μ l of streptavidin solution (10 ng/ μ l)

with 47 μ l PBS. The streptavidin solution was then added stepwise to the S1 solution in five aliquots of 2 μ l each, at 10-minute intervals, at room temperature and in the dark. After the last addition, the mixture was incubated for 1 hour under the same conditions to ensure complete tetramer formation.

2.8. Multiparameter flow cytometry

Based on the expression of specific CD markers, a 13-color panel was developed for analysis of B-cell subpopulations. Using this panel, we identified nine subpopulations further subdivided by IgM and IgG expression.

Table 1. Antibodies against human leukocyte antigens and fluorochromes used for multiparameter flow cytometric analysis.

| Специфичност на mAb | Флуорохром | Клон | Производител | Каталожен номер | Партиден номер |
|---------------------|-------------|---------|-----------------|--------------------------------------|--------------------|
| CD27 | BV480 | M-T271 | BD, Biosciences | 746296 | 1119770 |
| CD19 | BV605 | SJ25C1 | BD, Biosciences | 562653 | 2115078 |
| CD138 | BV650 | MI15 | BD, Biosciences | 743500 | 2272095 |
| CD45 | BUV496 | | BD, Biosciences | 569101 | 2014198 |
| CD20 | BV786 | 2H7 | BD, Biosciences | 568713 | 2279931 |
| IgD | BB515 | LA6-2 | BD, Biosciences | 565243 | 2101107 |
| CD38 | PE | HIT2 | BD, Biosciences | 560981 | 1153295 |
| CD24 | PE-CF594 | ML5 | BD, Biosciences | 562405 | 1229319 |
| CD3/CD14/CD16/CD56 | PerCP-Cy5.5 | | BD, Biosciences | 340949, 561116, 565421, 560842 | 2151708 1200688 |
| IgG | PE-Cy7 | G18-145 | BD, Biosciences | 561298 | 4319686 |
| IgM | APC | G20-127 | BD, Biosciences | 551062 | 2082257 |
| CD21 | R781 | B-ly4 | BD, Biosciences | 658170 | 2279913 |
| LIVE/DEAD | APC-Cy7 | | BD, Biosciences | | |

To determine the working concentration of monoclonal antibodies, serial dilutions were prepared. Each dilution was used to stain 1×10^6 cells. Samples were acquired on a FACS Aria III and analyzed with FlowJo v10.8.1. For each concentration, the median fluorescence intensity (MFI) of positive and negative populations was measured and the staining index was calculated. Staining indices were plotted and the dilution with the highest index was selected to achieve optimal resolution and specificity. Figure 3 illustrates the example of the staining index for CD27. Table 5 presents the titer and working volume for each antibody used in the 13-color panel for PBMC staining.

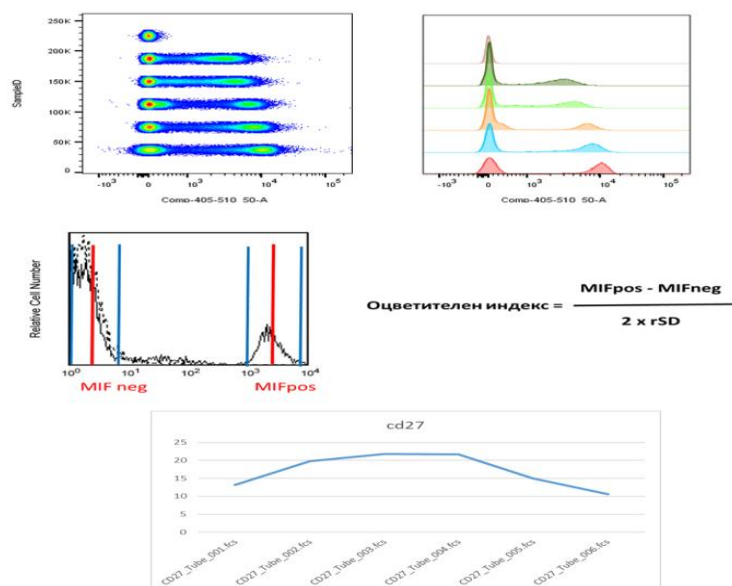


Figure 1. Determination of the CD27 staining index. The figure illustrates the approach for calculating the staining index by comparing the signals from CD27⁺ and CD27⁻ populations, showing dot plots and histograms, determining MFI values, and calculating the signal resolution formula.

Table 2. Working concentration of antibodies in the panel (μl per 1×10^6 cells). The table shows the optimized amounts of monoclonal antibodies needed to stain 1×10^6 cells used in multiparameter flow cytometric analysis.

| Parameter | Quantity per 1 sample in μl (1×10^6 cells) |
|-----------------|--|
| Staining Buffer | 35 μl |
| CD38 | 10 μl |
| IgM | 5 μl |
| IgG | 5 μl |
| CD19 | 2.5 μl |
| CD24 | 2.5 μl |
| IgD | 1.3 μl |
| CD3 | 1.3 μl |
| CD16 | 1.3 μl |
| CD21 | 1 μl |
| CD27 | 1 μl |
| CD138 | 1 μl |
| CD14 | 0.5 μl |
| CD20 | 0.5 μl |
| CD45 | 0.5 μl |
| SA BV711 | 1 μl |
| Live/Dead | 1 μl |

Transitional B-lymphocytes – CD19, CD20, CD24, CD38, IgD

Naïve B-lymphocytes – CD19, CD20, CD24, IgD, IgM

Mature B-lymphocytes

Memory B-lymphocytes – CD19, CD20, CD27⁺

Class-switched memory B-lymphocytes (CSMBs), IgG⁺ CSMBs, non-class-switched memory B-lymphocytes (NCSMBs); plasmablasts – CD19, CD27, CD38; plasma cells – CD27, CD38, CD138.

Table 3. 13-color panel for analysis of B-cell subpopulations with added S1 tetramers for detection of specific memory B-lymphocytes.

| mAb specificity | Fluorochrome | Clone | Manufacturer | Catalog number |
|--------------------------------|----------------|---------|------------------------|-----------------------------------|
| S1-Tetramer | BUV 395 | | BD, Biosciences | 564176 2069860 |
| S1-Tetramer | BV 421 | | BD, Biosciences | 563259 2258281 |
| “Decoy” Streptavidine | BV711 | | BD, Biosciences | 563262 1111072 |
| CD27 | BV480 | M-T271 | BD, Biosciences | 746296 |
| CD19 | BV605 | SJ25C1 | BD, Biosciences | 562653 |
| CD138 | BV650 | MI15 | BD, Biosciences | 743500 |
| CD45 | BUV496 | | BD, Biosciences | 569101 |
| CD20 | BV786 | 2H7 | BD, Biosciences | 568713 |
| IgD | BB515 | IA6-2 | BD, Biosciences | 565243 |
| CD38 | PE | HIT2 | BD, Biosciences | 560981 |
| CD24 | PE-CF594 | ML5 | BD, Biosciences | 562405 |
| CD3/CD14/ CD16/CD56 | PerCP-Cy5.5 | | BD, Biosciences | 340949, 561116, 565421, 560842 |
| IgG | PE-Cy7 | G18-145 | BD, Biosciences | 561298 |
| IgM | APC | G20-127 | BD, Biosciences | 551062 |
| CD21 | R781 | B-ly4 | BD, Biosciences | 658170 |
| LIVE/DEAD | APC-Cy7 | | BD, Biosciences | |

To identify S1-specific memory B-lymphocytes, biotinylated S1 protein conjugated with four streptavidin molecules was used. To increase specificity, two tetramers labeled with BV421 and/or BUV395 were included in the CD marker panel, together with a third “decoy” tetramer – streptavidin-BV711. Cells double-positive for SA-S1-BV421 and SA-S1-BUV395 and negative for the decoy streptavidin SA-BV711 were considered antigen-specific.

Cells for flow cytometric analysis were thawed as described above. The concentration of viable cells was determined with an automated cell counter. Fifty microliters of each tetramer were added to the cell suspension, followed by 30-minute incubation in the dark and washing with PBS (5 min, 400 × g). Cells were then stained with the antibody panel at working concentrations (15-minute incubation in the dark, followed by washing with PBS for 5 min at 400 rpm). Finally,

samples were fixed with 250 μ l Fix&Perm (BD Cytofix/Cytoperm) for 30 minutes at 4°C before acquisition.

Samples were acquired on a FACS Aria III flow cytometer using DIVA ver. 8 software. An analysis algorithm (gating strategy) was created to define B-cell subpopulations and SARS-CoV-2-specific memory B-lymphocytes and plasmablasts.

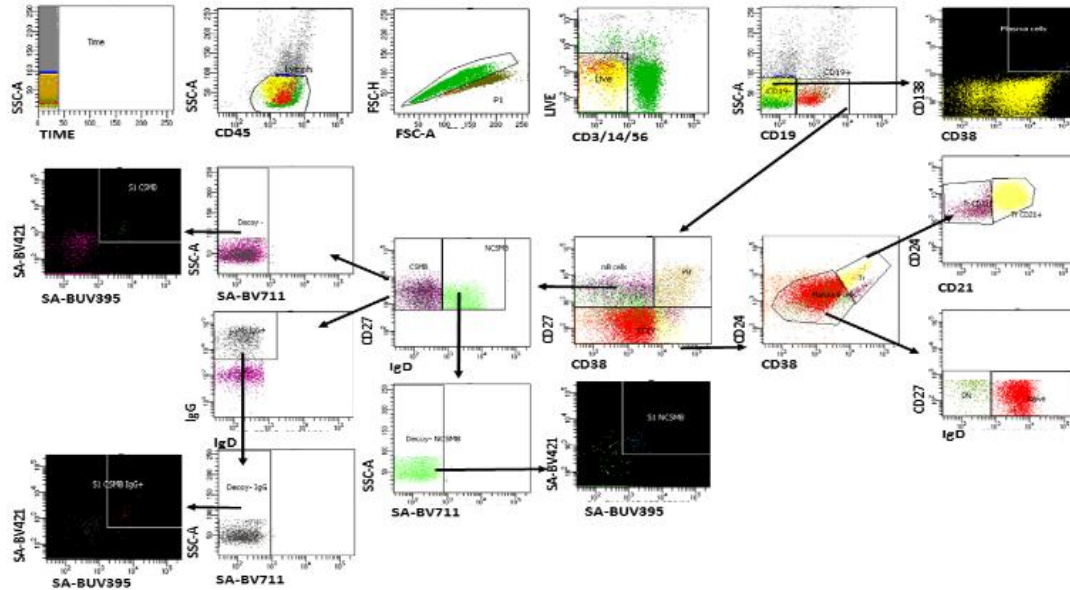


Figure 2. Algorithm (gating strategy) for analysis in DIVA ver. 8 software.

Initial gating included quality control based on time, selection of CD45⁺ lymphocytes (SSC-A vs CD45), singlet discrimination (FSC-H vs FSC-A) and exclusion of dead cells. CD19⁺ B-lymphocytes were identified after exclusion of CD14⁺ monocytes, CD3⁺ T-lymphocytes and CD56⁺ NK cells. Plasma cells were defined as CD19⁻CD138⁺CD38^{hi}. CD19⁺ B cells were further characterized according to CD27 and CD38 expression to distinguish plasma cells and memory B-cell subpopulations. Memory B cells (CSMB and NCSMB) were further subdivided based on CD27 and IgD expression, while CD21 and CD24 were used to better identify transitional B-lymphocytes. S1-specific memory B-lymphocytes were defined as double-positive for SA-S1-BV421 and SA-S1-BUV395 and negative for the decoy streptavidin SA-BV711. IgG⁺ S1-specific memory B-lymphocytes were identified within the class-switched memory subset.

2.8. Statistical analysis

Statistical analysis was performed using RStudio and SPSS (v.25). Normality of quantitative data was assessed by the Shapiro–Wilk test, followed by appropriate parametric or non-parametric methods. Data are presented as mean \pm SD for normally distributed variables or as median and interquartile range (Me, IQR) for non-normal distributions. Categorical variables are expressed as n (%).

Comparisons between independent groups were performed using the t-test or Mann–Whitney U test; for more than two groups, ANOVA or Kruskal–Wallis test with appropriate post-hoc corrections was applied. For repeated measures, paired t-test or Wilcoxon test were used, as well as repeated-measures ANOVA or Friedman test. Correlations were analyzed by Spearman’s ρ . Statistical significance was set at two-sided $p < 0.05$; in borderline cases ($p \approx 0.05$), clinical relevance and effect size were also considered.

V. RESULTS AND DISCUSSION

1. Demographic characteristics of the participants

1.1. Age distribution

Age distribution was analyzed separately for vaccinated participants, convalescents and healthy controls. Since the data did not follow a normal distribution, results are presented as median and interquartile range (IQR).

In the vaccinated group, the median age was 52 years, with an IQR of 20 years. Among convalescent COVID-19 patients, the median age was 47 years (IQR 19 years). In healthy controls, the median age was 48 years (IQR 15 years). These results indicate a relatively similar age structure across the groups, with slightly higher median age among vaccinated individuals. However, differences between groups fall within comparable interquartile ranges, suggesting a relatively homogeneous age distribution in the study sample. This facilitated comparability between groups when analyzing immunological parameters.

