

AMERICAN UNIVERSITY OF BEIRUT

DEVELOPMENT OF A LONG-LASTING HUMORAL
IMMUNE RESPONSE AGAINST THE Pfizer/BioNTech
mRNA-BASED VACCINE IN HEALTHY ADULT
PARTICIPANTS

by
SARA MOHAMAD ALI EL MASRI

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ABSTRACT

OF THE THESIS OF

SARA MOHAMAD ALI EL MASRI for Master of Science
Major: Microbiology and Immunology

Title: Development of a Long-Lasting Humoral Immune Response Against the Pfizer/BioNTech mRNA Based Vaccine In Healthy Adult Participants

Background: In December 2019, a cluster of pneumonia cases of unknown etiology was reported in Wuhan City, China. Later, the causative agent was identified as a new member of coronaviruses, and was named SARS-CoV-2. This virus quickly spread globally prompting the WHO to declare the outbreak a pandemic on March 11, 2020. The research to develop a vaccine for the novel coronavirus has taken place at an unprecedented fast pace, with clinical trials kicking off just a few months after the first case was identified. Currently, more than 180 vaccines candidates are under development with lack of evidence about the long term protection conferred by these vaccines. One of the vaccines imported by the Ministry of Health of Lebanon is the RNA-based vaccine (BNT162b2) developed by Pfizer/BioNTech. This vaccine was administered at the American University of Beirut Medical Center and other medical centers based on criteria and schedules set by the MOH.

Study aims: The purpose of this study is to determine the ability of the Pfizer/BioNTech mRNA-based vaccine to induce a protective humoral immune response in the healthy vaccinated adult population, and study the durability of the response in terms of longitudinal protection through the generation of protective neutralizing IgG, IgA, and IgM antibodies.

Methods: A total of 7 blood draws (collected at 7 time points: pre immune, 21 days post the first dose, and then 1, 3, 6, 9, and 12 months post the second dose) of a maximum of 10 ml blood each were collected from 12 participants by venipuncture. Blood was ficolled using a Ficoll-paque. The isolated plasma was used to perform ELISA, by which SARS-CoV-2 spike specific IgG, IgA, and IgM of the Alpha (B.1.1.7) variant were measured.

Results and discussion: BNT162b2 mRNA vaccination generates a robust IgG response with a peak at 1 month post the second dose. This response declined to disappear 6 to 9 months after the second dose. One participant has a detectable level of IgG at the 12th month after vaccination. IgA had a similar response as IgG but with a lower magnitude, whereas IgM levels were undetectable.

Conclusion: BNT162b2 mRNA vaccination generates a robust humoral immune response, that may last for at least 9 months post immunization, especially involving anti-SARS-CoV-2 IgG and IgA.

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CHAPTER I

INTRODUCTION

In December 2019, a cluster of pneumonia cases of unknown etiology was reported in Wuhan City, China [1]. The causative agent was soon after isolated from the bronchoalveolar lavage of infected individuals and identified as a novel enveloped single stranded RNA coronavirus [2]. The International Committee on Taxonomy of Viruses named this novel virus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and the World Health Organization (WHO) named the resulting disease as coronavirus disease 2019 (COVID-19) [3]. SARS-CoV-2 quickly spread globally prompting the WHO to declare the outbreak a pandemic on March 11, 2020. As of 22 April 2022, there were 505,817,953 confirmed cases of COVID-19, including 6,213,876 deaths, reported to WHO, and 1,095,959 cases with a cumulative death toll of 10,369 in Lebanon [4].

SARS-CoV-2 belongs to the family coronaviridae that also includes SARS-CoV and Middle East Respiratory Syndrome coronavirus (MERS-CoV), however, SARS-CoV-2 is phylogenetically closer to bat coronavirus BCoV RaTG13, suggesting that it originated in bats then it was transmitted to humans potentially through an intermediate host. The SARS-CoV-2 genome consists of six major open-reading frames that are common to coronaviruses, and several other accessory genes [2]. The entry of coronaviruses into host cells is mediated by the viral envelope spike (S) protein receptor-binding domain (RBD) region that binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of

host cells, resulting in the fusion of viral and host membranes and infection of target cells [5]. ACE2 is expressed in the bronchus and lung parenchyma, as well as in the heart, kidneys, and gastrointestinal tract [6]. SARS-CoV-2 infections cause a wide spectrum of clinical presentations ranging from asymptomatic infections to mild upper respiratory tract symptoms (fever, cough, and myalgia), to severe pneumonia, respiratory failure, and multiple organ dysfunction that can lead to death [7].

Preclinical studies have been performed to develop vaccines against SARS-CoV and MERS-CoV, but that work was halted and no vaccines against these coronaviruses have been licensed for use in humans [8]. However, it became clear that the most likely candidate antigenic target for coronavirus vaccines was the S protein because it is the only large surface protein, and it is responsible for ACE2 receptor binding and membrane fusion [5, 8, 9]. Therefore, antibodies (Abs) that bind to the S protein, especially to its RBD, are expected to prevent its attachment to host cells and thus neutralize the virus. This hypothesis was substantiated by studies performed on patients infected with SARS-CoV-2 who developed Abs against the S protein that have been shown to neutralize the virus [10, 11]. Based on this knowledge, the spike protein was identified as an antigenic target for the development of a vaccine against SARS-CoV-2 at a very early stage. Vaccine development is a lengthy process that can take up to 15 years, however, the development of the SARS-CoV-2 vaccine was accelerated at an unprecedented rate because of the need to slow down and reverse the pandemic, and because ample preclinical data were available from SARS-CoV and MERS-CoV vaccine studies that were applied to the SARS-CoV-2 vaccine development [12]. Traditional and new

approaches were used. An innovative approach used the RNA encoding the S protein of SARS-CoV-2 encapsulated in a lipid nanoparticle (Pfizer/BioNTech and Moderna). Once injected intramuscularly, the lipid component would transport the RNA inside the cells where it will be translated into the S protein and exposed extracellularly to be recognized as an antigen (Ag) by immune cells that mount an immune response against it.

One of the vaccines imported by the Ministry of Health (MOH) of Lebanon is the RNA-based vaccine (BNT162b2) developed by Pfizer/BioNTech. This vaccine was administered at the American University of Beirut Medical Center (AUBMC) and other medical centers based on criteria and schedules set by the MOH. BNT162b2 is a lipid nanoparticle-formulated, nucleoside-modified RNA encoding the SARS-CoV-2 full-length S protein, modified by two proline mutations to lock it in the prefusion conformation. The development of an adaptive immune response against SARS-CoV-2 using BNT162b2 has been demonstrated in the phase 1 clinical trial [13], however the duration of the immune response had not been determined. The purpose of this study is to determine the ability of the Pfizer/BioNTech mRNA-based vaccine to induce a protective immune response in the healthy vaccinated adult population, and study the durability of the response in terms of longitudinal protection through the generation of protective neutralizing IgG, IgA, and IgM Abs.

In my present work, we demonstrated the development of a robust humoral immune response against the Pfizer/BioNTech BNT162b2 mRNA vaccine that maintains immunity at least 6 months after immunization by 2 doses, and that might last for longer than 9 months in some participants.

CHAPTER II

LITERATURE REVIEW

A. Coronaviruses

Coronaviruses belong to the large coronaviridae family [14], and they constitute a diverse group of viruses that infect a wide range of animal hosts, with symptoms ranging from mild to severe in humans [15]. Due to the presence of a viral glycoprotein called the “spike” protein in many copies on the surface of this virus, the virus’s surface gives the viral particle a special crown-like halo [16]. Hence, the name of these viruses: coronaviruses.

1. Human Coronaviruses

Human coronaviruses are members of coronaviruses that are organized into four main subgroupings (genera): alpha, beta, gamma, and delta coronaviruses [17]. Among them, 5 human coronaviruses have been known in the last two decades to infect humans and causing pandemics, which are the severe acute respiratory syndrome coronavirus (SARS-CoV, 2003) [18], human coronavirus NL63 (HCoVNL63, 2004) [19], human coronavirus HKU1 (HCoV-HKU1, 2005) [20], Middle East Respiratory Syndrome Coronavirus (MERS-CoV,2012) [21], and SARS-CoV-2 (2019) [22]. Three of them have a zoonotic origin, and they include SARS-CoV, MERS-CoV, and SARS-CoV-2 that is responsible for the current pandemic (COVID-19) [23].

B. SARS-CoV-2 virus

SARS-CoV-2 is an enveloped, positive-sense, single stranded RNA beta coronavirus that shares 79% of nucleotide sequence identity with SARS-CoV, and 50% identity with MERS-CoV [24].

1. History and Discovery

In December 2019, many cases of pneumonia with unknown etiology were reported in Wuhan city, China [1]. The causative agent, isolated from bronchoalveolar lavage fluid specimens of the first pneumonia cases that were admitted to the intensive care unit Wuhan Jin Yin-Tan Hospital, was identified as a new member of the human coronavirus's family [2]. Later, this causative agent, was named by the coronaviridae study group of the international committee on taxonomy of viruses as SARS-CoV-2 [25]. At the beginning of its emergence, SARS-CoV-2 has caused an epidemic in China which soon spread globally and was declared as the COVID-19 pandemic on 11 March 2020 [12, 26]. Preceded by two pandemics of SARS-CoV and MERS-CoV in 2002 and 2012 respectively, this present outbreak of an acute respiratory disease associated with a coronavirus is the third documented emergence of a coronavirus from animals to humans in the past two decades [25].

2. Epidemiology and origin

SARS-CoV-2 appears to be the most contagious and infectious of the 3 beta coronaviruses (SARS-CoV, MERS-CoV and SARS-CoV-2) [27], putting it on the top of international health concerns. Globally, 505,817,953 confirmed cases of COVID-19, including 6,213,876 deaths were reported to WHO. And While COVID-19 is still

spreading, its origin is still debatable [4, 28]. This high mortality rate dwarfed the impact of SARS-CoV that caused 800 deaths and about 8000 infections [29]. The first source linked to SARS-CoV-2 outbreak was a food market selling live animals in Wuhan, China [30]. Genetically, there is evidence that SARS-CoV-2 has an animal origin, specifically a bat origin as it shares 96% of genome with two SARS-like CoVs, namely bat-SLCoVZXC45 and bat-SL-CoVZXX2 that were isolated from bats. However, no evidence shows where and when this virus has first entered humans [15, 30]. In addition, bats are known as a reservoir for a diverse range of coronaviruses including those that are closely related to SARS-CoV-2, responsible for the severe acute respiratory syndrome (SARS) CoV and Middle East Respiratory Syndrome CoV [31]. It was believed that due to the expedited urbanization and poultry farming, HCoVs have evolved to cross the animal barrier, benefiting from the high rate of genomic recombination characterizing these viruses [32]. Although the hypothesis of clear CoVs transmission from bats is not fully approved, the direct contact with intermediate animal hosts has been strongly suggested as a possible cause of transmission [30].

3. Structure, Genome and Genes

The entire genome of SARS-CoV-2 was characterized by an RNA based metagenomics next-generation sequencing approach. Its genome of 29,881 bp in length encodes 9860 amino acids [33], with genes fragments of structural and non-structural proteins. structural proteins are encoded by the S, E, M and N genes, whereas non-structural proteins are encoded by the ORF region and include the 3-chymotrypsinlike protease, papain-like protease, and RNA-dependent RNA polymerase [34].

4. SARS-COV-2 variants

As they spread between people over time, it's normal for viruses to change and evolve. Once these changes become significantly different from the original virus, they are named as “ variants”. Since SARS-COV-2, has been spreading globally, its variants have emerged and been identified in many countries around the world [35]. To date, four classes of SARS-CoV-2 variants were identified by the SARS-CoV-2 Interagency Group (SIG) Variant classification scheme, such as: Variant Being Monitored (VBM), Variant of Interest (VOI), Variant of Concern (VOC) and Variant of High Consequence (VOHC). Among the Variant Being Monitored (VBM), Alpha (B.1.1.7 and Q lineages), Beta (B.1.351 and descendent lineages), Gamma (P.1 and descendent lineages), Delta (B.1.617.2 and AY lineages), Epsilon (B.1.427 and B.1.429), Eta (B.1.525), Iota (B.1.526), Kappa (B.1.617.1), 1.617.3, Mu (B.1.621, B.1.621.1) and Zeta (P.2), can be mentioned [36]. Alpha (B.1.1.7) was the first identified major variant of concern, It was first identified in the United Kingdom in the fall of 2020. The variants Beta (B.1.351), first identified in South Africa, and Gamma (P.1), first identified in Brazil, both exhibit increased transmissibility, but not as much as the Alpha variant. Delta (B.1.617.2) is currently the most prevalent variant in the United States. Early data suggest that the Delta variant spreads almost twice as quickly as the original SARS-CoV-2 virus [37].

