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Humoral response during the COVID-19 pandemic: from infection to vaccination

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Humoral response during the COVID-19 pandemic: from infection to vaccination

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JURY

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Humoral response during the COVID-19 pandemic: from infection to vaccination

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the SARS-CoV-2. The disease emerged in Wuhan, China, in late 2019 in a cluster of patients with pneumonia. COVID-19 was swiftly declared a pandemic by the World Health Organization (WHO) on March 11, 2020. As of February 11, 2024, more than 774 million COVID-19 cases have been confirmed and around 7 million deaths have been recorded worldwide.

This unprecedented proliferation put considerable pressure on healthcare infrastructures globally, catalyzing extensive investigations to characterize the physiopathology of the virus and find appropriate treatments. The focal point of this thesis is the humoral response measurement in infected and/or vaccinated individuals. Each stage of the pandemic has been documented, from the release of commercial binding antibody assays in 2020 to the emergence of last variants that escape vaccine-induced neutralizing antibodies (NAbs) in late 2023.

Early in the pandemic, and despite the lack of formal regulatory approval, the healthcare community witnessed the introduction of a myriad of commercial assays designed to measure binding antibodies, essential for diagnosing past-infection or conducting seroprevalence surveys. The first objective was to evaluate and characterize the humoral response in infected patients. For this purpose, several binding antibody assays under different formats (total antibodies, immunoglobulin G [IgG], IgM, IgA), targeting different antigens (nucleocapsid [N], spike [S], receptor-binding domain [RBD]), and which could be quantitative, semi-quantitative, or qualitative, were carefully evaluated.

Accumulating evidence has highlighted the role of NAbs as the best correlate of protection (CoP) against SARS-CoV-2 infection. A pseudovirus-neutralization test was therefore developed by our team and continually adapted to accommodate the emergence of SARS-CoV-2 variants, including one of the most recent (*i.e.,* JN.1) in late 2023.

In response to the pandemic, major efforts have been made to produce and clinically validate new COVID-19 vaccines at an unprecedented speed. The CRO-VAX HCP study, a multicenter, prospective, and interventional study was designed to evaluate the humoral response among healthcare professionals having received two doses of

the BNT162b2 messenger ribonucleic acid (mRNA) COVID-19 vaccine. Two hundred and thirty-one volunteers from three medical centers in Belgium were enrolled. Samples were collected at regular intervals up to 6 months and kinetic models were rapidly developed.

Given the decrease of vaccine efficacy (VE) over time and the emergence of variants that can escape immunity, a third dose was soon recommended to boost immunity. A total of 155 volunteers from the CRO-VAX HCP study agreed to receive the booster and pursue our study. Breakthrough infection (BKI) occurrence in volunteers of two cohorts we followed (BNT162b2 and mRNA-1273) also allowed us to evaluate the protective role of the humoral response compared to a free-of-infection control group. We also documented the role of the humoral response as a CoP during the Omicron era.

Faced with a decreased VE correlated with a decline of the humoral response, a second and adapted booster was proposed by authorities. On September 2022, 54 participants of the CRO-VAX HCP study received the second and bivalent adapted BNT162b2 booster. The humoral response was evaluated and the NAbs against several variants were measured. BKI occurrence due to XBB.1.5 highlighted the importance of adapting vaccine formulation to circulating variants. Moreover, our study extended to evaluate the cellular response with an interferon gamma (IFNγ) release assay (IGRA). As compared to the humoral response, which significantly weakened over time, the cellular response remained quite stable and could therefore explained why individuals with low antibody titers can still be protected against severe disease.

This dichotomy of kinetics underscores the complexity of the immune response to SARS-CoV-2, advocating for continued exploration of CoP against the virus, particularly in the face of emerging variants.

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Réponse humorale pendant la pandémie COVID-19 : de l'infection à la vaccination

La maladie à coronavirus 2019 (COVID-19) est une maladie infectieuse causée par le SARS-CoV-2. La maladie est apparue à Wuhan, en Chine, fin 2019 chez un groupe de patients atteints de pneumonie. La COVID-19 a été rapidement déclarée comme étant une pandémie par l'Organisation mondiale de la santé (OMS) le 11 mars 2020. Au 11 février 2024, il y avait plus de 774 millions de cas confirmés de COVID-19 et environ 7,0 millions de décès dans le monde.