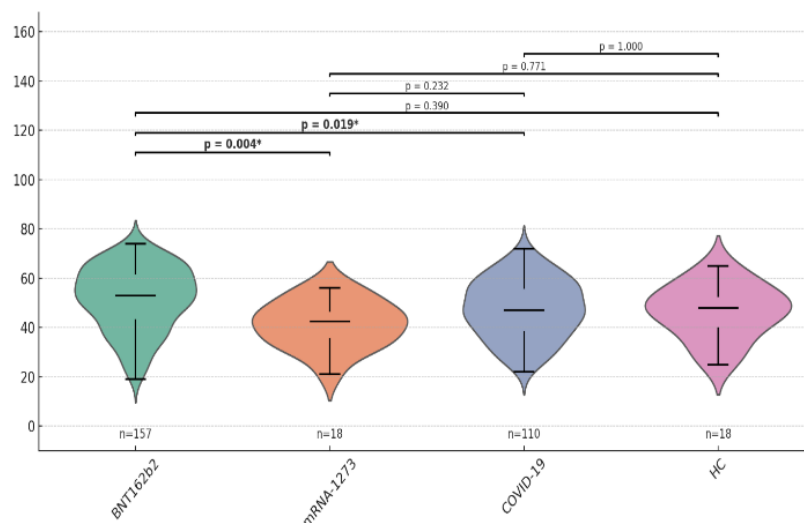


Figure 3. Distribution of participants by age. The analysis shows statistically significant differences between the BNT162b2 group and the mRNA-1273 ($p = 0.004$) and COVID-19 groups ($p = 0.019$). No significant differences were observed between the other groups ($p > 0.05$).

1.2. Sex distribution

In the vaccinated group, a total of $n = 175$ individuals participated, of whom $n = 120$ (68.6%) were women and $n = 55$ (31.4%) men. The convalescent COVID-19 group included $n =$

110 individuals, with n = 71 (64.5%) women and n = 39 (35.5%) men. The healthy control group consisted of n = 18 participants, of whom n = 10 (55.6%) were women and n = 8 (44.4%) men.

All study groups showed a predominance of women, which makes them comparable in terms of sex distribution for interpretation purposes.

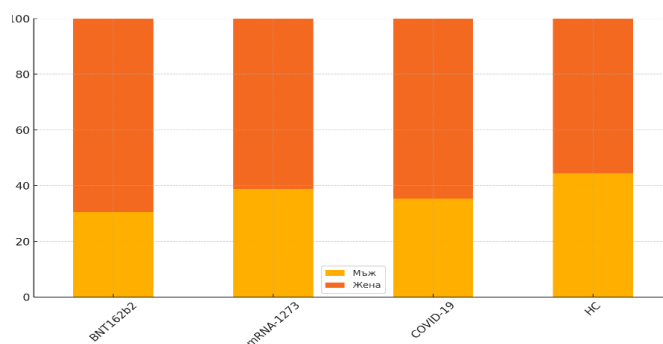


Figure 4. Distribution of participants by sex. The diagram illustrates the relative proportion of men and women in the BNT162b2, mRNA-1273, convalescent COVID-19 and healthy control groups. In all groups women predominate, most notably in the BNT162b2-vaccinated and COVID-19 groups.

Table 4. Distribution of participants by sex and age at the different time points. The table presents demographic characteristics of the BNT162b2, mRNA-1273 and convalescent COVID-19 groups, including age distribution, sex and number of participants at each follow-up time point.

| | Groups | | |
|------------------------------|-------------------|-------------------|-------------------|
| | BNT162b2 n=157 | mRNA-1273 n=18 | COVID-19 n=110 |
| Variables | | | |
| Age (years) (mean±SD) | 53.0, 19.5* | 41.4±10.9 | 47.4±11.8 |
| Sex, n (%) | | | |
| Males | 48 (30.6) | 7 (38.9) | 39 (35.5) |
| Females | 109 (69.4) | 11 (61.1) | 71 (64.5) |
| Time point 0, n (%) | 42 (26.8) | 6 (33.3) | 0 (0.0) |
| Time point 1, n (%) | 128 (81.5) | 13 (72.2) | 54 (49.1) |
| Time point 2, n (%) | 78 (49.7) | 9 (50.0) | 41 (37.3) |
| Time point 3, n (%) | 22 (14.0) | 3 (16.6) | 33 (30.0) |
| * median, IQR | | | |

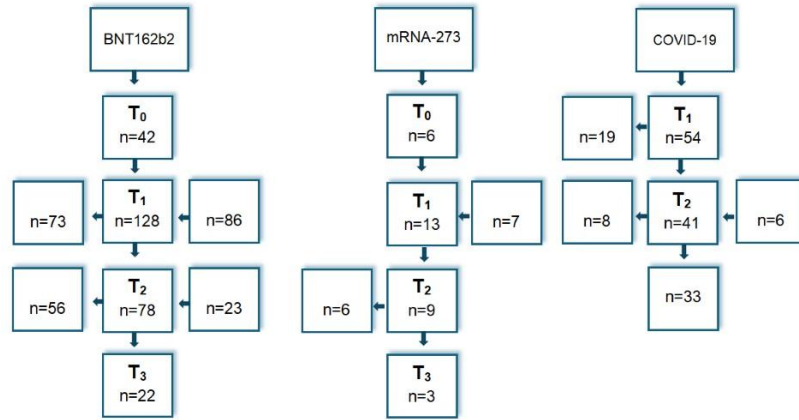


Figure 5. Distribution of participants in the study by time points. The diagram illustrates the number of participants in each of the three study groups at the different time points. For BNT162b2, participants were included at four time points: T₀ (before vaccination, n = 42), T₁ (1 month after full immunization, n = 128), T₂ (6 months, n = 78) and T₃ (12 months, n = 22), with some newly enrolled participants at later stages. The mRNA-1273 cohort was smaller, with T₀ (n = 6), T₁ (n = 13), T₂ (n = 9) and T₃ (n = 3). In the convalescent COVID-19 group, follow-up began at T₁ (n = 54), followed by T₂ (n = 41) and T₃ (n = 33). This distribution reflects the dynamics of recruitment and follow-up of participants.

2. Results for Objective 1.

2.1. Anti-SARS-CoV-2 RBD IgG

The dynamics of the humoral immune response were assessed by quantitative measurement of IgG antibodies against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein. Measurements were performed at four time points – T₀ (baseline), T₁ (1–2 months after the second vaccine dose), T₂ (6–7 months) and T₃ (12–13 months) in participants vaccinated with BNT162b2 or mRNA-1273; at three time points (T₁–T₃) in convalescent patients; and once in the control group. Healthy controls maintained levels below the detection limit and were not included in comparative analyses. Due to non-normal distribution, data are presented as median and IQR.

Before the second dose (T₀)

T₀ corresponds to the moment before administration of the second vaccine dose, i.e. during an ongoing but not yet completed vaccination course. It reflects the early phase of the humoral response after the first dose.

In participants who had received a first dose of BNT162b2, the median RBD-IgG level was 221.34 BAU/ml (IQR 330.56 BAU/ml). Values varied considerably – from low levels to concentrations close to the upper limit of the scale, reflecting individual differences in the initial response to the antigen. The group that had received a first dose of mRNA-1273 showed a lower median of 95.15 BAU/ml (IQR 215.87 BAU/ml), with a characteristic right-skewed distribution. This suggests that the immune response after the first dose is still in an early stage, although individual cases with higher reactivity are present.

These results demonstrate that a single dose of mRNA vaccine is not sufficient to achieve a maximal humoral response and that the full effect develops after completion of the vaccination schedule.

Peak response (T1)

One month after the second vaccine dose, both vaccinated groups showed a statistically significant increase in RBD-IgG antibodies ($p < 0.001$), reaching a peak at T1.

For BNT162b2, the median was 568.64 BAU/ml (IQR 303.89 BAU/ml), indicating a high immune response in most participants. The distribution was relatively symmetrical, suggesting a homogeneous serological profile post-vaccination.

For mRNA-1273, the highest median was observed – 671.89 BAU/ml (IQR 469.74 BAU/ml). Despite similar central tendency, this group exhibited a wider distribution, reflecting a broader individual range from moderate to very high values exceeding 1100 BAU/ml.

In the convalescent group, the median level was 448.84 BAU/ml (IQR 548.75 BAU/ml) – lower than in vaccinated participants, but with a much broader distribution, including values from 0 to over 1000 BAU/ml. This confirms the well-known heterogeneity of the natural immune response, which likely depends on disease severity and individual factors.

T2 – 6 months after exposure

At T2, all groups showed an expected decline in antibody levels, reflecting the decrease of circulating RBD-IgG, while measurable titers persisted in most participants.

Among BNT162b2-vaccinated individuals, there was a statistically significant decrease ($p < 0.001$) with a median of 137.84 BAU/ml (IQR 207.52 BAU/ml) – substantially lower than at T1, although some participants still exhibited high titers up to 764 BAU/ml. The distribution was strongly right-skewed, indicating clustering of values in the lower range.

In the mRNA-1273 group, a decline was observed but without statistical significance ($p = 0.216$): median 187.64 BAU/ml (IQR 312.78 BAU/ml), with better preserved serological activity at six months. The distribution was flatter, possibly reflecting a more robust and stable immune memory.

In convalescent COVID-19 patients, a statistically significant reduction was also recorded ($p = 0.002$), with a median of 133.57 BAU/ml (IQR 197.72 BAU/ml) – close to values in the BNT162b2 group but with a wider range. This again confirms the individual variability of the natural immune response.

T3 – 12–13 months after exposure

By the third time point, a more pronounced decline in RBD-IgG levels was noted, especially in the BNT162b2 group.

For BNT162b2, the median dropped to 71.56 BAU/ml (IQR 222.88 BAU/ml), and some participants had zero levels. This emphasizes the substantial reduction of circulating antibodies compared with T2 ($p < 0.001$).

For mRNA-1273, the median remained relatively high at 203.54 BAU/ml, with no zero values. Although an IQR could not be calculated due to the small number of observations, all values were in the medium to high range, indicating more sustained serological activity.

In convalescents, the median was 97.99 BAU/ml (IQR 198.73 BAU/ml), with a strongly right-skewed distribution – some individuals retained high antibody titers, while others had

undetectable RBD-IgG. This pattern suggests durable but heterogeneous humoral memory after natural infection. A statistically significant decline compared to T2 was observed ($p = 0.001$). The humoral response against SARS-CoV-2 is characterized by rapid generation of neutralizing antibodies after exposure, whether through vaccination or natural infection. In our study, IgG antibody levels against RBD peaked shortly after immunization (after the second dose of the vaccine) or shortly after the acute phase of infection, followed by a decline in the months thereafter. Our results show that by the sixth month after immunization with two doses, anti-RBD IgG titers decline sharply, although in most individuals they remain above the seropositivity threshold. The typical kinetic behavior after vaccination is biphasic: an initial rapid decline (with a calculated half-life of 2–4 months) and a subsequent more gradual decline.

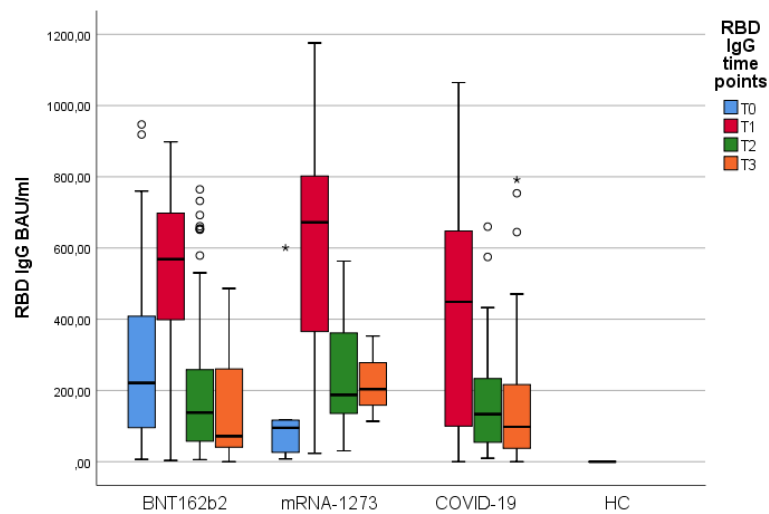


Figure 6. Distribution of anti-RBD IgG by groups at the respective time points. The figure illustrates the dynamics of the RBD-IgG antibody response over time in BNT162b2- and mRNA-1273-vaccinated individuals and in convalescents. A clear peak at T1 is followed by a statistically significant decline at T2 and T3, while seropositivity is largely preserved. The boxes represent the interquartile range, the horizontal line – the median; the whiskers indicate the minimum and maximum detectable values. Outliers are marked with circles.

