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Development of a T cell-based test for immune response diagnosis in COVID-19 infection

TESIS DOCTORAL

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D. Jordi Cano Ochando y D. Estanislao Nistal Villán, científico titular del laboratorio de referencia en Inmunología en el Instituto de Salud Carlos III y profesor de Ciencias Farmacéuticas y de la Salud de la Universidad San Pablo CEU respectivamente,

HACEN CONSTAR:

Que Doña María González Pérez ha realizado en el Instituto de Salud Carlos III y más concretamente, en el laboratorio de referencia en Inmunología bajo su dirección el trabajo "*Development of a T cell-based test for immune response diagnosis in COVID-19 infection*" con objeto de obtener el título de Doctor con Mención Internacional. Dicho trabajo es un compendio de tres artículos publicados que cumple con los requisitos exigidos por esta norma:

1. **Gonzalez-Perez M**, Montes-Casado M, Conde P, Cervera I, Baranda J, Berges-Buxeda MJ, Perez-Olmeda M, Sanchez-Tarjuelo R, Utrero-Rico A, Lozano-Ojalvo D, Torre D, Schwarz M, Guccione E, Camara C, Llópez-Carratalá MR, Gonzalez-Parra E, Portoles P, Ortiz A, Portoles J, Ochando J. **Development of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients After 1273-mRNA SARS-CoV-2 Vaccination**. Front Immunol. March 2022. 23;13:845882. doi: 10.3389/fimmu.2022.845882. PMID: 35401504; PMCID: PMC8983822.
2. García-Pérez J[1], **Gonzalez-Perez M[1]**, Castillo de la Osa M, Borobia AM, Castaño L, Bertrán MJ, Campins M, Portolés A, Lora D, Bermejo M, Conde P, Hernández-Gutierrez L, Carcas A, Arana-Arri E, Tortajada M, Fuentes I, Ascaso A, García-Morales MT, Erick de la Torre-Tarazona H, Arribas JR, Imaz-Ayo N, Mellado-Pau E, Agustí A, Pérez-Ingidua C, Gómez de la Cámara A, Ochando J, Belda-Iniesta C, Frías J, Alcamí J, Pérez-Olmeda M; **CombiVacS study Group. Immunogenic dynamics and SARS-CoV-2 variant neutralisation of the heterologous ChAdOx1-S/BNT162b2 vaccination: Secondary analysis of the randomised CombiVacS study**. EClinicalMedicine. July 2022. 1;50:101529. doi: 10.1016/j.eclinm.2022.101529. PMID: 35795713; PMCID: PMC9249304.
3. **Gonzalez-Perez M**, Baranda J, Berges-Buxeda MJ, Conde P, Pérez-Olmeda M, Lozano-Ojalvo D, Cámara C, Del Rosario Llópez-Carratalá M, Gonzalez-Parra E, Portolés P, Ortiz A, Portoles J, Ochando J. **Maintenance of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients after 1273-mRNA SARS-CoV-2 Vaccination**. Pharmaceuticals (Basel). April 2023. 11;16(4):574. doi: 10.3390/ph16040574. PMID: 37111331; PMCID: PMC10141011.

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“A todos los que se preguntan cómo hubiese sido nuestro destino si hubiéramos hecho algo diferente, les digo; **el mejor camino es el que ya has elegido,** ya que nunca sabrás que hubiera pasado en cualquier otro caso”- Papá

A mis padres.

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Abstract

Pandemics have been an inherent consequence of human society through the ages, and they are described as a continuous worldwide spreading infectious disease (disease outbreak) that affects many individuals at a particular time. There have been many pandemics throughout the history of humanity, including the famous black death pandemic caused by *Yersinia pestis* (1347-1351) or the Spanish flu caused by Influenza A (H1N1) from 1918 to 1919 among many others. The latest global pandemic of all is the well-known COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and originated in the city of Wuhan in December 2019. This worldwide disease outbreak quickly led to a public health emergency of international concern (March 2020) leaving, up to now, around 7 million registered deaths behind. The need for treatments and immunization tools for this new threat was crucial, so scientists around the world started working on the implementation of treatments and vaccine development against SARS-CoV-2, impacting the morbidity and mortality of this disease. Measurement of levels, duration, and efficacy of immune responses to SARS-CoV-2 in each country was decisive to establish, for instance, vaccine dose intervals, or to understand the long-term effects of vaccination within the population as well as to anticipate the risk of breakthrough infections due to the VOCs and VOIs.

The main objective of this present thesis was to establish the level of specific T-cell activation in SARS-CoV-2 vaccination in Spain with the development of a rapid T-cell-based test that used a non-processed sample, such as whole blood. To note, we decided to not only develop and test the T-cell-based assay in healthy volunteer samples but also a vulnerable cohort such as long-term hemodialysis patients (HD). COVID-19 mortality rates were higher in HD patients and therefore understanding their response towards vaccination was critical to protect them. Furthermore, the cellular immune response was not fully understood, so serological and cellular analyses were key to further reducing the hesitancy of COVID-19 vaccination in this immunocompromised group. Thus, in this thesis, we have contributed to several trials and longitudinal studies describing how SARS-CoV-2 vaccination (homologous and heterologous) has impacted differently in study groups such as healthy volunteers or immunocompromised individuals (HD patients), by quantifying not only their cellular immune response but also their humoral immune response.

Firstly, this work brought up additional information and clinical evidence in a multicentered, opened-label, randomized controlled phase II trial called CombiVacS study, about heterologous vaccination immunogenicity. In this scientific paper, there were over 676 healthy individuals enrolled with no history of SARS-CoV-2 infection. All of them had received a first and single dose of the ChAdOxI vaccine. Participants were randomly distributed in an

intervention (they received a second dose of BNT162b2) or a control group (continued follow-up observation) and both their cellular and humoral immune response was monitored in a longitudinally way (baseline, 14 days, 28 days, 56 days, 90 days and 180 days after heterologous boost). This published work reported for the first time how heterologous (different boost vaccine administration as previously administered, e.g., AZD1222 (first dose) vs BNT16b2 (second dose)) vaccination produced a robust cellular and humoral immune response with both acceptable and manageable reactogenicity profile.

Secondly, this thesis also englobes the monitoring of the cellular and humoral immune response in an immunocompromised cohort of patients that were hemodialyzed and had received a homologous vaccination (mRNA-based lipid nanoparticles) pattern in a longitudinal way (baseline, 10 days after first dose, 20 days after first dose, 10 days after second dose, 20 days after second dose, three months after second dose and three months after third dose) and compared them to a healthy volunteer cohort. Moreover, this study englobing hemodialyzed patients differentiated between individuals that had recovered from a SARS-CoV-2 infection from naïve individuals, to determine the utility and need of the second vaccination boost in COVID-recovered patients. Altogether, this work revealed how homologous (same boost vaccine administration as previously administered) vaccination in healthy volunteers and dialysis patients contributed to a good humoral and cellular immune response in up to 6 months follow-up after vaccination boosts.

Overall, this thesis has focused on performing all whole blood T-cell-based tests, measuring both IL-2 and IFN-γ secretion and complementing this information with IgG-Spike specific antibodies from plasma, from a vast number of Spanish individuals in a longitudinal way, giving rise to six publications (three of which are part of this thesis) that have added state-of-the-art knowledge to the evaluation of humoral and cellular immune response in the vaccination strategy worldwide.

Resumen

Las pandemias han sido una consecuencia inherente a la humanidad desde el principio de los tiempos y se definen cómo una epidemia de una enfermedad infecciosa que afecta a escala mundial a muchos individuos en un mismo tiempo. A lo largo de la historia de la humanidad, ha habido muchas pandemias, cómo por ejemplo la famosa pandemia de la peste negra que fue causada por *Yersinia Pestis* (1347-1351) o la gripe española que fue causada por el virus Influenza A (H1N1) desde 1918 hasta el 1919 entre otras muchas. La pandemia global más reciente conocida por todos es la COVID-19 causada por un virus respiratorio llamado SARS-CoV-2 y que se originó en la ciudad de Wuhan, China, en diciembre del 2019. Este brote se diseminó a escala mundial y rápidamente llevó a un estado de salud de emergencia internacional (marzo 2020) dejando atrás cerca de siete millones de muertos registrados a día de hoy. La necesidad de encontrar tratamientos y herramientas de inmunización fue clave, por ello, científicos de todo el mundo empezaron a trabajar en la mejora de tratamientos y en el desarrollo de vacunas para el SARS-Cov-2, lo cual impactó en la morbilidad y la mortalidad de esta enfermedad. La cuantificación de los niveles, la duración y la eficacia de las respuestas inmunes frente al SARS-CoV-2 en cada país, fue decisiva para establecer, por ejemplo, los intervalos de vacunación o para poder entender los efectos a largo plazo de esta en la población, así como para poder anticiparse al riesgo de futuros brotes de infección con la llegada de las nuevas variantes.

El objetivo principal de esta tesis fue establecer el nivel de activación específica de células T enfocado a la vacunación en España frente a SARS-CoV-2 desarrollando un test celular rápido que partiera de una muestra poco procesada, cómo es la sangre completa. Cabe destacar que no solo se decidió llevar a cabo este test en voluntaries sanos, si no, también en una cohorte vulnerable como los son los pacientes de hemodiálisis a largo plazo. La mortalidad de estos pacientes durante la COVID-19 fue más aguda y por ello, poder entender la respuesta inmune de estos individuos frente a la vacunación fue crítica para su protección. De hecho, la respuesta inmune celular no se conocía bien en este grupo inmunosuprimido y por este motivo, los análisis serológicos y de células T fueron clave para poder reducir la incertidumbre que hubo al vacunar a este conjunto de población. Por ello, esta tesis ha contribuido en varios ensayos y estudios longitudinales que han descrito como la vacunación frente a SARS-CoV-2 (tanto en pautas vacunales homologas cómo heterólogas) ha impactado en diferentes grupos de estudio cómo voluntarios sanos o individuos inmunocomprometidos (pacientes dializados) cuantificando no sólo su respuesta celular si no también la respuesta humoral.

Primero, este trabajo brindó información adicional y evidencia clínica de un ensayo de fase II multicéntrico, abierto, aleatorio y controlado denominado CombiVacS respecto a la inmunogenicidad de la vacunación heteróloga. En este artículo científico se englobaron más de 676 individuos sanos que no tenían historia clínica de haber pasado la infección por SARS-CoV-2. Todos ellos habían sido previamente vacunados con una sola dosis de la vacuna ChAdOxI. Los participantes fueron distribuidos de manera aleatoria en un grupo de intervención (recibieron una segunda dosis de la vacuna BNT162b2) y un grupo control (se continuó observándolos de manera normal) y a sendos grupos se les monitorizó tanto la respuesta celular como la humoral de una manera longitudinal (medición basal, 14 días, 28 días, 56 días, 90 y 180 días después de dosis de recuerdo heteróloga). Este trabajo publicado reportó que la vacunación heteróloga (dosis de recuerdo distinta a la primera dosis, como, por ejemplo: ChAdOxI (primera dosis) vs BNT16b2 (segunda dosis)) producía una respuesta celular y humoral robusta con un perfil de reactogenicidad tanto aceptable como manejable. Más específicamente, en el trabajo publicado bajo el nombre "*Immunogenic dynamics and SARS-CoV-2 variant neutralisation of the heterologous ChAdOx1-S/ BNT162b2 vaccination: Secondary analysis of the randomised CombiVacS study*" se concluyó que después de una pauta de vacunación heteróloga, y estudiando diferentes puntos en el tiempo, se siguieron patrones y dinámicas similares de producción de anticuerpos neutralizantes, producción de IgG específicas de Spike y secreción de citoquinas dependientes de respuesta de células T, comparado con los de una vacunación de régimen homólogo. Concretamente, después de la dosis de recuerdo heteróloga, se produjo una reducción pausada en la respuesta humoral y celular después de haberse producido un pico a día 14, lo cual es consistente con trabajos previos de vacunación homóloga con vacunas de mRNA como se menciona anteriormente. Este trabajo se llevó a cabo como análisis secundario o continuación al trabajo original denominado: "*Immunogenicity and reactogenicity of BNT162b2 booster in ChAdOx1-S-primed participants (CombiVacS): a multicentre, open-label, randomised, controlled, phase 2 trial"* el cual fue el primer ensayo de fase dos publicado a nivel mundial con datos inmunológicos de una pauta vacunal heteróloga.

Segundo, esta tesis también engloba la monitorización de la inmunidad celular y humoral en una cohorte de pacientes hemodializados que recibieron una pauta vacunal homóloga (vacunas de mRNA; 1273-mRNA y BNT162b2) en un análisis longitudinal (medición basal, 10 días después de la primera vacuna, 20 días después de la primera vacuna, 10 días después de la segunda dosis, 20 días después de la segunda dosis, 3 meses después de la segunda dosis y 3 meses después de la tercera dosis) y fueron comparamos con una cohorte de voluntarios sanos. Además, en este trabajo con pacientes hemodializados se diferenciaron individuos que habían pasado la infección frente a SARS-

CoV-2 frente a los que no, para poder determinar la utilidad y necesidad de una segunda dosis de recuerdo vacunal en aquellos que se habían recuperado de la COVID-19. Por consiguiente, este trabajo reveló como la vacunación homóloga (dosis de recuerdo igual a la primera dosis) en voluntarios sanos y en personas inmunocomprometidas ha contribuido a una respuesta humoral y celular de calidad en un periodo de hasta seis meses siguiendo pautas de dosis de recuerdo vacunales. Concretamente, estas investigaciones dieron lugar a dos trabajos posteriormente publicados y relacionados con pacientes inmunocomprometidos bajo el nombre de: "*Development of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients After 1273-mRNA SARS-CoV-2 Vaccination*" y de "*Maintenance of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients after 1273-mRNA SARS-CoV-2 Vaccination"* se concluyó que después de una pauta vacunal homóloga, los pacientes hemodializados consiguieron una inmunización similar e incluso superior a individuos sanos tanto con respecto a la inmunidad celular como a la humoral. Como se menciona anteriormente, cabe destacar que en estos trabajos se diferenció entre individuos que habían pasado una infección frente a SARS-CoV-2 (Recuperados de la COVID-19) y los que no (Naive), describiendo también las diferencias entre ambos, ya que tanto los pacientes como los individuos sanos que no habían pasado la enfermedad necesitaron una segunda dosis vacunal para aumentar significativamente la producción de IL-2 y de IFN-γ al igual que la de IgG específicas de Spike, sin embargo, tanto los pacientes dializados cómo los individuos sanos que habían pasado previamente la enfermedad presentaron una respuesta rápida y robusta desde la primera dosis de vacunación frente a SARS-CoV-2, correlacionándose estos resultados a algunos previamente vistos en otros estudios. También determinamos que esta respuesta humoral y celular se mantenía de manera firme en el tiempo en los pacientes hemodializados, sumándole importancia a la vacunación en esta cohorte.

Respecto a la metodología y de manera breve, en todos los ensayos realizados en los artículos científicos se obtuvo sangre periférica de cada individuo (tanto de individuos los sanos cómo de los pacientes hemodializados) en los tiempos establecidos de manera longitudinal, en tubos de heparina de litio para el test celular o en tubos especiales para la obtención de plasma para el estudio de anticuerpos específicos para la proteína Spike. Todos los individuos habían sido previamente vacunados; bien de manera heteróloga para uno de los estudios (Vacuna Astra Zeneca vs Vacuna Pfizer) o bien de manera homóloga (mismas pautas vacunales de vacunas de mRNA) y diferenciando entre individuos que ya habían pasado la COVID-19 o no. En todos los estudios, para el test celular, la sangre total fue mezclada con medio de cultivo y cultivada durante la noche junto con péptidos específicos de SARS-CoV-2 (péptidos S correspondiente a Spike o péptidos M, correspondiente a la proteína M de membrana) o un control de DMSO. Después del cultivo,

se recolectó el plasma para poder medir las citoquinas de interés. Las citoquinas de interés (IL-2 y de IFN- γ) se midieron a través de un ELISA automatizado mientras que los niveles de anticuerpos específicos para la proteína Spike (IgGs) se midieron a través de un ensayo comercial y un analizador automatizado y quimio luminiscente denominado ECLIA de la marca Roche o LIAISON ® XL de la marca DIASORIN. Además, para la medición de anticuerpos neutralizantes se procedió a la pre-incubación de plasma diluido con pseudovirus previamente titulados. La titulación de anticuerpos neutralizantes se obtuvo calculando la dosis inhibitoria al 50%, lo que resultaba de la reducción del 50% de la infección pseudoviral comparado con controles a los que no se les había añadido suero.

En conjunto, esta tesis se ha enfocado en la realización de todos los test celulares procedentes de sangre completa de una gran cantidad de individuos españoles de manera longitudinal en el tiempo. La realización de estos test celulares en la población fue crucial para poder entender y descifrar específicamente la respuesta celular tanto en casos de infección frente a SARS-CoV-2 cómo en la vacunación misma. Esto es así porque la protección que se obtiene frente a un cuadro severo de enfermedad o a la misma infección es probablemente muy dependiente de la activación coordinada de sendos brazos de la inmunidad adaptativa; tanto de la inmunidad humoral (producción de anticuerpos) cómo de la inmunidad celular. Es cierto que monitorizar de manera robusta la rama celular de las respuestas inmunes fue complejo durante los ensayos de pautas vacunales iniciales debido a la falta de ensayos sólidos que validaran esta información, pero hoy en día, muchos estudios han conseguido descifrar lo que las respuestas celulares aportan en la inmunización frente a SARS-CoV-2 gracias al cultivo de las células de interés con péptidos específicos (15-mers) frente a la proteína Spike o la proteína M de membrana. En nuestro caso, el uso de los cultivos con sangre total y estos péptidos y a través de la medición de la secreción de citoquinas proinflamatorias relacionadas con células T CD4+ y CD8+ (IL-2 y de IFN-γ) y complementando está información con niveles de IgG específicos de la proteína Spike en plasma, se han conseguido publicar seis artículos científicos (de los cuales tres componen esta tesis por compendio de publicaciones) que han añadido información de vanguardia a la evaluación de la inmunidad celular y humoral frente a la estrategia vacunal de SARS-CoV-2 e inmunización frente a la infección de manera mundial.

Abbreviations

ACE2	Angiotensin-converting enzyme 2
Ad	Adenovirus
AID	Activation-induced cytidine deaminase
AIM2	Absent in melanoma 2
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
BC	Before Christ
BCG	Bacille Calmette-Guérin
BCR	B cell receptor
BM	Bone marrow
DAMP	Damage-associated molecular pattern
CCL2	C-C motif ligand 2
CD	Cluster of differentiation
cGAMP	Cyclic GMP-AMP
cGAS	Cyclin GMP-AMP Synthase
CLR	C-type lectin receptor
COVID-19	Coronavirus disease 19
CPR	C-reactive protein
CSR	Class switch recombination
CXCL10	C-X-C motif chemokine ligand 10
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Envelope
ECDC	European center of disease prevention and control
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EMA	European Medicines Agency
FCS	Furin cleavage site
HBV	Hepatitis B virus
HD	Hemodialysis
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HV	Healthy volunteers
ICU	Intensive care unit
iE-DAP	γ-D-glu-meso-diaminopimelic acid

IFI16	Interferon Gamma-Inducible Protein 16
IFN-I	Type I interferon
IL	Interleukin
Ig	Immunoglobulin
IRF3/7	Interferon regulatory factor 3/7
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucin rich repeats
LTA	Lipoteichoic acid
M	Membrane
MDA5	Melanoma differentiation-associated gene 5
MDP	Muramyl dipeptide
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC	Molecular histocompatibility complex
mRNA	Messenger-RNA
N	Nucleocapsid
NK	Natural Killers
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain-3
NOD	Nucleotide-binding oligomerization domain
NSP	Non-structural protein
ORF	Open reading frames
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRR	Pathogen-recognition receptor
rAD26	Recombinant adenovirus 26
rAD5	Recombinant adenovirus 5
RBD	Receptor binding domain
RNA	Ribonucleic acid
RLR	RIG-I-like receptor
RORγt	Retinoic acid receptor related orphan receptor γt
S	Spike
SARS-CoV-2	Severe acute respiratory symptom coronavirus 2
ssRNA	Single stranded RNA
STING	Stimulator of interferon genes
T-bet	T-box expressed in T cells transcription factor
TCR	T cell receptor

TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serin 2
TNF	Tumor necrosis factor
T-reg	Regulatory T cells
VOC	Variants of concern
VOI	Variants of interest
WHO	World health organization

Introduction

I. The immune system: innate and adaptive immune response.

It was thousands of years ago when the earliest example of immunotherapy was noted in the Ebers papyrus (1550 BC), the first known medical treaty written in ancient Egypt. In it, it was mentioned the recommended treatment for tumors (known as swellings). Later in history, humanity also noticed an extraordinary phenomenon in the plague of Athens (430 BC) in which Thucydides, a Greek historian, recorded the outbreak and said: "*Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience and had now no fear for themselves; for the same man was never attacked twice – never at least fatally* (1)". This phenomenon is referred to the memory of the adaptive immune response towards an already presented antigen. The definition of immunology and the immune system as a distinct discipline as we know it today, appeared for the first time in the late nineteen century, although early immunologists faced fundamental problems explaining the vast repertoire of diverse antibodies that were generated to maintain immunity against pathogens (2-4). Nowadays, the immune system can be described as a complex and pervasive network of molecular and cellular components that protects your organism against infections and other diseases. The immune system can be divided into two main branches: the innate and the adaptive immune system.

The **innate immunity** is inherited and is the first non-specific line of defense against pathogens. This includes physical and chemical barriers (i.e., skin, and mucous secretions) and specific cells, such as macrophages, neutrophils, basophils, and Natural Killer (NK) cells. In addition to these specialized components, every cell from an organism has innate immunity mechanisms that participate in the defense against infections.

Although innate immunity lacks specificity, it can differentiate self from non-self-components from the organism. Briefly, the innate immune system gets activated through receptors that directly recognize pathogens or that signal for a cellular immune response. One of the best-known receptors of the innate immune system is the patter-recognition receptor (PRR) (5). They recognize repeating patterns of molecular structure in pathogens that are also known as Pathogen-associated Molecular Patterns (PAMPs) or Damage-associated Molecular patterns (DAMPs) in case of molecules released from self-damage cells. These include lipids, proteins, and nucleic acids such as the well-known lipopolysaccharides (LPS), bacterial DNA, or lipoteichoic acid (LTA). The signals derived from the activation of these PPRs lead to microbicidal and pro-inflammatory responses to fight the infection or control cell death. The main sub-families of PRRs are 1. Toll-like receptors (TLRs), 2. Retinoic acid-inducible gene 1 (RIG-I)-like receptors, 3. The nucleotide-binding

oligomerization domain (NOD)- Leucin Rich Repeats (LRR)-containing receptors (NLR), 4. the C-type lectin receptors (CLRs) and 5. DNA sensors such as Cyclin GMP-AMP Synthase (cGAS), Absent in melanoma 2 (AIM2) or Interferon Gamma-Inducible Protein 16 (IFI16) (6-8).

TLRs were one the first PPRs described in the innate immune system and were originally found in Drosophila in 1994 in the form of genes although its antipathogenic activity was described later on, due to its role in resistance to fungal infection (9, 10). TLRs can be either found in the external membrane of the cell, recognizing extracellular PAMPs, or located in the inner compartment of endosomes for intracellular recognition. TLR2 and TLR4 recognize viral compounds in the cell surface while TLR3, TLR7, TLR8, and TLR9 detect viral compounds in endosomes (11). These last ones induce the production of type I interferons (IFN-I), responsible for mounting anti-viral immune responses (12).

RIG-I-like receptors (RLRs) correspond to the intracellular type receptors being key sensors for an antiviral immune response (13), in addition to TLR7 and TLR9 as mentioned previously. RLRs can recognize viral nucleic acids directly in the cytosol and undergo activation through a conformational change. There are three RLRs family members; RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (14).

NOD-like receptors (NLRs) are also intracellular PRRs. Two of the most well-studied NLRs are NOD1 and NOD2 proteins. NOD1 recognizes diaminopimelic acid (γ-D-glu-meso-diaminopimelic acid (iE-DAP)) of the cell wall of Gram-negative bacteria mainly while NOD2 recognizes muramyl dipeptide (MDP) from the bacteria cell wall, and further identifies complete viral single-stranded RNA (ssRNA) (15).

C-type lectin receptors (CLRs) are members of the phagocytic PRRs (16), such as Dectin-1 (also known as CLEC7A). These CLRs recognize a broad variety of pathogens such as viruses, fungi, and mycobacteria through mannose specificity. As CLRs are phagocytic receptors, they bind to PAMPs leading to the internalization of pathogens towards cytoplasmic vessels for degradation and further control of infection through antigen presentation (17). Triggering all these different PRRs will induce a diverse innate immune response by the integration of complex signaling pathways that will tailor de immune response towards a specific pathogen and ultimately shape an effective and potent adaptive immune response.