5. Entry and replication

Viruses' entry into host cell is a major contributor to both viral infectivity and pathogenesis. This mechanism is a target of the host immune surveillance as well as human therapeutics strategies [29]. Initiated by host cell recognition, overcoming the host cell membrane barrier is the first step for a virus to enter the host cell and start the infection. For enveloped viruses, this process is accomplished by fusion proteins that are

endoproteolytically cleaved, resulting in a metastable complex of two subunits: the receptor binding and membrane fusion subunits. Upon binding to the host cell receptor, this fusion protein undergoes a conformational change resulting in the exposure of a hydrophobic fusion peptide and its insertion in the host membrane. Seeking for the most stable conformation, the refolding process leads to the liberation of free energy that mediate not only the apposition of viral and host cell membrane, but also the actual fusion of both membranes [38]. For SARS-CoV-2 this is accomplished by the S protein, a highly conserved glycoprotein among all human coronaviruses. The S protein is known by its function in ACE2 receptor recognition, viral attachment, and cell entry mediation [39]. The ACE2 receptor, is expressed in bronchus and lung parenchyma as well as in the heart, kidneys, and gastrointestinal tract [6]. With a size of 180-200 KDa, the S protein has an extracellular N- terminus domain, a transmembrane domain anchored in the viral membrane and a short intracellular C-terminal segment. This structural protein consists of 1273 amino acids (a.a.) that encodes 3 main regions: a signal peptide that extend till the 13th residue located in the N- terminus, and the two subunits S1 (14–685 residues) and S2 (686–1273 residues) that are responsible for host cell recognition and membrane fusion, respectively. The S1 subunit includes the N terminal domain (14–305 residues) and the receptor- binding domain (RBD, 319–541 residues), while the S2 subunit includes the fusion peptide (FP) (788–806 residues), heptapeptide repeat sequence 1 (HR1, 912–984 residues), HR2 (1163–1213 residues), transmembrane (TM) domain (1213–1237 residues), and the cytoplasmic domain (1237–1273 residues). For SARS-CoV, the recognition of the host cell is also accomplished by the spike protein that binds to the hACE2 receptor by its RBD domain included in the S1 region [39]. Measurements of SARS-CoV-2 binding affinity to the host cell's ACE2 receptor showed that it is also

mediated by a key factor: the RBD (receptor binding domain) located within the S1 subunit [40]. After binding, the S1/S2 are cleaved at their separation boundary by entry-activating proteases such as the cell surface protease TMPRSS2 and lysosomal proteases cathepsins [39]. In the case of SARS-CoV-2, in addition to these proteases, SARS-CoV-2 is proteolytically preactivated by the cleavage at the S1/S2 boundary, specifically at the PPC site by the furin protease allowing virus and host cell's membrane fusion. In one study investigating the role of the cleavage at the PPC site, SARS-CoV-2 mutant with a PPC site replaced by the equivalent region of SARS-CoV resulted in dramatically reduced cell entry efficiency, showing that such cleavage is critical for SARS-CoV-2 entry. In addition, this cleavage done at the virus's packaging phase is boosted by the function of two other proteases TMPRSS2 and lysosomal proteases cathepsins that have a cumulative effect on activating SARS-CoV-2 entry, leading to less dependence of SARS-CoV-2 on cellular proteases. The study of the crystal structure of the RBD complexed to hACE2 showed many differences between the RBD of SARS-CoV-2 and that of SARS-CoV. The binding affinity of SARS-CoV-2 RBD to the hACE2 receptor is higher than that of SARS-CoV. This RBD switches between two conformations: a standing-up position and a lying-down position for receptor binding and immune evasion respectively [29]. In contrast, the affinity of the whole S protein is higher in case of SARS-CoV than SARS-CoV-2, something that can be explained by which state the RBD is in, as it was found that SARS-CoV-2 RBD has a lying down status most of the time making it less exposed, a camouflage strategy that SARS-CoV-2 uses to evade the immune system, leading to severe illness and prolonged recovery duration [29].

6. Viral shedding

Understanding the full spectrum of a disease such as virus infectivity, transmission, and shedding kinetics is an important factor to understand this virus especially in case of an outbreak situation, where emerging organisms causing severe human diseases are in question [41]. For SARS-CoV-2 the peak of viral shedding is 24 to 48 hours prior to illness onset, an important factor favoring the possibility of virus transmission while the patient is still pre-symptomatic [42]. It has been demonstrated that pre-symptomatic and symptomatic infected patients have the same viral load [43], with a longer viral shedding in blood and feces in mild cases on glucocorticoid [44].

7. Transmission

Person-to-person transmission has been reported, occurring with close contact [45, 46]. This transmission is accomplished through large droplets or possibly via aerosolization [47]. The amount of virus to which a person is exposed to, depends on two factors: the volume of droplets in which the virus is included, and the time that separate the exhalation of infectious particles and exposure. As for the first factor, a higher risk of transmission is associated with small particles that remain in the airstream where large and heavy particles fall to the ground or other surfaces. Regarding the second factor, a higher gap between exhalation and exposure is associated with a higher loss of viral viability and infectiousness, influenced by environmental factors such as temperature, humidity, and ultraviolet radiation [48].

8. Symptoms

SARS-CoV-2 infections range from asymptomatic to severe [49]. At the onset of illness, the most common symptoms are fever (not necessarily present in all patients, even

hospitalized ones), cough (that may be productive or not), myalgia and fatigue (that may be profound). The upper respiratory tract may be affected in 20% of patients, manifesting as rhinorrhea, sneezing, and sore throat. Gastrointestinal symptoms such as abdominal pain, nausea, vomiting, and diarrhea are less common, manifesting in only 10% to 20% of patients. As for the less common symptoms, sputum production, headache and hemoptysis can be present [7, 50, 51]. The central nervous system may be involved at an early or a late phase of infection leading to a temporary loss of smell and taste [52]. There is a possibility of the symptoms worsening over the course of a week or more, progressing into sudden and catastrophic deterioration, leading to respiratory distress or shock [7].

9. Diagnosis

The typical incubation period of COVID-19 ranges between 14 days of exposure, and 5 days in most patients. Patients with an incubation period over 14 days are seen but rarely reported [45, 53]. Rapid and accurate detection of SARS-CoV-2 is mandatory to manage and limit COVID-19 pandemic. The current gold standard detection method is real-time reverse transcription–polymerase chain reaction (RT-PCR) that detects SARS-CoV-2 nucleic acid (RNA) in nasopharyngeal fluids [54, 55]. This method has a high sensitivity and specificity offering reliable diagnosis [56]. Antigen test is also available and works by specifically detecting 3-dimensional SARS-CoV-2 surface proteins [57]. This test is less sensitive than polymerase chain reaction, with a nearly equivalent specificity [58]. An advantage of the antigen test is the rapid turnaround [49].

10. SARS-CoV-2 disease and associated risk factors

Age has been reported as the most linked risk factor, where all age groups are susceptible to SARS-CoV-2 infection, but it appears to be more severe and causing higher death cases in old people [59]. As for sex, and based on the available data, SARS-CoV-2 is more often manifesting in females but has a higher severity in males [60]. In addition, some medical conditions can be associated with a higher risk of severe disease, such as chronic kidney disease, chronic lung diseases, chronic liver diseases, Diabetes mellitus type 1 and type 2, malignancy, hypertension, immunodeficiency, and sickle cell disease. However, some patients can develop severe disease with no comorbidities [61].

11. Treatment and treatment strategies

In addition to infection control measures and routine supportive care (i.e., oxygenation, ventilation, and fluid management), many attempts to discover a potential pharmacological therapy were made. At the beginning of the epidemic, the first treatment strategies have aimed to control the cytokine storm identified in COVID-19 patients with pneumonia [62]. The proposed medications include antivirals, monoclonal Abs, corticosteroid drugs, and immunomodulators. As for antivirals, the choice is made based on the pharmacologic mode of action and the stage of physiopathology [49]. Remdesivir, a viral polymerase inhibitor [63], has been used to treat COVID-19 patients specifically with FDA approval for both hospitalized and non-hospitalized patients who required supplemental oxygen or are at high risk of progressing into severe disease [64-66]. Monoclonal Abs that prevent the virus entry into host cells are also available, and included bebtelovimab [67], sotrovimab, casirivimab-imdevimab, and bamlanivimab-etesevimab. The emergency use has been approved in patients with mild to moderate

cases that can progress into severe disease. However, the administration of casirivimab-imdevimab, and bamlanivimab-etesevimab has been paused because of reduced activity showed with the new B.1.1.529 (Omicron) variant [68]. As for corticosteroids drugs, dexamethasone has been associated with a significant decrease in mortality rates among patients requiring supplemental oxygen [69]. One other therapy strategy is immunomodulators that are used to restore the immune system homeostasis in COVID-19 patients [70]. Antimalarials such as chloroquine (CQ) and hydroxychloroquine (HCQ) showed a summatory effect against SARS-CoV-2 infection when given with antivirals and azithromycin [71, 72]. On the other hand, subsequent studies have shown no significant efficacy but a higher risk of QT prolongation (extended interval between the heart contracting and relaxing), and cardiac arrhythmias, that lead to the withdrawal of FDA emergency use authorization [73]. Other strategies have been used such as anti-IL-6, anti-IL-1, anti-IL-2, as well as other cytokine targeted therapy and Janus kinase pathway [JAK] inhibition [74].

C. Immunity against SARS-CoV-2

The immune system is composed of a complex network of cells that cooperate in an orchestrated manner to fight a threat. It is broadly divided into two main components: the innate and adaptive immunity [75, 76]. Although the innate and adaptive immune responses are linked in such a powerful way, each consist of a set of cells with different characteristics and functions [77]. Understanding the immune response against SARS-CoV-2 is critical for the development of both novel vaccines and new therapeutic approaches [62]. Below are some recorded data about the immunity developed against SARS-CoV-2.

1. Innate immunity

The innate immunity is the first line of defense against pathogens, including SARS-CoV-2. It plays a role in limiting viral entry, translation, replication, and viral assembly. Also, it helps identifying and eliminating infected cells [63]. For the innate immunity to be activated, pathogen associated molecular patterns (PAMPs) of SARS-CoV-2 are recognized by pattern recognition receptors (PRRs), which activate an anti-viral signaling cascades. Distinct classes of specific PRRs are present and they include: Toll-like receptors (TLRs), retinoic acid-inducible Gene-I (RIG-I) receptors (RLRs), and C-type lectin receptors (CLRs) [78]. Since SARS-CoV-2 is a positive-sense single-stranded RNA (ssRNA) that uses double-stranded RNA (dsRNA) replication intermediates to replicate, those can be sensed by TLRs and RLRs in host cells [79]. The activation of these two PRRs lead to a production of type I and type III INFs [80]. TLR2 has been reported as an activator of the immune response against SARS-CoV-2. Once stimulated, it promotes the local integrity of epithelial barrier function, the suppression of excessive inflammation and tissue damage. On the other hand, TLR2 signaling may be associated with COVID-19 severity where its blocking by oxPAPC protects against SARS-CoV-2-induced lethality *in vivo*, showing a role of TLR2 in the development of COVID-19 disease. Other TLRs (TLR3, TLR4, TLR7) have shown an efficient role in the protection against SARS-CoV-2 infection [81-83]. The induced anti-viral signaling leads to the production of interferons, cytokines, and chemokines [84-86].

a. Interferons

Interferons are potent multifunctional cytokines secreted by various cell types [79]. The antiviral interferon (IFN) system is the first line of defense of the innate

immunity [87]. At a specific level, the IFNs type I and type III are known for the crucial role they play in innate immunity during viral infections [79]. Type I IFNs bind to the heterodimeric complex composed of IFNAR1 and IFNAR2, leading to the phosphorylation of STAT1 and STAT2 [88-90]. p-STATs 1 and 2 bind to IRF9 and enter the nucleus where they promote the transcription of hundreds of IFN-stimulated genes (ISGs) [91, 92]. Type III interferons, use the same signaling pathway to express the ISGs. After the engagement to their receptors, type I and type III IFNs activate overlapping intracellular signaling pathways and mediate the activation of the same sets of ISGs [79]. This activation of ISGs leads to the repression of viral replication through many mechanisms such as the degradation of the viral nucleic acids and the inhibition of viral transcription/translation. SARS-CoV-2 has been known for its capacity to frustrate the interferon response [79], where COVID-19 infection severity in adults has been associated with an impairment of antiviral response in the nasal epithelium and blood [93-95], showing an efficient counteracting of the antiviral system by SARS-CoV-2 [96-98]. On the other hand, it has been shown that IFN response may contribute to COVID-19 pathogenesis [99, 100].