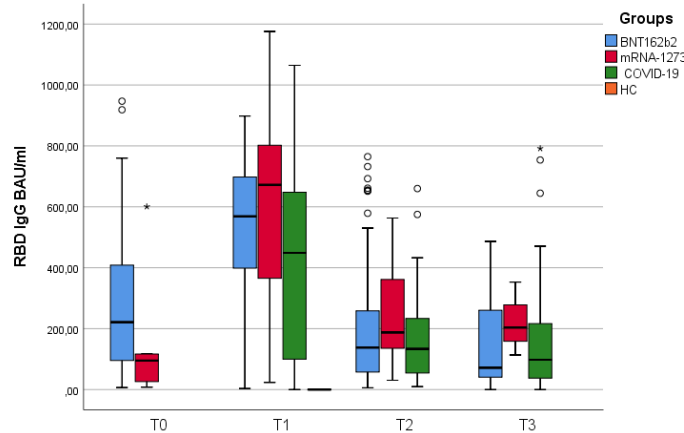


Figure 7. Comparison of anti-RBD IgG levels between groups at the respective time points. The figure presents the between-group comparison of RBD-IgG levels at T1, T2 and T3. The highest antibody levels are observed in the mRNA-1273 group at all time points, followed by BNT162b2. Convalescents show lower median values, but with a markedly broader dispersion, indicating that natural immunity is characterized by greater individual variability.

No statistically significant differences in mean anti-RBD IgG levels were observed between the different study periods.

Our study shows that mRNA-1273 induces higher and more persistent levels of RBD-specific antibodies compared to BNT162b2. Six months after vaccination with mRNA platforms, the humoral immune response is stronger in individuals who received mRNA-1273 compared to those who received BNT162b2, both in terms of antibody quantity and functional activity. The reported quantitative and functional differences in the humoral response between the two vaccinated groups can be explained by biological, pharmacological, and immunological factors. One of the most important is the different dose of antigen delivered: the mRNA-1273 vaccine contains 100 µg of mRNA per dose, while BNT162b2 contains 30 µg – more than a threefold difference. The increased antigen dose leads to longer and more intense expression of the S protein in vivo, which can generate stronger activation of follicular T-lymphocytes and a longer germinal reaction in the lymph nodes. This creates conditions for more effective affinity maturation of B-lymphocytes and selection of B lymphocyte clones with higher affinity to RBD epitopes – a mechanism that would explain the observed higher avidity and functional efficacy of antibodies in individuals vaccinated with mRNA-1273. The interval between the two doses of mRNA-1273 is longer – 28 days, compared to 21 days for BNT162b2, which could favor the maturation of germinal centers, leading to a better-formed secondary humoral response with longer-lasting serological persistence. The composition of the lipid nanoparticle used to encapsulate the mRNA may also be of additional importance. mRNA-1273 contains SM-102, and BNT162b2 contains ALC-0315. Although both formulations are effective, variations in particle stability, pharmacokinetics, and the degree of translation in different tissues may affect immunogenicity. Immunization with both mRNA vaccines leads to a potent humoral response with a high peak at

T1, followed by a decline in levels at T2 and T3. mRNA-1273 shows a higher median and more sustained values at later time points, while BNT162b2 shows a steeper decline and a higher proportion of participants with a waning response at 12 months. Previously infected individuals showed the widest individual variability, reflecting the nature of natural immunity. Such heterogeneity should be considered when modeling immune protection at the population level and when planning booster vaccinations.

2.2. Results from the assessment of virus-neutralizing antibody

The percentage inhibition of the interaction between the receptor-binding domain (RBD) of SARS-CoV-2 and the human ACE2 receptor was used as an indirect measure of the functional activity of neutralizing antibodies. The analysis was performed at the same time points for the four groups: individuals vaccinated with BNT162b2 or mRNA-1273, individuals who had previously had COVID-19 (unvaccinated), and healthy controls. In the control group, the results were below the limit of detection and were not included in the comparisons. Due to the lack of normal distribution, the results are presented as median and interquartile range (IQR).

At baseline T0, before the second dose of the vaccine was administered, BNT162b2 participants had a median inhibitory activity of 63.26% (IQR 47.98%), suggesting baseline immune reactivity in some of them. The mRNA-1273 group had a lower median of 44.29% (IQR 74.52%), with a wider distribution, reflecting heterogeneity in baseline immune status. Minimum values of 0% were observed in both groups, indicating a lack of neutralizing antibodies in some of the participants.

In T1, corresponding to the peak immune response after completion of the vaccination course or one month after recovery, there was a significant increase in the percentage of virus-neutralizing antibody inhibition. In those vaccinated with BNT162b2, the median reached 91.90% (IQR 13.61%), in those vaccinated with mRNA-1273 – 96.13% (IQR 29.89%), and in those who had previously had the disease – 91.25% (IQR 24.05%). The high values in all groups demonstrate effective induction of functional antibodies, both from vaccines and from natural infection. The lower variability in the BNT162b2 group suggests a more homogeneous response, while the wider interquartile range in mRNA-1273 and those who had previously been infected suggests greater individual variability. At time point T2 (six months), an expected decrease in neutralizing activity was observed. Despite the decline, all three groups—BNT162b2 (median 75.10%, IQR 39.63%), T1 vs T2 ($p < 0.001$); mRNA-1273 (median 78.90%, IQR 27.59%); and previously infected (median 73.40%, IQR 39.43%), T1 vs T2 ($p = 0.038$). High residual levels of functional antibodies are maintained. The data confirm the presence of a stable humoral response during the first six months. The highest median and smallest IQR are again reported for mRNA-1273, suggesting a sustained and more concentrated response in this group. In T3, there is an additional gradual decrease in serum neutralizing activity. In participants immunized with BNT162b2, the median falls to 47.90% (IQR 49.71). In comparison, the group of COVID-19 survivors maintained a high median of 81.02% (IQR 48.83%), indicating a more sustained natural immune response at this time point.

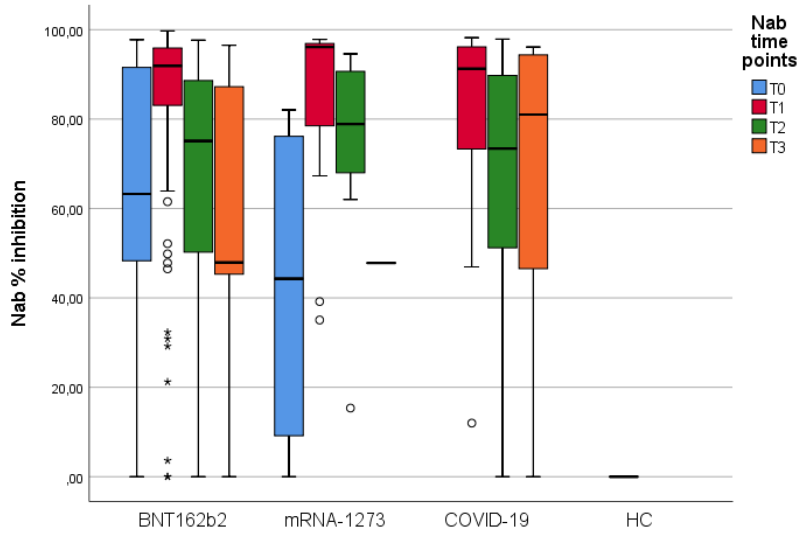


Figure 8. Degree of inhibition of virus-neutralizing antibodies by group in the respective periods. A distinct peak in T1 and a gradual decrease in T2 and T3, with higher values observed in the later periods in those who had previously been ill. During T2, the highest median and lowest IQR were reported for mRNA-1273.

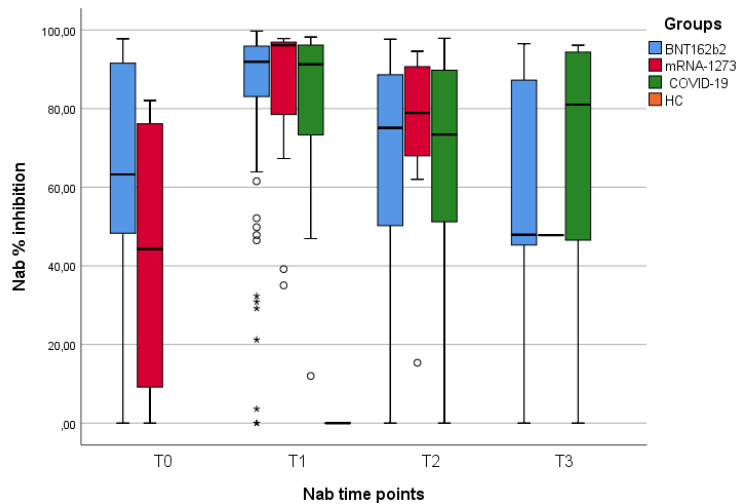


Figure 9. Comparison of the degree of inhibition of virus-neutralizing antibodies between groups in individual periods.

No statistically significant differences between the mean anti-RBD IgG values in the individual study periods were reported.

mRNA vaccines induce antibodies directed only against the spike protein, but in extremely high concentrations and with high neutralizing capacity. In the context of our three groups, this

means that those who have had the disease (mostly with mild to moderate symptoms) have lower peak RBD-IgG values than those who have been vaccinated, although their antibodies may persist for a relatively longer time (due to prolonged antigenic stimulation during infection). Some studies suggest precisely this—although the peaks after vaccination are higher, antibodies after infection may be characterized by a longer functional half-life. Ultimately, in both types of immunity, a significant weakening of the humoral component is observed 6–9 months after antigen exposure. Epidemiologically, this coincides with the emergence of breakthrough infections around this period. Previous infection reduces the risk of reinfection by 80–90% within 6–9 months, after which protection against infection wanes. Similarly, vaccine efficacy against symptomatic infection declines significantly after the 6th month. Based on growing evidence of a gradual decline in the humoral immune response after primary vaccination against SARS-CoV-2, international health institutions such as the CDC, ECDC, and WHO have issued consensus recommendations for the introduction of booster doses. The ECDC currently recommends booster doses, especially for older adults and vulnerable groups, due to the waning of neutralizing antibodies and the ongoing evolution of the virus.

3. Results from Objective 2

The assessment of cellular immunity, of antibody-synthesizing B-lymphocytes, is essential for understanding long-term protection after vaccination or infection with SARS-CoV-2. The number of spot-forming units (SFUs) recognizing the RBD of the virus was monitored using the ELISpot method. The results are presented as the number of RBD-specific antibody-synthesizing B-lymphocytes per 1,000,000 PBMC in each sample. The data were analyzed at four time points: T0 (before the second dose of vaccine), T1 (1 month after completion of the immunization course or recovery from illness), T2 (6 months), and T3 (12 months) for vaccinated participants and three time points for those who had previously had the disease.

- T0 – before the second dose of vaccine

This time point reflects the early cellular response after the first dose of vaccine. The predominant immune response is still in its initial phase. In the BNT162b2 group, the median RBD-specific antibody-producing B-lymphocytes is 14 SFU (IQR 26). Values between 0 and 42 were reported, with moderate positive asymmetry. This indicates partial activation of memory B-lymphocytes after the first dose in some participants. The mRNA-1273 group showed a median of 11 SFU, with a maximum value of 24. The absence of IQR (due to a limited number of observations) does not allow for a more accurate assessment of the dispersion, but the individual values suggest similar early immune activation. At this time point, no previously ill individuals were included.

- T1 – peak B-cell response

At this stage, the peak of B-cell memory is measured – after the second dose in vaccinated individuals or after recovery in those who have previously had the disease. BNT162b2 leads to a median of 150 SFU (IQR 171.5), with a maximum of 395 SFU; T1 vs T0 ($p < 0.001$). The distribution is moderately asymmetric and heterogeneous, indicating a high but individually variable response. For mRNA-1273, the median is 167.5 SFU (IQR 210.25), and the maximum reaches 433 SFU; T1 vs T0 ($p < 0.001$). A stronger positive asymmetry is observed, reflecting the presence of a subgroup with a particularly high response. Previously infected participants reached

a median of 129.5 SFU (IQR 99) – lower than the vaccinated participants, but within the expected strong post-infectious response. The control group showed no RBD-specific reactivity (all values = 0), which validates the specificity of the method.

- T2 – 6 months after immunization/infection

At six months, a physiological decrease in B-cell activity is observed.

The BNT162b2 group showed a median of 41.5 SFU (IQR 68.75), with a wide range and the presence of zero values, indicating a decline in response in some participants; T1 vs T2 ($p < 0.001$). mRNA-1273 demonstrated a more preserved response, with no statistically significant decrease – median 103 SFU (IQR 91.5), with a wider range of values and slight asymmetry towards higher values. Those who had previously been infected maintained a median of 60 SFU (IQR 95.75), with marked interindividual variability; T1 vs T0 ($p = 0.001$). Some had persistently high levels, highlighting the long-term effect of natural infection.