Cette vague d'infections sans précédent a exercé une forte pression sur le système de santé du monde entier. De nombreuses recherches ont été menées pour caractériser la physiopathologie du virus ainsi que pour identifier des traitements adaptés. Le sujet de cette thèse porte sur la mesure de la réponse humorale chez des individus infectés et/ou vaccinés. Chaque étape de la pandémie, depuis la sortie de kits commerciaux d'anticorps de liaison en 2020 jusqu'à l'émergence des derniers variants fin 2023 a été documentée.

Au début de la pandémie, les professionnels de la santé ont été confrontés à la commercialisation de nombreux kits conçus pour mesurer les anticorps de liaison. L'évaluation de la réponse humorale est notamment utile pour le diagnostic d'une infection antérieure ou pour les études de séroprévalence. Des centaines de tests ont été mis sur le marché sans approbation réglementaire formelle. Le premier objectif de nos recherches était d'évaluer et de caractériser la réponse humorale chez les patients infectés. Pour cela, plusieurs tests d'anticorps de liaison sous différents formats (anticorps totaux, immunoglobuline G [IgG], IgM, IgA), ciblant différents antigènes (nucléocapside [N], spicule [S], domaine de liaison au récepteur de la protéine de spicule), qu'ils soient quantitatifs, semi-quantitatifs ou qualitatifs ont été soigneusement évalués.

Le rôle des anticorps neutralisants comme meilleur corrélat de protection contre l'infection par le SARS-CoV-2 a rapidement été mis en lumière. Un test de neutralisation avec usage de pseudovirus a donc été développé par notre équipe et régulièrement adapté pour faire face à l'émergence de variants du SARS-CoV-2, dont l'un des derniers en date (*i.e.,* JN.1) fin 2023.

En réponse à la pandémie, des efforts considérables ont été déployés pour produire et valider cliniquement de nouveaux vaccins contre la COVID-19. L'étude CRO-VAX HCP est une étude multicentrique, prospective et interventionnelle conçue pour évaluer la réponse humorale dans une population de professionnels de santé ayant

reçu deux doses du vaccin COVID-19 BNT162b2 (ARN messager). Deux cent trente et un volontaires provenant de trois centres médicaux en Belgique ont été enrôlés. Des échantillons ont été collectés à intervalles réguliers jusqu'à 6 mois et des modèles cinétiques ont été rapidement développés.

Compte tenu de la diminution de l'efficacité du vaccin au fil du temps et de l'émergence de variants susceptibles d'échapper à l'immunité, une troisième dose a rapidement été recommandée par les autorités pour renforcer l'immunité. Au total, 155 volontaires de l'étude CRO-VAX HCP ont accepté de recevoir le rappel et de poursuivre l'étude. La survenue d'une infection malgré la vaccination dans deux cohortes (BNT162b2 et mRNA-1267) nous a également permis d'évaluer le rôle protecteur de la réponse humorale par rapport à un groupe témoin exempt d'infection. Nous avons également pu documenter le rôle de la réponse humorale en tant que corrélat de protection lors de la première vague dû à Omicron.

Faisant toujours face à une diminution de l'efficacité vaccinale avec le temps et à l'émergence de nouveaux variants, un deuxième rappel adapté a été proposé. En septembre 2022, 54 participants de l'étude CRO-VAX HCP ont reçu le deuxième rappel bivalent adapté de BNT162b2. La réponse humorale a été évaluée et les anticorps neutralisants contre plusieurs variants ont été mesurés. La survenue de certaines infections provoquées par le variant XBB.1.5 a mis en évidence l'importance d'adapter la formulation du vaccin aux variants en circulation. De plus, nous avons également mesuré la réponse cellulaire au moyen d'un test de libération d'interféron gamma (IGRA). Comparée à la réponse humorale qui diminue considérablement avec le temps, la réponse cellulaire est restée assez stable au fil du temps. Cela pourrait dès lors expliquer pourquoi les individus ayant de faibles titres en anticorps puissent toujours être protégés contre une forme sévère de la maladie.