DNA sensors are specialized molecules or complexes within cells that can detect and respond to the presence of DNA, serving critical roles in various biological processes. Along with TLRs, there are other important DNA sensors such as cGAS, which is a key DNA sensor especially in viral infections, AIM2, which acts as an inflammasome sensor detecting double-stranded DNA in the cytoplasm or IFI16, an activator of the stimulator of interferon genes (STING) pathway (18). Briefly, cGAS catalyzes the production of cyclic GMP-AMP (cGAMP), which activates the STING pathway, ultimately leading to the production of type I interferons and pro-inflammatory cytokines (19).

The **adaptive immunity** is acquired throughout life, and it is presented after the innate immune response appears. That is why it is considered as a later response in which both innate and adaptive immunity work together to eliminate pathogens. Unlike innate immune responses, adaptive responses are specific for antigens (microbial and nonmicrobial) and can mount immunologic memory through repeated exposures. Antigens are presented by specific cells from the innate immune system such as macrophages or dendritic cells, and this presentation involves the expression of an antigen molecule on the surface of such cell, the so-called antigen-presenting cell (APC) (20). Lymphocytes are the principal cells capable of recognizing and responding to antigens, thus mounting the adaptive immune response. This response can be humoral if depending on B lymphocytes or cell-mediated if depending on T lymphocytes.

The **humoral response** is mediated by B lymphocytes. Conventional B lymphocytes, also named B2 B cells, arise from bone marrow (BM) precursors (hematopoietic stem cells). Exceptionally, there are other types of B cells (B1 B cells) that arise from fetal liver (21). B2 B cells, upon acquisition of antigen specificity, migrate to peripheral blood being yet immature. They complete their maturation state once they arrive in the spleen.

Development of B cells happens firstly in the marrow, through gene rearrangement of immunoglobulin (Ig) heavy and light chain genes, which will potentially generate 9 different heavy chain types such as IgM, IgD, IgG1 to 4, IgA1 and IgA2, and IgE and 2 light chain types, such as κ and λ. The exons of these chains encoding antigen binding portions (variable region of the heavy chain) suffer chromosomal breakage and reassembly (cut and paste mechanism) through a process called V (variable) D (diversity) J (joining) recombination. VDJ recombination introduces the formation and resolution of double-strand breaks in adjacent pairs of segments, through deletion, inversion, or hybrid joint (inappropriate joining of a coding-end to a signal end) in the intervening DNA, ligating segments together to create a new product. First, the D and J segments join, and then the V segment is attached. (22) This combinatorial assembly and diversification strategy of the VDJ recombination combined with the variability at the junctions given by the slight variation of

loss or gain of a small number of nucleotides between segments, gives immunoglobulins and T-cell receptors a limitless repertoire to recognize a vast variety of antigens in mammals (23, 24). However, as a downside of VDJ recombination, there is the potential for autoreactive antibody generation. Luckily, there are checkpoints along the B and T cell development that serve as quality controls, preventing self-recognition.

Before exiting the BM, the IgM B cell receptor (BCR) is already expressed in the cell surface and ready for antigen recognition. At this point, a pre-B cell has three options; (I) Undergo positive selection initiating maturation and migrate to secondary lymphoid organs, (II) if positive selection signal is insufficient, it can continue to undergo light chain rearrangements, or (III) it can undergo receptor editing/apoptosis if the BCR is ligated to an antigen presented in the BM thus being autoreactive. Mature naïve B cells can encounter antigens and become activated, being differentiated into antibody-producing plasma cells or become a long-lived memory B cell, that can be activated in the future (25).

Briefly, B cell activation can happen through T-independent antigens or T-dependent antigens. T-independent antigen activation happens in the absence of T cells when a B cell can recognize certain antigens (polymerized proteins, polysaccharides, or glycolytic antigens), proliferate, and produce antibodies whereas T-dependent antigen activation requires a T follicular helper cell that, in form of costimulatory signals like ligands (CD40L) and cytokine production such as IL-4, IL-5, IL-6, IL-21, and IFN- γ, will make the B cell proliferate and either become a short-lived low-affinity secreting antibody plasma cell or form a germinal center that will host high-affinity and long-lived plasma cells that will secrete high levels of antibodies (5, 26). As mentioned before, when a B cell is activated by antigen recognition during immunization, it turns into a plasma cell that will secrete specific antibodies (Igs). The way a naïve B cell switches from the surface-expressed IgM to other Ig isotypes in the plasma cell is defined as class-switch DNA recombination (CSR) and it only occurs after a T-cell-B-cell activation. CSR happens in the lymph node, and it requires changes in the constant regions of the heavy chain gene of the antibodies. The key enzyme necessary for this switch initiation is called activation-induced cytidine deaminase (AID) and leads to a double-strand break within these regions. CSR is crucial for the maturation of antibody response that happens during infection or vaccination and a defect in it will result in various diseases such as hyper IgM syndrome, autoimmunity, or asthma (27).

Different classes of antibodies serve (I) as antigen receptors (BCR) within the cellular membrane, (II) can activate the classic complement pathway, or (III) are capable of neutralizing targeted antigens once they are secreted. There are 5 major Ig Isotypes (IgG, IgM, IgA, IgE, and IgD) that differ in the constant region of their heavy chains and have

different dominant effector functions. For instance, IgM and IgG3 are exceptional complement activators while IgG1, IgE, and IgA are great at binding to macrophages, promoting and mediating phagocytosis of pathogens through their Immunoglobulin Fc receptors (28).

Cellular immunity is mediated by T lymphocytes and their secretion products (cytokines) that fight against intracellular pathogens. Although T cells are developed and differentiated in the thymus, they also come from progenitors in the bone marrow and the fetal liver (29). They present a broad variety of unique antigen-binding receptors on their membrane known as T-cell receptors (TCR). TCRs can recognize a huge number of foreign peptides achieved by a harmonized series of genomic rearrangements of the α, β, γ and δ TCR chains that provide these receptors with an outstanding combinatorial diversity. This is described as an antigen-independent process and takes place in the thymus. Full differentiation of CD4+ and CD8+ single-positive T cells happens in the thymic medulla from which T cells exit towards circulation as antigen-naïve cells. As mentioned before, T-cell immunity activation requires APCs, such as dendritic cells (DCs) or macrophages. This antigen presentation happens through the major histocompatibility complex (MHC), a protein present on the surface of APCs. MHC proteins can be classified as class I or class II if found in all nuclear cells or if only found in specific cells like macrophages, DCs, and B cells respectively. Furthermore, B cells contribute directly to cellular immunity by serving as APCs, producing pro-inflammatory cytokines such as Interleukin-6 (IL-6) and Tumor necrosis factor (TNF)-α, and even modulating the immune response (Regulatory B cells) throughout the secretion of IL-10 (30). MHC class I presents intracellular peptides (fragments of desired antigen) such as viruses while MHC class II presents extracellular peptides that have been previously phagocytosed, such as foreign antigens or microbes (25, 28). MHC-antigen complex activates the TCR and stimulates T cells to differentiate into cytotoxic CD8+ T cells (MHC class I binding) or into helper CD4+ T cells (MHC class II binding). CD4+ helper T cells (Th) and CD8+ cytotoxic T cells orchestrate a response helping and eliminating reservoirs of infection. Specifically, CD8+ cytotoxic T cells are involved in the elimination of infected cells with microbes or foreign peptides (virus or tumor cells). Activation of these cells induces cytokine production and substance release inducing apoptosis of targeted cells.

CD4+ Th cells are involved in controlling and enhancing the immune response; thus, they do not kill cells directly. Their main role is to modulate and direct other cells to the site of infection, perform tasks, and regulate the immune response depending on the type needed. They also release cytokines as a way of immune communication with other cell types. After inflammation resolution, effector T cells die and are cleared by phagocytes, although a minority of these cells are kept as memory T cells. These memory T cells can be quickly

activated and expanded in a memory-like fashion (following encounter with the same antigen), differentiating in effector T cells (31). There are several types of Th cell response such as Th1, Th2, and Th17 depending on the release of cytokines.

Th1 cells differentiate from Th0 precursors under the effect of IL-12, IFN-γ and the T-box expressed in T cells transcription factor (T-bet) and are characterized by IFN-γ and IL-2 secretion. In contrast, **Th2 cells** differentiate by IL-4 and the GATA-3 transcription factor and are characterized by IL-4, IL-5, IL-10, and IL-13 production (25). Briefly, Th1 responses are directed towards activation of phagocytes, NK cells, and CD8+ T cells for elimination of intracellular pathogens and virally infected targets. Th2 responses enhance antibody production, hypersensibility, and parasite-induced immune responses (E.g., effective responses against parasites, like expulsion of helminths). **Th17 cells** differentiate through IL-6 and Transforming growth factor beta (TGF- β). They express the retinoic acid receptor-related orphan receptor γt (RORγt) and secrete IL-17 in its 5 homologous family molecules (IL-17A to IL-17F). Th17 cells are known to be important in autoimmune diseases (32).

There is also a subset of CD4+ T cells named **regulatory T cells** (Tregs). Tregs play an important role in the maintenance of self-tolerance. This cell subset was first discovered by Sakaguchi and colleagues in 1995 and was specifically marked by CD25 expression and produced Foxp3 as the master transcription factor (33, 34). Differentiation of Tregs is controlled by IL-2 and TGF- β and promotes Foxp3 expression. These cells are characterized by IL-10, TGF- β and IL-35 production (35).

II. Severe acute respiratory syndrome coronavirus (SARS-CoV-2): General characteristics, 2019 pandemic and Variants of Concern.

Coronaviruses belong to the *Nidovirales* order and can be divided into four genera (alpha, beta, gamma, and delta). They are a group of enveloped single-strained, positive-sense RNA viruses of around 30 kb that can infect different animals (avian and mammal hosts). Regarding their structure, they are spherical and are characterized by the presence of club-like spikes on their surface, giving them the appearance of a solar corona. They have helically symmetric nucleocapsid and contain four main structural proteins; spike (S), membrane (M), envelope (E), and nucleocapsid (N). They can cause upper respiratory disease that can be potentially lethal in humans (36).

The spread of the first-named 2019-nCov now known as Severe acute respiratory syndrome coronavirus (SARS-CoV-2), started in late December 2019 when authorities reported the first cases of severe pneumonia of an apparent viral cause, affecting a cluster of patients in Wuhan, China (37). SARS-CoV-2 is now known to be the causative pathogen of the coronavirus disease 19 (COVID-19), which recently led to a global pandemic, causing a significant threat to public health. Although the pathogenesis of SARS-CoV-2 infection in humans manifests with mostly influenza-like mild symptoms, it can also lead to severe respiratory failure, and it is highly contagious (38). As of April 2023, SARS-CoV-2 has resulted in more than 764 million confirmed cases and almost 7 million deaths (39) and has raised a threat to human health and public safety for the past 3 years.

As part of the *Coronaviridae* family, SARS-CoV-2 is a novel beta coronavirus that shares 79% genome sequence identity with SARS-CoV and 50% with Middle East respiratory syndrome coronavirus (MERS-CoV) (40). SARS-CoV-2 is a single-stranded positive-RNA virus of around 30 to 32 kilobases. It has a variable number of open reading frames (ORFs) that encode structural proteins such as the Nucleocapsid (N), membrane (M), envelope (E), spike (S), non-structural proteins contained in ORF1a and ORF1b (NSP1-NSP16) and some accessory proteins. (41, 42). When mentioning the structural proteins, the **S protein** is a type I transmembrane N-glycosylated protein that stands as one of the most important structural proteins of SARS-CoV-2. It is cleaved into N-terminal S1 (containing the receptor-binding domain (RBD)) and C-terminal S2 subunits and allows the entry of the virion into the host cell. The **N protein** is bound to the packed RNA inside the virion and facilitates virion assembly while the **M protein** remains distributed in the membrane surface, contributing to structural stability and functional expression of other structural proteins. They both play an essential role in viral replication (43, 44). Lastly, the **E protein** plays a major role in pathogenesis, virus assembly, and release (45) (**Figure 1**).

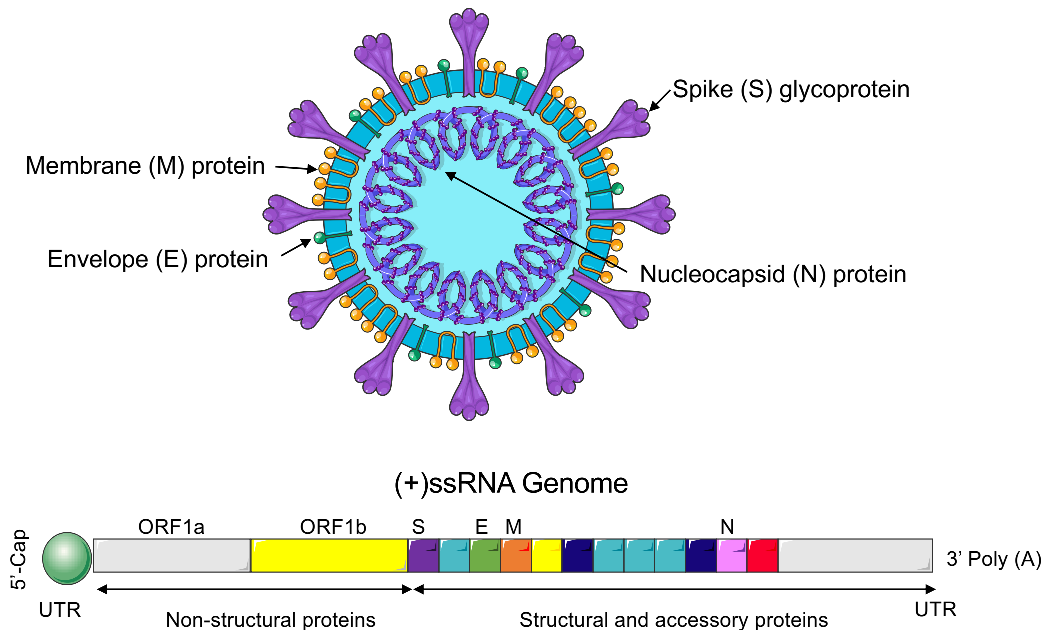

Figure 1. **Structure and genome of SARS-CoV-2.** Schematic representation of the structure and genomic arrangement of SARS-CoV-2.

Although SARS-CoV-2 origin remains unknown, phylogenetic analysis have described how SARS-Cov-2 is related and clustered with SARS-CoV and SARS-related coronaviruses found in bats (>96% of overall genetic similarity) although SARS-CoV-2 differs from all other coronaviruses from bats and pangolins in this species, due to years of sequence evolution (46). This information raises the hypothesis of SARS-CoV-2 being originated from bats (possible reservoirs) but it also suggests that these bat coronaviruses could likely be an evolutionary precursor rather than a direct progenitor of the virus (41).

During the pandemic, SARS-CoV-2 has been gaining infectivity and has been able to escape the buildup of immunity acquired either over-vaccination or infection, through variants of interest (VOI) and variants of concern (VOC). A VOI is considered when a virus that is circulating widely has mutations that could potentially turn into significant changes in its epidemiology. Furthermore, if this VOI is known to increase its transmission, cause more severe disease, or escape de body’s immune system, it becomes a VOC. These variants of the virus mutate their RBD and NTD of the spike protein affecting the angiotensin-converting enzyme 2 (ACE2) binding affinity and allowing the virus escape. Throughout the pandemic, five main VOCs were considered: Alpha, Beta, Gamma, Delta, and Omicron. Other variants such as Lambda and Mu were considered VOI. All these VOCs and VOIs harbor mutations close to the FCS region on the RBD.

The first VOC was reported in the United Kingdom (UK) and was named Alpha (January 2021). Alpha variant (B.1.1.7) was characterized by an N501Y mutation at the RBD region which highly increased ACE-2-RBD binding affinity, becoming more contagious than the previous strain. Furthermore, the Alpha strain is the VOC that increased the most its ACE2 affinity versus the ancestral RBD by 10-fold (47). Beta variant (B.1.351) was first identified in South Africa (May 2020) holding three RBD mutations: K417N, E484K, and the previously mentioned N501Y. The combination of these three conferred the beta variant a higher RBD-ACE2 affinity (2-fold) and, also promoted immune escape from neutralizing antibodies (mutation E484K) (48). Therefore, the beta variant became the leading strain in South Africa. In November 2020, the Gamma variant was reported in Brazil, being very similar to the Beta variant; their leading mutations on the RBD were: K417T, E484K, and N501Y. These mutations also led to an increase by 5-fold of the affinity in the RBD-ACE2 bond compared to the original strain (47). Delta variant (B.1.617.2) was originally identified in India (October 2020) and later became the dominant strain worldwide. It contained two RBD mutations: L452R and T478K, with a 2-fold higher affinity in the RBD-ACE2 bond and higher infectivity rates. Regarding human infection, the Delta variant required less incubation period and had a higher viral load than the ancestral viral strain (49). One of the most novel VOCs named by the World Health Organization (WHO) was Omicron (B.1.1.529) and it was first identified in South Africa and Botswana (November 2021) being the VOC with more mutations located in the S protein (around 32 mutations). 15 of those mutations were found in the RBD region. Of note, the Omicron variant retrieved the N501Y mutation back. Omicron variant spreads rapidly due to its increased transmissibility and can also mediate the escape from vaccine-induced neutralizing antibodies (50, 51). In 2022, the Omicron variant became the dominant strain worldwide, rapidly replacing the Delta variant with a 3.2 times higher transmissibility (52), but as of March 2023, the European Centre for Disease Prevention and Control (ECDC) had de-escalated certain no longer circulating Omicron lineages (BA.2, BA.4 and BA.5) (53). Furthermore, there is a shred of growing evidence that shows that COVID-19 from the omicron variant causes milder symptoms, having an attenuated pathogenesis (54), despite its higher spreading ability, which can be also partially explained due to boosted immunization with SARS-CoV-2 vaccines and to viral evolution. Spike protein evolution in SARS-CoV-2 variants is shown in **Figure 2.**

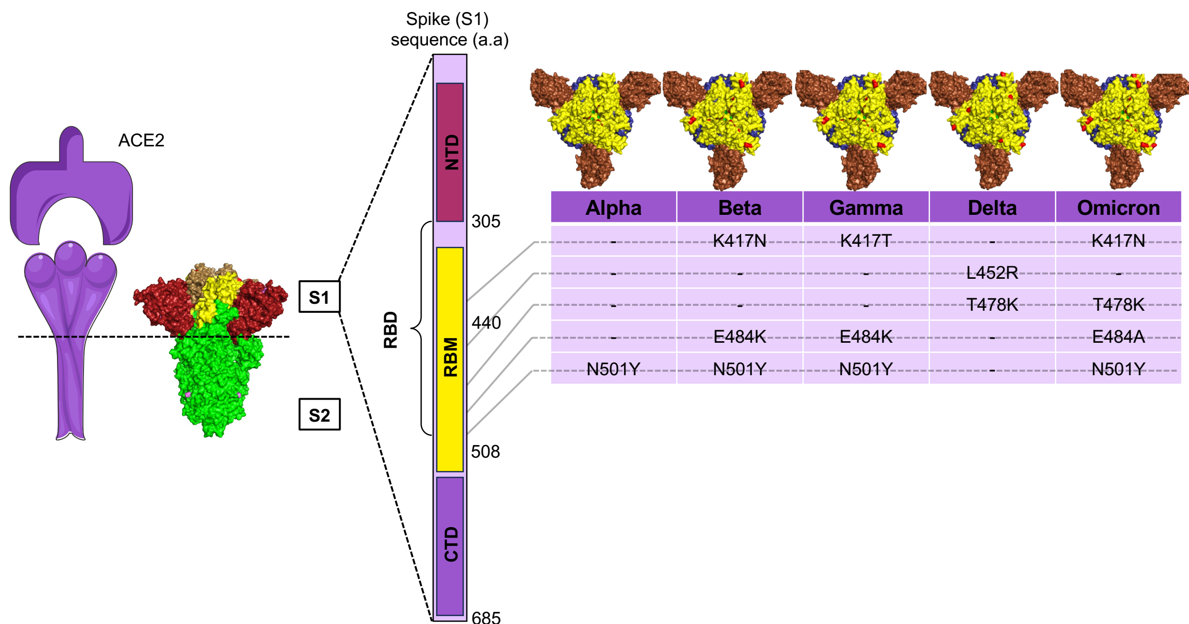

Figure 2. **Spike protein evolution of SARS-CoV-2 variants.** Schematic representation of the structure of the spike protein (S1 and S2) and the mutation's position in the RBD of the different variants.

Despite all the information about VOCs and the VOIs and how their mutations increase their RBD-ACE2 affinity or favor immune escape, the waning of neutralizing antibodies on clinical efficacy or vaccination is not yet clearly demonstrated. Some studies have described how protection against hospitalization or death remains high despite the bursting of new VOCs (55) while others have shown how certain variants can decrease the efficacy of vaccination (50, 56).

III. SARS-CoV-2 infection and pathogenesis.

When infecting humans, SARS-CoV-2 uses the ACE2 receptor to bind to epithelial cells in the respiratory tract (57) and is key for efficient infection. ACE2 is a protease (805 amino acids) with catalytic activity (carboxypeptidase) that cleaves a single amino acid from the C terminus of its substrates. As mentioned before, viral particles enter the cell through the S glycoprotein, a class-I viral fusion protein that covers the surface of SARS-CoV-2, giving it its crown-like appearance. This interaction of the RBD S1 subunit to the ACE2 receptor leads to the binding while the S2 subunit anchors the S protein to the membrane, promoting the virus-cell fusion process in the host cell (36). There are 20 residues of the ACE2 and 17 residues of the RBD creating a high affinity hydrophilic contact surface (58). Once the virus is strongly linked to the cell, viral entry is critically promoted by 3 proteases: Furin,

transmembrane protease serine 2 (TMPRSS2), and cathepsin L. The role of furin in the cleavage of the S1-S2 junction distinguishes SARS-CoV-2 from SARS-CoV cell entry. SARS-CoV, as well as other viruses from the *Sarbecovirus* subgenus S protein, do not rely on the furin S1-S2 cleavage during viral maturation (59). This process is necessary for membrane fusion to initiate and happens at the furin cleavage site (FCS), contributing to its efficient transmissibility in humans. After the S1-S2 boundary is broken, the fusion process must be activated by further cleavage of the S2 site. This process can be done either by TMPRSS2 on the cell surface or by cathepsin L in an endosome. TMPRSS2 is a type II protease that participates in the cleavage and activation of the S protein. It can be found in the gastrointestinal, respiratory, and urogenital epithelium, but is mainly co-expressed together with ACE2 in type II pneumocytes, especially at the higher level in the upper airways. (58, 60, 61). Cathepsin L, a non-specific protease with both endo and exopeptidase activity, can also mediate S2 cleavage, although SARS-CoV-2 activation happens mainly through TMPRSS2. This process occurs through ACE-2 boundary and further clathrin-mediated endocytosis (62). Viral cell entry is schematically shown in **Figure 3**.

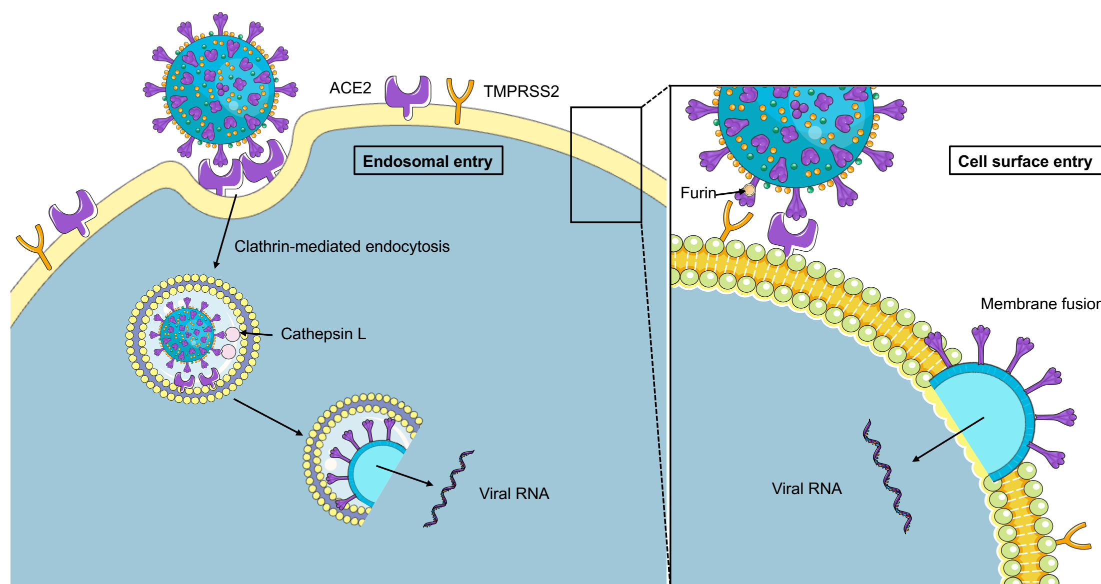

Figure 3. SARS-CoV-2 cell entry pathways. Overview of the endosomal entry and the cell surface entry of SARS-CoV-2 into the host. Endosomal entry is ACE2-dependent and requires endocytosis. Cell surface entry relies on TMPRSS2, furin, and ACE2 for membrane fusion.