T3 – 12 months

At the latest follow-up point, a further decrease in B-cell reactivity was observed. For BNT162b2, the median was 21 SFU (IQR 35), with several cases below the detection threshold. The distribution is slightly asymmetrical but stable. mRNA-1273 shows a median of 40 SFU, with maximum values up to 55 SFU. Although the lack of IQR complicates the analysis, individual values indicate more preserved B-cell memory compared to BNT162b2. Those who had previously been infected showed the highest median – 64.5 SFU (IQR 76.5); T2 vs T3 ($p = 0.001$). The distribution is symmetrical and uniform, suggesting that in some participants, the memory B-cell response is effectively maintained for up to 12 months after infection.

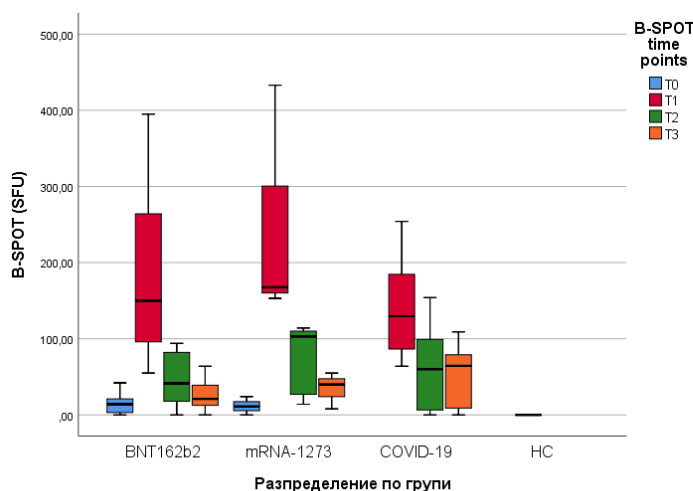


Figure 10. B-ELISpot results by group in the respective periods. The figure shows the dynamics of RBD-specific antibody-producing B-lymphocytes, with a marked peak in T1 after vaccination or infection, followed by a gradual decrease in their frequency in T2 and T3, with a more sustained response in mRNA-1273 and those who had previously been ill at later time points.

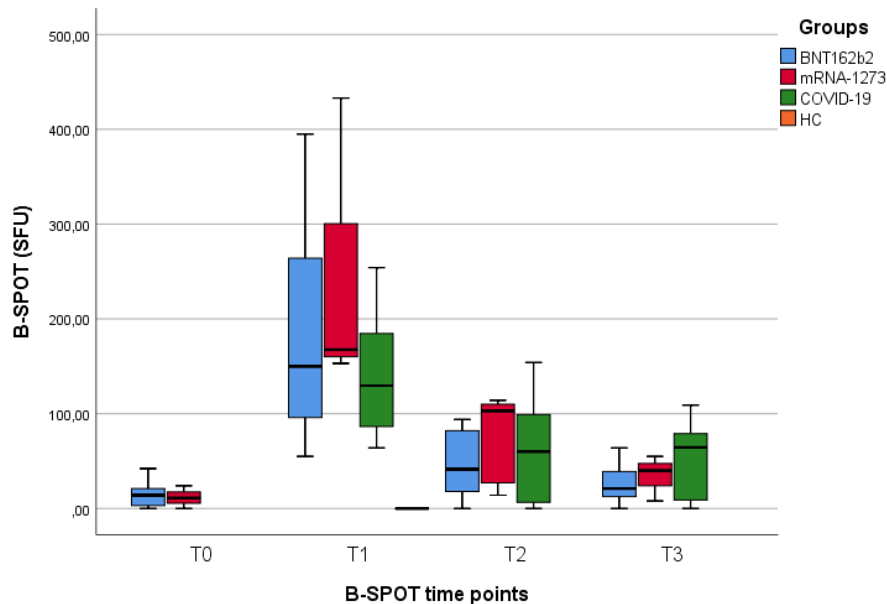


Figure 11. Comparison of B-ELISpot results between groups in individual periods.

No statistically significant differences between the mean SFU values in the individual study periods were reported.

Analysis of SARS-CoV-2-specific antibody-producing cells by ELISpot showed that:

Immunization with mRNA vaccines leads to a strong peak in B-cell memory, with a greater amplitude in mRNA-1273.

Six and twelve months later, a physiological decline is observed, but residual reactivity is maintained in most cases.

Previously infected individuals showed more sustained but lower peak in response, which persists long-term in some of them.

Both vaccinated and previously infected individuals show individual heterogeneity, which must be considered when assessing the need for booster doses and the duration of immune protection.

4. Results from Objective 3

During the study, the T-cell response was assessed using T-SPOT analysis, measuring the number of spot-forming units (SFU) in response to peptide stimulation in two different panels – Panel A (S peptide) and Panel B (N peptide). The analysis covered three main groups: vaccinated individuals (two different mRNA platforms), individuals who had previously had COVID-19, and healthy controls. The results are presented at time points – T1 (peak response), T2 (6 months), and T3 (12 months after vaccination or infection).

- Time point T1: Peak T-cell response

At T1, reported as the peak response after completion of the vaccination course, significantly elevated SFU values are observed in Panel A in all vaccinated participants. In individuals vaccinated with BNT162b2, the median SFU in Panel A reached 78 SFU (IQR: 110.5),

while results in the mRNA-1273 group showed a similar median of 91.5 SFU (IQR: 113). Among those who had previously been infected, the response was lower – 23 SFU (IQR: 64), although there were some high values. As expected, the control group showed no activity (0 SFU).

In Panel B, which measures response to N-peptide, there is a significant difference between the groups. In vaccinated individuals, values remained zero with single low values – median 2 SFU (IQR: 3) and 1.5 SFU (IQR: 3.75), while as expected in previously infected individuals, the median reached 25 SFU (IQR: 61), reflecting the expanded antigenic spectrum activating T cells after natural infection.

- Time point T2: Six months

At T2, a decline in T-cell response was observed in all groups. In vaccinated individuals, the median in Panel A decreased for BNT162b2 to 19 SFU (IQR: 45), T1 vs T2 ($p=0.46$) and 29.5 SFU (IQR: 86.5) for mRNA-1273, respectively. A reduction was observed in those who had previously been infected, but it remained relatively stable at 23.5 SFU (IQR: 26.5), suggesting longer-lasting T-cell immunity after infection.

In Panel B, vaccinees maintained a low, absent response – 2 SFU (IQR: 3) and 4 SFU (IQR: 10.5). In the group of previously infected individuals, the median remains elevated – 23 SFU (IQR: 33.25), showing a sustained response to N antigens after six months.

- Time point T3: 9–12 months

At the last follow-up point T3, the T-cell response in Panel A continues to decline. In the vaccinated group, the median drops to 14.5 SFU (IQR: 12.5), while the previously infected group maintains a moderate response – 17 SFU (IQR: 33.5). Although the absolute values are low, the presence of a response in the previously infected group suggests the existence of memory T-lymphocytes with a longer lifespan.

In Panel B, those who had previously been ill remained at a median of 9 SFU (IQR: 14.5). These results again demonstrate the broader epitope coverage and persistence of the T-cell response in individuals with naturally acquired immunity.

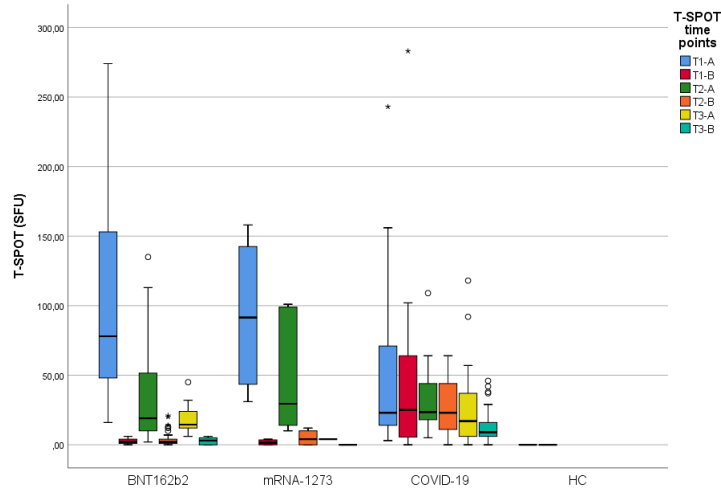


Figure 12. T-ELISpot results by group in the respective periods. The figure reflects the dynamics of the T-cell response, assessed by SFU in the panels for S and N peptides, with a distinct peak in T1, followed by a decline in T2 and T3, with the previously ill maintaining a more sustained and broader response than the vaccinated.

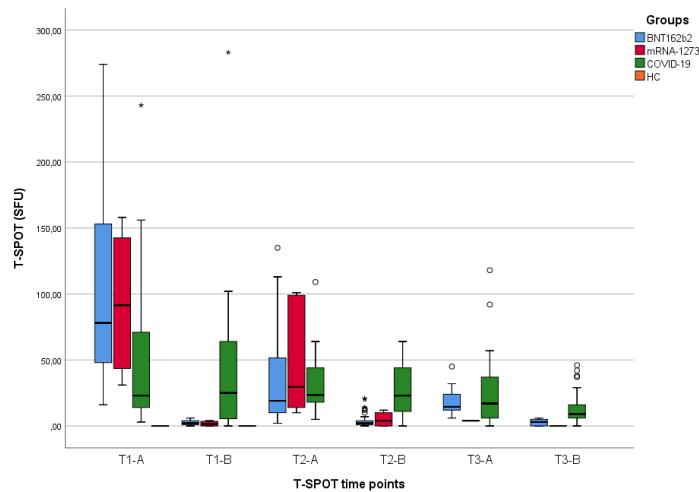


Figure 13. Comparison of B-ELISpot results between groups in individual periods.

Only in T1 were statistically significantly higher values found in the vaccinated group compared to those who had previously had the disease (p=0.021).

The T-SPOT analysis revealed a distinct peak in T-cell response after full vaccination (T1), followed by a gradual decline during T2 and T3. Regardless of the vaccine, by month 12 the T-cell response was weakened but still measurable, confirming the presence of immunological memory in the participants. While vaccination induces a strong response to S1-dominant peptides (panel A), natural infection elicits a more balanced and sustained response to other antigens as well (panel

B). This is reflected in the higher SFU values in Panel B at all time points for those who have had the disease.

Our study also focuses on the duration and dynamics of the T-lymphocyte immune response to SARS-CoV-2 after vaccination and natural infection, with the results showing some difference in the immune response. Both vaccination and natural infection lead to the formation of T-cell memory, which remains measurable over time, despite the observed decline in the number of specific T-lymphocytes after the peak response. These data confirm the importance of T-cell immunity for long-term protection against COVID-19, which is becoming increasingly critical with the emergence of new variants of the virus. Our observations of those vaccinated with mRNA vaccines show that the peak T-cell response after full vaccination is pronounced, with the response declining at 6 and 12 months but remaining measurable. These results are consistent with studies that also demonstrate that vaccine-induced T-cell responses, despite a significant decline over time, continue to provide protection against severe disease even with new variants of the virus. Studies show that six months after vaccination with mRNA vaccines, CD4⁺ T-cell responses and CD8⁺ T-cell responses remain stable, highlighting the durability of vaccine-induced T-cell memory.

The results from the group of recovered patients show that the T-cell response is longer lasting and less prone to weakening over time compared to vaccinated individuals. Our data demonstrate that six months and one year after infection, spike protein-specific T-lymphocyte levels remain significantly higher in those who have had the disease compared to those who have been vaccinated. This is consistent with studies showing that in recovered individuals, the T-cell response not only remains longer, but also expands to include different antigens of the virus, offering protection against new variants. A study by Dan et al. reports that in recovered individuals, the T-cell response to SARS-CoV-2 remains stable up to 8 months after infection, with about 92% of people retaining memory CD4⁺ T-lymphocytes and about 50% retaining memory CD8⁺ T-lymphocytes against the virus. This is particularly important in the context of a constantly mutating virus, as the broad spectrum of the T-cell response in recovered patients may provide greater protection against new variants of SARS-CoV-2.

Comparing the T-cell response in vaccinated and previously infected individuals confirms that hybrid immunity (combining vaccination and natural infection) can provide a stronger and longer-lasting T-cell response. Hybrid immune responses lead to a broader and more durable T-cell memory that includes not only a response to the spike protein but also to other viral antigens that are not subject to mutations in the new variants of the virus. This provides a basis for the assumption that hybrid immunity may be more effective in protecting against emerging variants of SARS-CoV-2, such as Delta and Omicron, which have shown evolution in the spike protein, making the humoral response less effective, while the T-cell response continues to provide protection.

T cells provide protection against severe forms of the disease and can prevent viral replication by eliminating infected cells. Although antibodies play an important role in the short term, when their levels begin to decline, the T-cell response remains critical for preventing severe disease and reducing viral load. Our results support these observations, showing that even after a significant decline in antibody levels, memory T-lymphocytes continue to circulate and provide

protection. This is important in the context of new variants of the virus that can escape neutralizing antibodies but not T-cell immunity.