Cette dichotomie de cinétique souligne la complexité de la réponse immunitaire du SARS-CoV-2, plaidant pour une exploration continue des corrélats de protection contre le virus, en particulier face aux variants émergents.

Main objectives

- Evaluation of the humoral response in patients who developed COVID-19:
  - *Defining the early kinetics of the humoral response.*
  - *Defining the long-term kinetics of the humoral response.*
  - *Comparing the measurement of binding versus neutralizing antibodies.*
- Evaluation of the humoral response in individuals who have been vaccinated with BNT162b2:
  - *Defining the early kinetics of the humoral response.*
  - *Defining the long-term kinetics of the humoral response.*
  - *Evaluating the impact of boosters on the humoral response.*
  - *Comparing the measurement of binding versus neutralizing antibodies.*
  - *Confirming the role of the humoral response as a correlate of protection against symptomatic disease.*
  - *Evaluating the immune escape of different variants.*
- Evaluation of the cellular response in individuals who have been vaccinated with BNT162b2 (bivalent booster only):
  - *Defining the early kinetics of the cellular response.*
  - *Defining the long-term kinetics of the cellular response.*

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Avant d'entamer votre lecture et de vous replonger dans cette période très particulière qu'a représenté la COVID-19 dans nos vies, je souhaiterais m'adresser aux personnes ayant contribué à l'aboutissement de ce projet.

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Figure IV.III.1.1: *(A) Evolution of NAbs against the BA.5 Omicron variant before and after the bivalent booster with a 6-month follow-up in a population of 51 healthy volunteers. GMT was 157 (95% CI: 112–219), 598 (470–761), 1,095 (903–1,327), 106 (83.4–134), and 47.4 (36.6–61.6) at baseline and after 14, 28, 90, and 180 days. (B) Kinetic models of the neutralizing capacity against the BA.5 Omicron variant. (C) Comparison of the neutralizing capacity against the D614G strain, the Delta and BA.5 Omicron variants, and the XBB.1.5 Omicron subvariant in a population of 30 healthy volunteers 6 months after having received the bivalent booster. GMT was 319 (95% CI: 241–423), 162 (119–220), 61.4 (42.7–88.2), and 29.5 (21.4–40.6) for the D614G strain, the Delta variant, the BA.5 Omicron variant, and the XBB.1.5 Omicron subvariant. The dotted line represents the positivity cut-offs for NAbs (dilution titer of 1:20). (D) Evolution of the cellular response by means of the measurement of IFNγ. GMT was 0.53 UI/mL (95% CI: 0.37–0.75), 0.95 (0.72–1.24), 0.87 (0.65–1.17), 0.65 (0.48–0.87), and 0.52 (0.34–0.79) at baseline and after 14, 28, 90, and 180 days. The positivity cut-off for IFNγ was 0.013 international units (IU)/mL. Geometric means and 95% CIs are represented. Only p values <0.05 were graphically represented.*

Figure IV.III.2.1:

Comparison of the neutralizing capacity against the D614G strain, the Delta, the BA.1 Omicron variant, the BA.5 Omicron variant, the XBB.1.5 Omicron subvariant, the BA.2.86 Omicron

subvariant, the FL.1.5.1 Omicron subvariant, and the JN.1 Omicron subvariant in a population of 30 healthy volunteers 6 months after having received the bivalent booster. The proportion of detectable NAbs was 100%, 100%, 93%, 93%, 67%, 67%, 50%, and 47%, respectively. GMT ±95% CI and percentage of positive samples are represented. The block dotted line represents the positivity cut-offs for NAbs (IC_{50} of 1:20). The grey dotted line represents the LOD of the assay (IC_{50} of 10). § = significantly higher compared to all other variants (p<0.0001). # = significantly higher compared to all other variants (p<0.0001) except for the D614G strain (p<0.0001).