b. IL-6 response

IL-6 is a pleiotropic cytokine that plays a major role in both adaptive and innate immunity [62]. In addition to its beneficial role in the direction of immune cells differentiation, plasma cells growth and Ab production, an excessive response of IL-6 is a characteristic of inflammatory diseases and autoimmunity [101] and is considered a potential pathogenic factor of acute respiratory distress syndrome (ARDS) in SARS-CoV-2 patients with pneumonia [102]. It has been demonstrated that SARS-CoV-2 has a

proclivity for the activation of macrophages leading to copious and sustained IL-6 production resulting in a cytokine storm, endothelial cell damage, and acute respiratory distress syndrome (ARDS). Data has shown that targeting IL-6 or its receptor may be a potential therapeutic strategy for SARS-CoV-2 infection [103-105]. Tocilizumab is the first immunomodulatory agent used for SARS-CoV-2 pneumonia treatment. It is a monoclonal Ab that targets the IL-6 receptor preventing IL-6 from binding to it [62].

c. Chemokines

Chemokines are informative inflammatory mediators that play many roles that consist of the recruitment of innate and adaptive immune cells to the site of infection, the enhancement of their cytotoxic function, as well as their ability to produce antiviral mediators, leading to pathogen clearance [106]. SARS-CoV-2 upregulate a specific profile of chemokines, where the levels of the chemokines CCL3, CXCL10, CCL5, and CCL20 were found to be high in COVID-19 patients [107]. Importantly, upregulation of CXCL17 (VCC-1) that attracts DCs and monocytes to the infected lungs was observed at the first stages of infection [108]. It is worth noting that disease severity correlates with an increase in CXCL10, which is absent in healthy individuals, suggesting an important role of this chemokine in early diagnosis that could be used as a potential predictor of disease outcome [109]. However, an excessive activation of chemokines is the main cause of hyperinflammation [107].

d. Cytokine storm

In addition to IFNs and IL-6, other pro-inflammatory cytokines are released such as: IL-1 β , TNF- α , IL-12, IL-17, IFN- γ and others [7, 94, 110]. Despite their important role in the maintenance of cellular homeostasis and infections clearance [63], a

dysregulated response of these pro-inflammatory cytokines leads to a cytokine storm, a life-threatening condition due to excessive cytokines production mediated by inflammatory cell death (PANoptosis) [111]. In case of COVID-19, this cytokine storm is mediated by TNF- α and IFN- γ combination, which mediate the inflammatory cell death or PANoptosis [112].

2. Adaptive immunity

Mainly composed of B cells, CD4⁺ and CD8⁺ T cells, this response is relatively slow, and takes around 6-10 days to develop effector cells from naïve cells. Virus specific B and T cells are present among a large pool of cells (>10⁹ cells each) that are specific for different molecular structures and sequences. After virus recognition, virus specific B and T cells are activated and expanded. Once developed into effector T cells (helper T cells and cytotoxic T cells) and effector B cells (Ab secreting cells, known as plasmablasts and plasma cells), these two components of the adaptive immunity work together to mount a considerable immune response against the pathogen. In the case of SARS-CoV-2 infection, this adaptive immunity is delayed because of the impairment of IFNs type I and type III, considered as the innate immunity alarm, needed to activate the adaptive immunity [77].

a. T cell response

T cell response was detected in almost all COVID-19 patients, and this include [77]:

i. CD4⁺ T cells response

With the capacity to differentiate into a range of helper and effector cells, CD4⁺ T cells can educate B cells, help CD8⁺ T cells, recruit innate cells, induce antiviral response, and induce tissue repair [77]. Once activated, virus specific CD4⁺ T cells differentiate into Th1 and T follicular helper cells (Tfh) subsets. Th1 cells are responsible for the production of IFN- γ and other cytokines that induce an antiviral state. Tfh cells are the main B cells educator, allowing their activation to produce specific SARS-CoV-2 neutralizing Abs, as well as long lasting memory B cells [113]. SARS-CoV-2 CD4⁺ T cells response is prominent above the CD8⁺ T cell response and seems to control the first stages of COVID-19 infection [114-116]. The magnitude and prevalence of SARS-CoV-2 specific CD4⁺ T depends on the level of SARS-CoV-2 expressed proteins, where S, M and nucleocapsid proteins were reported as the most important target for SARS-CoV-2 CD4⁺ T cells [114]. Patients with severe disease outcome who were admitted to the ICU has a diminished function of CD4⁺ T cells in terms of number and quality [117], where mild disease and accelerated clearance of the virus is associated with a rapid activation of SARS-CoV-2 specific CD4⁺ T cells [118]. However, an excessive activation of CD4⁺ T cells is associated with severe outcomes [119]. And while an increase in neutralizing Ab titers does not correlate with a reduced severity of disease, high frequency of Tfh cells has been correlated with a decrease in disease severity [116, 120-122]. In addition to the help of B cells, CD4⁺ T cells help CD8⁺ T cells. And while this role remains unclear, a potential role of IL-21, a canonical cytokine of Tfh cells, has been reported [123, 124]. CD4⁺ T cells has also a direct effector role, through Th1 cells, producing IFN- γ , the main produced cytokine by CD4⁺ T cells [114, 125, 126]. In addition, a robust secretion of IL-

22, known for its role in tissue repair especially for lung and gut epithelial cells, by SARS-CoV-2 specific CD4⁺ T has been reported [77].

ii. CD8⁺ T cells response

CD8⁺ T cells are important to clear viral infections by killing infected cells. It has been demonstrated that SARS-CoV-2 infection better outcomes were associated with the presence of SARS-CoV-2 CD8⁺ T cells [116, 127]. Following COVID-19 infection, SARS-CoV-2 specific CD8⁺ T cells secrete high levels of IFN- γ , granzyme B, perforin, and CD107a, molecules with potent cytotoxic effect [115, 116, 128]. It has been shown that exhausted CD8⁺ T cells, are more frequent and displayed a lower cytotoxicity and inflammatory features in patients with mild disease when compared to patients with severe illness [129]. In addition, CD8⁺ T cells were reported to be more frequent in the BALF of patients with mild disease compared to patients with severe disease [130].

B cell immunity

Abs, B cells, and plasmablasts that are responsible for Ab production are the main components of the humoral immunity induced by SARS-CoV-2 infection. Known by its short term, the immunity induced by coronaviruses is short compared to many acute viral infections such as yellow fever, measles, and polio. Virus-specific Abs produced by plasmablasts are the most important factor for a long-term protection against a reinfection by most viruses. This long-lasting immunity requires a good coordination between B and T cell responses to generate a durable Ab response. This coordination between B and T cells to mature the humoral immunity occurs within lymphoid tissue germinal centers

(GCs), where long-lived plasma cells as well as class-switched memory B cells are generated. These 2 factors play a major role in the rapid mounting of a secondary response after re-encountering of the same Ag. Upon infection with SARS-CoV-2, naïve B cells or potentially pre-existing cross reacting memory B cells because of previous HCoVs exposures are activated [131]. This leads to the production of SARS-CoV-2 specific Abs including IgG, IgA, and IgM. The first isotype to appear is the IgM isotype, with a median time of seroconversion between 11 to 13 days-post symptom onsets [132, 133]. After seroconversion, IgG and IgA isotypes appear. And while SARS-CoV-2 is still spreading, the rate at which IgG, IgA, and IgM Abs levels decay either post infection or vaccination remains a topic of some debate, and the aim of many longitudinal studies.

Immune system evasion strategies

Any virus causing a human disease should have at least one immune evasion strategy, or this virus will be qualified as “harmless”. Understanding the immune evasion mechanisms is important to decipher the virus pathogenesis, allowing the good understanding of challenges faced either by the immune system or by any vaccine candidate. SARS-CoV-2 has been largely known by his unusual capacity to evade the first line defense of the immune system represented by the IFN type I response, which is a major cause for the development of SARS-CoV-2 infection [77].

D. Vaccines Development

Already considered as a low priority concern, no vaccines against coronaviruses were licensed for use in humans, the reason for this lies in the mild symptoms caused by previous coronaviruses. Also, the developed vaccine should be quadrivalent to be

protective against four different viruses, even though, only a small portion of colds will be prevented because most of them are caused by other viruses [12]. As of January 11th, 2020, SARS-CoV-2 genetic sequence became available, making the development of a vaccine possible [134]. Thanks to the previously efforts made in designing a vaccine against the related highly pathogenic beta-coronaviruses SARS-CoV and MERS-CoV, the responsible agent of eliciting an efficient immune response became clear: the S protein, and more specifically the RBD encoded in that protein [135]. For these reasons, the research to develop a vaccine for SARS-CoV-2 took place at an unprecedented fast pace, with clinical trials kicking off just a few months after the first case was identified. Currently, more than 180 vaccines candidates are under development [12], among which several have entered the use phase, while others are still under development, with 148 and 195 vaccines in clinical and pre-clinical trials, respectively [136]. Based on the analyzed data, vaccines licenses range between urgent, temporally, or fully authorized by national regulatory authorities. And while some vaccines have been fully approved, other approvals are still pending [49]. In this process, both traditional and novel approaches were used. Among these strategies, inactivated SARS-CoV-2 virus vaccines (Sinovac Biotech), live attenuated SARS-CoV-2 vaccines (Codagenix, Serum Institute of India), recombinant protein vaccines based on the spike protein (Novavax), the RBD, or on virus-like particles (Medicago), replication-incompetent S protein-expressing adenovirus vector vaccines (AstraZeneca, Johnson and Johnson, Janssen, CanSino, Gamaleya Research Institute, ReiThera), replication-competent vector vaccines (Merck, Beijing Wantai Biological Pharmacy), and inactivated virus vectors vaccines that display the S protein on their surface (WHO), have been developed. These vaccines are given intramuscularly, to induce an immune response against SARS-CoV-2 S protein, or

intranasally in some cases, where they induce an immune response that resides in the mucosa of the upper respiratory tract, the major entry portal for SARS-CoV-2 [12].

Although they use different strategies, the aim of most vaccine candidate is to develop immunity against SARS-CoV-2 S protein by eliciting neutralizing Abs that bind to viral particles preventing their binding to the ACE2 receptor and their uptake by the human host cells, therefore, blocking the infection [137]. However, a significant role of the cellular immunity has been identified in the literature on the recovery of COVID-19 patients, which has promoted not only the vaccines strategies targeting T cell induction, but also the interest of studying the T cell immune response in vaccine's candidates during clinical trials [127, 138]. It is worth noting that although both N and S viral proteins have shown a potential immunogenic effect with an elicited T cell response, only the S protein was able to induce neutralizing Abs, making it the target for coronavirus vaccines [139, 140].

1. Nucleic acid-based vaccines

Therapeutic nucleic acids (TNAs) are recent approaches classified among the best drug discovery platforms, in addition to small molecules and Ab therapeutics [141]. The nucleic acid-based vaccines consist of either a DNA or RNA that once internalized by the host cell, they use the cellular machinery to be translated into the protein they are encoding [142]. Like other vaccines candidates, the DNA vaccines' main antigenic target in preclinical and clinical trials is the S protein, that can be presented in many forms: full length S protein, RBD, S1 subunit, or S2 subunit. DNA vaccines targeting the N protein, along with the S protein have also been developed [143]. Like the DNA vaccines, one new approach, have been propelled to the center stage of both biotechnology and

pharmaceutical industry, which is the use of mRNA-based vaccines in which the mRNA is delivered through nanolipidic particles (NLPs) [12, 144]. After vaccine's injection in the upper arm muscle, the mRNA enters the host cell and uses the cell's machinery to produce a harmless piece of the S protein. Later, this S protein will be displayed on the surface of host cells, triggering an immune response [145]. Nowadays, eight mRNA-based vaccines are in ongoing trials, among which two are considered as world leading vaccines led by both BioNTech/Pfizer and Moderna which are in the phase 3 [146]. The other six vaccines are CureVac, Sanofi/TranslateBio, Arcturus/Duke-NUS Medical School in Singapore, Imperial College London, Chulalongkorn University in Thailand, and Providence Therapeutics vaccines [147]. As of March 11, a new mRNA-based vaccine from the University of Melbourne has entered the phase 1 clinical trial [146].

The BNT162b2 vaccine developed by Pfizer-BioNTech, was the first mRNA-based vaccine to receive the use authorization in the United States, followed by a full FDA approval for candidates of 16 or above [49].