5. Results from Objective 4

Multiparametric flow cytometry allows precise identification of different subpopulations of B-lymphocytes based on their phenotype, surface marker expression, and antigen specificity. In the present study, a standardized gating strategy (Figure 4) was applied to select live CD19⁺ B-lymphocytes after excluding other lymphoid populations (T cells, NK cells, and monocytes) and dead cells. This allowed for the assessment of the relative distribution of B-cell subpopulations as a percentage of live lymphocytes in each study group and time point, as well as additional analysis of antigen-specific (S1) and class-switched memory cells. *Разпределение на CD19⁺ клетки.*

In those vaccinated with BNT162b2, the proportion of CD19⁺ lymphocytes showed some fluctuations over time. Before completion of the vaccination course (T0), the median is 6.07% (1.00–57.66%), with values decreasing to 5.69% (1.40–18.66%) in the first month after the second dose (T1). At T2, a similar median is observed – 5.58% (2.09–27.18%), while at 12 months (T3) there is a slight increase to 6.89% (3.04–27.18%). In those vaccinated with mRNA-1273, the distribution is more stable, with a median of 9.39% (5.75–10.55%) at T0, which decreases to 6.79% (3.99–7.80%) at T1. At T2, there was a decrease to 5.33% (3.79–14.48%), and at T3, a median of 7.93% (2.71–8.36%). In individuals who had previously had COVID-19, CD19⁺ cells peaked at T1 – 6.03% (1.13–98.16%), after which the median remained relatively stable: 6.27% (2.28–44.07%) at T2 and 6.41% (2.40–20.46%) at T3.

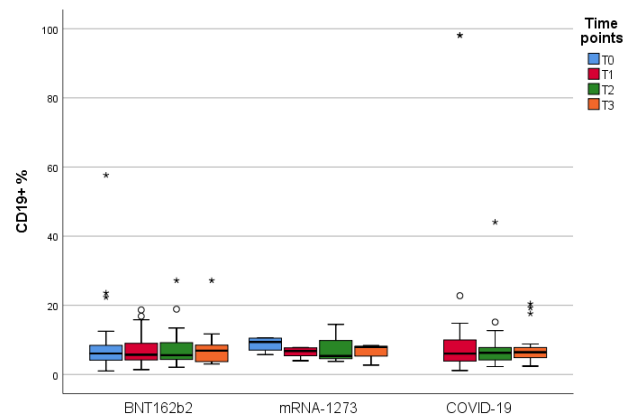


Figure 14. Distribution by groups of % CD19⁺ cells represented by the lymphocyte population. The figure shows the dynamics of CD19⁺ B-lymphocytes over time, with moderate fluctuations observed in all groups, without significant changes.

Tracking the relative proportion of CD19⁺ B-lymphocytes over time demonstrated similar dynamics across the different groups. Regardless of the vaccine (BNT162b2 or mRNA-1273), CD19⁺ cells undergo a moderate decrease in relative frequency after the peak of the immune response (T1), likely due to redistribution of cell subpopulations (e.g., transition to plasma cells or tissue migration). Of interest is the higher peak of CD19⁺ lymphocytes in T1 in previously infected

individuals, which may reflect activation of naive cells and heterogeneous reactivation during the convalescent period.

No statistically significant differences were found between the groups for the respective periods.

Distribution of memory B cells (mB cells).

The memory B cell pool in those vaccinated with BNT162b2 starts at a median of 25.26% (9.30–49.18%) at T0. After full vaccination, the values gradually increase: T1 – 27.39% (4.56–58.88%), T2 – 32.74% (8.08–64.34%). At T3, a decrease to 18.64% (10.48–57.89%) was reported. In those vaccinated with mRNA-1273, memory B-lymphocytes had a median of 31.09% (21.82–37.32%) at T0. At T1, a similar value was observed – 26.16% (19.14–50.31%), while at T2, the highest median was recorded – 39.58% (32.51–53.26%). At T3 – 28.38% (17.04–38.34%). Participants who had previously been ill showed the greatest diversity. The median at T1 was 21.60% (0.20–57.72%), with a statistically significant increase at T2 ($p=0.038$) – 32.60% (16.32–58.86%) and a slight decrease at T3 – 23.60% (12.41–87.77%). Individual values are highly dispersed, especially at the third time point.

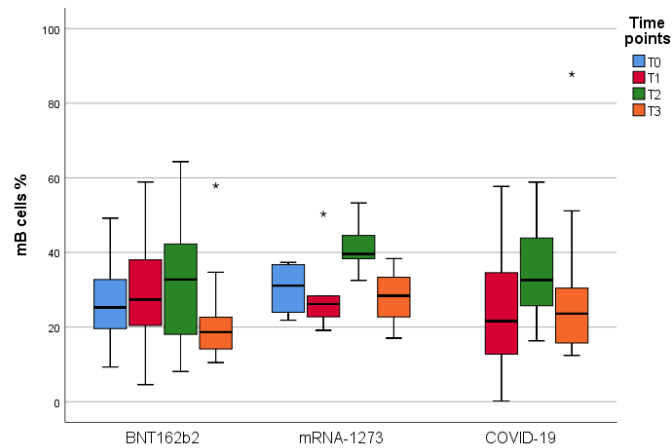


Figure 15. Distribution by groups of memory B-lymphocytes, presented as a percentage of CD19+ cells. The figure illustrates the dynamics of memory B-lymphocytes, with a peak observed in T2 and a gradual decline in T3.

- Switched class memory B-lymphocytes (represented as a percentage of memory B-lymphocytes).

In participants immunized with BNT162b2, the median CSMB at baseline (T0) was 17.99%, with values ranging from 4.50% to 44.44%. After the first dose (T1), the median remained stable at 16.75%, with values ranging from 3.48% to 34.22%. At T2, there was a slight increase to a median of 19.27% (3.46%–45.52%), followed by a marked decrease at T3, with a median of 11.74% and values ranging from 4.89% to 20.73%. In the mRNA-1273 group, the median CSMB

at T0 is 17.83% with a narrower range between 13.15% and 21.98%. The values remain at a similar level at T1 – median 16.41% (10.30% – 23.84%). A significant increase was reported at T2, where the median was 23.85% and the values ranged from 18.69% to 31.55%. At T3, a decrease was observed – median 13.34%, with a range from 10.07% to 21.24%. Among those who had previously been ill, the median CSMB at T1 is 11.96%, but the values are widely dispersed – from 0.15% to 60.70%. At T2, an increase is observed – median 18.78% and variation between 8.82% and 40.75%. At T3, high dispersion is again reported – the median is 13.83%, and the values vary from 9.19% to 84.68%.

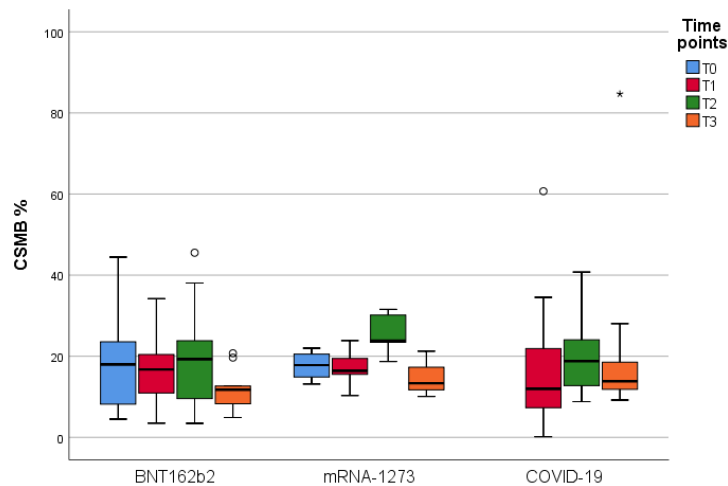


Figure 16. Distribution by groups of % CSMB cells, presented as a percentage of memory B-lymphocytes. In both vaccinated groups, a moderate increase is observed up to T2 and a decrease in T3, while in the previously infected group the values are more heterogeneous with separate high individual levels.

• IgG⁺ memory B-lymphocytes (CSMB IgG⁺)

In the group vaccinated with BNT162b2, the initial median IgG⁺ CSMB at T0 is 8.47%, with values ranging from 0.20% to 43.97%. One month after the second dose (T1), the median decreased to 5.57%, with a range between 0.38% and 18.86%. At T2, a slight increase was observed—the median was 6.96%, with a range from 1.61% to 19.77%. At the end of the follow-up (T3), the median increases to 8.64%, with values ranging from 2.14% to 19.11%.

Participants immunized with mRNA-1273 showed a median of 8.86% at baseline (T0), with a range from 4.40% to 9.62%. At T1, the values remained close to the median – 8.43% (4.27% – 12.54%). Interestingly, at T2, the median increased to 10.28%, with a range of 3.46% to 11.99%. Subsequently, at T3, the median decreased to 5.41%, with values between 3.51% and 9.84%. In participants who had previously been ill, the median IgG⁺ CSMB after recovery (T1) was 5.31%, with a strong dispersion – from just 0.08% to 22.92%. At T2, the median increased to 7.71%, with

values ranging from 3.79% to 25.97%. At T3, a new increase was reported – the median reached 7.83%, with an extremely wide range – from 2.87% to 59.55%.

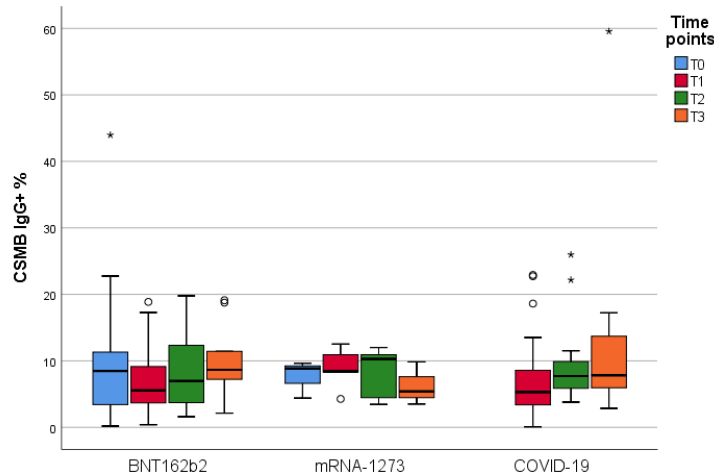


Figure 17. Distribution by groups of % CSMB IgG cells, presented as a percentage of memory B-lymphocytes. In both vaccinated groups, a moderate increase in T2 is observed, while in the previously infected group, the values are more heterogeneous with separate high individual levels.

- Non-class switched memory B-lymphocytes.

In participants vaccinated with BNT162b2, the median NCSMB at baseline (T0) was 6.18% with a reported range of 0.04% to 25.86%. One month after the second dose (T1), an increase was observed with a median of 10.52% and a range of 1.08% to 34.56%. The highest values were recorded at T2 – median 10.58% (2.99% – 40.31%), followed by a slight decrease at T3 – median 7.89% with a range of 5.59% to 38.22%; T0 vs T2 (p=0.036). In the group immunized with mRNA-1273, the values also show dynamics. The median at T0 is 11.79% with a range from 0.01% to 18.27%. At T1, a lower median of 8.84% (6.70%–26.47%) was reported. The values increased at T2, with the median reaching 14.41% with a reported variation between 8.03% and 34.56%. At T3, the median decreases again to 6.97%, with values ranging from 1.99% to 24.99%. In participants who had previously been ill, the median NCSMB at T1 is 6.65% with a wide range between 0.05% and 30.52%. At T2, an increase was reported – a median of 10.98% and values ranging from 5.44% to 42.01%. At T3, the median was 6.23% with a reported range from 0.05% to 39.13%.

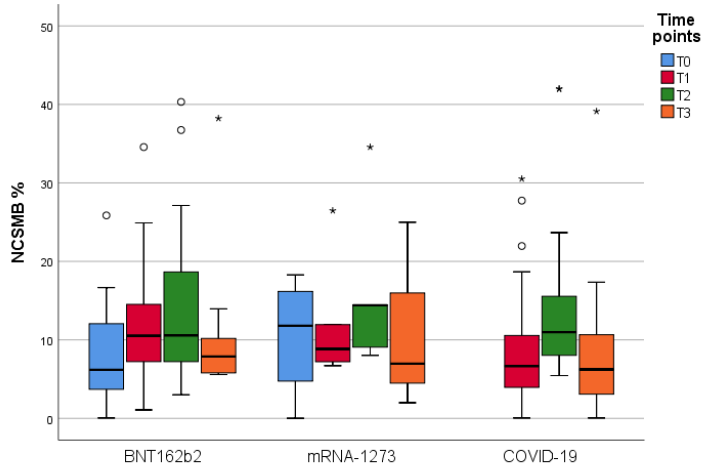


Figure 18. Distribution by groups of % NCSMB cells, presented as a percentage of memory B-lymphocytes. The figure shows an increase up to T2 in all groups and a subsequent decline in T3, with the greatest variability observed in those who had previously been ill.