Once the virus is inside the endosome, S2's site gets cleaved by cathepsins. When the virus enters the epithelial cells (ciliated cells, club cells, and basal cells), that co-express ACE2 and TMPRSS2 in abundance (63), it hijacks host cell machinery and starts actively replicating and migrating, reaching the lower respiratory tract, and therefore entering alveolar epithelial cells in the lungs. This process may lead to the activation of pro-inflammatory signaling and secretion of pro-inflammatory cytokines that serve as an alarm sign for both

innate and adaptive immune cells to the site of infection. Furthermore, these immune cells generate a positive feedback loop producing more cytokines to attack more immune cells.

Human SARS-CoV-2 pathogenesis can process from asymptomatic to mild constitutional symptoms to, in contrast, severe respiratory failure. The most common COVID-19 symptoms are fever, dry cough, headache, anosmia, and diarrhea (38, 64) but the risk of COVID-19 severity relies on comorbidities such as obesity, diabetes or hypertension, age, genetic factors, ethnicity, vaccination, and others (65). Furthermore, the severity of COVID-19 is intimately linked with a dysregulated immune response rather than a direct cytopathic viral effect. In severe COVID-19 there was a hyper-inflammatory response with a massive accumulation of pro-inflammatory cytokine-secreting cells called cytokine storm syndrome, considered the principal cause of death in COVID-19 patients (66, 67). This significantly high release of proinflammatory cytokines such as IL-6, Interleukin 10 (IL-10), Interleukin 2 (IL-2), and TNF-α among others led to the progression of acute respiratory distress syndrome (ARDS) and further multiorgan failure (68). Furthermore, highly increased concentrations of circulating chemokine (C-X-C motif) ligand-10 (CXCL10), chemokine (C-C motif) ligand 2 (CCL2), IL-2R, IL-6, TNF-α, C-reactive protein (CRP), ferritin and dysregulation of IFN-I response in serum were found in patients admitted in the intensive care units (ICUS) (69, 70)

IV. Host immune response towards SARS-CoV-2.

As the first line of defense, the innate immune response is primarily activated through PAMPs that are recognized by host PRRs as mentioned above. Specifically for SARS-CoV-2, TLR signal transduction is initiated. ssRNA fragments activate TLR7 and TLR8 and further activate Nuclear Factor κB (NFκB) for proinflammatory cytokine production (71). This step triggers the start of protein signaling cascades. Another key feature of an antiviral immune response is IFN-I signaling, which englobes the production of IFN-α and IFN-β leading to anti-viral, control of viral replication and immunomodulatory functions, ensuring host survival (72). SARS-CoV-2 genome is also sensed by RIG-I/MDA5 (cytoplasmic viral RNA sensors) and stimulates the IFN signaling pathway via IFN regulatory factor 3/7 (IRF3/7) which induces the production of IFN-I and chemokines (73, 74). Interestingly, SARS-CoV-2 presents an early delayed IFN-I and III response (74, 75). This delay is mediated by ORF3b and promotes the viral entry to the epithelial cells and replication of the virus (76). When infected, the accessory viral proteins from the virus along with the spike protein produce calcium/potassium ion channels that trigger the nucleotide-binding domain, leucine-rich–containing family, pyrin domain-3 (NLRP3) inflammasome pathway. This activation induces Interleukin 1-β (IL-1β) driven pyroptosis of the lung alveolar epithelium and releases DAMPS

that will be recognized by TLR2 and 4 therefore resulting in the production of more pro-inflammatory cytokines, IFN-I, and chemokines (77). Release of cytokines such as IL-6 or IL-1β recruit and activate cells from the innate immune system (Granulocytes, DCs, and macrophages) towards the lung (70). Paradoxically, when the innate immune response is exaggerated, it enhances viral replication because of the excessive tissue damage, increment of chemokine secretion, and higher recruitment of myeloid cells (78). To facilitate T cell responses, NK cells play key roles in controlling the viral infection IL-12, IL-18 secretion, and the Type I IFN-IFN-γ axis (79). Overall, effective control of SARS-CoV-2 infection is related to a rapid and strong innate immune response (interferon signaling) that will ultimately lead to a fast activation of the adaptive immune response, which will subsequently control the viral load. Therefore, an optimum adaptive immune response orchestrated by T and B cells is also needed for control and elimination of the virus as well as obtention of a robust and durable sterilizing immunity.

Regarding the humoral immune response, after antigen presentation by an APC, B cells from germinal centers can proliferate and differentiate into plasma cells, leading to high production of SARS-CoV-2-specific antibodies and memory B cells that have been found in most COVID-19-recovered patients and vaccinated individuals. After the germinal center response, plasma cells migrate to the bone marrow to produce neutralizing antibodies. It has been reported that live plasma cells can last from 7 to 11 months after stimulation and provide effective protection in mild SARS-CoV-2 infections (80, 81). Firstly, serum IgG (persist up to 6 months) and serum IgM (persist up to 4 to 6 weeks) subtype antibodies are produced against S protein, N protein, and the RBD, as in other acute viral infections (82, 83). Secondly, early development of neutralizing antibodies is key to controlling immunopathogenesis of SARS-CoV-2 infection (84), although the correlation between antibody titers and severity of disease has not yet been clearly established, early antibody production is associated with a better response towards infection compared to a later an exacerbated antibody production (in sever COVID-19 cases) that might not be effective towards infection. (80, 85).

In line with this, T-cell immunity also plays a decisive role in protection against severe acute infection, reinfection, and control of SARS-CoV-2 and other pandemic viruses such as SARS-CoV and MERS-CoV (86). Effective viral clearance and mild disease are deeply correlated with early and robust development of a CD8+ T cell immune response (87, 88). In the settings of COVID-19, lymphocytopenia (especially reduced CD4+ and CD8+ T cells) has been commonly observed in the blood of infected SARS-CoV-2 individuals, being predictive of disease severity (89, 90). Furthermore, as mentioned before, severe COVID-19 is characterized by a cytokine storm with significantly increased levels of TNF-α, IL-10, and especially IL-6 and IL-8 in serum, which negatively correlated with normal T cell counts (90,

91). CD4+ Type 1 responses are key for effective viral control. Importantly, CD4+ T cells dominate the Spike-specific cellular immune response, supporting antibody generation by B cells and further correlating T helper cells for memory-like properties (92). T cell memory response is characterized, for CD4+ T cells, with high secreting levels of IL-2 and IFN-γ production. For CD8+ T cells, with IFN-γ production (93). It is now known that potent T cell immunity can be maintained for at least 6 months after initial infection (94, 95), reaching around 0.5% (CD4+) and 0.2% (CD8+) of the memory T cell response repertoire (93).

V. Vaccines.

It was in the 18th century when vaccines first started to play a key role in human history as the practice of inoculation of material from human scabs of smallpox lesions started to become popular in Western Europe. Lady Montagu, wife of an English ambassador, successfully inoculated (practice of variolation) her child during a smallpox epidemic in England (96). These inoculations were not always effective and safe but seemed to induce protection of the disease in many cases. Right after this, in 1796, it was Edward Jenner who pioneered the concept of vaccine. He inoculated a child with cowpox obtained from lesions from the hand of a dairymaid, resulting in successful protection against human smallpox (97). This phenomenon set the basis for further development of new vaccines. After that point, vaccines have been developed over the centuries, transforming public health and providing specific immunity against plenty of diseases such as Rabies, Cholera, Measles, and Tuberculosis among many others (98). Studies have demonstrated that vaccination has significantly contributed to the decrease of childhood mortality worldwide (99, 100) and has reduced disease transmission over the years (101).

Vaccines can be defined as biological substances that safely provide acquired immunity and therefore protection against a harmful disease and/or infection upon a secondary encounter, through immune memory. Generally, vaccines are classified as live or non-living vaccines.

Firstly, live vaccines contain attenuated virulent microorganisms that have been obtained by serial passage and further selection of strains with an attenuated virulence (E.g., oral polio vaccine, measles-mumps-rubella (MMR), rotavirus vaccine or the Bacille Calmette-Guérin vaccine (BCG)). These vaccines have the potential to replicate sufficiently to induce an immune response in immunocompetent individuals but may not be suitable for an immunocompromised cohort (E.g., transplantation patients, HIV-infected individuals…).
Secondly, non-living vaccines contain purified components such as proteins or polysaccharides of a pathogen (E.g., HBV vaccine, meningitis, or pneumococcal vaccine) or

whole non-viable microorganisms (E.g., Whole cell *pertussis* vaccine or the cholera vaccine) and do not present a risk to immunocompromised individuals. They are normally combined with an adjuvant that will enhance the body's immune response towards a desirable antigen. Recently, modern non-living vaccines also include viral vectors, recombinant proteins, nucleic-acid-based RNA and DNA vaccines, and virus-like particles (101, 102), and they have become highly well-known due to their recent use to protect against COVID-19.

Depending on the type of vaccine, once an individual is vaccinated, it should be able to respond more rapidly and robustly towards the natural infection, becoming fully protected, as is the case of the Hepatitis B virus (HBV) vaccine, which effects have been demonstrated to remain long term upon booster doses. (103). Other examples of long-term immunization vaccines are the measles, mumps, and rubella (MMR) vaccine and meningococcal vaccination. This phenomenon does not happen with every vaccine type, as some need booster doses (for instance; tetanus, diphtheria (104) or polio vaccines) to sustain a threshold of antibody levels to be protective, although their immunity can last for decades. Vaccines are designed to interact mainly with the adaptive immune response compartment, mostly through the induction of antibodies via B cells but also induction of the T cells response, nevertheless, some vaccines are described to also interact with the innate immune response compartment, such as the BCG, giving the host a non-specific immune protection towards a second homologous or heterologous antigen, through a recently described concept called trained immunity (105). Vaccines are usually designed to prevent disease by stimulating or training the immune system in the general population and susceptible individuals. They can also be designed to therapeutically treat infectious diseases, allergies, or cancer. To confer protection, they must contain weakened or inactive parts of a particular agent (specific antigen) derived from the pathogen or that have been synthetically produced. They also contain adjuvants, preservatives, and stabilizers.

Overall, vaccines are designed to prevent life-threatening illnesses, they are a safe and reliable way to build protection against a certain disease and they offer added protection because of herd immunity.

VI. COVID-19 vaccination: development, efficacy, and safety.

The outbreak of the COVID-19 pandemic and some previous experiences from past emerged zoonotic coronavirus (SARS-CoV and MERS-CoV) led to the development of new vaccines and therapeutics that were critical to controlling SARS-CoV-2 spread and disease. Due to the importance of the RBD-ACE2 interaction in SARS-CoV-2 entrance to the host cell, the development of most COVID-19 vaccines was based on some aspects of the S

glycoprotein, to potentially prevent the viral infection of cells (106, 107). Other structural proteins could be suitable vaccine-target antigen candidates, such as the N protein considering its high immunogenic nature, stability, and conservation (108). The M protein also has a highly conserved nature and could also be used as a potential target for developing a SARS-CoV-2 vaccine.

Selection of an antigen for a vaccine is critical, as it will determine its efficacy in mounting a good humoral and/or cellular immune response. Up to date, **inactivated vaccines** make up a large portion of COVID-19 vaccines in China and India (E.g., CoronaVac and Covaxin respectively) but there are also novel vaccines in the market like **recombinant protein-based**, **RNA-based, DNA-based, and non-replicating viral vector vaccines**. Although inactivated vaccines hold a significant advantage in efficacy and safety, they also contain proteins and nucleic acids of the whole-viral antigen, which may lead to the production of irrelevant antibodies and therefore lower the specificity of antibodies against critical proteins (109). There was a need to induce specific and effective production of neutralizing antibodies against COVID-19 by the immune response that targets early stages of infection. In this regard, **mRNA vaccines** are nucleic acid sequences that encode the specific antigen synthesized *in vitro* and once injected, induce a strong cellular and humoral immune response by synthesizing the protein within the body. They also present some limitations, such as mRNA instability (labile and rapidly degraded) and high immunogenicity due to their ability to activate a variety of PAMPS (110). Vaccines such as BNT16b2 (Pfizer-BioNtech) and mRNA-1273 (Moderna) contain the S protein as the major antigenic target although in an enhanced approach. S protein stability was improved by the substitution of amino acids (K986 and V987) with two proline residues (S-2P) increasing the stability of the transmembrane-anchored S glycoprotein (111, 112). This engineered spike protein is included in a lipid nanoparticle and injected into the human body. Since December 2020, both BNT16b2 and mRNA-1273 mRNA-based vaccines have been authorized for widespread human use (113). Overall, both vaccines show high efficacy and very favorable safety profiles with no serious adverse events. Moderna vaccine reported a 94.1% efficacy rate in phase three clinical trial (111) while the Pfizer-BioNtech obtained a 95% credible interval of efficacy in a randomized efficacy trial (112).

Non-replicating viral vector vaccines also played an important role during COVID-19 vaccination. These vaccines encode pathogenic antigens that are cloned into viral vectors that have been genetically engineered so that they do not cause disease. Vaccines such as ChAdOx1 nCoV-19, Ad26.COV2.S and Sputnik V represent examples of vector vaccines. The advantages of virus vector vaccines are the promotion of an immune response of the viral vector itself, producing stronger responses than nucleic acid sequences alone.

ChAdOx1 nCoV-19 vaccine uses a DNA chimpanzee adenovirus as the viral vector encoding the S protein. Similar to AZD1222, Ad26.COV2.S vaccine contains adenovirus 26 CoV2 carrying the Spike protein gene. Likewise, the Gam-COVID-Vac vaccine follows the same adenoviral pattern, based on two adenoviruses (type 26 and 5). All three vaccines undergo the same mechanism of action, carrying information of SARS-CoV-2 S protein into the cell nucleus, where ultimately the cell machinery produces the Spike protein and induces humoral and cellular protection (114). All COVID-19 vectored vaccines had an acceptable safety profile, with a variable efficacy of around 70-90% for ChAdOx1 nCoV-19 vaccine (115, 116), and 66.9% efficacy for Ad26.COV2.S (117) vaccine and 91.6% for Sputnik V vaccine (118).

Due to SARS-CoV-2 vaccine development being an international health priority, all these vaccines were able to be released in less than a year, contributing to reduced numbers of infections, hospitalizations, and deaths globally. The most relevant COVID-19 vaccines administered worldwide, and their mayor characteristics are shown in **Table 1.**

Product name	Vaccine type	Antigen	Manufacturer
AZD1222 (ChAdOx1 nCoV-19 vaccine)	Non-replicating adenoviral vector	Chimpanzee Adenovirus encoding the full-length wild type S glycoprotein	Astra Zeneca/ University of Oxford
Ad26.COV2.S	Non-replicating viral vector	Non-competent recombinant adenovirus type 26 (rAD26) encoding the S protein	Johnson & Johnson
Gam-COVID-Vac (Sputnik V)	Non-replicating viral vector	Non-competent recombinant adenovirus type 26 and 5 (rAD26 and rAD5) encoding the full-length wild type S glycoprotein	Gamaleya Research Institute
BNT16b2 (Comirnaty)	mRNA-based lipid nanoparticle	Synthetic mRNA encoding the full-length S protein with 2P mutation in S2 subunit	Pfizer/BioNtech
mRNA-1273	mRNA-based lipid nanoparticle	Synthetic mRNA encoding the full-length S protein with 2P mutation in S2 subunit	Moderna
CoronaVac	Inactivated virus	Inactivated strain (CN02) grown in vero cells	Sinovac Biotech

Table 1. Selected COVID-19 vaccines used in global vaccination campaigns.

VII. COVID-19 vaccination in Spain.

The availability of an effective and secure COVID-19 vaccine in a short period that could be used in a vaccination strategy against SARS-CoV-2 was key to reducing the number of cases, hospitalizations, and deaths all over the world. As for the European Union and the European Commission, there was an agreement in purchasing and reserving certain vaccine doses (October 2020) with companies such as Astra Zeneca, Sanofi-GSK, Johnson & Johnson, Pfizer and Moderna, even before the end of clinical trials, ensuring that all Member States would have access to COVID-19 vaccination at the same time (119, 120).

In Spain, COVID-19 vaccination started the 27th of December 2020, after the European Medicines Agency (EMA) authorized the administration of the BNT16b2 Pfizer/BioNtech vaccine. Vaccination was first administered with a few available doses, to vulnerable groups such as front-line health care workers and individuals in residencies to at least provide partial immunization to minimize the severe consequences of the disease (121). Shortly after, in January 2021, the EMA also authorized the administration of the Moderna vaccine which was first inoculated in Spain on the 16 of January 2021, covering the full vaccination strategy from residents in nursery homes and health care workers as well as highly dependent people. At the same time, the Astra-Zeneca -Oxford vaccine was also authorized for administration by the EMA (January 2021) and was also first administered in Spain in February 2021. This vaccine had to be administered to younger people (from 18 to 55 years old) according to EMA's recommendations thus leading to a reorganization of the priority vaccination groups in our country. ChAdOx1 doses were given to less than 55-year-old healthcare workers and essential personnel (E.g., Military forces or teaching professionals). Once the main priority groups were vaccinated, Moderna and Pfizer vaccine doses were given to 80-year-old individuals or older, starting a massive program of COVID-19 vaccination (February 2021). In March 2021, the EMA authorized the administration of the Johnson & Johnson vaccine (Janssen Pharmaceutica), becoming available for administration in Spain in April 2021 and accelerating the vaccination campaign (122).

In March 2021, the Astra-Zeneca vaccine was temporarily suspended in Austria, Denmark, Norway, and Iceland after some raising concerns about a potential side effect considering blood clots: some isolated thrombosis cases (123). Unfortunately, the Astra-Zeneca vaccine administration was suspended definitively in Spain in late April 2021, leaving over 2 million Spanish people who had been previously vaccinated with ChAdOx1, without a boost dose. To solve this problem, a study that was led by the *Instituto de Salud Carlos III* in Madrid, and in which our laboratory took part, developed the first heterologous vaccination trial reported, that would describe the immunogenicity and reactogenicity of heterologous vaccination schedule in over 600 individuals (124).

Overall, Spain has been one of the leading countries with higher COVID-19 vaccination acceptance with over 92.7% of the population (12 years old and older) at least 1 dose vaccinated and over 90.8% of the population with a full vaccination schedule completed (125, 126).

VIII. T cell-based test for immune responses against SARS-CoV-2.

Throughout the pandemic, and after the development of COVID-19 vaccines, there was a need to systematically measure and monitor the level and duration of protective immune responses in the population. The conventional marker often used to measure immune protection of COVID-19 vaccines, or natural SARS-CoV-2 infection was the quantification of SARS-CoV-2 Spike-specific or Nucleocapsid-specific IgGs and neutralizing antibodies (127), but long-term protection from viral infection also englobed cellular immunity, so measurement of specific memory T cell responses must have been performed too. Furthermore, the correlation between humoral measurements and T cell responses was not always straightforward (E.g., High immune T cell responses could match with non-seroconverters or low-neutralizing individuals) (128), so specific T cell responses might be present in individuals with an antibody deficiency. It is important to consider that the assessment of T cell responses was more complex given the fact that cellular immunity is being activated through multiple human leukocyte antigens (HLA) alleles and due to the difficulties accessing T cells to study in humans, since blood is the only easily accessible sample, while many T cells are resident in tissues (lymph nodes). However, antigen-specific T cell immune responses have been commonly measured with Enzyme-linked immunospot (ELISpot) ever since it was described four decades ago (129). This technique is highly quantitative, it can be standardized across laboratories and can assess IFN-γ (or other cytokines) or granzyme B release. Furthermore, it is a very affordable technique that has been used in the study of vaccine-induced responses and immune monitoring of clinical trials (130). One of the most common applications of the ELISpot assay is the IFN-γ ELISpot and it has been broadly used to evaluate CD4+ and CD8+ T cell response towards an antigen. Other ELISpot applications are Granzyme B ELISpot, TGF-β1 ELISpot, Fluorescent ELISpot, or many other cytokines (IL-2, IL-1β, IL-12, etc.) that could be detectable with an ELISpot platform. Despite the advantages of this gold-standard technique, there are some disadvantages including the need to isolate peripheral blood mononuclear cells (PBMCs) or lack of quantification of the secreted cytokine. Thus, regarding SARS-CoV-2, new techniques have focused on the identification of specific memory T cells.

Nowadays, measurement of SARS-CoV-2 specific T cell response has been studied using incubation of SARS-CoV-2 specific synthetic peptides (131-133) and it has been demonstrated that SARS-CoV-2 specific peptides can activate CD4+ and CD8+ T cells *in vitro* through HLA-class I or class II presentation. Likewise, these peptides activated SARS-CoV-2 specific T cell response in PBMCs and whole blood. Activation was measured by quantification of specific pro-inflammatory cytokines, such as IL-2 and IFN-γ by enzyme-linked immunosorbent assay (ELISA), in plasma, after overnight stimulation. This augmentation of IL-2 and IFN-γ correlated with the number of SARS-CoV-2-specific T cells quantified with gold-standard technique ELISpot and confirmed with intracellular cytokine staining (132). Thus, these data confirmed that the addition of peptides to whole blood allowed SARS-CoV-2 specific T cell quantification. Importantly, our research group has contributed to the development of a fast and simple method to measure SARS-CoV-2 specific T cell responses in whole blood (124, 134, 135) and to the application of this new technique in Spain in the context-of vaccination trials and longitudinal studies to asses both SARS-CoV-2 specific T cell response and the humoral immune response.

Briefly, the T-specific cell-based test is grounded in the activation of CD4+ and CD8+ T cells through SARS-CoV-2 specific synthetic peptide pools 15-mers such as the spike-specific SARS-CoV-2 peptide. Once the blood was extracted from individuals, whole blood was stimulated with pools of SARS-CoV-2 peptides (E.g., S or M peptides) overnight in an *in vitro* culture, and supernatant from cultures (plasma) was collected for further cytokine (IFN-γ and IL-2) and spike-specific IgG measurements as shown in **Figure 4**. For cytokine measurements, an automated ELISA platform was used, called ELLA™. For spike-specific IgG measurements, a fully automated chemiluminescent analyzer was used called LIAISON® XL.

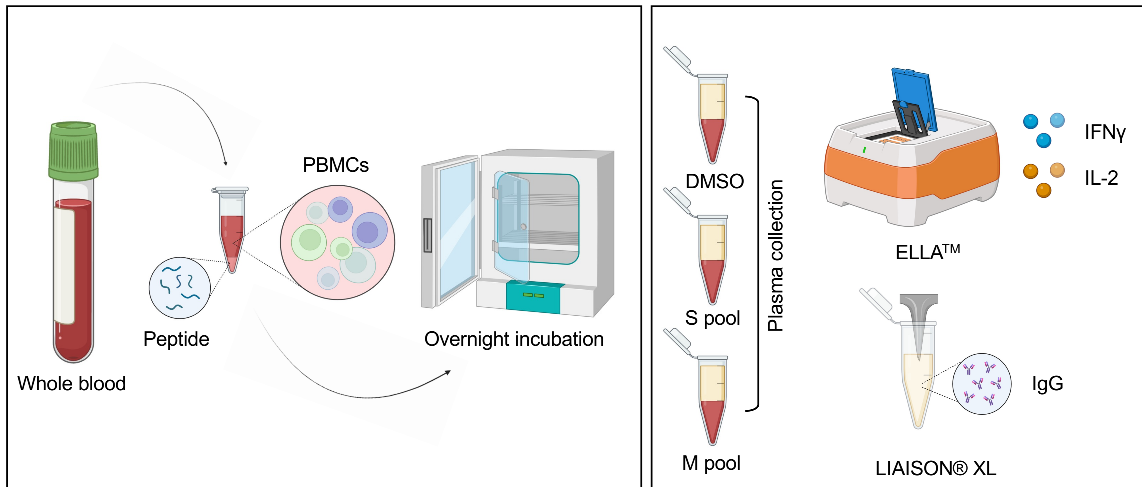

Figure 4. Overview of the schematic methodology for a SARS-CoV-2 T-cell-based test. Whole blood was collected and cultured in independent Eppendorf tubes with different SARS-CoV-2 peptides or DMSO as a negative control. After overnight incubation, plasma was collected from the different tubes and stored for further cytokine or IgG measurements.