2. Vaccines' effectiveness

To be considered successful, a vaccine should produce both specific and efficient neutralizing Abs against SARS-CoV-2 [137]. Based on data compilation done by the CDC on the effectiveness of various vaccines (Pfizer/BioNTech, Moderna, Johnson and Johnson, AstraZeneca), most of them have shown an efficacy above 90% [148]. As for the mRNA-based vaccines, excellent results have been achieved by both companies, Pfizer/BioNTech and Moderna, while developing their BNT162b2 and mRNA-1273 vaccines, respectively. The full-length of the S sequence with the proline substitutions K986P/V987P was utilized. The Pfizer-BioNTech vaccine (BNT162b2 vaccine) achieved

95% efficacy against COVID-19 [149]. A high efficacy has been also reported against both the B.1.1.7 and B.1.351 variants with a value of 87% and 72.1% respectively [150]. This efficacy of vaccines cannot be compared head-to-head, as each one of vaccines candidate has its design strategy. Accordingly, every vaccine licensed for emergency use by the WHO is effective and protects against COVID-19 complications including severe symptoms and hospitalization [151]. As for the effectiveness over time, initial evidence of decreasing efficacy has been suggested, especially with the new circulating delta variant. However, the vaccination remains highly efficient in preventing the SARS-CoV-2 infection and the related complications that can lead to death [148, 152-154]

3. Major pitfalls in vaccine development

Mainly caused by an “unwanted” enhancement of the immune response, vaccines can lead to undesirable outcomes detrimental to the host. These adverse effects are mainly mediated by Ab-dependent enhancement (ADE) of viral uptake and Th2 immunopathology.

ADE– antibody-dependent enhancement of viral infection

ADE is a process where the virus specific Abs are used to help leverage the viral infection. In this case the Virus-Ab immunocomplexes are attached to cells bearing the Fc receptor, facilitating their internalization, and promoting infection [155, 156]. Based on previously compiled data on SARS-CoV and MERS-CoV vaccines, where neutralizing Abs targeting the RBD of the S protein have showed virus entry mediation into Fc receptor-expressing human cells *in vitro*, the hypothesis of a possible manifestation of ADE in SARS-CoV-2 has been highlighted [157]. Recently, it has been

reported that unlike SARS-CoV and MERS-CoV, the RBD of SARS-CoV-2 S protein is able to induce a high neutralizing Ab without causing ADE in animal immunization studies [158]. On the other hand, two studies have shown a positive correlation between the severity of COVID-19 and the high titer of total Abs, with a specific increase in IgG [159, 160]. However, the relevance of ADE in COVID-19 is still in doubt [137].

Th2 immunopathology

One study correlated the severity of SARS-CoV-2 infection with an enhanced immune response of CD4⁺ T cells biased toward the Th2 subset leading to allergic reactions (e.g., IL-4, IL-5, and IL-13) and an infiltration of eosinophils into the lungs [161].

CHAPTER III

MATERIALS AND METHODS

A. Ethics statement

This study was approved from the institutional review board (IRB) of the American University of Beirut (BIO-2021-0115). An informed consent was obtained from each enrolled participant.

B. Study design and population

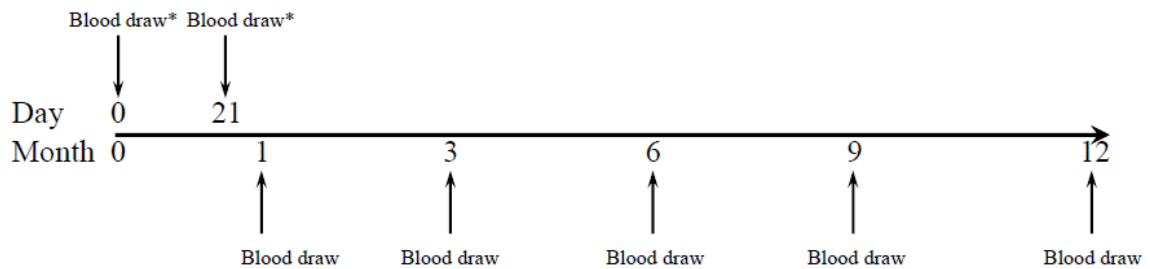
Adults of 18-55 years of age, who are healthy, planning to take the vaccine and have no history of SARS-CoV-2 infection were recruited. People with medical history of Covid-19, who contracted SARS-CoV-2 after vaccination (with the prime and/or third dose -first boost dose-), treated with immunosuppressive therapy, and diagnosed with an immunocompromising condition are excluded. Co-morbid conditions such as hypertension, cardiovascular disease, chronic lung disease, diabetes, and cancer are also exclusion criteria. Twelve candidates who met the above-mentioned conditions were included in the study.

C. Recruitment process

Flyers were used to recruit participants who were planning to take the Pfizer/BioNTech RNA-based vaccine. Those who met the inclusion criteria and showed interest in participating after reading the flyer were included, and the purpose of the study was explained to them.

D. Specimen collection

A total of 7 blood draws of a maximum of 10 ml each were collected from 12 participants by venipuncture, in tubes containing sodium-heparin as anticoagulant. Blood was withdrawn on day 0 before the prime dose was given, on day 21 after the prime dose was given and before the boost dose was given, then 1 month, 3, 6, 9, and 12 months after the boost dose was is given, as detailed in the below timeframe (Figure 1).



*Before administering the prime and boost vaccine doses.

Figure 1. Time points at which the blood samples from our study participants were collected.

E. Plasma and Peripheral Blood Mononuclear Cells Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient sedimentation using Ficoll-Paque (GE healthcare). Blood was layered onto 4 ml of Ficoll solution in a 15ml sterile tube. Tubes were centrifuged at 2000 rounds per minute (rpm) for 30 minutes. Four separated layers were obtained in each tube: plasma at the top, followed by the buffy coat that consists of the PBMCs, the Ficoll-Paque media, and the erythrocytes along with granulocytes at the bottom (Sigma-Aldrich Protocol). The plasma was collected into aliquots and stored at -80°C for a later use. The PBMCs were collected using a graduated pipette and placed in a sterile tube of 15 ml. The PBMCs were then washed 3 times with RPMI (10% FCS, 1% Peni/Strep, 1% L-glutamine, 1% HEPES). Cells

were counted under an optic microscope using a hemocytometer. The total number of cells is calculated using the following formula:

Total number of cells/ml = (amount of cells/number of squares counted) * dilution factor * 10,000.

The PBMCs were then cryopreserved in a recovery cell medium (FCS, 10% DMSO) and stored at -20°C. Next day cell samples were transferred to the -80°C.

F. Enzyme-Linked Immunosorbent Assay (ELISA)-coating concentration optimization

Ninety-six well plates (Nunc, Denmark) were coated with 50 µl per well of His-tagged recombinant SARS-CoV-2 full S protein (S1+S2) of the Alpha (B.1.1.7) variant (HEK293-derived) (R&D SYSTEMS), at 4 different concentrations: 0.5, 1, 2 and 4 µg/ml protein solution, corresponding to 25, 50, 100, and 200 ng/well, respectively, in PBS overnight at 4°C. The next day, the protein solution was aspirated, and plates were blocked at room temperature (RT, 20–25°C) for 1 h with 200 µl of a blocking buffer (3% non-fat milk powder in PBS/0.1% Tween 20). Pre vaccine samples and 1 month post the second dose samples of one of the participants, were heat-inactivated at 56°C for 1 hour, then plasma samples were diluted with a dilution of 1/100 in a diluent buffer (1% non-fat milk powder, PBS/0.1% Tween 20). Fifty µl of sera were added per well in duplicates for 2 h at room temperature. Wells were washed 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). Fifty µl horseradish peroxidase (HRP)-conjugated anti-human IgG (BioLegend) diluted to 1:3000 in a diluent buffer (PBS/0.1% Tween 20) were added per well for 1 h at RT. Wells were washed 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). Plates were developed with 50 µl of 3,3',5,5'-Tetramethylbenzidine (TMB;

BioLegend) substrate up to 10 min at RT, followed by the addition of 25µl 2 M Sulfuric Acid stop solution. Absorbance at 450 nm was measured on TriStar2 S LB 942 Multimode Reader (Berthold Technologies) using the absorbance at 570 nm to account for background. The average of duplicate wells was calculated.

¶. Enzyme-Linked Immunosorbent Assay optimization (ELISA)- sera dilution determination

Ninety-six well plates (Nunc, Denmark) were coated with 50 µl per well of His-tagged recombinant SARS-CoV-2 full S protein (S1+S2) of the Alpha (B.1.1.7) variant (HEK293-derived) (R&D SYSTEMS) with a 0.5 µg/ml (25 ng/well) protein solution in PBS overnight at 4°C. The next day, the protein solution was aspirated, and plates were blocked at room temperature (RT, 20–25°C) for 1 h with 200 µl of a blocking buffer (3% non-fat milk powder in PBS/0.1% Tween 20). For IgG detection, pre-immune and 1 month post immune samples were diluted with 5 different dilutions of 1/100, 1/300, 1/900, 1/2700 and 1/8100 in a diluent buffer (1% non-fat milk powder, PBS/0.1% Tween 20). For IgA and IgM detection the tested dilutions were 1/10, 1/50, 1/100, 1/300, 1/900 and 1/2700, 50 µl of sera were added per well in duplicates for 2 h at room temperature. Wells were washed 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). HRP-conjugated anti-human IgG, IgA, or IgM (IgG and IgA from BioLegend, IgM from Southern Biotechnology) were diluted to 1:3000 in a diluent buffer (PBS/0.1% Tween 20), and 50 µl were added per well for 1 h at RT. Wells were washed again 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). Plates were developed with 50 µl of TMB substrate (BioLegend) up to 10 min at RT, followed by the addition of 25µl 2 M Sulfuric Acid stop solution. Absorbance at 450 nm and was measured on TriStar2 S LB 942

Multimode Reader (Berthold Technologies) using the absorbance at 570 nm to account for background. The average for the wells duplicates was calculated.

H. Enzyme-Linked Immunosorbent Assay- plasma samples

Ninety-six well plates (Nunc, Denmark) were coated with 50 µl per well of His-tagged recombinant SARS-CoV-2 full spike protein (S1+S2) of the Alpha (B.1.1.7) variant (HEK293-derived) (R&D SYSTEMS) of 0.5 µg/ml (25ng/well) protein solution in PBS overnight at 4°C. The next day, the protein solution was aspirated, and plates were blocked at room temperature (RT, 20–25°C) for 1 h with 200 µl with a blocking buffer (3% non-fat milk powder in PBS/0.1% Tween 20). All plasma samples were heat-inactivated at 56°C for 1 hour, then the plasma samples were diluted in diluent buffer (1% non-fat milk powder, PBS/0.1% Tween 20), with a starting dilution of 1/1000 for IgG and 1/100 for IgA and IgM. The plasma samples were then diluted three-fold and 50 µl of plasma were added per well in duplicates for 2 h at room temperature. Wells were washed 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). HRP-conjugated anti-human IgG, IgA, or IgM (diluted 1:3000) were added in a diluent buffer (PBS/0.1% Tween 20), and 50 µl per well were added for 1 h at RT. Wells were washed again 3 times with 200 µl wash buffer (PBS/0.1% Tween 20). Plates were developed with 50 µl of TMB substrate up to 10 min at RT, followed by the addition of 25µl 2 M Sulfuric Acid stop solution. Absorbance at 450 was measured on TriStar2 S LB 942 Multimode Reader (Berthold Technologies) using the absorbance at 570 nm to account for background. The average for the wells duplicates was calculated.

I. Statistics

GraphPad version 8 was used for statistical analyses and generation of graphs, area under the curve (AUC), ROC curves, and t-tests. For statistical analyses * $p < 0.05$, ** $p < 0.001$, *** $p = 0.001$ and **** $p < 0.0001$ were considered statistically significant.

CHAPTER IV

RESULTS

A. Sero-status of samples

In this study, the total number of plasma samples collected from participants was 71 samples including plasma samples from vaccinated healthy participants (Figure 1). SARS-CoV-2 sero-status was determined using SARS-CoV-2 rapid test, to detect if any of the participants is positive for SARS-CoV-2 at the beginning of the study, and all were negative (data not shown).

B. Optimization of ELISA protocol

During the optimization of the ELISA conditions, it's important to reach a protocol where sufficient Ag is present to capture the Ag-specific Abs, covering all the plate's surface in a monolayer with a minimum non-specific background [162]. Hence, plates were initially coated with SARS-CoV-2 full length (S1+S2) His-tag recombinant protein at a concentration range of 0.5, 1, 2 and 4 µg/ml corresponding to 25, 50, 100 and 200 ng per well respectively, to determine the minimum amount that gives the maximum signal. To determine this optimal amount, pre immune and 1 month post immune plasma samples from a randomly-selected participant were used at a 1:100 dilution. The secondary HRP-conjugated anti-human IgG detection Ab was used at 1:3000 dilution. The detection methodology used for our ELISA was colorimetric where the amount of color in each well is read by a spectrophotometer translated into optical density (OD). The four tested concentrations had almost the same OD₄₅₀ with respective values of 1.029, 1.085, 1.16 and 1.185 for the concentrations of 25, 50, 100 and 200 ng per well (Figure 2).