- Mature B-lymphocytes

In participants immunized with BNT162b2, the proportion of mature B-lymphocytes started with a median of 3.00% (0.26–24.36%) before the second dose (T0). At T1, values decreased slightly to a median of 2.71% (0.41–15.79%), with the downward trend continuing at T2 – 2.56% (0.54–10.18%). At T3, values recovered to 3.61% (1.39–8.71%). In those vaccinated with mRNA-1273, the median of mature B-lymphocytes at T0 was 3.85% (2.42–5.43%), remaining relatively stable at T1 – 3.10% (1.57–4.36%). At T2, there was a slight decrease to 1.71% (1.51–5.28%), and at T3, the median recovered to 2.91% (1.15–4.75%). Previously infected individuals showed significant individual variability. At T1, the median is 2.61% (0.12–71.49%), with the extremely high maximum indicating possible late expansion or an active subpopulation in some of the participants. At T2, the values stabilize at 2.43% (0.04–15.44%), and at T3 – 3.78% (0.44–14.54%).

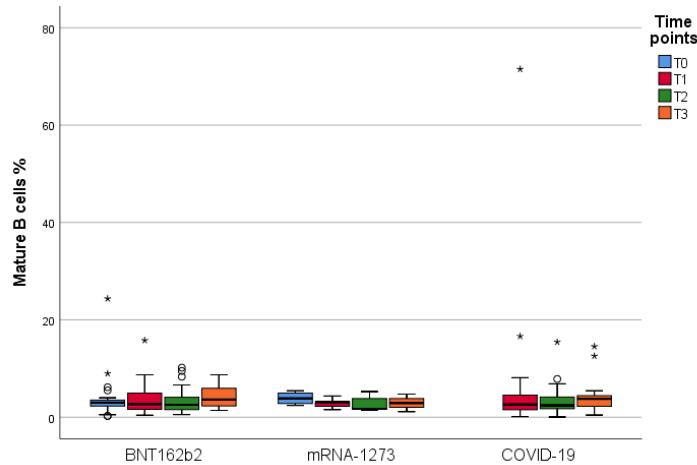


Figure 19. Distribution by groups of % mature B-lymphocytes. The figure illustrates relatively stable values with slight fluctuations over time.

- Transitional B-lymphocytes

At baseline (T0) in those vaccinated with BNT162b2, the median transitional B lymphocyte count was 0.58%, with values ranging from 0.06% to 5.90%. At T1, a decrease was observed, with the median reaching 0.36%, ranging from 0% to 3.72%. At T2, the median increased slightly to 0.45%, with values ranging from 0.09% to 2.48%. At T3, there is a further increase in the median to 0.71%, with values ranging from 0.14% to 2.80%. Participants immunized with mRNA-1273 show a median of 0.86% at T0, with a range from 0.57% to 1.92%. At T1, a similar median of 0.66% was observed, with a range between 0.18% and 1.51%. Time point T2 showed a slight decrease – the median was 0.61%, and the values ranged from 0.18% to 0.85%. At T3, the values remain the same, with a median of 0.54% and a range from 0.17% to 1.21%. In those who have recovered, the median of transitional B-lymphocytes after recovery (T1) is 0.46%, with values ranging from 0.04% to 2.90%. At T2, a decrease was observed, with a median of 0.28% and a range of 0.008% to 2.80%. At T3, the median increased slightly to 0.49%, with values ranging from 0.03% to 1.34%.

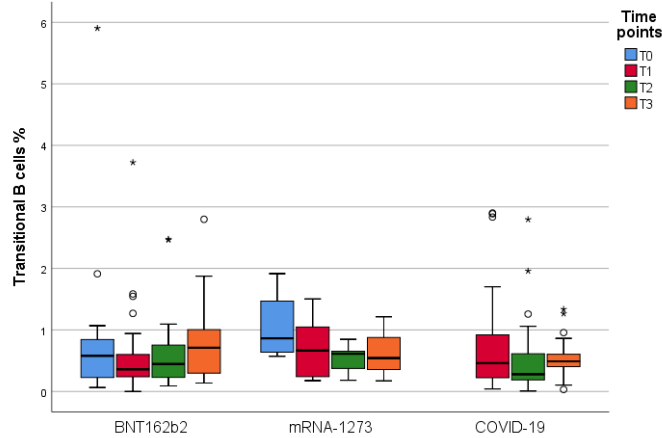


Figure 20. Distribution by groups of % transitional B-lymphocytes. The figure shows low and relatively stable levels of transitional B cells in all groups, with minimal variations over time.

• Naive B-lymphocytes

At baseline (T0), the median naive B lymphocyte count in participants immunized with BNT162b2 was 2.26%, with values ranging from 0% to 22.76%. At T1, the median was similar – 2.36%, with a range from 0.27% to 12.97%. At T2, the median increases slightly to 2.39%, with values ranging from 0.32% to 9.38%. At T3, a moderate increase is observed – the median reaches 3.37%, with a range between 1.30% and 7.72%. In participants immunized with mRNA-1273, the median of naive B-lymphocytes at T0 is 3.13%, with reported values ranging from 0% to 5.01%. At T1, the median is close – 2.79%, with a range from 1.15% to 3.69%. At T2, a decline was reported – the median was 1.58%, with values between 1.25% and 4.30%. Time point T3 showed a moderate recovery – the median reached 2.71%, with a range from 0.69% to 4.56%. In those who have had the disease, the median of naive B-lymphocytes at T1 is 2.34%, with values ranging from 0.17% to 70.31%, indicating the presence of isolated extreme values. At T2, a slight decrease is observed – the median is 2.17%, with a range from 0.60% to 12.34%. At T3, the median reaches 2.75%, with reported values ranging from 0% to 13.29%.

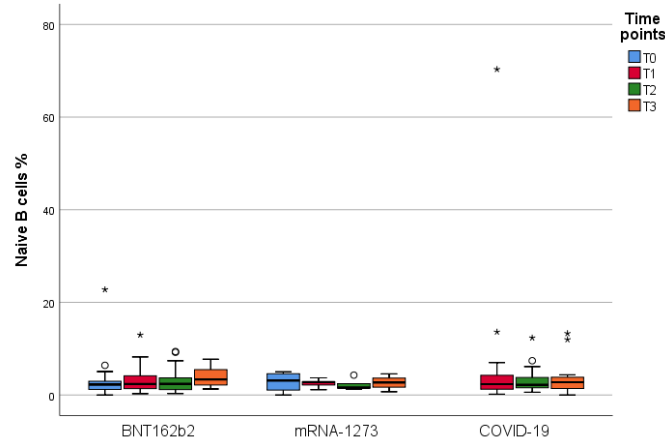


Figure 21. Distribution by groups of % naive B-lymphocytes. Stable levels of naive B cells with minimal fluctuations over time are shown.

- Plasmablasts

In those vaccinated with BNT162b2 at T0, the median plasmablast count is 0.21%, with a reported range of 0.045% to 3.47%, indicating marked individual variability even before immunization. After the second dose (T1), the median drops to 0.12%, and the range of values is between 0% and 1.49%. In T2, an increase is observed (p=0.013) – the median is 0.23%, with variations from 0.03% to 1.52%. In T3, the values remain low, with a median of 0.14% and values ranging from 0.03% to 0.26%. In participants immunized with mRNA-1273, the median plasma cell count at T0 is 0.31%, with values ranging from 0.12% to 0.84%. At T1, the median remains similar at 0.32%, with a range from 0.09% to 0.54%. At T2, the median value was 0.22%, with variations between 0.14% and 0.87%. In T3, a slight stabilization is observed – the median is 0.33%, with a narrow range from 0.29% to 0.40%, which suggests limited post-vaccination activity of plasma cells at this time. In those who have had the disease, the median plasma cell count in T1 is 0.19%, with values ranging from 0.005% to 2.70%, suggesting an acute post-infectious immune response in some individuals. In T2, the median remains relatively low at 0.22%, but with a wide range from 0.05% to 7.17%, which again highlights the heterogeneity of the immune response. In T3, a more limited response is observed – the median is 0.13%, with values between 0.05% and 1.78%.

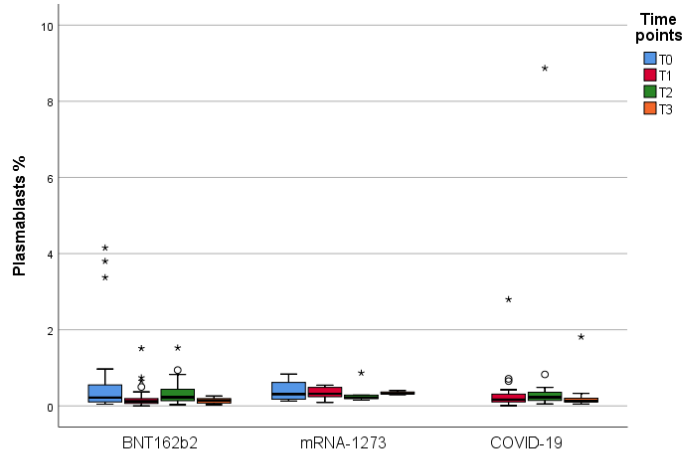


Figure 22. Distribution by groups of % plasmablasts. The figure shows low frequencies of plasmablasts in all groups and time points, with short-term fluctuations after vaccination or infection, with the most pronounced individual variability observed in the recovered individuals.

- Plasma cell distribution

In BNT162b2 vaccinees, the median plasma cell count at T0 is 0.13% (0.02–1.59%), decreasing to 0.11% (0.00–0.97%) at T1 and remaining close to that value at T2 – 0.09% (0.01–0.63%) and again slightly increased to 0.11% (0.01–0.41%) at T3. For mRNA-1273, the median at T0 is 0.15% (0.01–0.53%), followed by a decrease to 0.09% (0.05–0.29%) at T1. At T2, an increase to 0.16% (0.07–0.60%) was observed, with a similar value at T3 – 0.16% (0.08–0.51%). In previously ill individuals, plasma cells reach their highest values at T1 – median 0.16% (0.01–5.10%). At T2, the median is 0.16% (0.00–2.41%), and at T3 – 0.10% (0.03–8.77%), with significant individual variability.

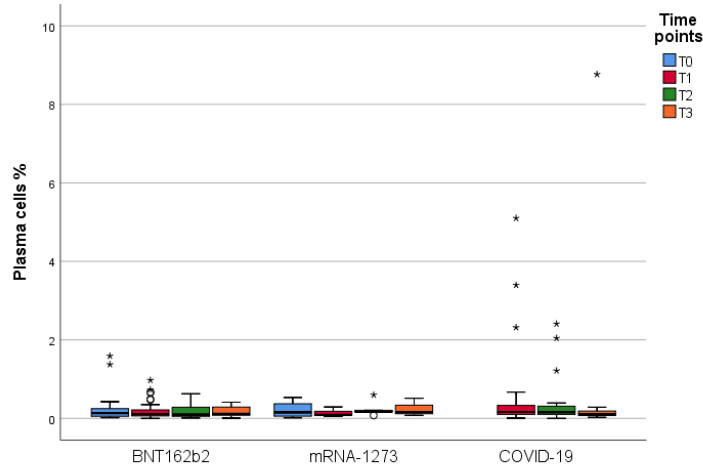


Figure 23. Distribution by groups of % plasma cells. Low and relatively stable levels of plasma cells in vaccinated individuals, while in those who have had the disease, there is greater individual variability and isolated higher values, especially in the early post-infection period.

Using multiparametric flow cytometry in the study, we characterized the main B-cell subpopulations in peripheral blood: total B-lymphocytes (CD19⁺), plasma cells, transitional, naive, mature, and memory B-lymphocytes (Ig class-switched and non-switched). A small number of plasmablasts normally circulate in peripheral blood, but after immune stimulation (vaccination or infection), their proportion temporarily increases dramatically. Our results show a clear peak of CD19⁺CD27^{hi}CD38^{hi} plasmablasts shortly after antigen exposure, followed by a rapid decline. Vaccinated individuals (especially after the second dose) demonstrate a transient increase in plasmablasts after immunization. This is consistent with results reported by other authors, according to which individuals vaccinated with BNT162b2 show a peak in IgG⁺ plasmocytes around the seventh day after the second dose. During this period, up to 0.5–1% of circulating B-lymphocytes are plasma cells secreting anti-Spike antibodies, which is significantly above baseline values. A positive correlation has been established between the frequency of plasmablasts on day 7 and the number of memory B-lymphocytes formed at month 6, suggesting that an early plasmablastic response may predict long-term humoral memory.

Memory B-lymphocytes (identified as CD19⁺CD27⁺) undergo more gradual changes over time. Naive B-lymphocytes activated by antigen either differentiate directly into plasmablasts or enter the germinal centers of lymph nodes, where they divide, undergo somatic hypermutation and affinity selection, and emerge as high-affinity memory cells or long-lived plasma cells. This process takes time, which is why memory B-lymphocytes begin to appear in the periphery weeks after immunization/infection, and their number may increase in the following months. Our research shows that during the first month after vaccination, there is an initial increase in memory B-lymphocytes, while at 3 and 6 months, there is a clear increase in their fraction relative to the baseline. This is consistent with observations that in vaccinated individuals, the frequency of spike-

specific memory B-lymphocytes increases between 3 and 6 months post-vaccination. The proportion of memory B-lymphocytes increases between 3 and 6 months, indicating active formation of new memory cells during this period. In those who have had the disease, there is a similar trend with an increase in the percentage of memory B-lymphocytes around 6 months after infection. Our results reflect this trend – a gradual increase followed by a plateau.