Figure IV.III.2.1: *Comparison of the neutralizing capacity against the D614G strain, the Delta, the BA.1 Omicron variant, the BA.5 Omicron variant, the XBB.1.5 Omicron subvariant, the BA.2.86 Omicron subvariant, the FL.1.5.1 Omicron subvariant, and the JN.1 Omicron subvariant in individuals who developed a BKI following administration of the bivalent booster or not. Blood was collected 6 months after having received the bivalent booster. GMT ±95% CI are represented as well as the fold-change between groups. The block dotted line represents the positivity cut-offs for NAbs (IC_{50} of 1:20). The grey dotted line represents the LOD of the assay (IC_{50} of 10).*

List of abbreviations

ACE2	Angiotensin-converting enzyme 2
AU	Arbitrary unit
AUC	Area under the curve
BAU	Binding antibody unit
BKI	Breakthrough infection
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Maximal concentration
COI	Cut-off index
CoP	Correlate of protection
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
Ct	Cycle threshold
CV	Coefficient of variation
E	Envelope
(E)CLIA	(Electro)chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
EMA	European Medicine Agency
FDA	Food and Drug Administration
GISAID	Global Initiative on Sharing Avian Influenza Data
GMT	Geometric mean titer
HCW	Healthcare workers
IC_{50}	Half maximal inhibitory concentration
ICU	Intensive care unit
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGRA	Interferon gamma release assay
IQR	Inter-quartile range
IS	International standard
IU	International unit
LFA	Lateral flow assay
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification

LRT	Lower respiratory tract
mCoP	Mechanistic correlate of protection
MERS-CoV	middle east respiratory syndrome coronavirus
MLV	Maloneymurine leukemia virus
mRNA	Messenger ribonucleic acid
N	Nucleocapsid
NAAT	Nucleic acid amplification test
NAb	Neutralizing antibody
nCoP	Nonmechanistic correlate of protection
NP	Nasopharyngeal
NTD	N-terminal domain
ORF1	Open reading frame 1
p	p-value
POC	Point of care
PRNT	Plaque reduction neutralization test
pVNT	Pseudovirus neutralization test
r	Coefficient of correlation
R^2	Coefficient of determination
RDT	Rapid detection test
RBD	Receptor-binding domain
RdRP	RNA-dependent RNA polymerase
RLU	Relative light unit
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rRT-PCR	Real-time reverse transcription polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
S	Spike
S1	Spike protein subunit 1
S2	Spike protein subunit 2
SARS-CoV-1	Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SOP	Standardized operating procedure
sVNT	Surrogate virus neutralization test
$T_{1/2}$	Half-live
TAT	Turnaround time
T_{max}	Time to maximal concentration
URT	Upper respiratory tract
VE	Vaccine efficacy
VOC	Variant of concern
WHO	World Health Organisation
WT	Wild-type

“This manuscript is a compilation of selected scientific articles written during my cursus, but these have been updated or adjusted if this was necessary to avoid redundancy and ease the reading. On that note, I wish insightful reading”

INTRODUCTION

The Surge of SARS-CoV-2

Coronaviruses (CoVs) are single-stranded ribonucleic acid (RNA) enveloped viruses and belong to the *Coronaviridae* subfamily. The HKU1, the NL63, the OC43, and the 229E are four common endemic CoVs that typically infect the upper respiratory tract (URT), causing common cold symptoms (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020; Gorbalenya et al., 2020; Lamers & Haagmans, 2022). In the last two decades, three zoonic CoVs have infected humans, *i.e.,* the SARS-CoV-1 (initially called "SARS-CoV") reported in 2002, the middle east respiratory syndrome CoV (MERS-CoV) in 2012, and the "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) in late 2019. SARS-CoV and the SARS-CoV-2 share 79% sequence similarity across the genome. These three viruses can replicate in the lower respiratory tract (LRT) and provoke acute respiratory distress syndrome (Lamers & Haagmans, 2022).

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the SARS-CoV-2 virus (Coronaviridae Study Group of the International Committee on Taxonomy of, 2020). The disease emerged in Wuhan, China, in December 2019 in a cluster of patients with pneumonia and rapidly evolved into a pandemic (Chan et al., 2020). COVID-19 was declared a pandemic by the WHO on March 11, 2020. As of February 11, 2024, more than 774 million confirmed COVID-19 cases and around 7 million deaths have been reported around the globe (WHO, 2024).