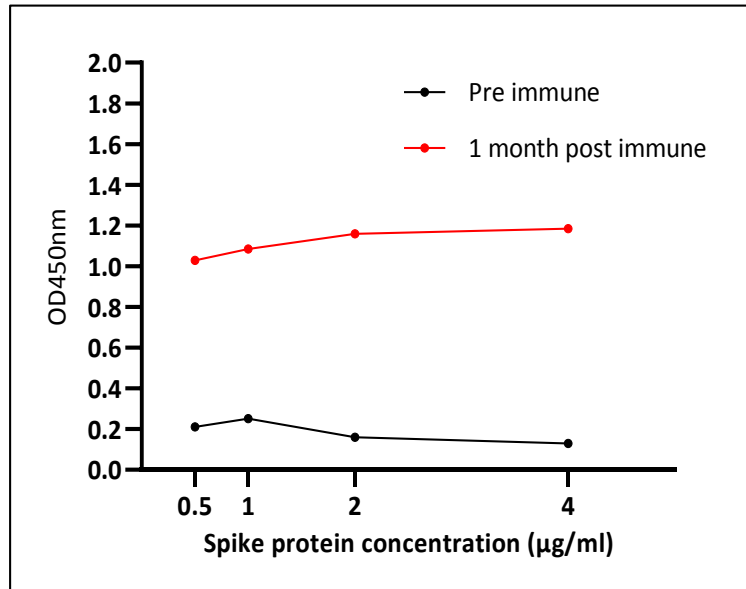


Figure 2. IgG values for the 2 plasma samples pre immune and 1-month post immunization for a randomly selected participant, using the four concentrations of 0.5, 1, 2, and 4 µg/ml corresponding to 25, 50, 100 and 200 ng per well, respectively, of S protein and 1/100 dilution of plasma.

The difference between the four ODs was small, reaching a saturating signal with the 100 ng per well concentration. We concluded that coating the plate with 25 ng per well was optimal to reach an almost maximum of OD using the minimum concentration of S protein.

To determine the appropriate dilution for the candidates' plasma and to ensure maximum capture of SARS-COV-2 specific Abs, the same candidate's plasma were serially-diluted from 1:100 to 1:8100 for IgG detection (Figure. 3) and from 1:10 to 1:2700 for both IgA and IgM detection (Figure. 4).

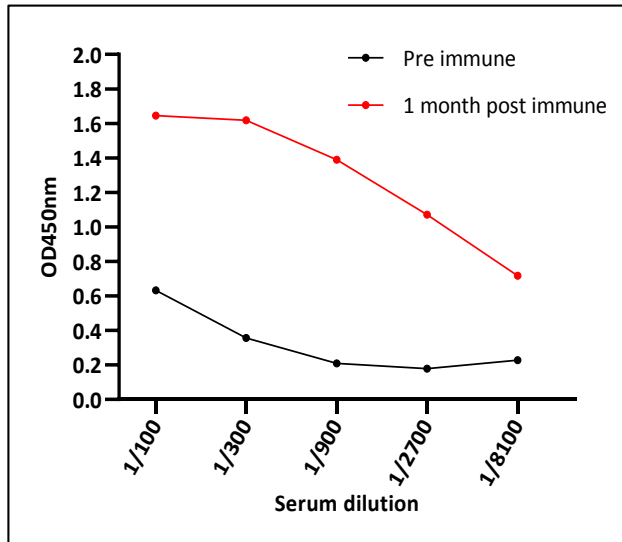


Figure 3. IgG values for the 2 sera samples pre immune and 1-month post immunization for a randomly selected participant, serially diluted with dilutions that range between 1:100 to 1:8100 using 25 ng per well of S protein.

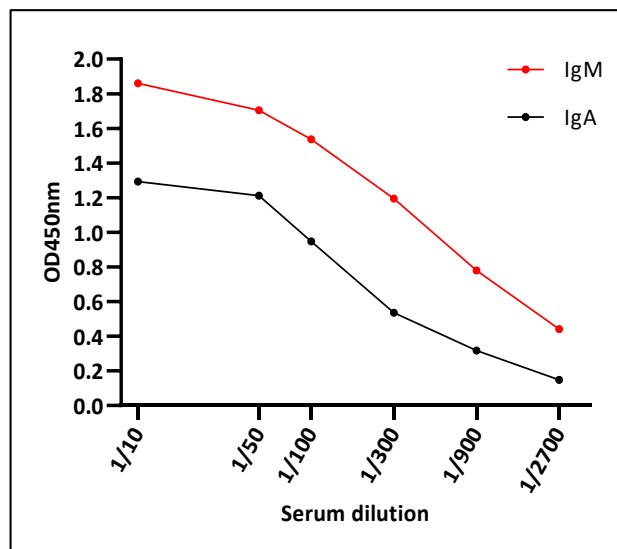


Figure 4. IgM and IgA values for the 1-month post-immunization sample for a randomly chosen participant, serially diluted with dilutions that range between 1:10 to 1:2700 using 25 ng per well of S protein.

The HRP-conjugated anti-human IgG, anti-human IgA, and anti-human IgM were used at 1:3000 dilution. Based on the obtained ODs, the chosen dilutions that gave a detection signal that is not saturated on the spectrophotometer upon dilution were 1:1000 for IgG, and 1:100 for both IgA and IgM.

After optimizing the amount of S protein Ag and starting dilution of the plasma to be used, we went on to analyze the levels of IgG, IgA, and IgM in the pre-immune and 1 month post immune plasma of all 12 participants (Figure 5. A, B, C).

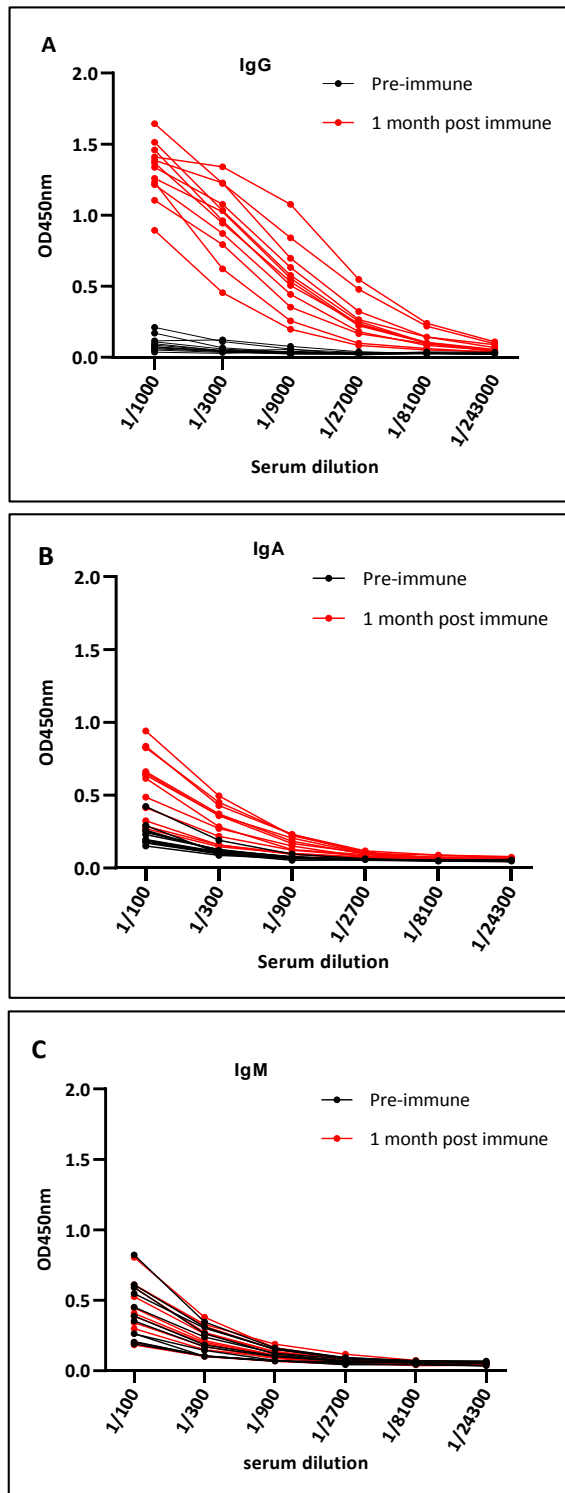


Figure 5. ELISA validation. The optical densities (OD) at 450nm for each three-fold, serially diluted plasma sample were measured in the indirect developed in house-ELISAs. ELISAs measuring IgG (A), IgA (B) and IgM (C) respectively, in serial dilution against the spike (S) protein. Each curve represents a single sample collected before taking the vaccine (black) or 1 month after the second dose (red).

Pre immune and 1 month post immune plasma samples were three-fold serially diluted from 1:1000 to 1: 243000 for IgG detection (Figure 5. A), and from 1:100 to 1: 24300 for both IgA and IgM detection (Figure 5. B, C) and then the levels of anti-S Abs for each participant were determined by ELISA. This showed high levels of anti-S IgG in all participants one-month post-immunization with minimum background in the pre-immune plasma of all participants. The levels of anti-S IgA were also increased, albeit to lesser levels than anti-S IgG, whereas the levels of anti-S IgM were not significantly different in 1 month post-immune and pre-immune plasma of each participant. To obtain an accurate determination of the levels of anti-S Ab in the samples, the area under the curve (AUC) was determined for each sample by calculating the area under curve for each pre immune and 1 month post immune values for the same participant at every single dilution. The calculated AUCs were plotted and demonstrated an average of 5-fold increase in the levels of IgG (range 2 to 11 fold), 1.5-fold increase in the levels of IgA (range 1.2 to 1.8 fold), but no significant increase in the levels of IgM (Figure 6. A, B, C).

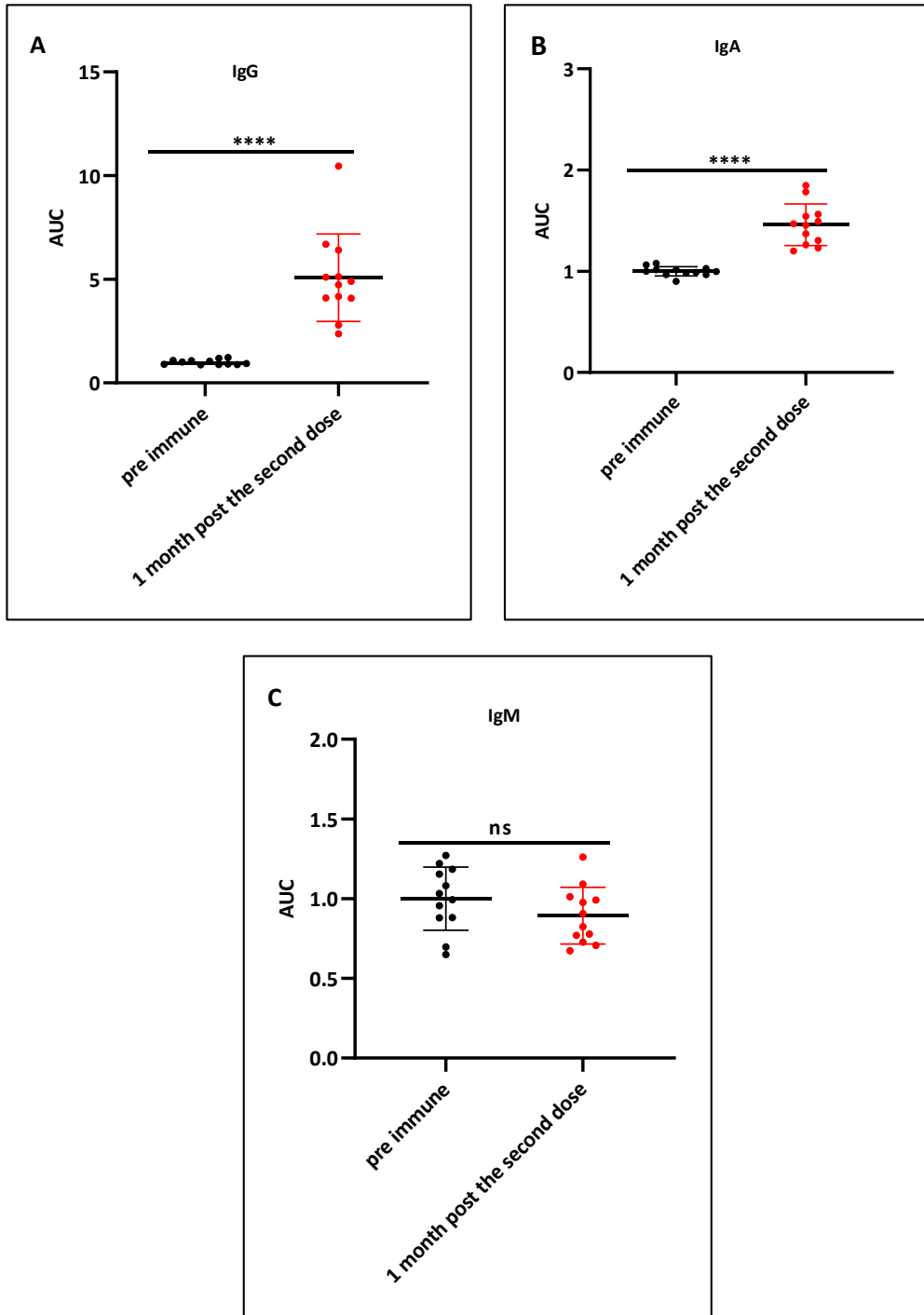


Figure 6. Area under the curve (AUC) of IgG (A), IgA (B), and IgM (C) respectively, measuring the distance between the pre immune and 1 month post immune values for each participant at each dilution . The significance of AUCs were also represented with **** $p < 0.0001$, ns, not significant.