This T-cell-based test allows large-scale studies to report and evaluate specifically the cellular immune response towards COVID-19, in contrast to the traditional methods (ELISpot and flow cytometry), as it is a rapid and highly accessible assay that can be easily reproduced.

To conclude, this thesis work has contributed to more than six scientific publications in the last three years, three of which are part of this PhD study. These articles have been published in high-impact journals such as **eClinical Medicine**, **Frontiers in Immunology,** and **Pharmaceuticals**, all of them part of a quartile 1 (Q1) in 2021. All the information described in the articles was highly valuable, as it brought complete up-to-date data regarding SARS-CoV-2 infection and immunization when needed, throughout the COVID-19 pandemic.

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Scientific publications.

Immunogenic dynamics and SARS-CoV-2 variant neutralisation of the heterologous ChAdOx1-S/BNT162b2 vaccination: Secondary analysis of the randomised CombiVacS study

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Summary

Background **The CombiVacS study was designed to assess immunogenicity and reactogenicity of the heterologous ChAdOx1-S/BNT162b2 combination, and 14-day results showed a strong immune response. The present secondary analysis addresses the evolution of humoral and cellular response up to day 180.**

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[3] Study group members are listed in Appendix 1.

Methods Between April 24 and 30, 2021, 676 adults primed with ChAdOx1-S were enrolled in five hospitals in Spain, and randomised to receive BNT162b2 as second dose (interventional group [IG]) or no vaccine (control group [CG]). Individuals from CG received BNT162b2 as second dose and also on day 28, as planned based on favourable results on day 14. Humoral immunogenicity, measured by immunoassay for SARS-CoV-2 receptor binding domain (RBD), antibody functionality using pseudovirus neutralisation assays for the reference (G614), Alpha, Beta, Delta, and Omicron variants, as well as cellular immune response using interferon-γ and IL-2 immunoassays were assessed at day 28 after BNT162b2 in both groups, at day 90 (planned only in the interventional group) and at day 180 (laboratory data cut-off on Nov 19, 2021). This study was registered with EudraCT (2021-001978-37) and ClinicalTrials.gov (NCT04860739).

Findings In this secondary analysis, 664 individuals (441 from IG and 223 from CG) were included. At day 28 post vaccine, geometric mean titres (GMT) of RBD antibodies were 5616·91 BAU/mL (95% CI 5296·49–5956·71) in the IG and 7298·22 BAU/mL (6739·41–7903·37) in the CG ($p < 0·0001$). RBD antibodies titres decreased at day 180 (1142·0 BAU/mL [1048·69–1243·62] and 1836·4 BAU/mL [1621·62–2079·62] in the IG and CG, respectively; $p < 0·0001$). Neutralising antibodies also waned from day 28 to day 180 in both the IG (1429·01 [1220·37–1673·33] and 198·72 [161·54–244·47], respectively) and the CG (1503·28 [1210·71–1866·54] and 295·57 [209·84–416·33], respectively). The lowest variant-specific response was observed against Omicron-and Beta variants, with low proportion of individuals exhibiting specific neutralising antibody titres (NT50) >1:100 at day 180 (19% and 22%, respectively).

Interpretation Titres of RBD antibodies decay over time, similar to homologous regimes. Our findings suggested that delaying administration of the second dose did not have a detrimental effect after vaccination and may have improved the response obtained. Lower neutralisation was observed against Omicron and Beta variants at day 180.

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Keywords: SARS-CoV-2; Heterologous vaccination; Neutralisation; Variants; Antibodies

Research in context

Evidence before this study

We did not perform a systematic search of the literature because of the rapidly developing situation. In summer 2021, the Delta variant of SARS-CoV-2 emerged and replaced the circulating Alpha strain. In November 2021 the Omicron variant was first described, and became predominant by March 2022 worldwide. In parallel, in summer 2021 different works pointed to a decrease in vaccine protection after six months of immunisation, in particular against new variants, due to waning of neutralising antibody activity. Together, these observations open the debate on a third dose booster that was adopted in many countries over the final months of 2021.

Added value of this study

The present results provide additional evidence on late immunogenicity – up to 6 months – of the heterologous ChAdOx1-S/BNT162b2 vaccination regime. Further, SARS-CoV-2 variant-specific elicited immunity is also reported here.

Implications of all the available evidence

Antibody titres decay over time, but delay in second dose administration has no deleterious effect on the immune response; on the contrary it resulted in better humoral responses. Functionally, all individuals exhibited neutralising titres >1:100 against G614 reference strain at day 28 after vaccine and a relevant proportion (76%) did so at day 180. However, variant-specific neutralisation was variable, with the lowest activity observed at day 180 against Omicron variant followed by Beta and Delta (19%, 22% and 56% with NT50>1:100, respectively). These findings support the use of heterologous regimes, which is consistent with that arising from homologous schemes, and a third dose strategy in patients previously immunised with a combination of adenovirus- and mRNA-based vaccines.

Introduction

Early after the SARS-CoV-2 outbreak started – rapidly evolving to a worldwide pandemic – active immunization emerged as the key priority of global healthcare policies. Most scientific efforts have focused in vaccines development, which successfully resulted in three

homologous two-dose vaccines and one single-dose vaccine available for use in the European Union between late 2020 and early 2021. Notwithstanding, rare severe thrombotic with thrombocytopenia events related to ChAdOx1-S vaccine (Vaxzevria, AstraZeneca) and shortage in supplies had an impact on European vaccination plans and drove an interest in heterologous regimes. The combination of ChAdOx1-S and the mRNA vaccine BNT162b2 (Comirnaty, BioNTech) has been the first heterologous scheme studied. Results on 14-day[1] and 28-day[2] immunogenicity showed that robust humoral and cellular immune responses were elicited. Accordingly, and as planned in the protocol, participants included in the control group were offered to receive BNT162b2 as a second dose.

Concurrently, public health plans to control the pandemic faced a recurrent issue, namely the periodic outbreak of new SARS-CoV-2 variants. To date, numerous variants have been identified, of which Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) are currently considered as variants of concern (VoC), the latter beginning in summer 2021 and soon becoming dominant.[3] More recently a new variant — Omicron (B.1.1.529) — was first described in Botswana and South Africa. Because of its high transmissibility, Omicron is displacing Delta as the dominant variant in most world countries in less than 1 month, representing a new challenge in the control of pandemic.[4] Not surprisingly, dynamics over time of variant-specific immune response induced by vaccination is a hot matter of research. Several works have reported a deterioration of vaccine-induced antibody response and a waning of protection against infection with either homologous regimes.[5,6] By variants, a 3·5-fold to 14-fold reduction of serum neutralisation titres against Beta variant from vaccinated individuals has been reported.[7–9] Further, ChAdOx1-S vaccine results evidenced undetectable neutralisation activity against Beta variant in 60% of vaccinated individuals and decay by a factor of up to 31·5% in the remaining 40%.[10] Preliminary data from Omicron variant were even more worrying as a 14 to 30-fold reduction in neutralisation susceptibility elicited by immunisation was reported.[11,12]

Notwithstanding this, the impact of waning neutralising antibodies on clinical efficacy is not clear. Some studies found that protection against hospitalization or death persisted at a robust level,[6] while others showed that efficacy notably decreased, or even failed, against Beta[10,13] and Delta[6] variants. A consequent question is whether this reduction makes the variant resistant to vaccination. Some results on Beta variant comparing sera from naturally infected and vaccinated individuals have suggested that those vaccinated retain protective levels of humoral immunity,[7] while others evidenced no efficacy in mild and moderate disease.[10] It must be mentioned that effectiveness may be influenced by the interval between doses, with longer time associated to an enhanced antibody response[14] and higher effectiveness, as well as by cellular immunity, given the relevant role of CD4+ and CD8+ responses found in COVID-19 patients and cross-reactivity observed in unexposed individuals.[15,16] Also, as suggested for Omicron variant, a decreased virulence could contribute to lower the rates of hospital admissions and death.[17,18]

In addition, decay of variant-specific antibody titres is shifting focus towards the need of a third dose, especially in those with weakened immune systems such as older adults[19] and immunosuppressed patients.[20,21] Considering the available evidence, the European Medicines Agency (EMA) has issued recommendations for an extra dose with mRNA vaccines and is completing conclusions on booster doses for people with a normal immune system.[22]

Increasing evidence on immunity dynamics aiming to answer these questions derives mostly from homologous vaccines, while heterologous regimes are still less studied. Here we present additional results of the CombiVacS study[1] addressing a) total and neutralising antibody dynamics of heterologous ChAdOx1-S/BNT162b2 vaccines combination, and b) immune response against different SARS-CoV-2 variants, including Delta and Omicron variants. This analysis also aims to provide valuable data to the debate on extra booster doses.

Methods

Study design and participants

Data from the 12-month, phase 2, open-label, randomised, controlled CombiVacS study are included in this secondary analysis. Full descriptions of the methods as well as safety and initial immunogenicity analyses have been previously published in detail.[1] Full study protocol is provided in Appendix 1 (p 26).

Healthy, or clinically stable, adults from 18 to 59 years old with no history of SARS-CoV-2 infection who had been vaccinated with a single dose of ChAdOx1-S between 8 and 12 weeks before screening were enrolled in the CombiVacS study to evaluate immunogenicity and reactogenicity of a second dose of the mRNA COVID-19 vaccine BNT162b2.

All participants provided written informed consent before enrolment. The trial complies with the principles of the Declaration of Helsinki and Good Clinical Practice. This study was approved by the Spanish Agency of Medicines and Healthcare Products (AEMPS) and by the Ethics Committee at University Hospital La Paz.

Randomisation and masking

Briefly, participants were randomly assigned (2:1) to receive one intramuscular injection of BNT162b2 (interventional group, IG) or maintain observation (control group, CG). Since the main immunogenicity objective was met, and reactogenicity was acceptable,[1]

participants included in the control group were offered to receive BNT162b2 as a second dose at day 28, as planned in the protocol. A systematic randomisation stratified by study site, gender and age (18-49 years, and 50-59 years) was used. The randomization list was centrally generated with the SAS software for Windows (version 9.4; SAS Institute Inc., Cary, NC, USA), and imported into the secure Research Electronic Data Capture platform (REDCap version 8.7.4; Vanderbilt University, Nashville, TN, USA) used for the study electronic case report form (eCRF).

Procedures

Study procedures have been described in full previously.[1] In brief, at randomization clinical assessments were performed and blood samples for safety and immunology collected from all participants. Concurrently, participants in the interventional group were administered 0·3 mL BNT162b2 dose as a single intramuscular injection (day 0), whilst individuals from control group were vaccinated on day 28 of study. Planned follow-up visits for safety and immunologic purposes were scheduled on days 7, 14, 28, 90, 180 and 360. All vaccinated participants were on-site monitored for safety for at least 15 minutes. Safety procedures included both direct report from individuals during the post-vaccine observation period and online report using an electronic diary throughout the study follow-up period.

To the present analysis, the commercial electrochemiluminescence immunoassay (ECLIA) Elecsys® Anti-SARS-CoV-2 S assay (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect antibodies (including IgG) specific to the SARS-CoV-2 spike protein receptor binding domain (RBD-S protein) on the Cobas e411 module (Roche Diagnostics GmbH, Mannheim, Germany),[23] with a measuring range from 0·4 to 250 U/mL (up to 2,500 U/mL with onboard 1:10 dilution and up on 12,500 with onboard 1:50 dilution). Values higher than 0·8 BAU/mL were considered positive.

Measurement of neutralising antibodies titres in a predefined subset of 198 participants was carried out by preincubation of diluted plasma samples with titrated pseudoviruses (10 ng p24Gag per well) generated by co-transfection of pNL4-3ΔenvRen and an expression vector for the different viral spikes (pcDNA3.1-S-CoV2Δ19-G614, -Alpha, -Beta, -Delta, or -Omicron) and added to Vero E6 cells in 96-well plates. Viral infectivity 48 hours post infection was assessed by measuring luciferase activity (Renilla Luciferase Assay, Promega, Madison, WI, USA) using a 96-well plate luminometer LB 960 Centro XS³ (Berthold Technologies, Oak Ridge, TN, USA). The titre of neutralising antibodies was calculated as 50% inhibitory dose (neutralising titre 50, NT50), expressed as reciprocal of four-fold serial dilution of heat-inactivated sera (range 1:32–1:131·072) resulting in a 50% reduction of pseudovirus infection compared to control without serum. Samples below the detection threshold (1:32 serum dilution) were given 1:16 value. Positive and negative controls were included in the assays and non-specific neutralisation was assessed using a related pseudovirus expressing the vesicular stomatitis virus envelope (VSV-G). Cellular immune response was measured in participants from two pre-selected sites by quantification of interferon-γ (IFN-γ) and interleukin-2 (IL-2) present in plasma on overnight stimulation of whole blood cultured with pools of SARS-CoV-2 spike peptides (2 μg/mL) or dimethyl sulfoxide control. Cytokines were quantified using the next-generation enzyme-linked immunosorbent assay (ELISA) tool, Ella (ProteinSimple, San Jose, CA, USA). Full details on the pseudo-virus neutralisation assays and cellular immunity quantification are provided in the Appendix 1 (pp 18-19).

Outcomes

Outcomes included in the present secondary analysis were humoral immune response to vaccination as per antibodies titres and neutralising antibody titres at days 28, 90 and 180 after the BNT162b2 dose. Of note, in the control group outcomes at day 28 post-vaccine correspond to day 56 of study, and outcomes at day 180 correspond to day 152 post vaccine. In the control group, no outcomes were planned at day 90, according to the protocol. Alpha-, Beta-, Delta-, and Omicron-specific neutralising antibody titres at days 28 and 180 post-dose have been analysed in both study groups. Cellular response defined as inflammatory IFN-γ and IL-2 cytokines production against SARS-CoV-2 spike peptide pools at days 28 and 180 post-BNT162b2 dose were also assessed.

Statistical analysis

To the present analysis, the immunogenicity population included all the participants who were randomly assigned, completed all applicable visits, and for whom serological samples were available at the baseline visit and on days 28, 56 (only applicable to control group), 90 (only applicable to interventional group) and 180. Laboratory data cut-off was on November 19, 2021. Day-28 variables (i.e. humoral immunogenicity by RBD-specific IgG analysis, neutralising activity of SARS-CoV-2-specific antibodies, and cellular immunity) were analysed in 658, 194 and 114 individuals, respectively. Missing values from later visits were not imputed (Appendix 1 p 17).

Data were presented as geometric mean and 95% confidence interval (CI) or, for categorical variables, number, and percentage, unless otherwise stated. For serological measurements, difference at each time point was evaluated using ratio of geometric means. Since the outcome variable, i.e. antibodies against SARS-CoV-2 spike protein RBD on day 28 post-dose, was restricted

by technical limitations, a truncated regression model was used. The model incorporated right censoring values, raw data response with distribution lognormal data, and treatment effect (interventional group versus control group) adjusted by sex, age, and time between vaccine doses. Additionally, reverse cumulative distribution curve (RCDC) was plotted. A stratified analysis by sex, age and interval between vaccine doses was done for the humoral and cellular immunity endpoints. Laboratory parameters with values below detection limit were replaced by a value equal to the lowest limit divided by two. All analyses were carried out using the statistical software SAS, version 9·4. Sample size was calculated for primary efficacy endpoint – i.e. antibody titres at day 14 –, and was also considered appropriate to evaluate most of secondary endpoints (Appendix 1 p 14). Sample size calculation methods as well as independent data monitoring committee procedures have been described previously.[1]

Composition of the independent data monitoring committee is provided in the Appendix 1 (p 23). This study was registered with EudraCT (2021-001978-37) and ClinicalTrials.gov (NCT04860739).

Role of the funding source

The funder – Institute of Health Carlos III (ISCIII), a public research organization – designed the trial in cooperation with the Spanish Clinical Trials Platform (SCReN), a public network of clinical trial units at the Spanish National Health System funded by the ISCIII. Trial coordination, participant recruitment and data analysis were performed by SCReN. All immunological procedures were performed at ISCIII. All authors reviewed and approved the original draft. All authors had full access to the full data in the study and accept responsibility to submit for publication.

Results

Of 676 participants enrolled and randomised between April 24th and 30th, 2021, 450 individuals were assigned to the interventional group, receiving BNT162b2 as second dose, and 226 were to the control group, maintaining observation. Participant's flow up to day 14 of study has been fully described previously.[1] 441 participants from the interventional group completed day 28 of study and 418 completed day 180. 223 individuals from control group received BNT162b2 vaccination at day 28; 212 and 199 of them completed day 56 of study (day 28 post vaccination) and day 180 (day 152 post vaccine), respectively (Figure 1).

Baseline and demographic characteristics of the population are summarised in Table 1. 378 (57%) individuals were women and 289 (43%) were men. 431 (65%) participants were aged 18–49 years, and the mean age of both groups was 44·03 years (SD 8·82). In the control group, mean (SD) interval between ChAdOx1-S and BNT162b2 administration was 89·03 days (5·92). Neither differences between groups were found in demographic characteristics at day 180 (Appendix 1 p 15).

Median time in collection of day-28 post- BNT162b2 dose blood sample was similar in both study groups (28 days [interventional] vs. 27 [control]), however variability was higher in individuals from the control group (range 21-38 days [interventional] vs. 16-42 [control]) (Appendix 1 p 2).

Results on immunogenicity dynamics in both groups show a decay in titres of antibodies specific to the SARS-CoV-2 S-protein RBD over time (Appendix 1 pp 3-4). In the interventional group, geometric mean titres (GMT) of S-RBD antibodies decreased from 7739·21 BAU/mL (95% CI 7371·53–8161·96) at day 14 after BNT162b2 second dose (fully reported earlier)[1] to 5616·91 BAU/mL (95% CI 5296·49–5956·71) at day 28, 2303·28 BAU/mL (95% CI 2141·66–2477·1) at day 90 and 1142·0 BAU/mL (1048·69–1243·62) at day 180. Of note, waning was slower from third to six month (mean lognormal difference -0·303 [95% CI -0·324–(-)0·283]) than from first to third (-0·389 [95% CI -0·405 –(-)0·374]). The regression model for outcome variables to 180 days by treatment (interventional group versus control group) and adjusted by covariables is showed in the Appendix 1 p 16. Interestingly, immunogenic response in the control group was significantly stronger at day 28 after second dose (7298·22 BAU/mL [95%CI 6739·41–7903·37]) than in the interventional group ($p < 0·0001$). Likewise, antibody levels remained higher at day 180 in the control compared to interventional group (1836·4 BAU/mL [95%CI 1621·62–2079·62]; $p < 0·0001$) (Figure 2; Appendix 1 p 3). This effect was also observed in stratified analyses by sex and age (Appendix 1 pp 5–6). Adjusted differences in day-28 and day-180 lognormal RBD values in the interventional vs. control group resulted from the regression model were -0·0881 (95% CI -0·1239–(-)0·0523 [$p < 0·0001$]) and -0·1760 (95% CI -0·2277–(-)0·1242 [$p < 0·0001$]), respectively. In addition, the effect of delayed vaccination in the control group is linear over time, resulting in a difference of S-RBD antibody levels in the test vs. control group of -0·1329 (95% CI -0·1831–(-)0·0826) over the follow-up period.

Consistently with waning of antibody titres observed in the interventional group, a decay in neutralising antibodies was also evidenced. At day 14, GMT of neutralising antibodies was 1905·69 (95%CI 1625·65–2233·98) in the interventional group, which decreased to 1429·01 (95%CI 1220·37–1673·33) at day 28, to 480·68 (95%CI 398·27–580·13) at day 90 and to 198·72 (95%CI 161·54 –244·47) at day 180. In the control group, neutralising antibody titres were similar to the interventional group both 28 days after second dose (1503·28 [95%CI 1210·71 –1866·54]) and at day 180 (295·57 [95%CI 209·84 –416·33]) (Figure 3; Appendix 1 p 7). RCDC for

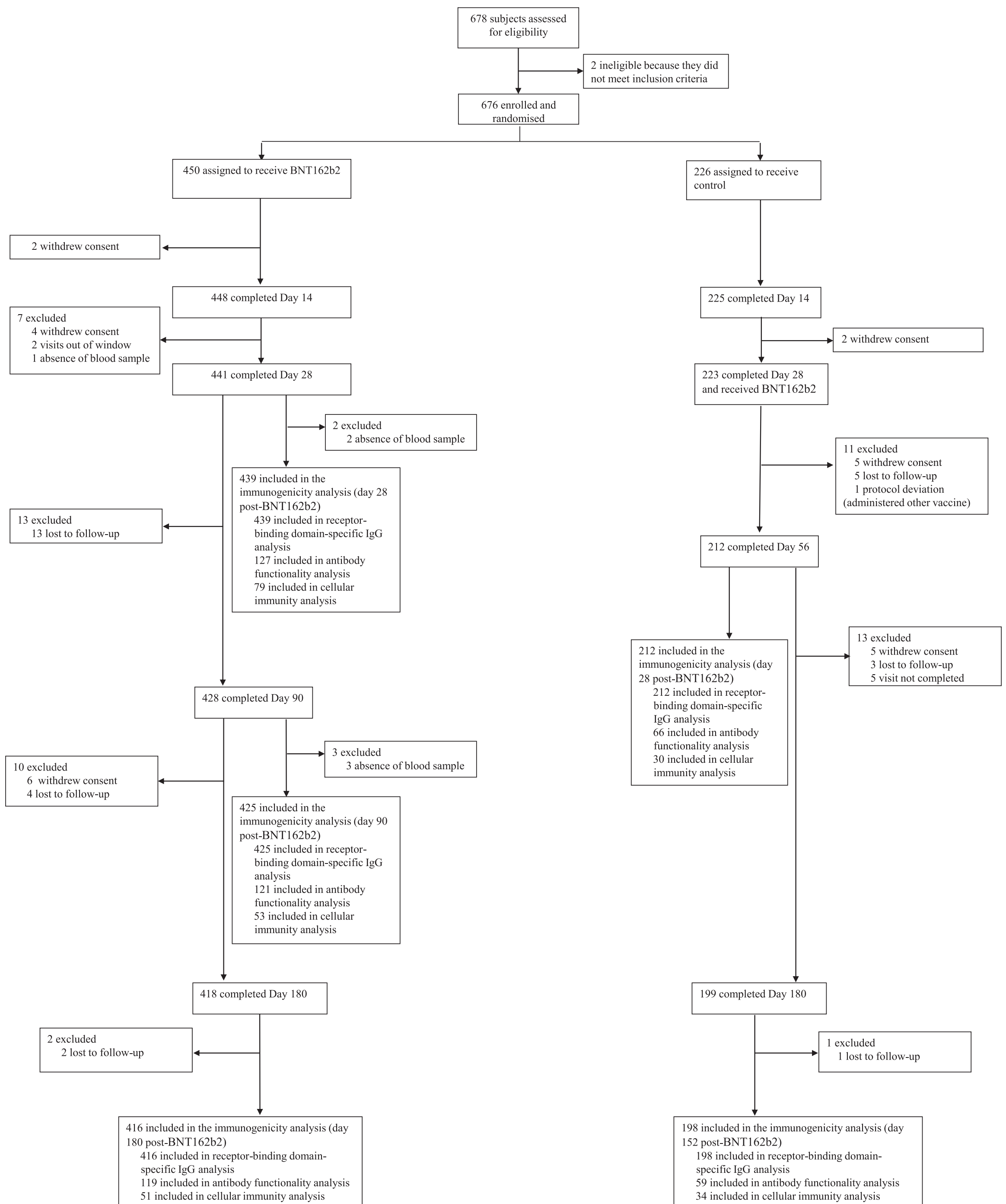

Figure 1. Trial profile.

neutralising antibodies in both study groups are shown in Appendix 1 (p 8). All patients from both interventional and control group exhibited high neutralising activity (NT50 >1:100) against the reference variant G614 28 days post- BNT162b2 dose; a threshold that has been recently described[24] as associated with vaccine efficacy. Yet decreased, a relevant proportion of individuals (76%) exhibited NT50 >1:100 at day 180 of study. (Appendix 1 p 9).