Tracking the dynamics of the main B-cell subpopulations using multiparametric flow cytometry reveals a complex but consistent picture of the humoral immune response in the three groups studied—those vaccinated with BNT162b2, mRNA-1273, and those who had previously had SARS-CoV-2. A similar overall pattern was observed in all groups: early activation of naive and transitional B-lymphocytes, followed by an increase in the memory pool and a gradual decline in plasmablastic and plasma cell activity. The predominant expansion of class-switched (IgG⁺) memory cells indicates effective antigen stimulation and long-term humoral memory.

At the population level, vaccinated and previously infected individuals show a similar organization of their B-cell subpopulations in the long term: a small proportion of plasmablasts (except immediately after antigenic stimulation), a stable percentage of memory cells, and a dominant pool of naive cells. The differences are most evident in the short periods after exposure—vaccinated individuals have clearly defined peaks of plasmablasts after doses, while in those who have had the disease, such a peak would be during the symptomatic phase (outside the observed convalescent points). In terms of memory B cells, both groups develop strong memory.

6. Results from Objective 5

Dynamics of S1-specific class-switched memory B-lymphocytes (S1 CSMB)

The longitudinal assessment of S1-specific class-switched memory B-lymphocytes (S1 CSMB) reveals distinct differences in the temporal profile of the response in individuals vaccinated with BNT162b2 or mRNA-1273, as well as in convalescent subjects.

In participants immunized with BNT162b2, the median frequency of S1-specific CSMB at baseline (T0) is 0.07%, with an observed range from 0.007% to 1.25%. This indicates the presence of a pre-existing pool of reactive cells prior to completion of the vaccination course. One month after the second dose (T1), the values unexpectedly decrease – the median is only 0.06%, with an extremely wide range from 0% to 8.45%, suggesting a highly individualized response. Subsequently, at T2 (month 6), the median slightly declines to 0.05% (0.004–0.30%), and by T3 (month 12) an even lower value is recorded – a median of 0.03% with minimal variation (0.012–0.081%).

In the mRNA-1273 group, S1 CSMB cells exhibit a relatively stable and overall higher expression compared with BNT162b2. At T0, the median is 0.097%, with a range of 0.010% to 0.213%. At T1, the values are similar – a median of 0.072% (0.023–0.292%). At T2, the median is 0.059% (0.054–0.256%), and by T3 it remains stable at 0.047% (0.022–0.077%). These data indicate better consistency of the response over time, without extreme outliers, which is suggestive of a more durable and uniform B-cell immune profile with this platform.

In convalescent participants, following the acute infectious episode, a peak is observed at T1 – the median reaches 0.075% (0.005–5.32%). This reflects intense generation of antigen-specific memory lymphocytes in the early convalescent phase. At T2, a further increase is recorded – the median is 0.28% with a broad range (0.034–2.26%), which may be related to ongoing

antigenic stimulation or tissue recirculation. By T3, however, a decline is evident – the median decreases to 0.020%, with a range from 0% to 0.52%; T2 vs T3 ($p < 0.001$).

These findings underscore that although all three groups generate S1-specific CSMB cells, their long-term profile depends on the nature of the immunological stimulus – vaccination versus infection. mRNA-1273 induces the most stable and homogeneous response, whereas BNT162b2 is characterized by greater interindividual variability. The post-infectious response is the most dynamic but also displays the greatest dispersion.

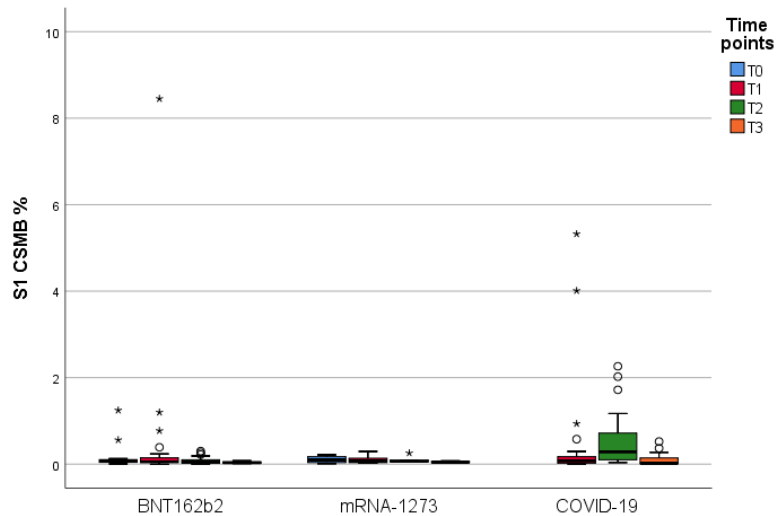


Figure 24. Distribution of S1 CSMB cells by group, expressed as a percentage of memory B-lymphocytes. mRNA-1273 induces a more stable and uniform response, BNT162b2 displays greater interindividual variability, whereas convalescent individuals demonstrate the most dynamic yet highly heterogeneous immune profile.

At T2, statistically significantly higher levels of S1 CSMB were observed in convalescent individuals compared with those vaccinated with BNT162b2 ($p < 0.001$).

S1-specific IgG⁺ class-switched memory B-lymphocytes (S1 CSMB IgG⁺)

The longitudinal assessment of S1-specific IgG⁺ CSMB-lymphocytes revealed notable differences in both the magnitude and durability of the response across the three study groups, with overall low frequencies but distinct temporal dynamics.

In participants vaccinated with BNT162b2, the baseline (T0) median was 0.028% (0.003–1.245%), indicating a very low initial presence of antigen-specific IgG⁺ memory B cells. After the second vaccine dose (T1), a modest increase was recorded, with the median reaching 0.051% (0.000–8.228%), accompanied by a wide range consistent with strong interindividual variability. At T2 and T3, the median gradually declined to 0.037% (0.000–0.222%) and 0.028% (0.011–0.061%), respectively, with a narrower distribution, suggesting a short-lived expansion followed by attenuation of the S1-specific IgG⁺ memory response.

In the mRNA-1273 group, a similarly low initial median of 0.065% (0.000–0.077%) was observed at T0. At T1, the median was 0.031% (0.027–0.292%), followed by a stable median of 0.051%

(0.043–0.137%) at T2. At T3, a slight decrease to 0.035% (0.021–0.062%) was recorded. Although the values remained low overall, they were more consistent over time compared with the BNT162b2 group.

Convalescent individuals demonstrated the greatest variability. At T1, the median was 0.043% (0.002–8.257%), reflecting marked interindividual differences in the early post-infectious period. At T2, the median increased to 0.094% (0.013–1.165%), followed by a decline at T3 to 0.013% (0.000–0.453%); T2 vs T3 ($p=0.005$). Although some individuals displayed high frequencies, most remained within low ranges, indicating that activation of S1-specific IgG⁺ memory B-lymphocytes after natural infection strongly depends on the individual immunological profile and clinical presentation.

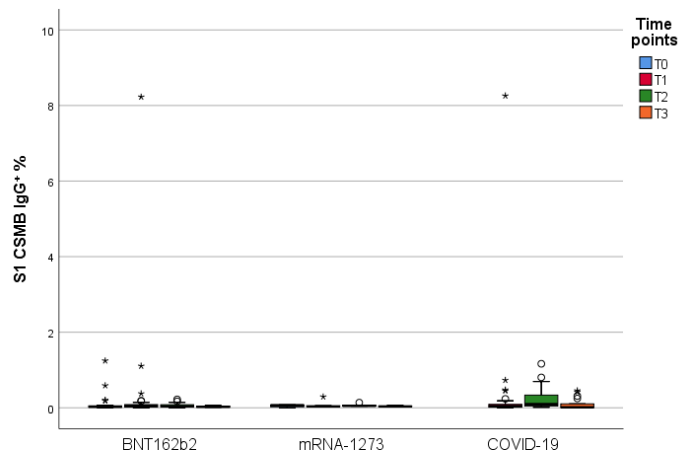


Figure 25. Distribution of S1 CSMB IgG⁺ cells by group, expressed as a percentage of memory B-lymphocytes. S1-specific IgG⁺ CSMB cells exhibit a more sustained response in the mRNA-1273 group, greater interindividual variability in the BNT162b2 group, and the most dynamic yet heterogeneous profile in convalescent individuals, with a statistically significant increase at T2.

At T2, statistically significantly higher levels of S1 CSMB were observed in convalescent individuals compared with those vaccinated with BNT162b2 ($p=0.021$).

S1-specific non-class-switched memory B-lymphocytes (S1 NCSMB)
S1 NCSMB represent a functionally important yet quantitatively limited population, which was likewise monitored over time following vaccination or natural infection. The analysis reveals low baseline levels and only transient expansion, followed by a marked decline, particularly in the vaccinated groups.

In participants vaccinated with BNT162b2, the baseline (T0) median was 0.036% (0.002–0.324%). Although at T1 the median reached 0.027% (0.000–4.644%), the value remained low while the wide range indicated substantial heterogeneity in individual immune responses. Subsequently, a

sharp decrease was observed — to 0.011% (0.000–0.439%) at T2 and 0.018% (0.000–0.052%) at T3, indicating limited durability of this population following vaccination.

The mRNA-1273 group displayed similar trends but at even lower values. At T0, the median was 0.017% (0.006–0.047%), increasing to a maximum of 0.041% (0.001–0.143%) at T1. In the subsequent time points, low levels persisted, with medians of 0.025% (0.020–0.137%) at T2 and 0.016% (0.007–0.100%) at T3. This again underscores the limited and short-lived nature of the NCSMB response following vaccination, regardless of platform.

Among individuals with prior SARS-CoV-2 infection, the median at T1 was 0.039% (0.000–0.371%), comparable to vaccinated groups. However, at T2 a clear increase was observed — up to 0.107% (0.004–2.484%), accompanied by a very wide range, followed by a decline to 0.022% (0.000–0.520%) at T3; T2 vs T3 ($p=0.004$). These findings suggest that natural infection triggers a more pronounced, albeit transient, expansion of S1-specific NCSMB-lymphocytes in a subset of individuals.

Overall, S1-specific NCSMB-lymphocytes remain sparsely represented and inconsistent over time, regardless of the type of antigenic stimulus — vaccination or natural infection. Only some convalescent individuals exhibit meaningful expansion, but it is not sustained long term. These results support the concept that long-lived memory responses to SARS-CoV-2 are predominantly mediated by class-switched memory B cells, while NCSMB play a more limited role and persist as a small fraction of the total S1-specific B-cell pool.

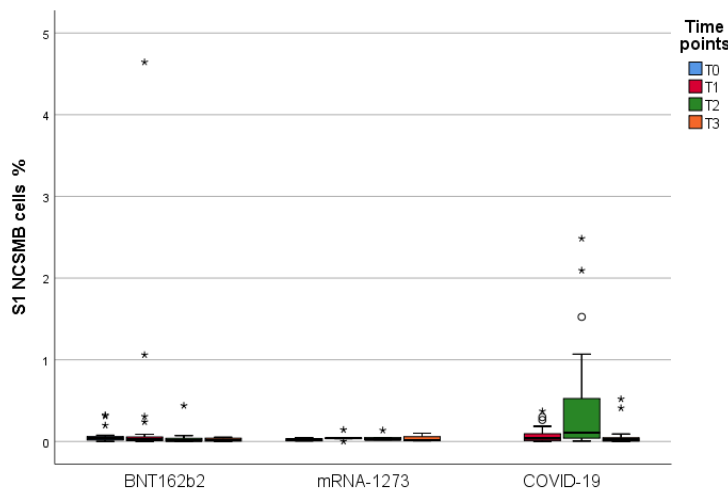


Figure 26. Distribution of S1 NCSMB cells by group, expressed as a percentage of memory B-lymphocytes. The figure illustrates low and short-lived levels of S1 NCSMB in vaccinated individuals, with a weak and inconsistent response, whereas convalescent subjects exhibit a transient expansion at T2 that does not persist long term.

At T2, statistically significantly higher levels of S1 CSMB were observed in convalescent individuals compared with those vaccinated with BNT162b2 ($p<0.001$).

S1 PB

S1-specific plasmablasts (PB) represent a short-lived yet functionally important population

associated with intensive antibody secretion following antigenic stimulation. The data reveal clear differences in the temporal distribution and magnitude of responses across the studied groups.

In the BNT162b2-vaccinated group, S1-specific plasmablasts were already detectable at baseline (T0), with a median of 0.41% (0.00–6.08%), reflecting activity triggered by the first vaccine dose. At T1, all participants in this group showed absence of S1 PB (0.00%). At T2, the population reappeared with a median of 0.12% (0.00–12.25%), followed by a sharp decline at T3 to 0.05% (0.00–0.67%). The high dispersion and asymmetric distribution at T2 suggest that only a subset of individuals respond with a detectable expansion of S1-specific plasmablasts.