We then calculated the receiving operator characteristic (ROC) values to assess the accuracy and determine the cut-off value that offers maximum sensitivity and specificity of this assay (Figures 7, 8, 9). The developed and optimized ELISA for IgG demonstrated high accuracy with an AUC equal to 1; 95% confidence interval of 1.000 to 1.000 (Figure 7). As per analysis, the cut-off value that provides 100% sensitivity and 91.67% specificity is 0.19 for IgG. A range of OD450 readings and their associated sensitivity and specificity percentages are shown (Table 1).

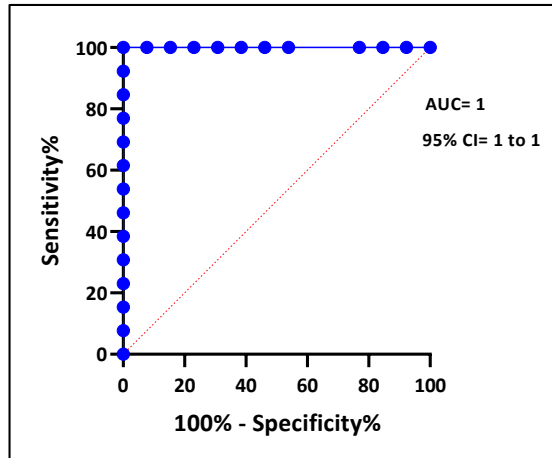


Figure 7. Receiver operating characteristics (ROC) analysis of IgG. ROC analysis was performed for positive samples collected 1 month after the second dose.

	Sensitivity%	95% CI	Specificity%	95% CI
> 0.1900	100.0	75.75% to 100.0%	91.67	64.61% to 99.57%
> 0.5500	100.0	75.75% to 100.0%	100.0	75.75% to 100.0%
> 1.000	91.67	64.61% to 99.57%	100.0	75.75% to 100.0%

Table 1. Range of cut-off values for IgG detection and their associated sensitivity and specificity with 95% confidence interval (CI) as obtained from ROC analysis.

For IgA, the optimized ELISA had a high accuracy with an AUC equal to 0.9583 ± 0.03658 , 95% confidence interval of 0.8866 to 1.000 (Figure 8). Based on this analysis, the cut-off value that provides 100% sensitivity and 83.33% specificity is 0.2650. A range of OD450 readings and their associated sensitivity and specificity percentages are also shown (Table 2).

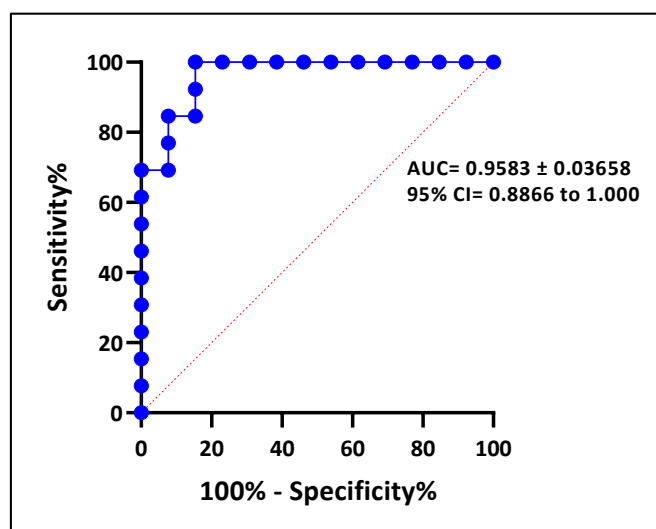


Figure 8. Receiver operating characteristics (ROC) analysis of IgA. ROC analysis was performed for positive samples collected 1 month after the second dose.

	Sensitivity%	95% CI	Specificity%	95% CI
> 0.2560	100.0	75.75% to 100.0%	75.00	46.77% to 91.11%
> 0.2650	100.0	75.75% to 100.0%	83.33	55.20% to 97.04%
> 0.2790	91.67	64.61% to 99.57%	83.33	55.20% to 97.04%

Table 2. Range of cut-off values for IgA detection and their associated sensitivity and specificity with 95% confidence interval (CI) as obtained from ROC analysis.

These high specificity and sensitivity were not the case in the optimized ELISA for IgM detection, since much less specificity and sensitivity than IgG and IgA was obtained using the ROC analysis. The AUC was equal to 0.5556 ± 0.1212 and confidence interval of 0.3181 to 0.7930. As per analysis, the cut-off value that provides fair sensitivity and specificity (53,85% for both) is 0.3980 (Figure 9). A range of OD450 readings and their associated sensitivity and specificity percentages are shown (Table 3).

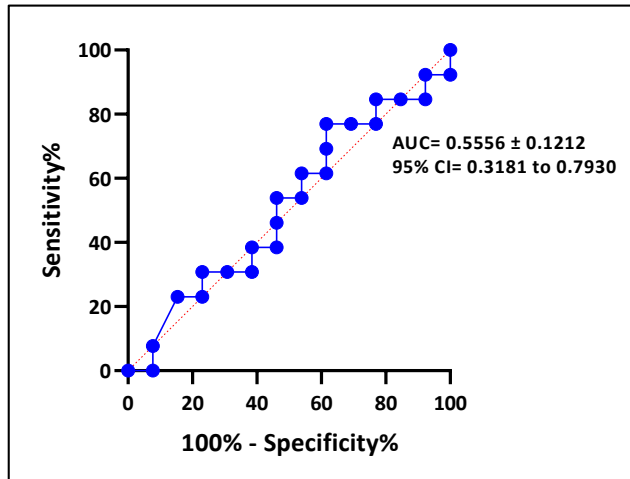


Figure 9. Receiver operating characteristics (ROC) analysis of IgM. ROC analysis was performed for positive samples collected 1 month after the second dose.

	Sensitivity%	95% CI	Specificity%	95% CI
> 0.3875	58.33	31.95% to 80.67%	50.00	25.38% to 74.62%
> 0.3980	58.33	31.95% to 80.67%	58.33	31.95% to 80.67%
> 0.4270	50.00	25.38% to 74.62%	58.33	31.95% to 80.67%

Table 3. Range of cut-off values for IgM detection and their associated sensitivity and specificity with 95% confidence interval (CI) as obtained from ROC analysis.

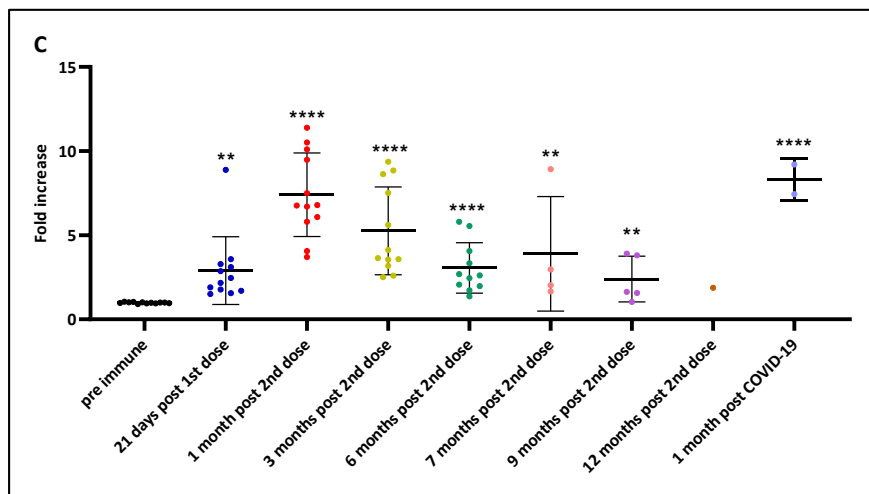
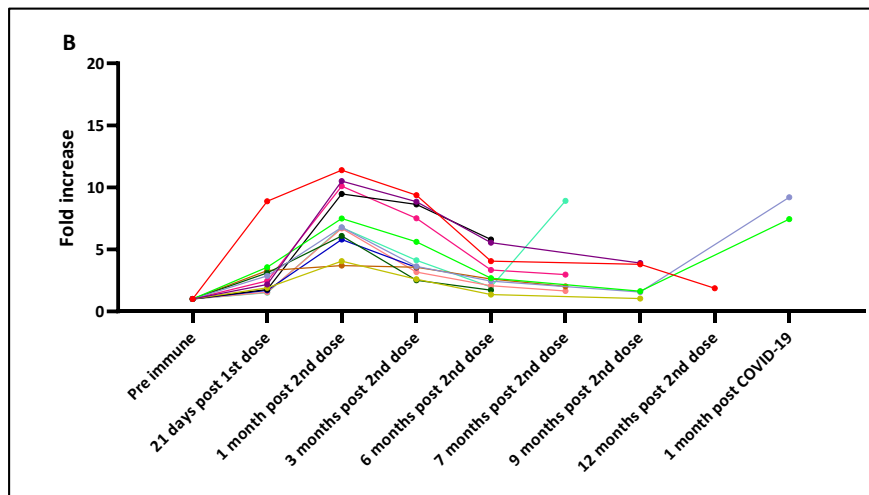
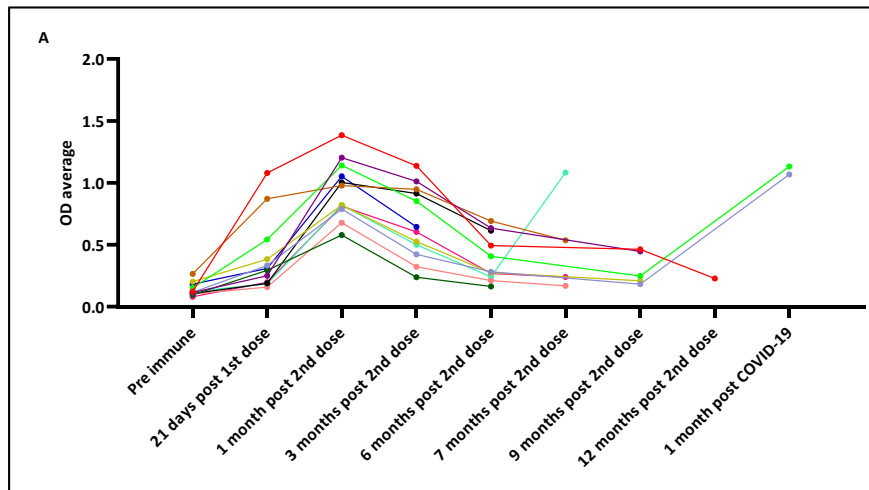
Collectively, the presented data showed that our ELISA protocol described in this study provides a valid protocol that can detect anti-SARS-CoV-2 specific Abs in human sera with high sensitivity and specificity for IgG and IgA, but not for IgM.

C. Longitudinal humoral immune response by PfiZer/BioNtech BNT162b2 mRNA vaccine

After optimizing our in-house ELISA conditions, SARS-COV-2 specific IgG, IgA, and IgM Ab values were determined longitudinally up to 12 months after the second dose. Prior to vaccination, SARS-COV-2 specific IgGs were undetectable with OD values below the already determined cut-off value equal to 0.19 (Figure 10. A).