Differences between variants and original G614 strain at different time points were analysed (Appendix 1, p 10). By SARS-CoV-2 variants, the poorest

	Interventional group (n = 450)	Control group (n = 226)[d]	Overall (n = 676)
Sex, n (%)			
Male	193 (43%)	101 (45%)	294 (43%)
Female	257 (57%)	125 (55%)	382 (57%)
Age (years), mean (SD)	43·93 (8·88)	44·10 (8·82)	43·98 (8·85)
Age group, n (%)			
18-49 years	293/450 (65%)	144/226 (64%)	437/676 (65%)
Male	123/293 (42%)	65/144 (45%)	188/437 (43%)
Female	170/293 (58%)	79/144 (55%)	249/437 (57%)
50-59 years	157/450 (35%)	82/226 (36%)	239/676 (35%)
Male	70/157 (45%)	36/82 (44%)	106/239 (44%)
Female	87/157 (55%)	46/82 (56%)	133/239 (56%)
Days between vaccines, mean (SD)	61·16 (5·73)	89·03 (5·92)	70·33 (14·32)

***Table 1*: Baseline characteristics of the population.**
[d] Based on 223 subjects (3 subjects withdrew consent before being immunized).

neutralisation capability at day 28 post-vaccination was found with Omicron variant in both interventional and control groups (GMT 144·84 [95%CI 116·65–179·85] and 204·84 [95%CI 151·99–276·06]). A decrease in NT50 was also observed for Beta variant in both interventional and control groups (GMT 293·21 [95%CI 234·8–366·15] and 483·89 [95%CI 352·53–664·2]). Of note, titre of Omicron and Beta-neutralising antibodies at day 28 in the control group was higher than in the interventional group (p = 0·0641 and p = 0·0102, respectively), whilst no differences between groups were evidenced for Alpha- and Delta-neutralising antibody titres (Figure 4; Appendix 1 p 11). NT50 against Delta variant on day 28 after second dose was 717·13 (95%CI 587·27–876·13) in the interventional group and 837·14 (95%CI 609·7–1149·41) in the control group. Overall, day-28 NT50 was above 1:100 in 94% to 100% patients against all variants excepting Beta (88% patients) and Omicron (69%) (Appendix 1 p 12).

At day 180 Omicron-neutralising antibody titres decayed to 34·46 (95%CI 27·72–42·85) and 61·52 (95%CI 43·66–86·71) in the interventional and control

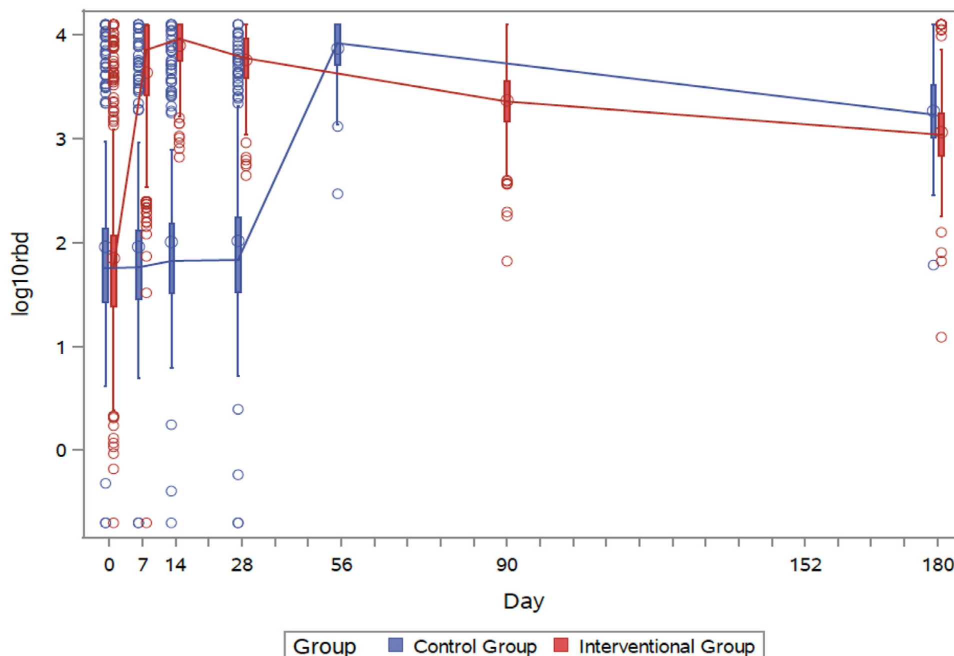

Figure 2. RBD (anti-spike) antibody titres measured in both interventional (red) and control (blue) groups over time. Interventional group was immunised at day 0 and control group at day 28. Accordingly day 180 corresponds to day 152 post vaccine in the control group.

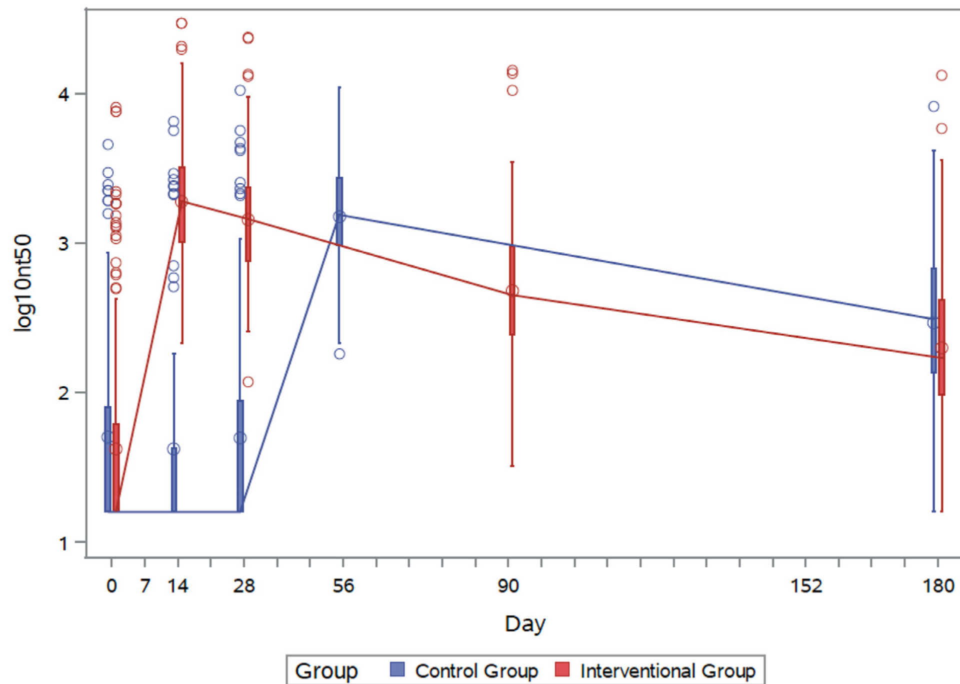

Figure 3. Neutralising antibodies titres (NT50) measured in both interventional (red) and control (blue) groups over time. Interventional group was immunised at day 0 and control group at day 28. Accordingly, day 180 corresponds to day 152 post vaccine in the control group.

groups, respectively ($p = 0{\cdot}0038$). Beta-neutralising antibody titres decayed to 37·08 (95%CI 29·76–46·2) and 76·24 (95%CI 53·99–107·67) in the interventional and control groups, respectively ($p = 0{\cdot}0004$). Delta NT50 decayed to 94·57 (95%CI 71·83–124·52) and 192·89 (95%CI 126·93–293·12) at day 180 in the interventional and control groups, respectively ($p = 0{\cdot}0043$) (Figure 4; Appendix 1 p 11).

Regarding dynamics of functional spike-specific T-cell response, an increase in levels of both IFN-γ and IL-2 after vaccination is followed by a progressive waning over time. In the interventional group, maximum IFN-γ production was observed at day 14 post-dose. Levels decreased to 380·93 pg/mL (95% CI 309·07–469·5) at day 28 and 223·8 pg/mL (166·25–301·28) at day 180. In the control group, IFN-γ levels 28 days after BNT162b2 dose were 485·32 pg/mL (343·51–685·68), and decreased to 171·23 pg/mL (120·15–244·02) at day 180. Similarly, IL-2 concentrations were maximum at day 28 post-vaccination in both the interventional and control groups (244·07 pg/mL [95% CI 204·89–290·74] and 299·2 [217·81–411·01], respectively) and progressively decayed until day 180 (171·54 [133·08 –221·12] and 170·25 [122·24–237·11], respectively). Of note, day-180 levels of both IFN-γ and IL-2 were higher than those present at baseline (Figure 5; Appendix 1 p 13).

Discussion

Our results provide evidence that humoral immune response of patients vaccinated with the heterologous ChAdOx1-S/BNT162b2 regime decays over time after peaking at day 14 post- BNT162b2 dose. A decline ranging 25%-27% in total RBD and neutralising antibody levels was observed after 28 days, which increased up to 70%-75% on day 90 and 86%-90% on day 180. This waning of immunogenicity was expected consistently with previous reports from COVID-19 mRNA vaccines[5,25,26] that reported antibody half-life of 28-33 days.[26] A similar decline of about 80% in antibody levels was found with homologous BNT162b2 regime at 90 days after the second dose[5] as well as with homologous mRNA-1273 regime, albeit the latter to a lesser extent (decline around 60%).[25] With homologous vaccination with ChAdOx1, antibodies are induced at lower levels than with homologous RNA regimens or heterologous vaccination of ChAdOx1 with BNT162b2[27] or mRNA-1273,[28] although it has been reported that antibodies decay with a slower kinetics.[29] Furthermore, these results are consistent with known kinetics of humoral immune response against acute viral infections, in which extrafollicular short-lived plasmablasts contribute to early antibody production – IgM, IgG, IgA –, while a secondary increasing contribution of germinal centre-derived plasma cells – with longer lifespan and larger secretory capacity – leads to secretion

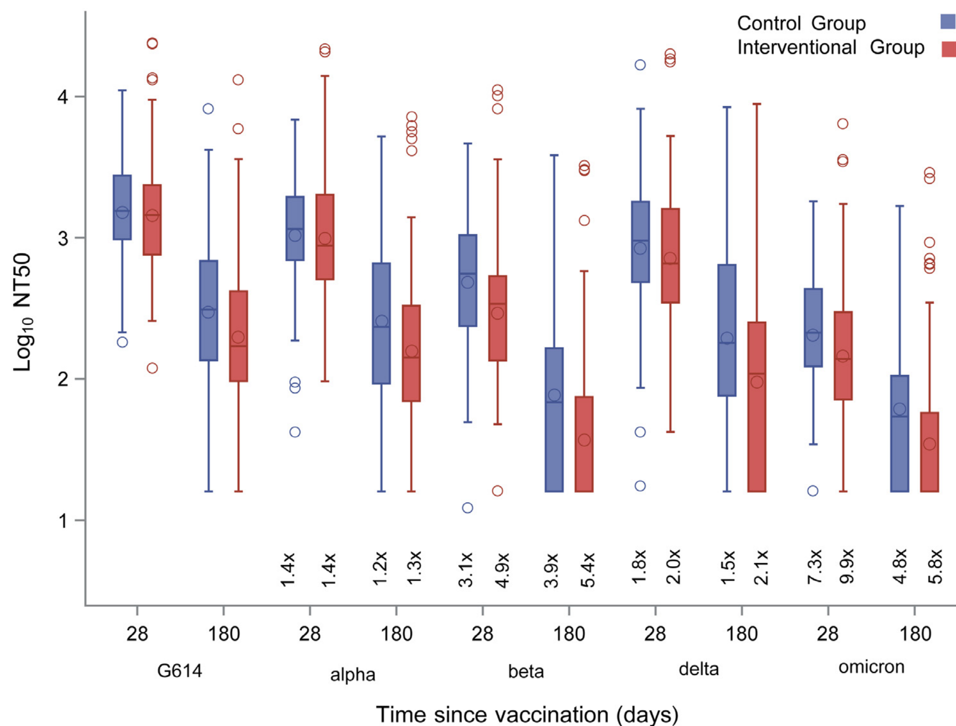

Figure 4. Neutralising antibodies titres (NT50) against SARS-CoV-2 variants measured in both interventional (red) and control (blue) groups at days 28 and 180 after BNT162b2 administration. Interventional group was immunised at day 0 and control group at day 28. Accordingly, day 180 corresponds to day 152 post vaccine in the control group.

Dashes and circles inside boxes indicated the median and arithmetic mean, respectively. Box limits indicate the interquartile range (IQR). Whiskers are adjusted to maximal and minimal values if lower than 1.5 times the IQR. Further outliers are indicated as circles.

of class-switched antibodies, mainly IgG. Considering that half-life of IgM is substantially shorter than IgG, a decay in antibody titres is common once the extrafollicular response is resolved. However they will rapidly rise if memory B cells are re-exposed to viral antigens in the future.[30] In this regard, we observed a slowing-down in SARS-CoV-2 antibody decay from month 3 to month 6, consistent with previous reports.[26]

BNT162b2 administration to the control group 28 days later than the interventional one did not result in worse or weaker antibody responses 28 days after immunization. Actually, S-RBD antibodies and all variant-specific neutralising titres were higher — S-RBD and Beta-specific titres significantly higher— in the control group four weeks after vaccination suggesting a benefit of second dose delay. However, lack of antibody determination 14 days after immunization — when top levels of antibodies are reached — does not allow to perform a parallel kinetics of S-RBD and neutralizing antibodies between CG and IG to fully demonstrate that delayed administration of BNT162b2 results in better antibody responses.

Importantly, our results suggest that high levels of protection against Delta-variant persisted in both IG and CG at day 28 after the second dose of the heterologous ChAdOx1-S/BNT162b2 scheme.

Regarding differences found at day 180 between control and intervention group it must be noted that this measurement carried over the 28-day delay in BNT162b2 administration to the control group (measured at day 152 — instead of 180 — after dose). A second explanation for these differences could be related with vaccination delay itself, supporting an apparent benefit for longer intervals between doses as found previously.[14,31] Indeed, results from regression models pointed in this direction. Such delay could favour the maturation process of memory B cells from germinal centres (GC), over which B cells accumulate somatic mutations in their variable region leading to selection of those with higher affinity for a given viral antigen.[30] A recent study has demonstrated that antigen-driven activation of memory B cells persisted and matured up to 6 months after SARS-CoV-2 infection.[32] The observation that at day 28 neutralisation activity in the control group against Beta was higher — and trended to higher against Omicron — while no differences were observed between groups for G614 reference strain, Alpha and Delta variants suggests that the 4-week delay in vaccination

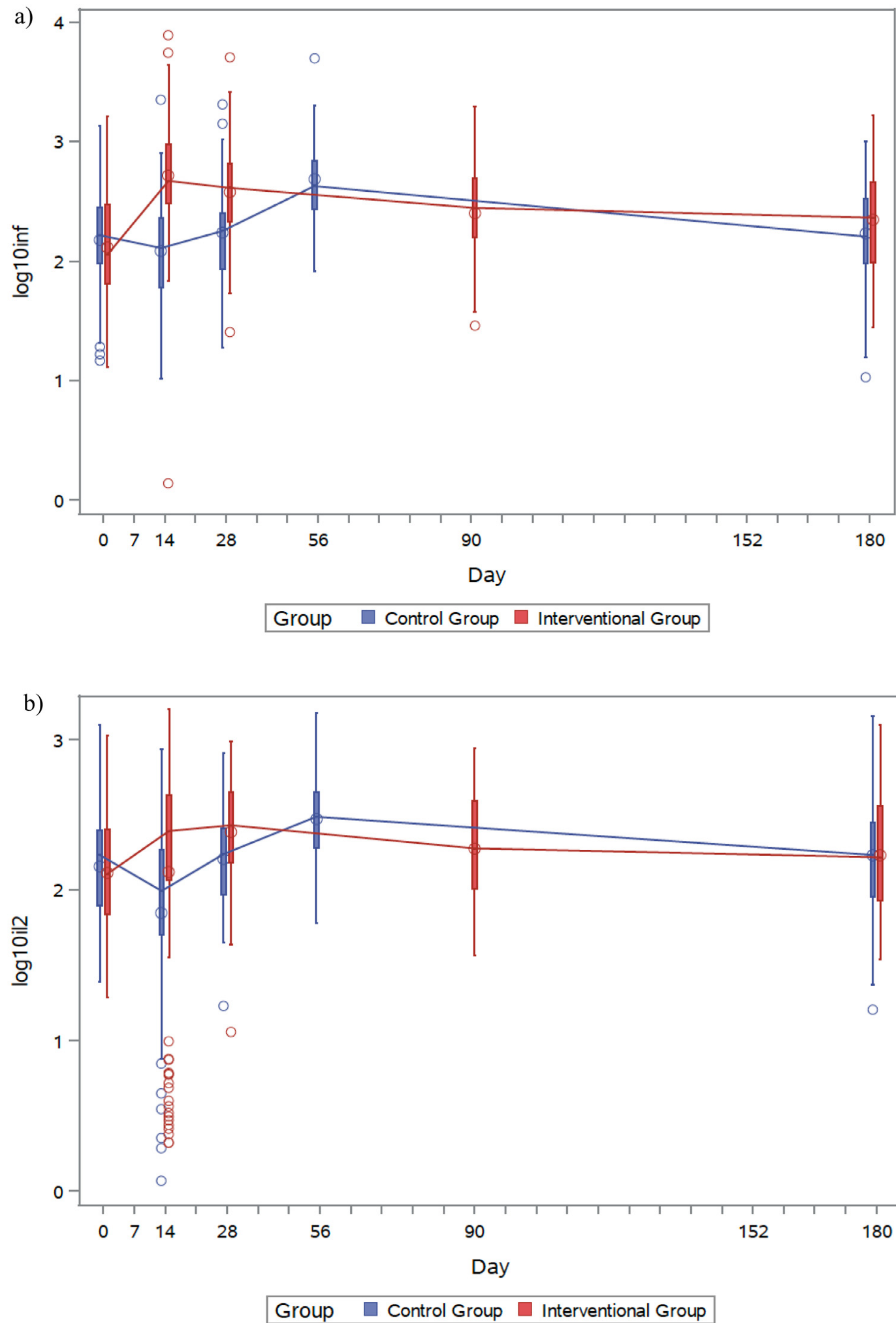

Figure 5. IFN-γ (a) and IL-2 (b) measured in both interventional (red) and control (blue) groups over time. Interventional group was immunised at day 0 and control group at day 28. Accordingly, day 180 corresponds to day 152 post vaccine in the control group.

window for the control group in our study might have contributed to a better affinity maturation against 'difficult' variants such as Beta and Omicron. Regarding cellular responses, similar decay of IFN-γ and IL-2 were found in both groups at different time points which is consistent with the generation of memory T lymphocytes in which maturation process and selection of Tc receptor affinity is not dependent on somatic mutation.

Notwithstanding this, the sharp decay in RBD antibodies and neutralisation titres observed at day 180 as compared to day 14 and 28 support the use of a third immunization to reach higher protection levels, particularly considering the high infectivity potential of the Omicron variant.[33] Actually, despite the persistence of immune memory, antibody decay increases the risk of SARS-CoV-2 infection and a third dose becomes necessary to achieve protection against asymptomatic and symptomatic infections, particularly in aged groups above 60 and patients with risk factors for developing severe COVID-19 as immune suppression.[19,20] It has been described that boosting with a third dose of mRNA vaccines generate potent neutralization of Omicron, shortening the difference in neutralization titres with other variants.[34] Thus, increased protection of a booster dose would be related not only to higher levels of neutralizing antibodies but to antibody maturation leading to the generation of antibodies with increased affinity to their targets. These qualitative changes are particularly important against escape variants as Omicron and represent an added value for a third dose. Unfortunately, very recently it has been described a waning effectiveness of a third dose of BNT162b2 against hospital admission after 3 months due to the Omicron variant.[35]

As previously described,[1] the main limitation of the CombiVacS study is the absence of a control group completing the homologous ChAdOx1-S scheme to compare with the heterologous ChAdOx1-S/BNT162b2 regimen. This arm would had been very useful to compare antibody waning and neutralization activity in individuals vaccinated with homologous or heterologous vaccine regimes. Besides, the abovementioned 4-week delay between both study groups in BNT162b2 administration led to capture 5-month, rather than 6-month, post dose data in the control group. Although this limitation may have influenced some differences observed at day 180, overall results are consistent between groups. As mentioned, lack of antibody determination at day 14 in the CG limits the interpretation of the results. Also, we have found a low proportion of high-responder outliers, in particular before immunization with the second vaccine dose. We cannot rule out asymptomatic SARS-CoV-2 infection between both vaccine doses leading to a “booster-like” response after first immunization.

In conclusion, follow-up of individuals included in the CombiVacS trial that were immunised with heterologous ChAdOx1-S/BNT162b2 confirm waning of humoral and cellular responses over time, nevertheless a relevant proportion of individuals exhibited neutralising activity > 1:100 six months after full vaccination excepting against Beta and Omicron variants. These results support the use of a third dose six months after regular vaccination to enhance immune response, particularly against new VoCs, as Omicron. Further studies addressing immunogenicity using different heterologous vaccination schemes are warranted.

Contributors

Trial conceptualisation was done by C.B.-I., J.A., M.P.-O., A.M.B., A.J.C., J.F., J.R.A. and M.C. A.J.C., J.F., and A.Ag. developed the study methods. J.A., M.P.-O., A.M. B., J.F., L.C., M.J.B., J.G.-P., M.C., A.P., M.G.-P., E.A.-A., M.T., A.As., N.I.-A., E.M., C.P.-I., J.O., M.C.-O., M. B., P.C., L.H.-G., I.F., H.E.D.T. and J.R.A. were study investigators. M.T.G.M., D.L., J.G.-P. and A.G.C. ensured data verification. J.G.-P., D.L., J.A., M.P.-O., M. T.G.M., J.O., A.M.B. and A.J.C. were responsible for the present secondary statistical analysis. C.B.-I., J.A., M.P.-O., J.G.-P., M.G.-P., A.M.B., L.C., M.C., M.J.B., A.P., J. O., J.F., and J.R.A. supervised the study. C.B.-I. was responsible for funding acquisition. J.A., M.P.O., J.O., J. G.-P. and A.M.B. wrote the original draft of this Article. All authors reviewed and edited the manuscript, and approved the manuscript for submission. All authors reviewed and approved the original draft. All authors had full access to the full data in the study and accept responsibility to submit for publication.

Data sharing statement

Individual participant data will be made available when the trial is complete, on request to the corresponding authors. After approval of a proposal, data will be shared through a secure online platform.

Declaration of interests

JA has received fees for educational programs from Gilead, MSD, GSK and Janssen outside of the submitted work. MC has participated in advisory boards and has received research funding from GSK, Sanofy Pasteur, Pfizer, Novavax and Janssen.CB-I is the deputy general manager of the Instituto de Salud Carlos III. JRA has received fees from Janssen, outside of the submitted work. AMB is principal investigator of clinical trials sponsored by GlaxoSmithKline, Daiichi-Sankyo, Janssen, and Farmalider, outside of the submitted work. All other authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2022.101529.

Appendix 1. CombiVacS Vaccine Trial Group

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Development of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients After 1273-mRNA SARS-CoV-2 Vaccination

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Long-term hemodialysis (HD) patients are considered vulnerable and at high-risk of developing severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection due to their immunocompromised condition. Since COVID-19 associated mortality rates are higher in HD patients, vaccination is critical to protect them. The response towards vaccination against COVID-19 in HD patients is still uncertain and, in particular the cellular immune response is not fully understood. We monitored the humoral and cellular immune responses by analysis of the serological responses and Spike-specific cellular immunity in COVID-19-recovered and naïve HD patients in a longitudinal study shortly after vaccination to determine the protective effects of 1273-mRNA vaccination against SARS-CoV-2 in these high-risk patients. In naïve HD patients, the cellular immune response measured by IL-2 and IFN-ɣ secretion needed a second vaccine dose to significantly increase, with a similar pattern for the humoral response. In contrast, COVID-19 recovered HD patients developed a potent and rapid cellular and humoral immune response after the first vaccine dose. Interestingly, when comparing COVID-19 recovered healthy volunteers (HV), previously vaccinated with BNT162b2 vaccine to HD patients vaccinated with 1273-mRNA, these exhibited a more robust immune response that is maintained longitudinally. Our results indicate that HD patients

develop strong cellular and humoral immune responses to 1273-mRNA vaccination and argue in favor of personalized immune monitoring studies in HD patients, especially if COVID-19 pre-exposed, to adapt COVID-19 vaccination protocols for this immunocompromised population.

Keywords: COVID-19, SARS-CoV-2 vaccine, hemodialysis, chronic kidney disease, cellular response, humoral response

INTRODUCTION

Progression of chronic kidney disease (CKD) leads to the need of kidney replacement therapy such as hemodialysis (HD). Long-term HD patients are at higher risk of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection associated with coronavirus 19 disease (COVID-19) (1–3). In addition, the overall mortality of SARS-CoV-2 increases from 3.2% in healthy individuals to >20% in HD patients (1, 4, 5). As HD prevalence is increasing worldwide (6), HD patients represent a public health problem and specific considerations should be given to immunization of HD patients against SARS-CoV-2 infection.