In individuals vaccinated with mRNA-1273, the baseline level (T0) showed a median of 1.59% (0.00–3.31%), comparable to BNT162b2. At T1, no S1 PB were detected (0.00%). At T2, the median reached 0.24% (0.00–0.75%), and at T3 it increased modestly to 0.38% (0.12–0.62%), which may indicate a more moderate but somewhat more sustained response compared with BNT162b2. This group does not display sharp peaks, but rather a more uniform response with limited amplitude.

The most pronounced response was observed in convalescent individuals. Although no S1 PB were detected at T1 (0.00%), a marked expansion occurred at T2, with a median of 3.22% (0.13–16.83%), reflecting intense plasmablast activation in the post-infectious period. At T3, this population declined but remained detectable in a subset of participants, with a median of 0.12% (0.00–3.46%) and a wide, highly asymmetric distribution; T2 vs T3 ($p=0.014$).

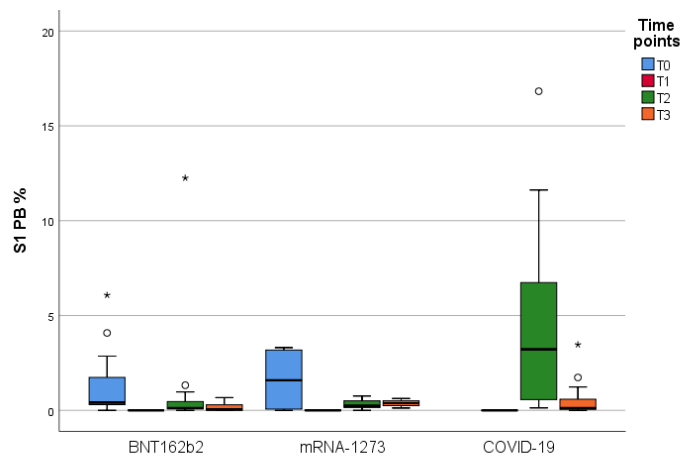


Figure 27. Figure 29. Distribution of S1 PB by group. The figure illustrates the transient appearance of S1-specific plasmablasts following vaccination or infection, with a more moderate and uniform response in the mRNA-1273 group, a more fluctuating profile in the BNT162b2 group, and the strongest yet transient expansion in convalescent individuals at T2.

At T2, statistically significantly higher levels of S1 CSMB were observed in convalescent individuals compared with those vaccinated with BNT162b2 ($p < 0.001$).

Using flow cytometric analysis, we further characterized three subtypes of S1-specific B-lymphocytes: CSMB, NCSMB, and PB. CSMB represent the classical long-lived memory cells that have undergone affinity maturation within germinal centers. NCSMB retain the IgM isotype (often together with surface IgD) and are typically generated through extrafollicular pathways; they are considered more “primary” memory cells. PB are effector antibody-secreting cells circulating in the blood that specifically recognize the S1 domain. The results are presented as a percentage of memory B-lymphocytes over time. This detailed characterization provides insight into the qualitative composition of B-cell memory formed in each of the study groups.

mRNA vaccines induce robust germinal center reactions supported by T follicular helper cells, resulting primarily in the formation of class-switched memory B-lymphocytes. Accordingly, vaccinated individuals are expected to display a high proportion of S1-specific CSMB and a low proportion of NCSMB. Supporting this, Ciabattini et al. reported that memory B-lymphocytes six months after BNT162b2 vaccination are predominantly IgG⁺ class-switched cells. Our data show that the percentage of S1-specific CSMB increases after vaccination and remains the dominant component of antigen-specific memory up to the 6-month time point. In contrast, NCSMB are poorly represented among vaccine recipients.

In convalescent individuals, the profile is more heterogeneous. SARS-CoV-2 infection activates both follicular and extrafollicular B-cell pathways. Particularly in the context of marked inflammation, part of the B-cell compartment differentiates rapidly outside germinal centers, giving rise to IgM⁺ “naive-like” memory cells. Such cells have been described in various infections as atypical or marginal zone-like B cells that express memory markers (e.g., CD27⁺) but retain the IgM isotype. Pape et al. note that extrafollicular memory B cells are most frequently IgM-expressing. Therefore, it is expected that individuals recovering from COVID-19 would exhibit a measurable proportion of S1-specific NCSMB. Our data indeed demonstrate a higher proportion of NCSMB in convalescent individuals compared with vaccinated groups, especially during the early months after infection. Over time, some of these IgM memory cells may further differentiate or decline, yet they remain detectable six months post-infection.

Simultaneously, natural infection also generates class-switched memory (CSMB) through canonical germinal center pathways — particularly in cases involving prolonged or severe disease. Six months after infection, both IgG⁺ and IgM⁺ memory B cells remain present. Memory B cells persist up to one year after exposure and include functionally diverse subsets associated with higher BCR avidity, such as CD21⁻/CD27⁻, CD21⁻/CD27⁺, and CD21⁺/CD27⁺ phenotypes, indicating substantial plasticity and multiple functional capacities upon re-exposure to antigen. Specifically, cells with the phenotypes CD27⁺IgD⁺IgM⁺ or CD27⁺IgD⁺IgM⁻ (the so-called “intermediate memory phenotype”) have been linked to strong neutralizing potential. These memory cells, often described as marginal-zone-like B cells, possess intermediate features between naive and classical memory B cells. Such observations support the notion that IgM memory B-lymphocytes generated during infection are important contributors to the overall neutralizing capacity of the memory pool. Analysis of the qualitative composition of B-cell memory highlights the potential durability and adaptability of immunity. The predominance of class-switched memory (IgG/IgA) in vaccinated

individuals suggests that, upon re-exposure, they are poised to rapidly produce high-efficiency antibodies and are likely protected against severe disease. However, the relatively smaller IgM memory compartment may render them slightly more vulnerable to highly divergent variants - underscoring the importance of continual vaccine updates (bivalent or variant-adapted vaccines) to enrich memory against circulating strains.

In convalescent individuals, the coexistence of both IgG and IgM memory forms a dual-layered defense: IgG memory provides rapid high-avidity responses, while IgM memory offers broader reactivity and may recognize evolutionarily distant variants. This may help explain why hybrid immunity (infection plus vaccination) is associated with exceptional protection, as it integrates the strengths of both memory pathways.

VI. CONCLUSION

In conclusion, the data from this study highlight the complex and dynamic nature of the immune response to SARS-CoV-2 and underscore the fundamental importance of immunological memory for long-term protection. Despite the decline in serum antibody levels several months after vaccination or infection, the persistence of memory B and T-lymphocytes demonstrates that both humoral and cellular memory can maintain clinical protection even when circulating antibody titers are low. Notably, vaccination with mRNA-1273 results in higher and more durable levels of RBD-specific antibodies compared with BNT162b2 an observation consistent with findings from independent cohort studies. This advantage may be attributed to the higher mRNA dose, the longer dosing interval, and the immunomodulatory properties of the lipid nanoparticle formulation.

The multiparametric analysis of B-cell subpopulations emphasizes that both vaccination and infection lead to the formation of stable S1-specific memory. However, natural infection induces a more diverse B-cell repertoire, including IgM⁺ memory cells that can participate in affinity maturation upon re-exposure to variant antigens. In contrast, vaccinated individuals predominantly generate class-switched (IgG⁺) high-avidity memory cells. This distinction helps explain why hybrid immunity—arising from the combination of infection and vaccination—confers broader and more resilient protection against viral evolution.

The T-cell component of immune memory also warrants special attention, as it remains stable over time and exhibits lower susceptibility to mutations in the Spike protein. Convalescent individuals display broader T-cell responses directed not only against Spike, but also against internal viral proteins. This expanded antigenic specificity may contribute to improved protection against emerging variants. Thus, the durability of cellular immunity reinforces its role as a critical protective mechanism, particularly when the humoral component wanes.

The accumulated data support the need for booster doses, especially among vulnerable populations, as well as the future personalization of vaccination schedules based on age, prior immune status, and infection history. Additionally, monitoring functional parameters—such as avidity, epitope specificity, and cellular phenotype—should be incorporated into future strategies for assessing immune status.

In the context of ongoing viral evolution and the emergence of variants with enhanced immune evasiveness, such as XBB.1.5, BQ.1.1, and BA.2.86, there is a clear need for vaccines with updated antigenic composition. mRNA platforms allow rapid adaptation and have already

enabled the deployment of bivalent vaccines containing components from both the original Wuhan strain and currently circulating variants. Studies indicate that such adapted vaccines restore serum neutralizing activity and enhance T-cell responsiveness. Beyond bivalent formulations, pan-sarbecovirus vaccines targeting conserved epitopes within the S2 domain are being explored as a long-term solution with potential cross-protective capacity against the broader β -coronavirus family. These vaccines aim to reduce the need for frequent updates by stimulating broader and more durable immune memory.

Taken together, the current evidence suggests that future advances in SARS-CoV-2 immunoprophylaxis will depend not only on the quantitative magnitude of the immune response but also on the ability to flexibly adapt vaccine platforms, integrate individual-level factors, and deepen our understanding of the qualitative determinants of immune memory.

VII. CONCLUSIONS

1. Immunization with both mRNA vaccines induces a strong humoral response, characterized by an early peak followed by a gradual decline up to the 12th month. A more durable profile is observed with mRNA-1273 compared with BNT162b2, while convalescent individuals exhibit greater interindividual variability.
2. Neutralizing activity follows a similar kinetic pattern, with mRNA-1273 maintaining higher and more homogeneous levels over time, whereas convalescent individuals demonstrate a more sustained residual response at the end of the observation period.
3. Immunization with mRNA vaccines induces a pronounced peak in B-cell memory, particularly in antibody-secreting cells, followed by a physiological decline between the sixth and twelfth month. Residual reactivity is preserved in most participants for at least one year after exposure to the viral antigen. Convalescent individuals show a weaker but more durable response.
4. The T-cell response peaks after complete immunization and remains detectable up to month 12, indicating the formation of long-lived memory T-lymphocytes capable of producing IFN- γ .
5. Multiparametric flow-cytometric analysis reveals an early transient activation of naïve and transitional B cells, followed by a sustained increase in memory populations and expansion of IgG⁺ class-switched cells, consistent with effective antigenic stimulation and the establishment of long-lasting humoral memory.
6. Longitudinal tracking of S1-specific B-cell subpopulations demonstrates distinct temporal profiles in vaccinated versus convalescent individuals: mRNA-1273 elicits a more stable and homogeneous response, whereas BNT162b2 and naturally infected individuals show more pronounced dynamics and variability. Long-term memory is maintained primarily by IgG⁺ class-switched B-lymphocytes.

VIII. CONTRIBUTIONS

1. A multiparametric flow-cytometry panel for B-cell heterogeneity was developed, enabling simultaneous and detailed analysis of multiple B-lymphocyte subpopulations (CD19⁺ cells, memory B cells, CSMB, IgG⁺ CSMB, NCSMB, transitional B cells, plasmablasts, and plasma cells). The panel was optimized through a clearly defined gating strategy, ensuring high reproducibility and sensitivity.
2. S1-specific memory B-lymphocytes were described for the first time in a Bulgarian population using tetramer-based flow-cytometric analysis.
3. For the first time at the national level, a comprehensive and long-term assessment of the humoral and B-cell immune response was conducted following mRNA vaccination (BNT162b2 and mRNA-1273) and natural SARS-CoV-2 infection. It was demonstrated that the mRNA-1273 vaccine induces a more durable and highly specific B-cell response compared with BNT162b2.
4. It was established that antigen-specific memory B-lymphocytes persist for at least 12 months after vaccination and natural infection, regardless of fluctuations in the antibody response.
5. An integrated laboratory approach for monitoring immune status was proposed, with potential applications in both clinical practice and research. The developed analytical platform can be used to evaluate population-level immunity in Bulgaria and to optimize vaccination strategies for specific groups, including patients with primary and secondary immunodeficiencies.

IX. PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS RELATED TO THE TOPIC OF THE PRESENT DISSERTATION

1. **Bozhkova M.**, Raycheva R., Petrov S., Dudova D., Kalfova T., Murdjeva M., Taskov H., Shivarov V. Humoral and Memory B Cell Responses Following SARS-CoV-2 Infection and mRNA Vaccination. *Vaccines* 2025, 13(8), 799; <https://doi.org/10.3390/vaccines13080799>
2. **Bozhkova M.**, Gardzheva P., Rangelova V., Taskov H., Murdjeva M. Cutting-edge assessment techniques for B cell immune memory: an overview. *Biotechnology & Biotechnological Equipment*. 2024;38(1):2345119. <https://doi.org/10.1080/13102818.2024.2345119>
3. **Bozhkova M.**, Petrov S, Velyanova T, Stoycheva M, Murdjeva M. Immunological Enigma: A Case Report of COVID-19 Survival in a Patient With Human Immunodeficiency Virus, Hepatitis C Virus, and Tuberculosis Co-infection. *Cureus*. 2024 Sep 17;16(9):e69588. doi: 10.7759/cureus.69588. PMID: 39421098; PMCID: PMC11484182.

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