The pre immune values were considered as the baseline where all participants are considered as seronegative. At the 21 days after the first dose time point, total IgG anti full length S protein increased an average of 2.898 fold with a range between 1.51 and 8.8 folds (Figure 10. B). These levels of IgG reached a peak 1 month after the second dose, with a fold increase averaging at 7.406 fold and ranging between 3.7 and 11.39 folds, but then slightly went down, to reach the baseline in 5 participants at 6 months, and in 2 participants 9 months after the second dose. The ELISAs of all participants were conducted till the 3 months post the second dose time-point, and all samples showed a decrease in the total IgG response to reach an average of 5.261 fold and a range of 2.512 to 9.37 fold higher than background. One participant got infected few days before reaching the 6 months post the second dose time-point, and this participant was excluded from additional collection. Two other participants maintained a slightly persistent IgG response 9 months after a fully vaccination, with a fold increase of 3.81 and 3.9 but a tendency towards a waning IgG response. Of these 2 participants, one has reached the 12 months post the second dose time point at which a very low IgG immunity was present. Two participants have maintained a slight IgG response at both 6 months and 7 months post the second dose time points with a fold increase of 5.802 and 2, respectively. These 2 participants were later on excluded from the study and no additional samples were

collected, because one of them has been infected, while the other one has received the third dose. Two participants of our study had a positive PCR results, 11 months after the second dose, and samples 1 month after infection from both have been collected and the levels of IgG were determined showing a strong increase in the IgG response. In one participant, the recovered immune response was similar to the value of 1 month after the second dose with a fold increase of 7.44, while the other one has recored a recovered IgG response that was even stronger than the 1 month time point levels with a 9.2 fold increase (higher than the first one that was 6.79). Because of the decision issued by the AUB, university students had to take the third dose, where samples referring to 7 months post the second dose were collected from 4 participants who have not reached yet the 9 months post the second dose time point. At the 7 months post the second dose time-point, one participant had a strong IgG response even stronger than the one reported 1 month after the second dose with a fold increase of 8.923, greater than 6.758 reported 1 month post the second dose. This sudden increase in the IgG value was explained by the presumption of an aysmptomatic SARS-COV-2 infection. The t-test was performed to determine wether our results are significant or not, showing a high significant correlation between each value compared to the pre vaccination's values, with * $p < 0.05$, ** $p < 0.001$ and **** $p < 0.0001$ (Figure 10. C,D).



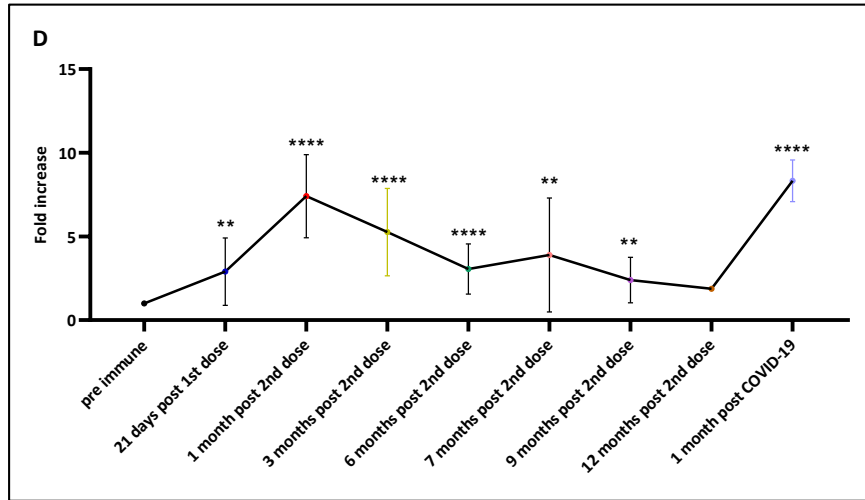
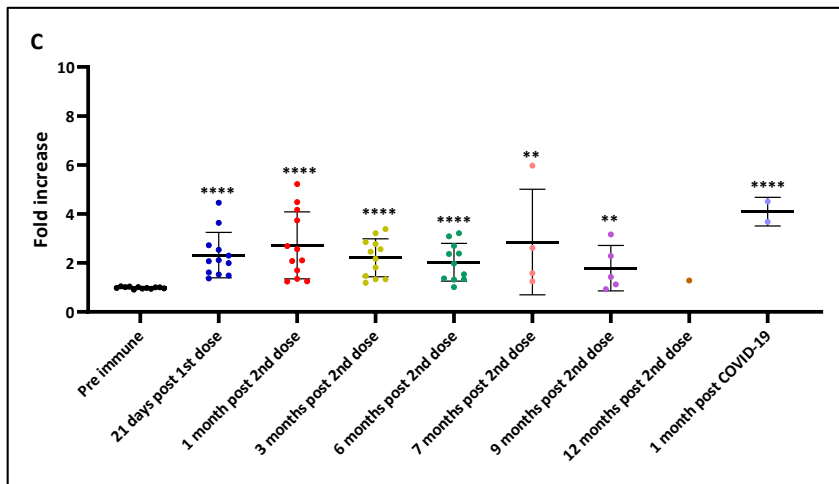
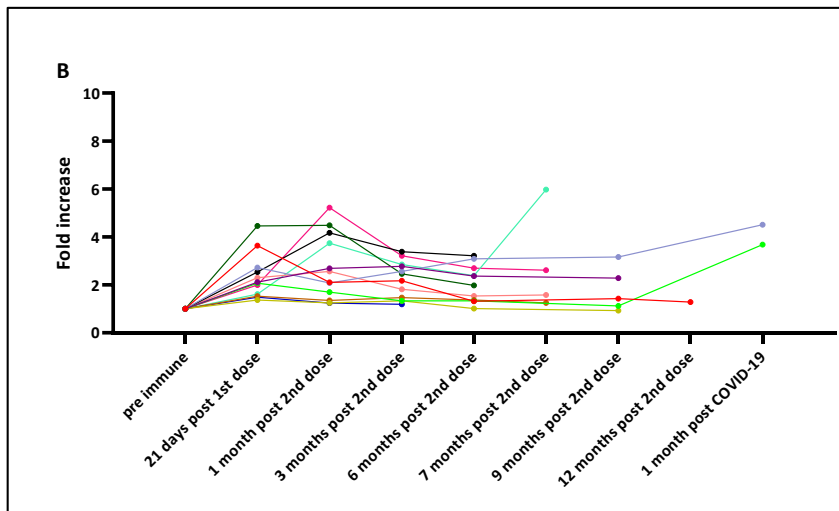
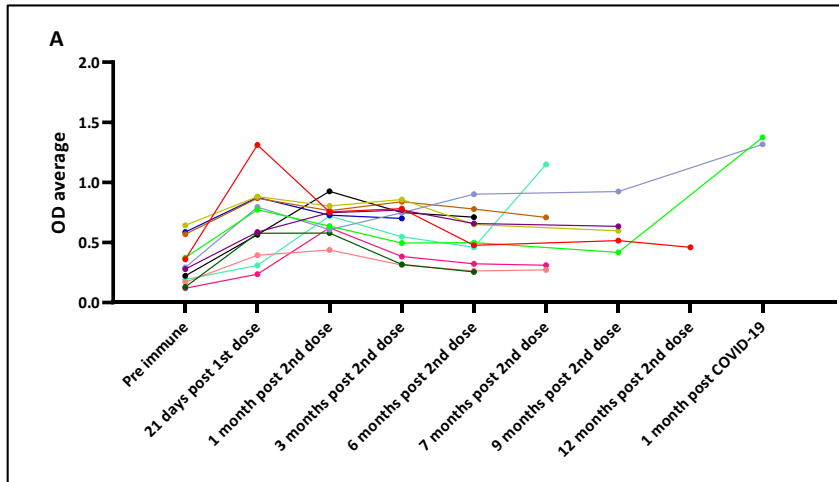


Figure 10. A. Longitudinal SARS-CoV-2-specific serum IgG Ab responses ODs in Pfizer-BioNTech BNT162b2 vaccinated participants, at all-time points. B. Fold increase in IgG values, for all participants at all-time points, compared to the baseline. C, D. Statistical significance of IgG fold increases, for all participants. ** $p < 0.01$, **** $p < 0.0001$.

SARS-COV-2 specific IgA levels, were also measured (Figure 11. A). Based on the ROC analysis the cut off value that will determine whether participants are seronegatives or seropositives for SARS-COV-2 spike specific IgA is 0.265 (Table 2). Highly heterogeneous profiles were reported but a statistically-significant average fold increase for all participants of 2.319 and 2.715 fold increase was seen 21 days post the first dose and 1 month post the second dose time points, respectively. Five participants reached a peak 21 days after the first dose with a fold increase ranging between 1.37 and 4.46 (Figure 11. A, B, C, D). These IgA values have slightly decreased to almost reach the baseline 9 months after the second dose. Among this group, 1 participant was infected by SARS-COV-2, 11 months after the second dose, where IgA levels have been recovered reaching a second peak with an increase of 3.68 folds, higher than the first one reported for the pic of 21 days post the first dose, with an increase of 2 folds from baseline. The IgA values of 5 participants have increased reaching a peak 1 month after the second dose, with a fold increase ranging between 2.56 and 5.22. Among these 5 participants, 3 participants have reserved an IgA signal detection that remained 6 and 7 months post the second dose, this signal had a decreasing tendency that most probably will disappear 9 months after the second dose. The fourth remaining participant had a persisting IgA signal at the 6 month post the second dose time point, with a 3.21 fold increase. Only one participant had a peak 9 months post the second dose, where additional increase in IgA levels by 4.51 folds from baseline has been observed due to a COVID-19 infection 11 months after the second dose. It's worth noting that the background values for IgA were high due to the long time we took to develop the plates in order to detect a good signal (Figure 11. A,B,C,D).



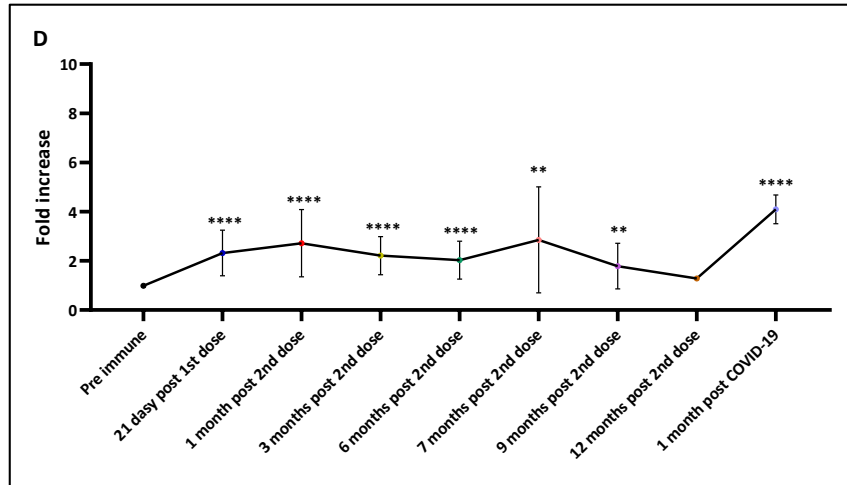
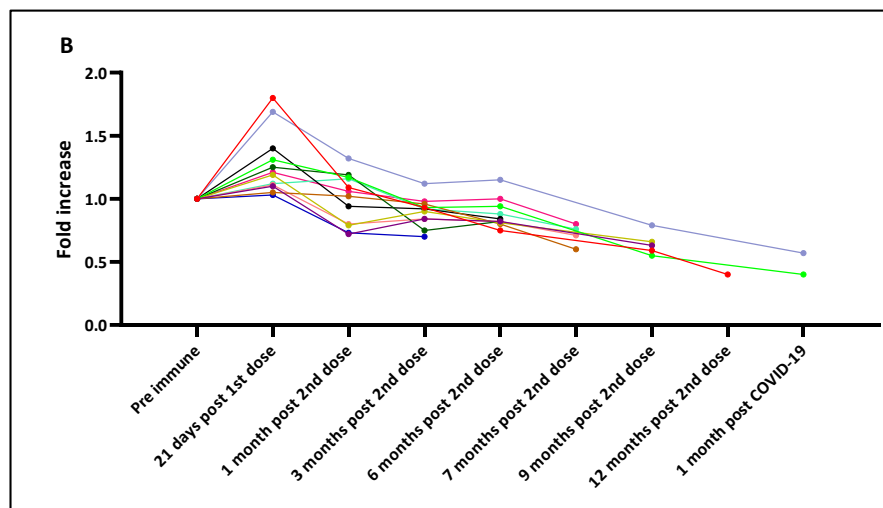
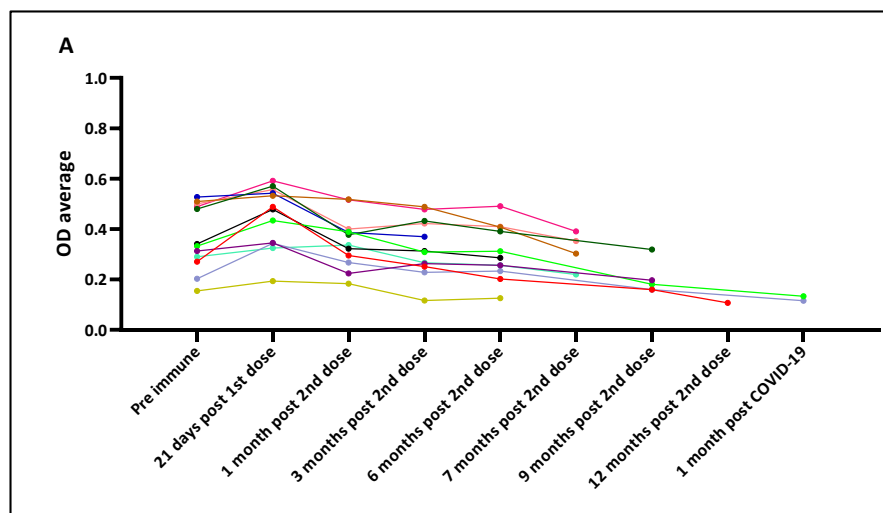


Figure 11. A. Longitudinal SARS-CoV-2-specific serum IgA antibody responses in Pfizer-BioNTech BNT162b2 vaccinated participants, at all-time points. B. Fold increase in IgA values, for all participants at all-time points, compared to the baseline. C, D. Statistical significance of IgA fold increase for all participants. ** $p < 0.01$, **** $p < 0.0001$.