Recent humoral immunity studies on natural SARS-CoV-2 infection in HD patients reported that, while 75% seroconverted shortly after infection, >70% of these patients exhibit a rapid decline of IgG specific antibodies (7), indicating that HD patients develop short-term humoral immunity after COVID-19. On the contrary, cellular immunity data indicates that COVID-19 convalescent HD patients exhibit higher frequencies of SARS-CoV-2 reactive memory T cells ($CD4^{+}CD154^{+}CD137^{+}$) that express IFN-γ and IL-2 in comparison to patients with normal kidney function, although this increase did not reach statistically significance in a single time-point observational case-control study (8).

Regarding vaccination in HD patients, previous studies demonstrated deficient immune responses against the Hepatitis B and Pneumococcus vaccines in high-risk groups (9–12). Interestingly, recent data reported two patients that did not respond to the Hepatitis B vaccine, did not mount antibody responses to the COVID-19 vaccine and developed severe COVID-19 infection after vaccination (13). This highlights the immunocompromised state of HD patients and the need for monitoring their specific humoral and cellular response against SARS-CoV-2 following vaccination. In this respect, some studies have reported (i) lower response rate to the vaccine, (ii) lower anti–spike antibody level and neutralizing capacity, and (iii) higher rate of COVID-19 infection compared to healthy volunteers (HV) after SARS-CoV-2 vaccination (14–16).

While the humoral immune response to COVID-19 mRNA vaccines in HD patients are currently under investigation, only a few studies have simultaneously studied the humoral and cellular immune responses in HD patients following vaccination. Bertrand and colleagues reported that 89% of HD patients developed anti-spike SARS-CoV-2 antibodies, while 100% displayed specific T cells response after full vaccination (17). More recent studies by Strengert and colleagues described significantly reduced IgG titers and IFN-γ release when compared to HV (18). These apparent contradictory studies may be due to differences in the study design, as data was obtained in a single time-point, arguing in favor of longitudinal experiments to fully understand the kinetics of the immune response of HD patients after SARS-CoV-2 vaccination.

Here, we investigated the effects of the 1273-mRNA SARS-CoV-2 vaccine on the humoral and cellular immune responses in a longitudinal study shortly after vaccination that included COVID-19 recovered and naïve HD patients and further compared the results with non-dialyzed healthy volunteers (HV).

METHODS

Study Design

All 39 long-term hemodialysis (HD) patients were recruited at Hospital Puerta de Hierro and Fundación Jimenez Diaz (Madrid, Spain) between April and June, 2021. COVID-19 recovered patients were classified by RT-PCR and confirmed by their ability to react against membrane (M) peptide pools *in-vitro*. COVID-19-recovered patients (n=20) were 45% women and 55% men (mean age 65 years [SD 13.23]). Naïve patients (n=19) were 52.63% women and 47.36% male (mean age 64 years [SD 12.39]). All HD patients received the mRNA-1273 vaccine (Moderna). The study also includes 92 healthy volunteers (HV) that were fully vaccinated with BNT162b2 (Pfizer). The mean time since COVID-19 was 9.6 ± 3.1 months in HD patients. COVID-19-recovered healthy volunteers (HV) (n=45) were 76% women and 24% men (mean age 44.3 years [SD 16.90]). Naïve healthy volunteers (n=47) were 78% women and 22% men (mean age 39.9 years [SD 14.73]). The mean time since COVID-19 was 6.9 ± 4.1 months in HV. Blood samples were longitudinally collected at different time points: pre-vaccination (Pre), ten (d10) and twenty (d20) days after the first dose, followed by ten (d30) and twenty (d40) days after the second dose. HD patient characteristics are displayed in **Table 1**.

Ethics Statement

Ethical approval of the study was obtained from the relevant authority - the Internal Review Board of Hospital Puerta de Hierro and Fundación Jimenez Diaz. Written informed consent was obtained from all participants prior to starting the study.

Abbreviations: HD, Hemodialysis; HV, Healthy Volunteers; COVID-19, Coronavirus disease 2019; IFN-γ, Interferon gamma.

TABLE 1 | Naïve and COVID-19 recovered HD patients on long-term hemodialysis present similar characteristics, except for diabetic nephropathy as a cause of end-stage renal disease and smoking.

Total	Naïve HD patients N=19	N (partial)	COVID-19 HD patients N=20	N (partial)
Characteristics				
Gender				
Male	47.36%	9	55%	11
Female	52.63%	10	45%	9
Age (Mean± SD)	64 ± 12.39		65.25 ± 13.23	
Active smoking (yes)	15.8%	3	0,15%	3
HD time, months (Mean± SD)	96.11 ± 102.48		81.41 ± 72.21	
Use of EPO	89.4%	17	100%	20
Previous kidney transplantation	26.31%	5	35,00%	7
Comorbidities				
Obesity	15.79%	3	30%	6
Hypertension	89.47%	17	95%	19
Diabetes mellitus	31.58%	6	45%	9
Ischemic heart disease	31.58%	6	15%	3
Dyslipidemia	68.42%	13	60%	12
Cause of end-stage renal disease				
Diabetic nephropathy	28.57%	2	40%	8
Hypertensive nephrosclerosis	5.26%	1	10%	2
IgA nephropathy	0		5%	1
Membranoproliferative glomerulonephritis	5.26%	1	10%	2

SARS-CoV-2 Peptides

PepTivator ® SARS-CoV-2 Peptide Pools (Miltenyi Biotec, Germany) of the Spike protein (S1, S+, and S) and the Membrane (M) protein were used to perform whole blood cultures.

Whole Blood Cell Culture With SARS-CoV-2 Peptide Pools

Lithium heparinized blood samples were collected before the start of dialysis. On the same day, 320µl of whole blood were mixed with 80µl of RPMI and stimulated with PepTivator ® SARS-CoV-2 Peptide Pools (S; 2µg/ml, M; 2µg/ml) or a DMSO control. After 16-20 hours of culture, supernatant (plasma) was collected and stored at -20°C for further cytokine quantification (19, 20).

Cytokine Measurements and Analysis

Cytokine concentrations in the supernatants (plasma) were quantified using ELLA with microfluidic multiplex cartridges measuring IFN-ɣ and IL-2 release following the manufacturer's instructions (ProteinSimple, San Jose, California). The cytokine levels present in plasma stimulated with DMSO were subtracted from the corresponding Peptide-pool stimulated samples as previously reported (20). Values higher than 32.7 pg/ml and 36.8 pg/ml were considered positive for IL-2 in naïve HV and HD patients, respectively. Values higher than 9.0 pg/ml and 27.6 pg/ml were considered positive for IFN-ɣ in naïve HV and HD patients (**Supplementary Figure 1**).

Spike-Specific IgG Quantification

Liaison ® SARS-CoV-2 TrimetricS IgG assay (DiaSorin, Stillwater, MN, USA) was used for semiquantitative detection of IgG directed against Spike glycoprotein in human plasma sample on the LIAISON XL (DiaSorin, Saluggia, Italy) with a measuring range from 4.81 BAU/ml to 2080.00 BAU/ml. As described by the manufacturer's instructions, values over 2080.00 BAU/ml were diluted 1:20. Values over 33.8 BAU/ml were considered positive.

ROC Curves

ROC curves and AUC values for predicting vaccination status based on IL-2 and IFNg levels were calculated using the ROCit (v2.1.1) R package in an R 4.0.3 environment (21). Predictions were generated by using the pre-vaccination time point as the control group and the twenty days post second dose (d40) time point as the case group. Optimal cutoffs were determined in ROCit using Youden's index.

Statistics

For IgG, IFN-ɣ and IL-2 determination, statistical comparison between groups was performed using ANOVA test in GraphPad Prism version 9.1.1 (GraphPad Software, La Jolla, CA) P (*≤0.05, ** ≤0.01, ***≤ 0.001, ****≤0.0001). Data are reported as mean ±SEM.

RESULTS

Differential Cellular Immunity Effects of mRNA Vaccination in COVID-19 Recovered and Naïve HD Patients

In naïve HD patients without previous SARS-CoV-2 infection (**Figure 1A**) secretion of IFN-ɣ and IL-2 associated with cellular immunity was significantly increased after the second dose of the mRNA vaccine (d30). This suggests that IFN-ɣ and IL-2

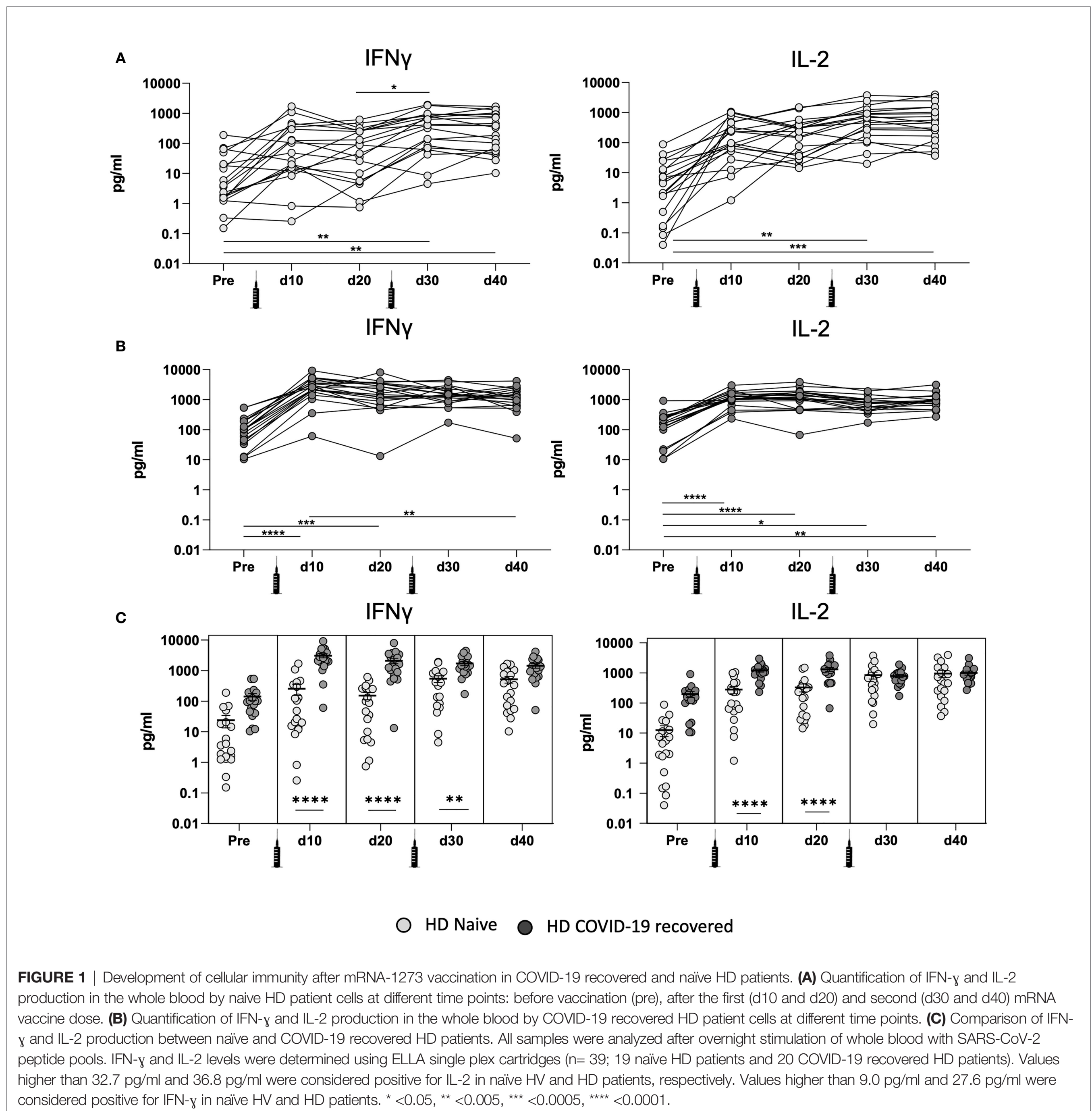

FIGURE 1 | Development of cellular immunity after mRNA-1273 vaccination in COVID-19 recovered and naïve HD patients. **(A)** Quantification of IFN-ɣ and IL-2 production in the whole blood by naive HD patient cells at different time points: before vaccination (pre), after the first (d10 and d20) and second (d30 and d40) mRNA vaccine dose. **(B)** Quantification of IFN-ɣ and IL-2 production in the whole blood by COVID-19 recovered HD patient cells at different time points. **(C)** Comparison of IFN-ɣ and IL-2 production between naïve and COVID-19 recovered HD patients. All samples were analyzed after overnight stimulation of whole blood with SARS-CoV-2 peptide pools. IFN-ɣ and IL-2 levels were determined using ELLA single plex cartridges (n= 39; 19 naïve HD patients and 20 COVID-19 recovered HD patients). Values higher than 32.7 pg/ml and 36.8 pg/ml were considered positive for IL-2 in naïve HV and HD patients, respectively. Values higher than 9.0 pg/ml and 27.6 pg/ml were considered positive for IFN-ɣ in naïve HV and HD patients. * <0.05, ** <0.005, *** <0.0005, **** <0.0001.

secreting cells need the second vaccine dose to reach full immunity.

We next evaluated the cellular response in HD patients with previous SARS-CoV-2 infection (**Figure 1B**) and our results indicate that these patients achieved their peak of IFN-ɣ and IL-2 associated T cell responses ten days after the first vaccine dose (d10). Interestingly, the second dose did not significantly further increase the production of IFN-ɣ or IL-2, suggesting that only one dose may be necessary to achieve protection mediated by cellular immunity in COVID-19 recovered HD patients. These results suggest that HD patients with pre-existing immunity develop a more rapid and sustained cellular immune response against SARS-CoV-2 spike peptide pools after the first dose of the vaccine, consistent with our recent investigation in healthy volunteers (HV) (20).

When comparing the cellular immune response between HD patients with and without previous SARS-CoV-2 (**Figure 1C**) there are significant differences between IFN-ɣ and IL-2 after the first vaccination dose, but we did not observe differential cytokine secretion 20 days after the second vaccine dose (d40).

Overall, the data suggests that HD patients develop potent cellular immunity in response to SARS-CoV-2 vaccination.

Differential Humoral Immunity Effects of mRNA Vaccination in COVID-19 Recovered and Naïve HD Patients

In naive HD patients without previous SARS-CoV-2 infection (**Figure 2A**), the IgG-specific humoral immunity was significantly increased only after the second vaccine dose. This suggests that naïve HD patients may exhibit a similar cellular and humoral response patterns.

Next, we evaluated the humoral response in HD patients with previous SARS-CoV-2 infection (**Figure 2B**). These patients achieved their peak of IgG levels 20 days after the first vaccine dose (d20). In line with the cellular immune response, the second dose of the vaccine did not significantly increase the levels of IgG, suggesting that only one dose is necessary to achieve the peak humoral immunity in COVID-19 recovered patients.

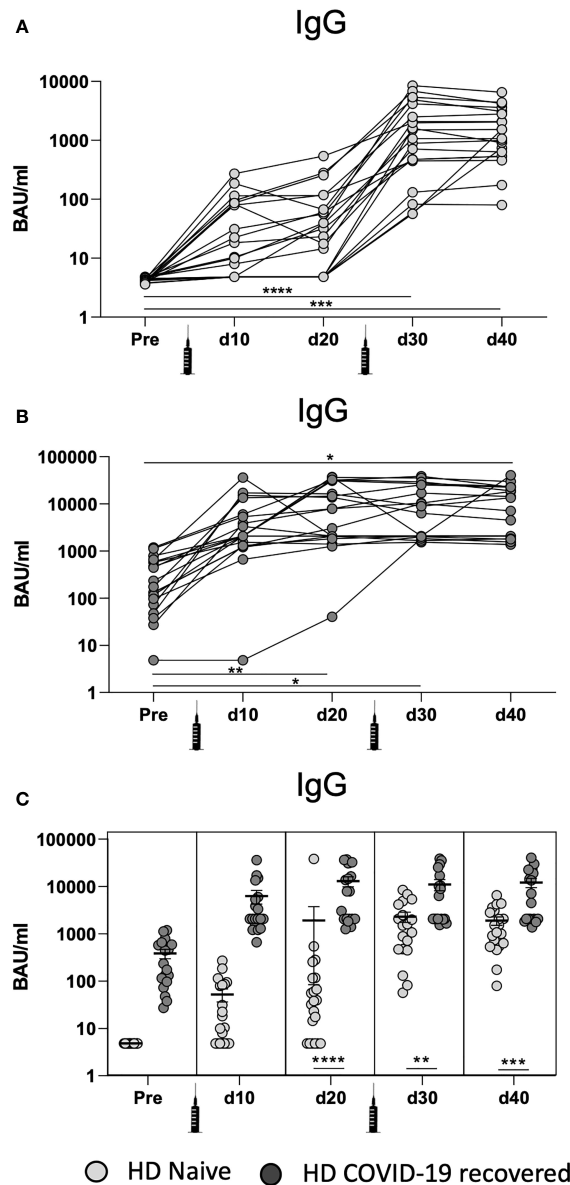

FIGURE 2 | Development of humoral responses after mRNA-1273 vaccination in COVID-19 recovered and naïve HD patients. **(A)** Quantification of SARS-CoV-2 spike-specific IgG serum levels in naive HD patients at different time points: before vaccination (pre), after the first (d10 and d20) and second (d30 and d40) mRNA vaccine dose. **(B)** Quantification of SARS-CoV-2 spike-specific IgG serum levels in COVID-19 recovered HD patients at different time points. **(C)** Comparison of SARS-CoV-2 spike-specific IgG serum levels in naïve and COVID-19 recovered HD patients. Samples were measured with Liaison ® SARS-CoV-2 TrimetricS IgG assay. Values higher than 33.8 BAU/ml were considered positive. * <0.05, ** <0.005, *** <0.0005, **** <0.0001.

When comparing the humoral immune response between HD patients with and without previous SARS-CoV-2 (**Figure 2C**), we observed that significant differences between IgG levels occur 20 days after the first vaccine dose (d20) and they are maintained longitudinally (d30 and d40). These results show that, while cellular immunity peaks 10 days after the first vaccine dose (d10) (**Figure 1B**), humoral IgG levels arise 20 days after the first vaccine dose (d20). Overall, the data indicate that HD patients develop potent humoral immunity in response to SARS-CoV-2 vaccination.

Differential Correlation Between Humoral and Cellular Immunity in COVID-19 Recovered and Naïve HD Patients

In naïve HD patients, we observed a significant correlation between the overall humoral (IgG) and the cellular (IFN-ɣ and IL2) immune responses (**Figure 3A**). Our results are comparable to other studies examining vaccination responses to BNT162b2, which observed correlation between T-cell and B-cell responses in naïve HD patients (Spearman's rho=0·56) (18). HD COVID-19 recovered HD patients also displayed a significant correlation between the humoral (IgG) and the cellular (IFN-ɣ and IL2) immune responses (**Figure 3B**). In conclusion, **Figure 3** indicates a strong correlation between the cellular and humoral immunity in both COVID-19 recovered and naïve HD patients.

Humoral and Cellular Immunity in HD Patients and HV Individuals

We next compared the humoral and cellular immune response between mRNA-1273 vaccinated HD patients (100 µg/dose) and BNT162b2 vaccinated healthy volunteers (HV) (30 µg/dose) with and without previous SARS-CoV-2 infection. Our results demonstrate that naïve HD patients without previous SARS-CoV-2 infection (**Figure 4A**) exhibit a significant increase in IFN-ɣ and IL-2 production 20 days after the second vaccine dose (d40), in comparison with HV. We further compared the cellular response in COVID-19 recovered patients with previous SARS-CoV-2 infection (**Figure 4B**) and our results indicate a significant increase in IFN-ɣ and IL-2 production after the first vaccine dose (d10), which is maintained longitudinally. Overall, these results indicate that naïve HD patients exhibit a significant increase in the cellular immune response in comparison with HV. However, this observation is likely be associated with the mRNA vaccine dosage on the magnitude of the induced cellular immune response.

Finally, we compared spike-specific IgG levels in both vaccinated HD patients and HV individuals with and without previous SARS-CoV-2 infection. Our results demonstrate that naïve HD patients without previous SARS-CoV-2 infection (**Figure 4C**) exhibit similar IgG levels to HV and no significant differences between HD and HV were observed. On the contrary, HD patients with previous SARS-CoV-2 infection (**Figure 4D**)

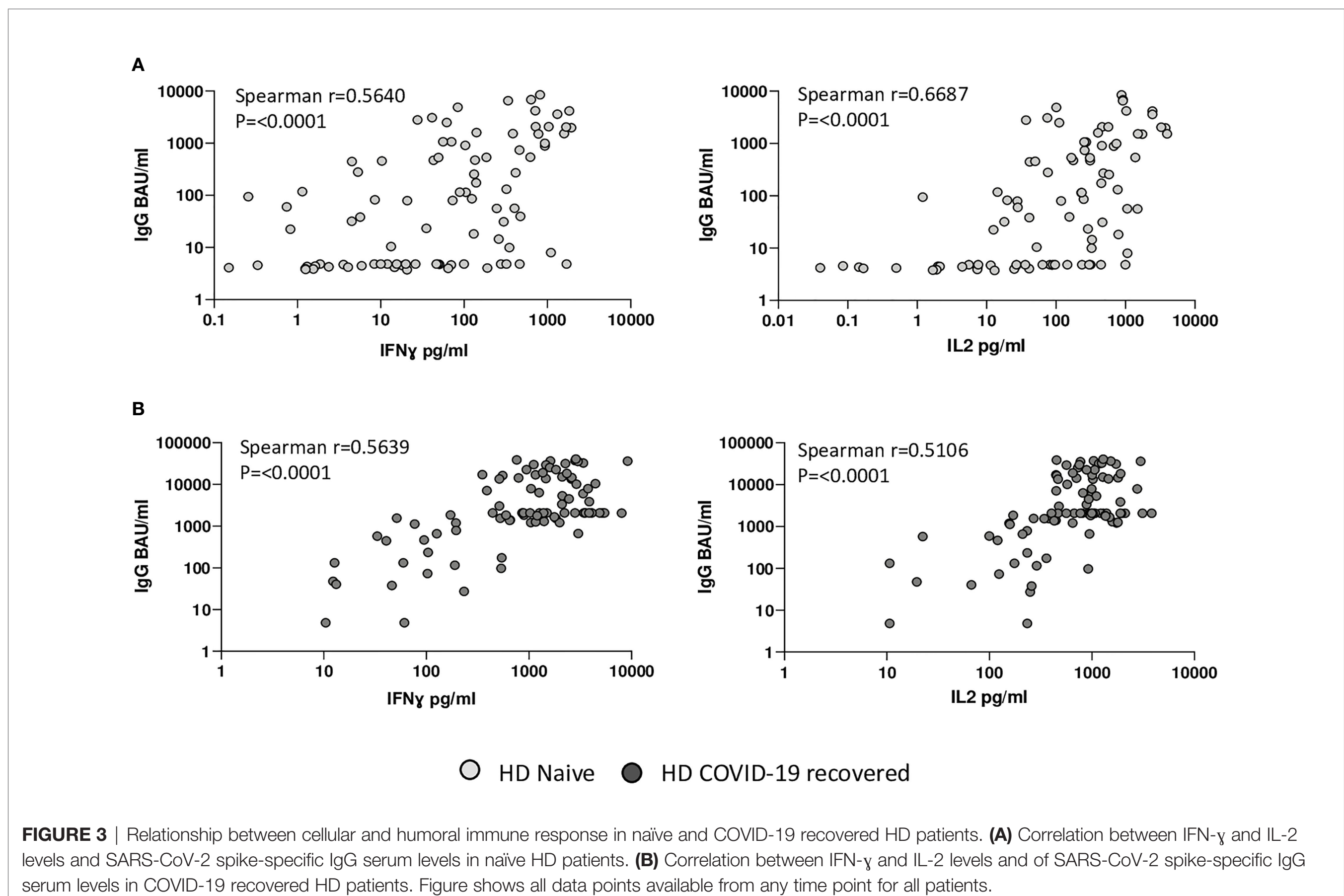

FIGURE 3 | Relationship between cellular and humoral immune response in naïve and COVID-19 recovered HD patients. **(A)** Correlation between IFN-ɣ and IL-2 levels and SARS-CoV-2 spike-specific IgG serum levels in naïve HD patients. **(B)** Correlation between IFN-ɣ and IL-2 levels and of SARS-CoV-2 spike-specific IgG serum levels in COVID-19 recovered HD patients. Figure shows all data points available from any time point for all patients.

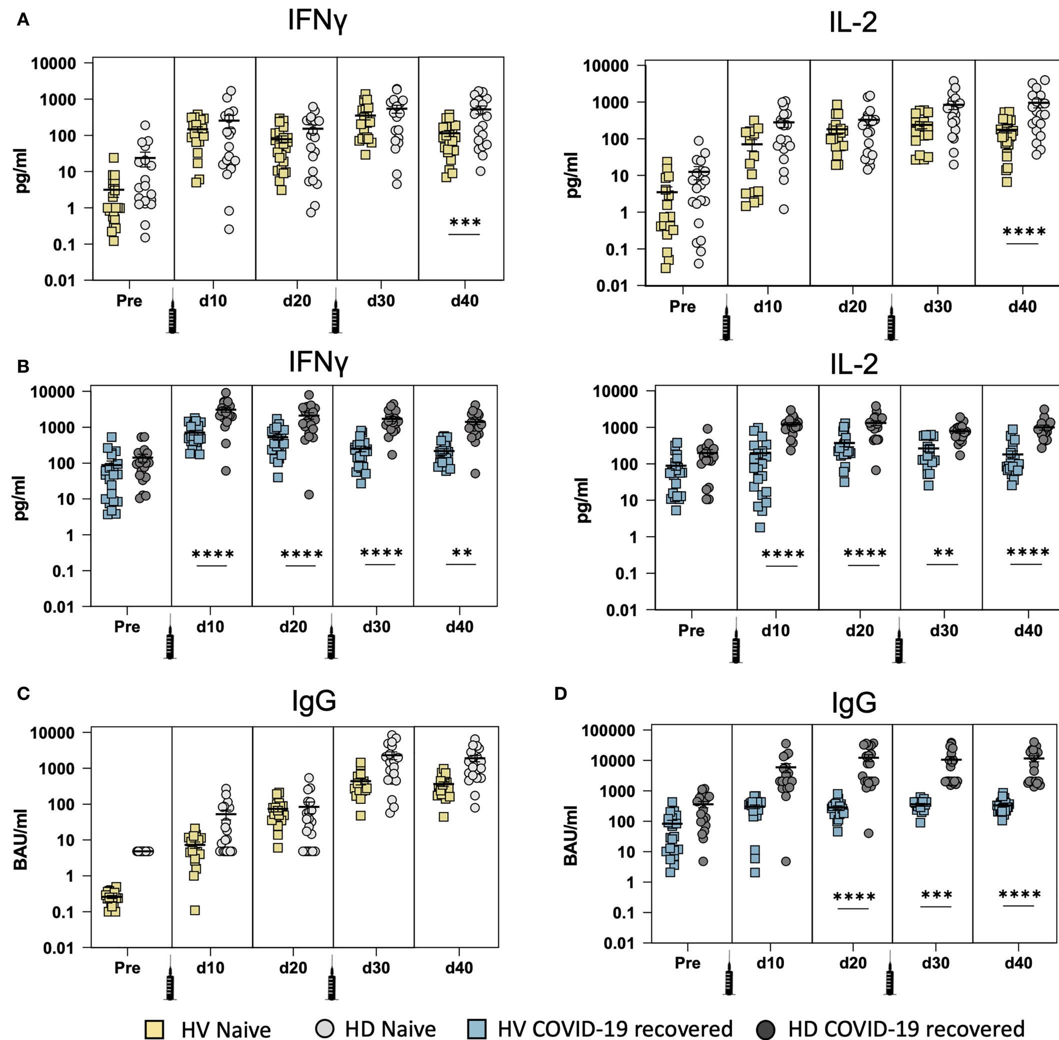

FIGURE 4 | Development of cellular and humoral immunity after SARS-CoV-2 vaccination in COVID-19 recovered and naïve hemodialysis (HD) patients vaccinated with mRNA-1273 vaccine (100 µg/dose) and in healthy volunteers (HV) vaccinated with BNT162b2 (30 µg/dose). **(A)** Comparison of IFN-γ and IL-2 production between naïve HD patients and HV at different time points: before vaccination (pre), after the first (d10 and d20) and second (d30 and d40) mRNA vaccine dose. **(B)** Comparison of IFN-γ and IL-2 production in COVID-19 recovered HD patients and HV at different time points. **(C)** Comparison of SARS-CoV-2 spike-specific IgG serum levels in naïve HD patients and HV. **(D)** Comparison of SARS-CoV-2 spike-specific IgG serum levels in COVID-19 recovered HD patients and HV. ** <0.005, *** <0.0005, **** <0.0001.

display a significant increase in spike-specific IgG production after the first dose of the vaccine (d20) that is maintained longitudinally. This confirms that COVID-19 recovered HD patients develop strong humoral immunity in response to SARS-CoV-2 vaccination.

DISCUSSION

In this study, we investigated the effects of the mRNA-1273 vaccine on the SARS-CoV-2 specific cellular and humoral immune responses in HD patients with and without previous SARS-CoV-2 infection in a longitudinal study shortly after vaccination and compared the results to a HV cohort. Our results indicate that naïve HD patients without previous SARS-CoV-2 infection develop an effective cellular and humoral immune response after the second dose of the vaccine, while HD patients with previous SARS-CoV-2 infection exhibit a potent and rapid immune response after the first dose. Interestingly, HD patients display an overall significant increase in the production of IFN-γ, IL-2 and IgG in comparison to HV.

Previous studies evaluating the humoral response in naïve HD patients reported a favorable but profoundly lower SARS-CoV-2 spike protein antibody response in comparison with a non-dialysis cohort (i.e. median 253 versus 1756 U/mL, $P < 0.001$) (22–24). Consistent with these results, Simon et al. described that, while 80% of HD patients developed a humoral

immunity (>29 U/ml), these patients had significantly lower anti-SARS-CoV-2 S antibody titers than control patients 21 days after vaccination (median was 171 U/mL for dialysis patients and 2500 U/mL for controls) (16). Similar frequencies of seroconversion were observed by others, in which 20-30% of patients on dialysis had a suboptimal humoral response to vaccination or were non-responders (14, 25, 26). On the contrary, other studies have described strong humoral immunity in response to complete vaccination in naïve HD patients reporting a remarkably high seroconversion rate of ≥95% (27–29), although a direct comparison between the IgG values in HD *vs*. HV was not reported. One possible explanation for the different conclusions in the above studies may be the limited number of HD patients enrolled in some of the studies. Our results are in line with studies suggesting high seroconversion rates in naïve HD patients, but further demonstrate similar IgG levels when compared to HV.

HD patients with previous SARS-CoV-2 infection develop robust humoral responses and earlier studies have reported similar seroconversion rates and IgG levels between HD patients and healthy volunteers with previous infection (HD: 51475 U/mL; HV: 10650 U/mL, P = 0.024) (22). In addition, others have reported that COVID-19 recovered HD patients reach their IgG peak levels after the first dose in comparison to naïve HD patients (29). These observations are consistent with our results, which demonstrate that COVID-19 recovered HD patients exhibit strong and fast humoral immunity after the first vaccine dose. However, we observed a significantly higher humoral response in HD patients when compared with HV, which has not been previously reported and argues in favor of additional studies that distinguish between naïve and SARS-CoV-2 infected HD patients.

With regards to cellular immunity, some studies reported a lower IFN-ɣ production three weeks after the second vaccine dose in naïve HD patients compared to HV, as only 71% of HD patients responded to SARS-CoV-2-specific *in vitro* T cell activation by interferon-ɣ release assay (IGRA) (18). Comparable frequencies of decreased IFN-ɣ production were observed by other authors. Schrezenmeier and colleagues reported that 67% of naïve HD patients displayed significantly lower levels of IFN-ɣ release than healthy controls (93%) (24), while similar findings were observed using flow cytometry by Broseta and colleagues, in which activated $CD4^+$ T cells expressing intracellular IFN-ɣ were observed only in 62% of naïve HD patients (28). Other studies have noted no difference between healthy controls and HD patients with regards to cellular immune response (30). On the contrary, recent studies have described that naïve HD patients exhibit an adequate T cell immunity five weeks after the second vaccine dose as assessed by IGRA and flow cytometry (27). Consistent with these results, Bertrand and colleagues described that all naïve HD patients develop T cell immune response in after the second vaccine dose measured by ELISpot (17). Our results are consistent with the later studies which report high percentages of T cell immunity after vaccination, but we further extend those findings and provide qualitative IFN-ɣ and IL-2 production measurements, which indicate for the first time that HD patients produce significantly higher pro-inflammatory cytokines than HV. We did not find prior studies that compared the cellular immunity in response to SARS-CoV-2 vaccination between COVID-19 recovered and HD patients without previous infection with SARS-CoV-2.

Taken together, we conclude that HD patients mount strong cellular and humoral immune responses after mRNA-1273 SARS-CoV-2 vaccination despite their immunocompromised condition. Unexpectedly, longitudinal immune monitoring of HD COVID-19 recovered patients revealed a potentially excessive cellular immune response that may be associated with a pro-inflammatory syndrome observed in HD patients in comparison to HV. While naïve HD patients may benefit from a third vaccine dose as described by Bensouna et al (31), COVID-19 recovered HD may be at risk of developing T cell exhaustion arguing in favor of personalized immune monitoring studies in HD patients. In Bensouna's study, HD patients with a history of symptomatic COVID-19 were excluded and the third vaccine dose appeared to have a diminished benefit in patients who had already developed good humoral responses after two vaccine doses. Interestingly, in 4 patients that were positive for anti-nucleocapsid antibodies, the levels of anti-spike humoral response decreased after the third vaccine dose (anti-spike after the 2nd dose, 165,565 AU/ml; anti-spike after the 3rd dose, 116,110 AU/ml), which suggests that HD patients with previous SARS-CoV-2 infection may be spared from additional booster vaccine doses. Other studies have described an enhanced humoral response after the third dose in HD patients independently of previous SARS-CoV-2 infection but specifically in those with lower antibody titers after the second dose (32). Our study provides a broader assessment of the efficacy and dynamics of SARS-CoV-2 vaccination in HD patients, providing evidence that boost vaccination may not be necessary for HD patients with a history of previous SARS-CoV-2 infection.

As a limitation to our study, our HD patients were vaccinated with mRNA-1273 (Moderna) while HV individuals were vaccinated with BNT162b2 (Pfizer). Some studies have indicated that dialysis patients vaccinated with BNT162b2 had higher prevalence of no detectable or diminished IgG response, compared with patients vaccinated with mRNA1273 (33). In addition, Kaiser and colleagues described that patients vaccinated with mRNA-1273 display a 3-fold significantly higher spike-specific IgG titers (34). Furthermore, a lower seroconversion rate has been described in naïve HD patients vaccinated with BNT16b2 vaccine (88%) compared to mRNA-1273 vaccine (97%) (27). However, the absolute indicators of cellular and humoral immunity in HD and HV of our study are comparable, as we used the same methodological approaches to obtain the data.

To our knowledge, this is the first longitudinal study investigating the differential effects of cellular and humoral immunity in response to mRNA vaccination, distinguishing between HD patients' previous history of SARS-CoV-2 infection and comparing the results with a HV cohort. Our work aims at providing additional scientific evidence and understanding of the immune response to SARS-CoV-2 infection and vaccination to further reduce the hesitancy of COVID-19 vaccination in HD patients (35).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical approval of the study was obtained from the relevant authority - the Internal Review Board of Hospital Puerta de Hierro and Fundación Jimenez Diaz. Written informed consent was obtained from all participants prior to starting the study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MG-P, MM-C, PC, IC, JB, and MB-B performed cellular assays. MP-O performed humoral assays. RS-T and DL-O, DT and MS organized the database and performed the statistical analysis. EG, CC, ML-C, EG-P, PP, AO, JP, and JO contributed to conception and design of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.845882/full#supplementary-material

Supplementary Figure 1 | Optimal cutoff values for IFN-ɣ and IL-2. **(A)** Receiver operating characteristic (ROC) curves for predicting vaccination status from IFN-ɣ and IL-2 levels in HV and HD patients with and without prior COVID-19 infection. Area under curve (AUC) and optimal cutoff values are displayed. **(B)** Bar plot with optimal cutoffs for predicting vaccination status from IFN-ɣ and IL-2 levels in HV and HD patients with and without prior COVID-19 infection. **(C)** Histogram displaying individual values of IFN-ɣ and IL2 in HV and HD patients with and without prior COVID-19 infection before vaccination (pre) and twenty days after the second vaccination dose (post). Vertical lines indicate the optimal cutoff values.

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Communication

Maintenance of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients after 1273-mRNA SARS-CoV-2 Vaccination

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Abstract: Continuous evaluation of the coronavirus disease 2019 (COVID-19) vaccine effectiveness in hemodialysis (HD) patients is critical in this immunocompromised patient group with higher mortality rates due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The response towards vaccination in HD patients has been studied weeks after their first and second SARS-CoV-2 vaccination dose administration, but no further studies have been developed in a long-term manner, especially including both the humoral and cellular immune response. Longitudinal studies that monitor the immune response to COVID-19 vaccination in individuals undergoing HD are therefore necessary to prioritize vaccination strategies and minimize the pathogenic effects of SARS-CoV-2 in this high-risk group of patients. We followed up HD patients and healthy volunteers (HV) and monitored their humoral and cellular immune response three months after the second (V2+3M) and after the third vaccination dose (V3+3M), taking into consideration previous COVID-19 infections. Our cellular immunity results show that, while HD patients and HV individuals secrete comparable levels of IFN-γ and IL-2 in ex vivo stimulated whole blood at V2+3M in both naïve and COVID-19-recovered individuals, HD patients secrete higher levels of IFN-γ and IL-2 than HV at V3+3M. This is mainly due to a decay in the cellular immune response in HV individuals after the third dose. In contrast, our humoral immunity results show similar IgG binding antibody units (BAU) between HD patients and HV individuals at V3+3M, independently of their previous infection status. Overall, our results indicate that HD patients maintain strong cellular and humoral immune responses after repeated 1273-mRNA SARS-CoV-2 vaccinations over time. The data also highlights significant differences between cellular and humoral immunity after SARS-CoV-2 vaccination, which emphasizes the importance of monitoring both arms of the immune response in the immunocompromised population.

Keywords: COVID-19; chronic kidney disease; hemodialysis; SARS-CoV-2 vaccine; 1273-mRNA vaccine; humoral response; cellular response

1. Introduction

End-Stage Renal Disease (ESRD) patients undergoing hemodialysis (HD) are considered immunocompromised due to their vulnerability to severe acute respiratory syndrome

coronavirus 2 (SARS-CoV-2) infection and their increased risk of COVID-19 mortality [1]. Vaccine effectiveness against SARS-CoV-2 is crucial for the protection of HD patients [2], especially after the SARS-CoV-2 B.1.1.529 (Omicron) variant, which partially escapes the majority of existing SARS-CoV-2 neutralizing antibodies [3,4] and has been reported to increase the number of hospitalizations among vaccinated adults [5]. Several reports have determined the ability of SARS-CoV-2 vaccines to generate immunity in HD patients and recommend implementing booster doses from highest to lowest priority-use groups [6–8].

Previous studies demonstrated a substantial increase in the antibody levels of naïve and COVID-19-recovered HD patients shortly after the second and third vaccine dose [9,10]. Others investigated the dynamics of post-vaccination antibody and T-cell responses for up to two months to determine the most appropriate timing for delivery of a booster dose. Results demonstrated comparable levels of total RBD antibodies and T-cells fifteen days and three months after the second vaccine dose between HD and HV [11]. This research group also investigated the immune response in HD patients, 90% without previous infection, and observed a booster effect on anti-RBD and neutralizing antibodies to different variants and a significant increase in SARS-CoV-2-S-IFN-γ-producing T-cells 46 days after receiving the third homologous mRNA vaccine dose [12]. More recent studies compared the immune response of non-infected naïve HD patients, who received four vaccine doses, with COVID-19-recovered HD patients, who only received three doses of the SARS-CoV-2 mRNA vaccine. The results indicated that, while there were no differences in the production of the proinflammatory cytokines interleukin-2 (IL-2) and tumor necrosis factor (TNF) by T-cells, better humoral immunity was observed in the convalescent-vaccinated compared to vaccinated-only HD patients [13]. These results suggest that the cellular and humoral immune responses provide different information regarding the immunological status of vaccinated HD patients that do not necessarily correlate with each other. Table 1 summarizes some of the most relevant and related studies.

Table 1. Summary of studies evaluating the humoral and cellular immune response in HD patients.

Article	Studied Type of Response	Follow Up	Type of Patients	Vaccine Type	Outcome
Paal M. (2021) [6]	Humoral	3–6 weeks after V2	Control and HD patients; Naïve and COVID-19-recovered	mRNA vaccines [1]	Control individuals had significantly higher Ab titers compared to HD patients
Stumpf J. (2021) [7]	Humoral and cellular	Baseline, 3–4 weeks after V1, 4–5 weeks after V2	Control and HD patients; Naïve.	mRNA vaccines [1]	HD patients present a higher seroconversion rate compared to similar tested medical personnel
Bensouna I. (2021) [9]	Humoral	After V2 and 3 weeks after V3	HD patients; Naïve and COVID-19-recovered	BNT162b2	V3 substantially increased Ab titers in HD patients compared to V2
Panizo N. (2022) [11]	Humoral and cellular	Baseline, Day 15, and 3 months after V2	Control and HD patients	mRNA vaccines [1]	HD patients develop similar humoral response compared to controls. No differences were found in cellular immune responses
Panizo N. (2022) [12]	Humoral and cellular	46 days after V3	Control and HD patients	mRNA vaccines [1]	Boosted humoral and cellular responses in HD patients
Mirioglu S. (2023) [10]	Humoral	1 and 3 months after V2	HD patients; Naïve	BNT162b2 and Coronavac	HD patients had induced humoral response after booster dose
Anft M. (2023) [13]	Humoral and cellular	158 days after V4	HD patients; Naïve and COVID-19-recovered	mRNA vaccine	HD patients present high humoral and cellular responses

[1] mRNA- 1273 and BNT162b2, V1: First vaccine dose, V2: Second vaccine dose, V3: Third vaccine dose, V4: Fourth vaccine dose, Ab: Antibody.

Here, we monitored the long-term effects of SARS-CoV-2 vaccination in both, the cellular and humoral immune response in HD patients. Specifically, we evaluated the production of IFN-γ and IL-2 in the whole blood after stimulation with SARS-CoV-2

peptide pools and the IgG directed against Spike glycoprotein in HD patients and Healthy Volunteers (HV) with (COVID-19 recovered individuals) or without (naïve individuals) previous infection of SARS-CoV-2. Our results indicate that both naïve and COVID-19-recovered HD patients mount cellular and humoral immune responses comparable with HV individuals after the second and third dose of the 1273-mRNA SARS-CoV-2 vaccine.

2. Results

We first monitored the cellular immune response in naïve subjects without previous SARS-CoV-2 infection by evaluating the production of IFN-γ in the whole blood after spike-specific peptide pool stimulation. Comparing the production of IFN-γ between HD patients and HV individuals, we observed a similar IFN-γ production between these two groups at both time points, V2+3M ($p = 0.35$) and V3+3M ($p = 0.73$) (Figure 1A). However, when comparing the production of IFN-γ between the two time points, we observed a significant decrease at V3+3M in HV individuals ($p = 0.008$). This suggests that the durability of cellular immunity decreases more rapidly in healthy individuals without previous SARS-CoV-2 infection after a booster dose.

We next measured the production of IFN-γ in COVID-19-recovered subjects. Comparing the production of IFN-γ between HD patients and HV individuals, we observed that, while similar amounts of IFN-γ were produced between these groups at V2+3M ($p = 0.69$), there was a significant IFN-γ decrease in HV individuals compared to HD patients at V3+3M ($p = 0.003$) (Figure 1B). When comparing the production of IFN-γ between the two time points, we also observed a significant decrease at V3+3M in HV individuals ($p = 0.001$). This suggests that the durability of cellular immunity is maintained in HD patients with a previous SARS-CoV-2 infection after a booster dose.

Next, we measured the production of IL-2 in naïve subjects. Comparing the production of IL-2 between HD patients and HV individuals, we observed a similar IL-2 production between these two groups at both time points, V2+3M and V3+3M ($p = 0.33$). However, when comparing the production of IL-2 between the two time points, we observed a significant decrease at V3+3M in HV individuals ($p = 0.011$) (Figure 1C). These results are consistent with data from Figure 1A, suggesting a decrease in the durability of the cellular immunity in healthy individuals without a previous SARS-CoV-2 infection after a booster dose.

Finally, we compared the production of IL-2 in COVID-19-recovered subjects. Comparing the production of IL-2 between HD patients and HV individuals, we observed that, while similar amounts of IL-2 were produced between these groups at V2+3M ($p = 0.15$), there was a significant IL-2 decrease in HV individuals compared to HD patients at V3+3M ($p = 0.0008$) (Figure 1D). When comparing the production of IL-2 between the two time points, we also observed a significant decrease at V3+3M in HV individuals ($p \leq 0.0001$). Overall, the cellular immunity results indicate that HD patients are able to mount and maintain a robust cellular immune response over time, while HV individuals decrease their ability to secrete both IFN-γ and IL-2 after a booster dose.

We also monitored the humoral immune response in naïve subjects without previous SARS-CoV-2 infection by measuring IgG binding antibody units specific against the Spike glycoprotein (Figure 1E,F). Comparing the IgG levels between naïve HD patients and HV individuals, we observed a similar antibody production between these two groups at both time points, V2+3M ($p = 0.43$) and V3+3M ($p = 0.72$) (Figure 1E). However, when comparing the IgG levels at the two time points, we observed a significant increase in antibody production from V2+3M to V3+3M in both HD patients ($p = 0.045$) and HV individuals ($p = 0.002$). This suggests that booster doses significantly increase the cumulative antibody responses after repeated vaccinations. Comparing the IgG levels between previously infected HD patients and HV individuals, we observed that HD patients show significantly higher IgG levels compared to HV individuals at V2+3M ($p = 0.009$). However, these differences were not significant at V3+3M between both groups ($p = 0.63$), indicating

that both COVID-19-recovered HD patients and HV individuals maintain their humoral response long-term after boosting (Figure 1F).

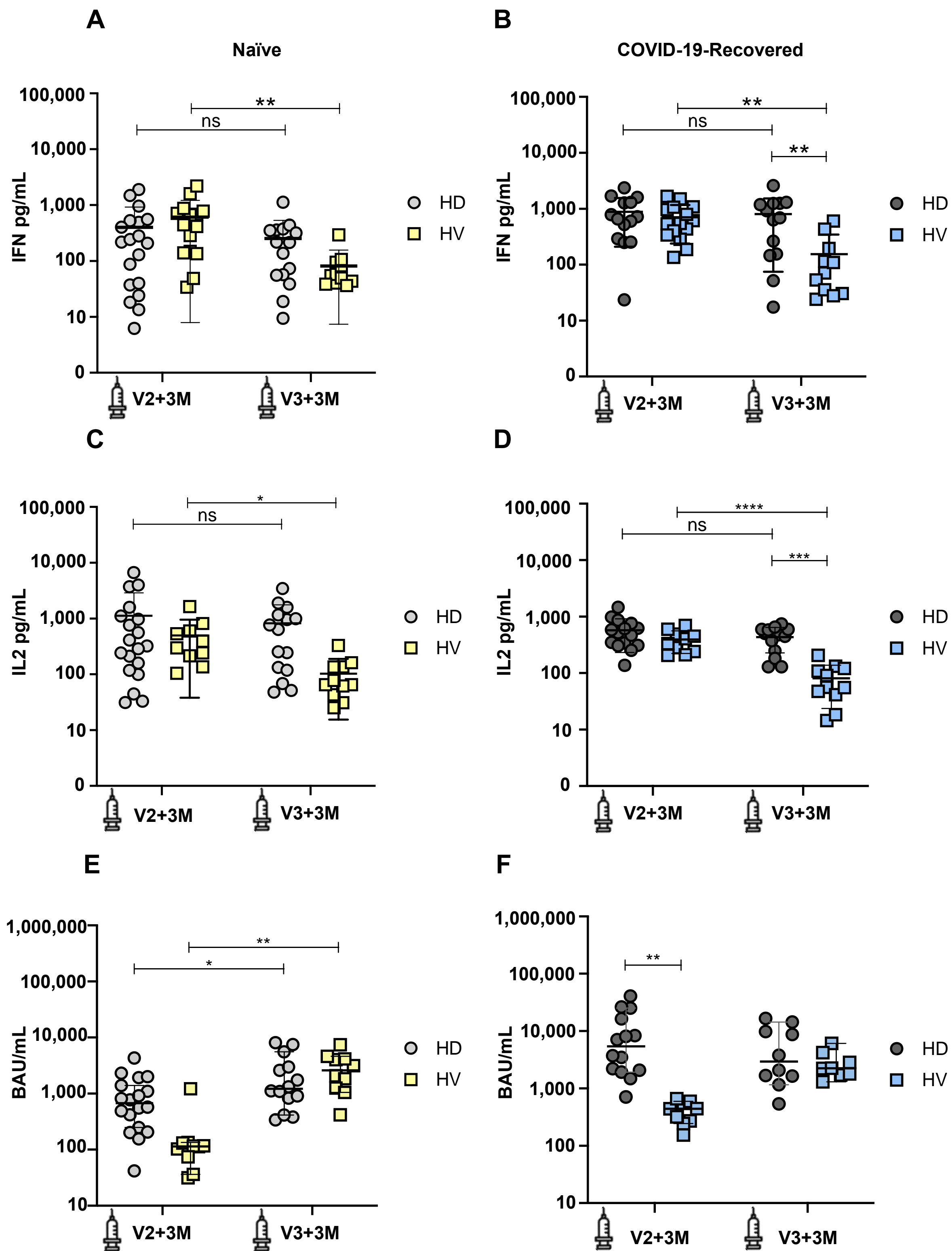

Figure 1. Development of cellular and humoral immune responses after SARS-CoV-2 vaccination in COVID-19-recovered and naïve hemodialysis (HD) patients and healthy volunteers (HV) 3 months after second (V2+3M) and 3 months after third (V3+3M) vaccination dose. (**A**) IFN-γ production in naïve HD patients (light grey symbols) and HV individuals (yellow symbols) at V2+3M and V3+3M.