SARS-COV-2 specific IgM levels, were also measured (Figure 12. A). All IgM profiles were almost the same, with a peak detected 21 days after the first dose that had a statistically significant but very low levels of increase 21 days after the first dose only, but dropped back down to baseline levels 1 month after the second dose (Figure 12. A, B, C, D).



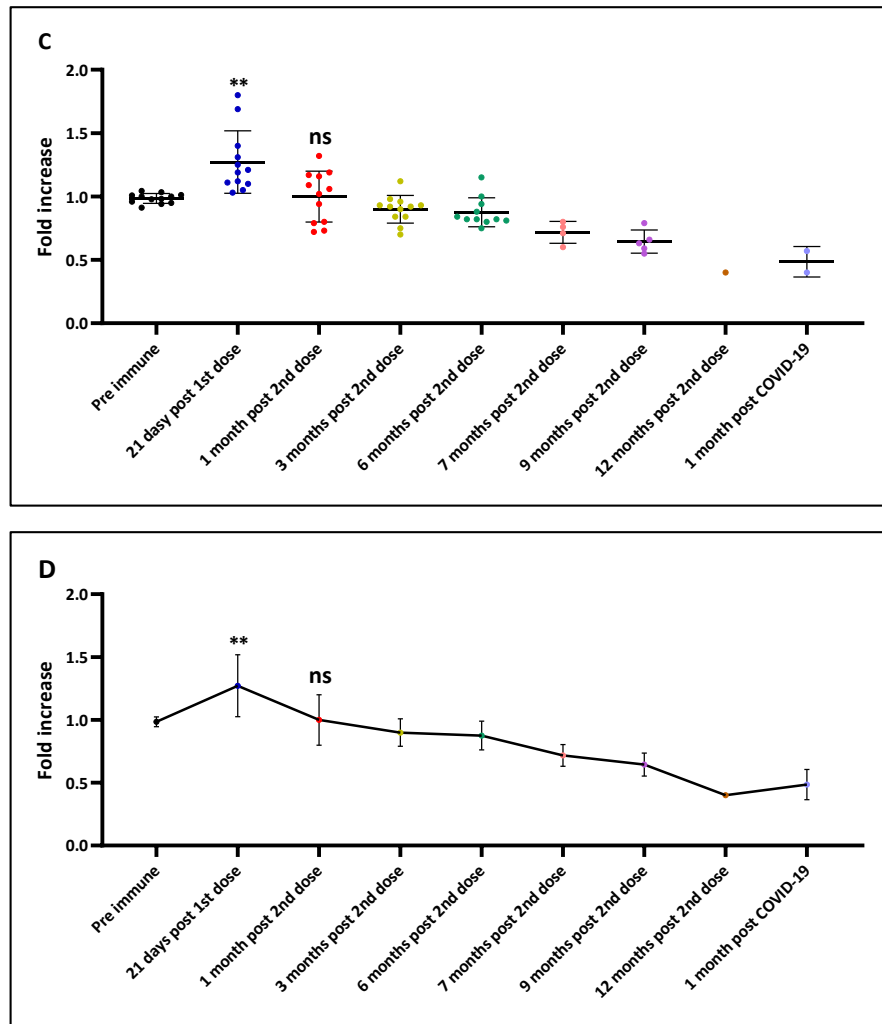


Figure 12. A. Longitudinal SARS-CoV-2-specific serum IgM Ab responses in Pfizer-BioNTech BNT162b2 vaccinated participants, at all-time points. B. Fold increase in IgM values, for all participants at all-time points, compared to the baseline. C, D. Statistical significance of IgM fold increases for all participants. ** $p < 0.01$, ns, not significant.

CHAPTER V

DISCUSSION

While SARS-CoV-2 is still spreading all around the globe, this spread has posed a threatening challenge for the human population. Early control measures such as social distancing, travel restriction and educational institutes closure have been imposed. However, mitigating measures have been taken in many affected countries, by gradually lifting these restrictions and relax regulations [163-165]. To achieve a “back- to- normal” plan, active surveillance, isolation of new cases and clusters, and sero-prevalence estimation among populations are mandatory [166]. The serology-based techniques play a major role at this level, where large scale and high throughput screening are yielded. In addition, they are easy to perform, with no intervention of biosafety containment level 3 facilities or highly specialized equipment. Therefore, the development of serology-based rapid point-of-care kits is considered an easy process [162]. In this study, we have developed and optimized an in-house ELISA based on the SARS-CoV-2 full length S protein (S1+S2) that enables the specific and sensitive detection of viral specific human IgG and IgA. In our optimized conditions, plates were coated with 25 μ g per well of S protein, with samples screened at 1/1000 dilution for IgG and 1/100 for IgM and IgA, and the ELISA was developed using a 1/3000 appropriate secondary Ab dilution. For IgG detection, our developed ELISA provides 100% sensitivity and 91,67% specificity, and high accuracy with an AUC of 1 (95% confidence interval of 1.00 to 1.00) (Figure 7, Table 1). As for IgA detection, our optimized ELISA had also a high accuracy with an AUC equal to 0.9583 ± 0.03658 and 95% confidence interval of 0.8866 to 1.000 (Figure 8, Table 2).

On the level of IgM detection our ELISA showed both poor sensitivity and specificity (Figure 9, Table 3). This is due to the fact that that IgM are the first immunoglobulins isotype to appear but also to disappear because of isotype switching into IgG, IgA or even IgE. In our conditions of optimization, the positive plasma samples were the 1 month post immune sera samples, an enough duration for the IgM to switch and disappear from the plasma samples, leading to almost the same OD values between the pre immune and the 1 month post the second dose samples. This problem might be surpassed by the very early collection of samples one week post the first dose, where IgM would still be present. This early detection of IgM leads to a significant increase in the difference between the negative and positive controls considered as pre immune and 1 month post the first dose, respectively, leading to an increase in both sensitivity and specificity of the optimized ELISA. The accuracy of this in-house optimized ELISA can be improved by further analysis that assess the cross-reactivity with Abs against other relative coronaviruses (MERS-CoV and HCoV HKU1) [162].

The dynamics of the Ab response after SARS-CoV-2 infection have been explored in several studies. Many of them have evaluated Abs, especially IgG, in periods ranging from 6 to 8 months, which confirmed the presence of IgG. A small number of studies have evaluated the dynamics at 10 months [120, 167]. The major difference between our work and others is that our study evaluated IgG, IgA and IgM at seven different time points following vaccination rather than natural infection. The only participant who was not infected during the study and did not take the third dose (the first booster), and thus was included for 12 months till the completion of the study demonstrated that IgG could be slightly detected at this time point.

This study demonstrated a high humoral immune response against the SARS-COV-2 S protein, which is considered as the most immunogenic among other SARS-COV-2 Ags. In terms of IgGs, the participants showed a robust response 1 month post the second dose that lasts up to 6 months in most cases and may last to up to 9 months in a small group of participants, with a waning response after this time point. Only one participant reached the 12 months post immune time point, where a very low IgG detection has been reported. Other studies evaluating the longitudinal humoral immunity induced by the BNT162b2 mRNA-vaccine, found an elevated IgG response 3 weeks post the first dose (21 days after the first dose), that increased significantly after the second vaccination dose, and lasts up to six months, to start declining after this time point [168]. It's worth noting that a SARS-COV-2 infection preceded by a BNT162b2 mRNA vaccination leads to a robust recovered immunity that may be higher than the first one developed one-month post immunization. In our study, an infected participant 11 months after the immunization showed a 9.2-fold increase from baseline, which is higher than the first value reported at the pic that was 6.79 folds, showing an important role of vaccination in priming the immune system for possible infections. One other participant demonstrated a recovered IgG response at 7 month post immunization, with a fold increase of 8.923 higher than 6.758 recorded 1-month post immunization. This patient had not reported SARS-CoV-2 infection, and we believe that this increase in IgG titers might be due to an asymptomatic infection that was detected by our study. These data indicate the capacity of the PfiZer/BioNtech BNT162b2 mRNA vaccine to boost the elicited IgGs titer after an infection, which is especially beneficial for groups who are prone to a rapid waning of humoral immunity. Most importantly, these results prove a developed memory post PfiZer/BioNtech BNT162b2 mRNA vaccination.

On the other hand, the study of the IgA response that represents the mucosal immunity is of high importance. For the IgA response, the results were more heterogeneous with peaks at either 21-days post the prime dose or 1-month post the boost dose. Despite this heterogeneity, the IgA response followed the same trend as the IgG response, albeit with less intensity. Interestingly, participants who were infected after a prior vaccination have developed a robust IgA response with fold increases that were even higher than the fold increases values at peaks. This shows the importance of the Pfizer/BioNTech BNT162b2 mRNA vaccination to generate a more robust mucosal immunity as IgA it is the most important Ab that provides defense at the mucosal sites, which represents one of routes of entry of SARS-CoV-2 [169].

IgM was negative in all tested pre-vaccination samples, and almost undetectable at all-time points after vaccination. One explanation is the rapid waning of IgM levels where their appearance is restricted to only the first stages of immunization, explaining their absence in the 21 days post the first dose and 1 month post the second dose samples. This explains also the undetected values of IgM 1 month after COVID-19 infection in the 3 participants who got infected during this study. A Longitudinal study of the humoral immunity induced by the BNT162b2 mRNA-vaccine showed only IgM sero-positivity in 52% of samples collected 1 week before the second dose (which means 2 weeks after the first dose), and in 82% of samples collected 1 week after the second dose [168]. This study showed a higher detection of IgM in samples collected only 1 week after immunization with the second dose, better than the detection 2 weeks after first dose. With this in mind, we believe that the low to undetectable levels of IgM in our study at due to the time point of collection, rather than the sensitivity of the ELISA.

A. Interpretation

Our findings highlight the importance of tracking the humoral immune response longitudinally to assess the kinetics and durability of the conferred protection by BNT162b2 mRNA vaccination in order to implement re-vaccination, especially in individuals prone to a weaker humoral immune response. In addition, in time of vaccine shortage, participants with proven protective immunity can decide to opt out of getting a booster shot to allow individuals with no or low protective immunity to obtain the booster shot. Furthermore, participants with a high serum IgG levels could decide to donate serum that can be used in the treatment of patients with severe COVID-19 symptoms and no signs of a robust protective immunity.

B. Limitations

The present study has some limitations. With the aim to follow all participants over one year (12 months), many SARS-CoV-2 variants appeared. It is not clear what variant infected a few of the participants in our study, but their infection occurred during the surge of the new B.1.1.529 (Omicron) variant. A sample of 1 month after infection was collected and showed a re-increase of IgG and IgA levels in the infected participants. Regardless of the variant they got infected with, this indicates that the vaccine is efficient at priming the immune system for a rapid response against infections with the same strain, or potentially against other variants. One other limitation is the obligatory third dose (first booster) that AUB placed with restricted entry to the university for those who do not comply with this condition of taking a third dose, where many participants had to take the first booster. A sample before taking the third dose has been collected, after which these

participants have been excluded from this study. These two factors prevented us from following all the participants over the intended 12 month period.

C. Conclusion

BNT162b2 mRNA vaccination generates a robust humoral immune response, that may last for 9 months or even longer post immunization, especially involving anti-SARS-COV-2 IgG and IgA, and is magnified by the second dose. More importantly, vaccination with the BNT162b2 primes the immune system for a strong and fast response upon natural infection with SARS-CoV-2.

D. Future work

In our future work, the neutralization potential of S-specific Igs will be assessed. In addition, a detailed Immunophenotyping on SARS-COV-2 spike protein specific B cells will be done, to determine their specific subsets. More importantly, B cells isolated from the participants will be stimulated *in vitro* and their ability to secrete S-specific IgGs will be determined with time. All the proposed experiments will identify the longevity of the memory B cell response against the SARS-CoV-2 S protein, generated by vaccination with the BNT162b2 mRNA vaccine.

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