(**B**) IFN-γ production in COVID-19-recovered HD patients (dark grey symbols) and HV individuals (blue symbols) at V2+3M and V3+3M. (**C**) IL-2 production in naïve HD patients and HV individuals at V2+3M and V3+3M (**D**). IL-2 production in COVID-19-recovered HD patients and HV individuals at V2+3M and V3+3M. (**E**) SARS-CoV-2 spike-specific IgG serum levels in naïve HD patients and HV individuals at V2+3M and V3+3M. (**F**) Comparison of SARS-CoV-2 spike-specific IgG binding antibody units (BAU) in COVID-19-recovered HD patients and HV individuals at V2+3M and V3+3M. Values higher than 33.8 BAU/mL were considered positive. <0.05 (*), <0.005 (**), <0.0005 (***), and <0.0001 (****). Data are shown as mean ± SEM.

3. Discussion

In this study, we examined the effects of SARS-CoV-2 vaccination on the humoral and cellular specific immune responses in HD patients compared to HV individuals with (COVID-19-recovered) or without (naïve) previous SARS-CoV-2 infection, three months after the second (V2+3M) and after the third (V3+3M) vaccination dose. Our results indicate that both naïve and COVID-19-recovered HD patients maintain strong cellular and humoral immune responses after receiving a third dose (booster), which is comparable or higher (significant increased at V3+3M for IFN-γ and IL-2) to HV individuals.

Several studies have described that most HD patients can mount competitive immune responses [7,14,15]. Considering the humoral immune response alone, a recent cohort study reported the induction of robust and durable humoral immune response three months after receiving the BNT162b2 vaccine in naïve HD patients, following a two-dose immunization scheme [10]. Previous studies from David Navarro's laboratory evaluated both the T-cell and Spike-specific reactive antibody responses in HD patients fifteen days and three months after two doses of mRNA vaccines (mRNA-123 and BNT162b2). In line with our results, they observed that HD patients develop SARS-CoV-2 antibody responses comparable to healthy controls (HC) (i.e., 95% rate of HD patient responders at 3M vs. 100% of HC responders at 3M) [9]. In addition, no differences between CD4+ or CD8+ T-cell responses were observed across groups, although we reported higher IFN-γ and IL-2 production in HD patients with previous SARS-CoV-2 infection compared to controls at V3+3M. It is likely that differences across studies regarding the clinical characteristics of patients, the time points under study, and the methodological approaches to evaluate T-cell immunity may, in part, explain the discrepancy. More recent data from Navarro's laboratory confirmed the ability of HD patients to produce high levels of IgG production 46 days after the booster (anti-RBD antibodies were detected in 39/40 HD patients). Furthermore, SARS-CoV-2 specific-IFN-γ-producing CD8+ and CD4+ T-cell responses were detected in 35 and 36/37 of HD patients, respectively, indicating that mRNA COVID-19 vaccines induce a booster effect on both humoral and cellular immune responses in this immunocompromised group [12]. Similarly, Anft and colleagues recently described a stable cellular immunity with no differences in the production of proinflammatory cytokines (IL-2 and TNF) between four times vaccinated, non-infected HD patients compared to three times vaccinated, infected HD patients. However, a significant fade of neutralizing antibodies after SARS-CoV-2 vaccination in naïve HD patients (25%) compared to COVID-19-recovered HD patients (62.5%) was observed [13]. These results indicate significant differences between the humoral and cellular immune responses and highlight the importance of measuring both arms of the immune response in HD patients. Our results are consistent with these studies that report potent humoral and cellular immune responses in HD patients but further extend those findings, comparing HD data with HV and differentiating between patients with/without previous SARS-CoV-2 infection.

A limitation of our study is the small sample size and the differences in vaccines between groups; HD patients were vaccinated with mRNA-1273 (Moderna), while HV individuals were vaccinated with BNT162b2 (Pfizer). Several studies have described that BNT162b2 vaccination induces diminished seroconversion compared to mRNA-1273 vaccination [7,16,17]. Nevertheless, the absolute indicators of the cellular and humoral

immunity in HD and HV are comparable in our study, as we used the same methodological approaches to obtain the data.

We conclude that HD patients develop potent cellular and humoral immune responses after COVID-19 vaccination over time, which is critical to lower the rate of COVID-19-related hospitalizations in this vulnerable group of patients [18]. While the precise mechanisms behind the robust immune response induced by SARS-CoV-2 vaccination, we hypnotize that trained immunity, which has previously been associated with COVID-19 vaccination and infection [19,20], may be responsible, in part, to the delicate balance between the protective and the inflammatory state of HD patients [21]. Although further studies are required to demonstrate the relationship between protection and specific T-cell or serological immune responses, the development of strong cellular and humoral immune responses reported here may help guide future vaccination strategies in immunocompromised groups of patients.

4. Materials and Methods

4.1. Experimental Design

In this study, peripheral blood from HD patients was drawn before hemodialysis (n = 38), while in HV individuals (n = 30) it was prospectively collected. The second vaccination dose of HD patients and Healthy Volunteers occurred in May 2021. The third vaccination dose of HD patients and Healthy Volunteers occurred in October 2021. All blood extractions were performed approximately 90 days after second vaccination dose and 90 days after third vaccination dose. All individuals were based in the Comunidad de Madrid, Spain. Healthy volunteers were obtained from Hospital Universitario La Paz in Madrid and HD patients were obtained from Hospital Universitario Puerta de Hierro in Madrid. Blood extractions from HD patients [Naïve (n = 19), COVID-19-recovered patients (n = 19)], HV individuals [naïve (n = 15), and COVID-19-recovered HV (n = 15)] were collected three months after the second (V2+3M) and three months after the third vaccine dose (V3+3M). Tables 2 and 3 summarize HD patient and HV individuals' characteristics.

Table 2. Naïve and COVID-19 recovered HD patients' characteristics.

Characteristics	Naïve HD * Patients N = 19	N (Partial)	COVID-19 HD * Patients N = 19	N (Partial)
Male gender	47.4%	9	55.0%	10
Age, years (Mean ± SD)	64.0 ± 12.4	-	65.3 ± 13.2	-
Active smoking	15.8%	3	15.0%	3
HD vintage, months (Mean ± SD)	96.1 ± 102.5	-	81.4 ± 72.2	-
Use of EPO *	89.4%	17	100.0%	19
Previous kidney transplantation	26.3%	5	35.0%	7
Comorbidities	-	-	-	-
Obesity	15.8%	3	30.0%	6
Hypertension	89.5%	17	95.0%	18
Diabetes mellitus	31.6%	6	45.0%	9
Ischemic heart disease	31.6%	6	15.0%	3
Dyslipidemia	68.4%	13	60.0%	12
Cause of end-stage renal disease				
Diabetic nephropathy	28.6%	2	40.0%	8
Hypertensive nephrosclerosis	5.3%	1	10.0%	2
Glomerulonephritis	5.3%	1	15.0%	3

* HD: Hemodialysis, EPO: recombinant Erythropoietin treatments.

4.2. SARS-CoV-2 Peptide Pools and Whole-Blood Culture Assays

Lithium-heparinized blood samples were collected before the start of dialysis. On the same day, 320 µL of whole blood was mixed with 80µL of RPMI and stimulated with PepTivator ® SARS-CoV-2 Peptide Pools (S; 2 µg/mL, M; 2 µg/mL) or a DMSO control. After 16–20 h of culture, supernatant (plasma) was collected and stored at −20 °C for further

cytokine quantification, as previously reported [14]. For previous SARS-CoV-2 infection detection, whole blood cultures were incubated with a peptide pool against SARS-CoV-2 membrane (M) protein (2 μg/mL).

Table 3. Naïve and COVID-19 recovered HV individuals' characteristics.

Characteristics	Naïve HV *N* = 15	N (Partial)	COVID-19 HV *N* = 15	N (Partial)
Male gender	13.33%	2	6.66%	1
Age, years (Mean ± SD)	46 ± 14.38	-	46 ± 16.98	-
Active smoking	13.33%	2	13.33%	2
Comorbidities	-	-	-	-
Obesity	13.33%	2	13.33%	2
Hypertension	40%	6	46.66%	7
Diabetes mellitus	6.66%	1	13.33%	2
Ischemic heart disease	0%	0	0%	0
Dyslipidemia	46.66%	7	33.33%	5

4.3. Spike-Specific IgG Quantification and Analysis

To study the specific serologic response against SARS-CoV-2, plasma from HD patients and HV was collected. The Liaison ® SARS-CoV-2 TrimetricS IgG assay (Diasorin, Stillwater, MN, USA) was used for semiquantitative detection of IgG directed against the Spike glycoprotein. Values over 33.8 BAU/mL were considered positive.

4.4. Cytokine Quantification and Analysis

Cytokine concentrations in the supernatants (plasma) were quantified using ELLA with microfluidic multiplex cartridges measuring IFN-γ and IL-2 release following the manufacturer's instructions (ProteinSimple, San Jose, CA, USA). The cytokine levels present in plasma stimulated with DMSO were subtracted from the corresponding Peptide-pool stimulated samples, as previously reported [22].

4.5. Statistics

Statistical analyses were performed by Two-Way ANOVA and Šídák's multiple comparison tests. Normality of data was tested using D'Agostino and Pearson tests for normal distribution. Paired t test and unpaired t test were also used as appropriate, using Graphpad PRISM 9.01 (Graphpad Software, La Jolla, CA, USA).

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Informed Consent Statement: Written informed consent was obtained from all participants prior to starting the study. The patients/participants provided their written informed consent to participate in this study.

Data Availability Statement: The original data contribution presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

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Discussion and future work

In this present thesis, we developed a new T cell-based test to measure the effects of SARS-CoV-2 infection and vaccination-specific cellular and humoral immune response in different scenarios: heterologous vaccination, COVID-recovered vs naïve patients, hemodialyzed patients, and healthy volunteers. The obtained results were published in three scientific publications.

Firstly, in the publication under de name “Immunogenic dynamics and SARS-CoV-2 variant neutralization of the heterologous ChAdOx1-S/ BNT162b2 vaccination: Secondary analysis of the randomized CombiVacS study”, results provide evidence that a heterologous ChAdOx1-S/BNT162b2 vaccination regimen was effective long-term in naïve healthy individuals. Humoral immune response peaked at day 14 post-BNT16b2 dose but declined over time. A significant decline (25%-27%) in total RBD and neutralizing antibody levels was observed after 28 days, which further increased up to 70%-90% by day 180. In line with these results, data obtained from the novel T-cell-based test revealed that both groups exhibited a similar decay in IFN-γ and IL-2, indicating the generation of memory T lymphocytes.

This decline was also observed in homologous vaccination regimens (BNT162b2 and mRNA-1273) but to a lesser extent and although homologous ChAdOx1 vaccination induced lower antibody levels, it had a slower decay kinetics. The antibody decay was consistent with the known kinetics of humoral immune response towards acute viral infections when, upon the initial response, the waning of the antibody titer normally happens once the initial response is resolved, but rapidly rises upon re-exposure (136, 137). This sharp waning in RBD antibodies and neutralizing titers may suggest a potential benefit of a third dose, especially against the highly infectious Omicron variant.

Also, this paper indicates that the administration of BNT162b2 28 days after, compared to the control group, did not result in a worse antibody response. Furthermore, S-RBD antibodies and variant-specific neutralizing titers were higher in the control group, suggesting the potential benefit of a delayed second dose. Regarding the Delta variant, protection against it persisted in both the intervention and the control group 28 days after the second dose administration.

Overall, this work highlights the importance of understanding the dynamics of immune responses over time in a vaccination context and suggests strategies for optimizing vaccine effectiveness towards emerging SARS-CoV-2 variants.

Secondly, the publication under the name “Development of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients”, examines the impact of SARS-CoV-2 mRNA vaccination on both humoral and cellular immune responses in hemodialysis (HD) patients compared to healthy volunteers (HV) with a clear distinction between those with and without prior SARS-CoV-2 infection. Findings were assessed longitudinally.

Although previous studies have reported lower spike protein antibody responses in a hemodialyzed cohort (138, 139) and lower IFN production after the second vaccine dose compared to a healthy cohort (140), key findings in this study showed how naïve HD patients developed an effective cellular and humoral immune response after the second vaccine dose, showing a significant increase on the production of IFN-γ, IL-2, and Spike-specific IgG compared to HV. Furthermore, this phenomenon was more robust in COVID-19-recovered patients, where this increase was noticed rapidly after the first vaccine dose with an exhibition of a strong and fast humoral and cellular immune response. This higher immunity in HD patients compared to HV had not been reported in previous studies, but it highlights the importance of boost doses in this immunocompromised cohort.

Overall, this study indicates that HD patients exhibit strong immune responses towards the mRNA-1273 vaccine, despite their immunocompromised condition. It suggests that while naïve HD patients may benefit from a third vaccine dose, COVID-19-recovered HD patients may not require additional booster doses. The findings contribute to a better understanding of the immune response and highlight the importance of tailored vaccination strategies in this specific patient population.

Lastly, as a follow-up of this last scientific publication, the article under de name “Maintenance of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients after 1273-mRNA SARS-CoV-2 Vaccination” assessed the effect of SARS-CoV-2 mRNA vaccination in the same cohort previously described but three months after the second dose and three months after the third dose administration. Findings revealed that, again, both naïve and COVID-19-recovered HD patients displayed a robust cellular and humoral immune response after the booster dose (third dose). These responses were comparable or in some cases such as in COVID-19 recovered HD patients, even higher than those observed in HV individuals. These results were correlated with previous studies which also indicated that HD patients can mount competitive immune responses towards SARS-CoV-2 (141, 142).

Both studies concluded that HD patients developed strong and enduring cellular and humoral immune responses after COVID-19 vaccination and presented different dynamics depending on their prior SARS-CoV-2 infection. This fact is crucial for reducing the risk of COVID-19-related hospitalizations in this vulnerable population. Although the precise mechanisms behind the robust immune response towards SARS-CoV-2 vaccination in HD patients are not yet understood, we speculated that trained immunity might be responsible, in part, for the phenomenon due to its association with COVID-19 vaccination and infection (143, 144).

Future perspectives and work for T cell-based tests are promising and involve development and application in, for instance, biomarker discovery, vaccine development, or early disease detection. Briefly, T cell-based tests, such as the one on SARS-CoV-2, can **improve diagnosis accuracy** enhancing the sensitivity and specificity towards a specific pathogen by refining antigen selection and improving detection methods to ensure accurate identification of specific T cell responses. They can also take part in **biomarker discovery** as ongoing research seeks to identify novel T-cell biomarkers associated with various diseases, including infectious diseases, autoimmune disorders, and cancer. These biomarkers can provide valuable diagnostic and prognostic information. Furthermore, they can play a crucial role in **personalized medicine** since tailoring treatment strategies based on an individual's T-cell response profile can optimize therapeutic outcomes and minimize adverse effects.

Likewise, T-cell tests are instrumental in **vaccine development and monitoring** in the evaluation of the immunogenicity of vaccines. They can provide insights into the long-term efficacy of vaccines and help identify which populations may benefit from booster doses, such as immunocompromised patients. These tests can be particularly valuable for **assessing immune responses in individuals with compromised immune systems**, such as transplant recipients or patients undergoing immunosuppressive therapy. In the cancer field, T cells are fundamental to helping identify the tumor thus T cell-based assays could help identify tumor-specific T-cell-based responses, guide the selection of appropriate immunotherapies, and monitor treatment effectiveness.

Furthermore, T-cell-based tests may offer the potential for **early disease detection**, even before the manifestation of symptoms. This can be crucial for diseases where early intervention significantly impacts the outcome. Moreover, T-cell assays could be used for tracking infectious disease outbreaks as they can be deployed for surveillance during infectious disease outbreaks, aiding in the assessment of population immunity and helping guide public health interventions.

These T-cell-based tests are normally rapid and easy to develop, leading to portable and user-friendly techniques that contribute to the revolution of diagnostic capabilities, especially in resource-limited settings and during pandemics. Additionally, as T-cell-based tests are ease-to-use assays, establishing standardized protocols and validation criteria for them is critical for their widespread adoption in clinical practice. Certainly, the integration of other omics (genomics, transcriptomics, and proteomics) with T-cell-based tests could further provide a deeper understanding of immune responses and disease mechanisms.

Overall, the development and innovation of T-cell-based tests in immunology are key in diagnosis, personalized medicine, and or the understanding of immune responses in health and disease.

Conclusions

In this present thesis, we were able to develop a T-cell-based test for immune response diagnosis against SARS-CoV-2 and test it in different scenarios (immunocompromised cohort and heterologous vaccination) obtaining the next conclusions:

1. There is a progressive decline in humoral immune response in patients receiving the heterologous ChAdOx1-S/BNT162b2 vaccination regimen which is consistent with previous reports.
2. Administering the BNT162b2 dose to the control group 28 days later did not lead to worse antibody responses. The delayed administration resulted in higher levels of specific antibodies and neutralizing titers, suggesting a potential benefit of second dose delay.
3. High levels of protection against the Delta variant persisted in both groups (interventional and control) at day 28 after the second dose of the heterologous ChAdOx1-S/BNT162b2 scheme, highlighting the effectiveness of this vaccination approach against this variant.
4. The observed sharp decline in RBD antibodies and neutralizing titers at day 180 emphasizes the potential need for a third immunization to reach higher protection levels. Boosting may be particularly crucial for individuals above 60 and those with risk factors for severe COVID-19.
5. Patients on hemodialysis (HD) without prior SARS-CoV-2 infection develop robust cellular and humoral immune responses after the second dose of the mRNA-1273 vaccine. This indicates that the vaccine is effective in generating immune protection in this immunocompromised population.
6. HD patients with a history of SARS-CoV-2 infection exhibit a strong and rapid humoral and cellular immune response after the first vaccine dose. Additionally, they display significantly higher levels of IgG compared to healthy volunteers (HV) and HD patients without previous infection.
7. HD patients produce significantly higher levels of pro-inflammatory cytokines, including IFN-γ and IL-2, compared to HV after vaccination at all time points.
8. HD patients with a history of previous SARS-CoV-2 infection may not require additional booster vaccine doses, as they exhibit a robust immune response after the first dose, however, for naïve HD patients, a third vaccine dose may be beneficial.

Conclusiones

En esta tesis hemos sido capaces de desarrollar un test celular dirigido al diagnóstico de la respuesta inmune frente a SARS-CoV-2 y probarlo en diferentes escenarios (en una cohorte de pacientes inmunosuprimidos y también en el contexto de vacunación heteróloga) obteniendo las siguientes conclusiones:

1. Existe un descenso progresivo de la respuesta humoral en pacientes que recibieron vacunación heteróloga ChAdOx1-S/BNT162b2, lo cual es consistente con otros estudios.
2. La administración de una dosis de BNT162b2 al grupo control 28 días más tarde que al grupo de intervención no derivó en una peor respuesta de anticuerpos. La administración tardía resultó en niveles más elevados de anticuerpos específicos y neutralizantes, lo que sugirió un beneficio potencial en esta demora.
3. Los altos niveles de protección frente a la variante Delta persistieron en sendos grupos (intervención y control) a día 28 después de la segunda dosis en un régimen heterólogo, remarcando la efectividad de este enfoque vacunal frente a esta variante.
4. El fuerte descenso de los anticuerpos RBD y anticuerpos neutralizantes a día 180 resalta la necesidad potencial de una tercera dosis de inmunización para obtener unos niveles de protección mayores. Las dosis de recuerdo podrían ser particularmente cruciales para los individuos mayores de 60 años y aquellos con factores de riesgo para la COVID-19 severa.
5. Los pacientes hemodializados (HD) sin infección a SARS-CoV-2 previa desarrollan una respuesta celular y humoral robusta después de la segunda dosis de mRNA-1273. Esto indica la efectividad de la vacuna en general una protección inmunológica en esta población inmunocomprometida.
6. Pacientes HD con un historial de infección de SARS-CoV-2 muestran una respuesta humoral y celular fuerte y rápida después de la primera dosis de vacunación. Además, muestran valores significativamente más altos de IgG en comparación con voluntarios sanos y pacientes HD sin infección previa.
7. Los pacientes HD producen niveles significativamente más altos de citoquinas pro-inflamatorias, incluyendo IFN-γ e IL-2, en comparación a individuos sanos después de la vacunación en todos los tiempos.
8. Los pacientes HD con infección previa de SARS-CoV-2 podrían no requerir una dosis de recuerdo, ya que exhiben una respuesta inmune robusta después de la primera dosis, aunque, para pacientes que no han pasado la infección, una tercera dosis de recuerdo sería beneficiosa.

List of scientific publications and contributions.

1. **Gonzalez-Perez M**, Sanchez-Tarjuelo R, Shor B, Nistal-Villan E, Ochando J. **The BCG Vaccine for COVID-19: First Verdict and Future Directions**. Front Immunol. March 2021. 8;12:632478. doi: 10.3389/fimmu.2021.632478. PMID: 33763077; PMCID: PMC7982405.

Review
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2. Borobia AM, Carcas AJ, Pérez-Olmeda M, Castaño L, Bertran MJ, García-Pérez J, Campins M, Portolés A, **González-Pérez M**, García Morales MT, Arana-Arri E, Aldea M, Díez-Fuertes F, Fuentes I, Ascaso A, Lora D, Imaz-Ayo N, Barón-Mira LE, Agustí A, Pérez-Ingidua C, Gómez de la Cámara A, Arribas JR, Ochando J, Alcamí J, Belda-Iniesta C, Frías J; **CombiVacS Study Group. Immunogenicity and reactogenicity of BNT162b2 booster in ChAdOx1-S-primed participants (CombiVacS): a multicentre, open-label, randomised, controlled, phase 2 trial.** Lancet. 2021 Jul 10;398(10295):121-130. doi: 10.1016/S0140-6736(21)01420-3. Epub 2021 Jun 25. Erratum in: Lancet. August 2021. 14;398(10300):582. PMID: 34181880; PMCID: PMC8233007.

Impact factor: 202.731 (Q1) *
Contribution: Experimental procedures.

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4. **Gonzalez-Perez M**, Montes-Casado M, Conde P, Cervera I, Baranda J, Berges-Buxeda MJ, Perez-Olmeda M, Sanchez-Tarjuelo R, Utrero-Rico A, Lozano-Ojalvo D, Torre D, Schwarz M, Guccione E, Camara C, Llópez-Carratalá MR, Gonzalez-Parra E, Portoles P, Ortiz A, Portoles J, Ochando J. **Development of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients After 1273-mRNA SARS-CoV-2 Vaccination**. Front Immunol. March 2022. 23;13:845882. doi: 10.3389/fimmu.2022.845882. PMID: 35401504; PMCID: PMC8983822.

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7. **Gonzalez-Perez M**, Baranda J, Berges-Buxeda MJ, Conde P, Pérez-Olmeda M, Lozano-Ojalvo D, Cámara C, Del Rosario Llópez-Carratalá M, Gonzalez-Parra E, Portolés P, Ortiz A, Portoles J, Ochando J. **Maintenance of Potent Cellular and Humoral Immune Responses in Long-Term Hemodialysis Patients after 1273-mRNA SARS-CoV-2 Vaccination**. Pharmaceuticals (Basel). April 2023. 11;16(4):574. doi: 10.3390/ph16040574. PMID: 37111331; PMCID: PMC10141011.

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