The SARS-CoV-2 virus is made of structural (membrane, N, envelope [E], and S), non-structural (viral replication and transcription complex), and accessory proteins. The S protein is composed of two subunits (S1 and S2). The S1 subunit binds to the angiotensin-converting enzyme 2 (ACE2) receptor of the host cell and the S2 subunit mediates membrane fusion (Lamers & Haagmans, 2022). Once bound to ACE2, the S protein is cleaved by the transmembrane serine protease at the S2' site (furin cleavage motif), leading to the fusion of viral and host membranes, and to the release of the viral ribonucleoprotein complex into the host cell (**Figure 0.1**).

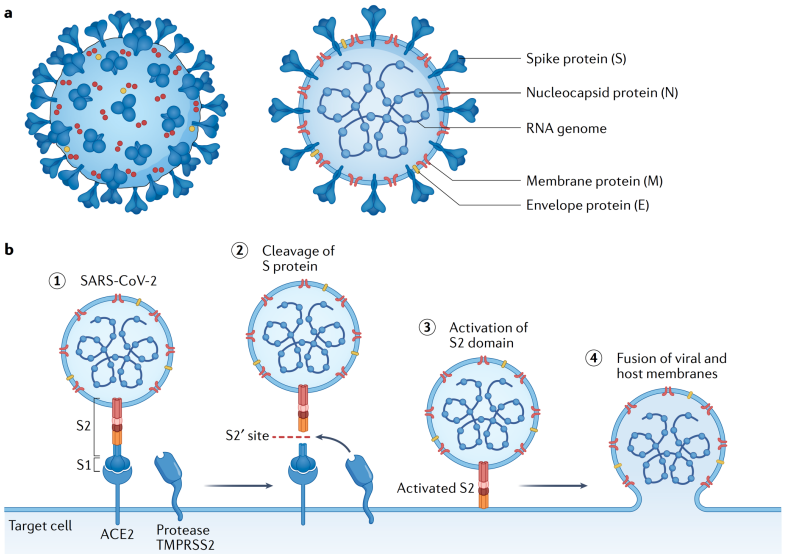

Figure 0.1: *Description of the SARS-CoV-2 virus (a) and mechanism of virus entry (b) (reproduction authorized by Prof. Bart L. Haagmans; Lamers & Haagmans 2022 Nat Rev Micro).*

Multiciliated cells (in the URT) are the first cells with a high ACE2 expression targeted by SARS-CoV-2 during natural infection.

This virus is mainly transmitted via respiratory droplets and aerosols, with an incubation period around 4 to 5 days before symptom onset. Patients suffering from COVID-19 may be asymptomatic or may develop a mild, moderate, or severe disease form (Marshall et al., 2020), but most patients developed a mild to moderate respiratory disease (Lamers & Haagmans, 2022). The SARS-CoV-2 infection profile is complex due to its non-specific nature, and sometimes complicates its diagnosis because its symptoms overlap with those of other viral infections. Most common symptoms include cough, headache, myalgia, fever, and diarrhea.

As in any viral infection, the role of innate and adaptative responses is crucial to fight against the virus. A robust and rapid multicomponent immune response is associated with infection resolution and improved clinical outcomes (Kent et al., 2022). The role of the innate response is to rapidly restrict viral replication in infected cells, to create an antiviral state with recruitment of specific effector cells to limit the spread, and to prime the adaptive response of B cells (which produce antibodies), CD4+ and CD8+ T cells (Sette & Crotty, 2021). In the context of SARS-CoV-2, the virus effectively avoids or delays the innate response, and the adaptive response is therefore primed later. In non-severe cases, the delayed occurrence of T cell and antibody responses is still associated with successful infection resolution. If the delay of the innate response (and thus the adaptive response) is too long (defective innate immunity and/or effective virus evasion), the risk of developing a severe form of the disease is

significantly increased (Sette & Crotty, 2021). Severe COVID-19 cases are characterized by an increased lung infection rate, high serum levels of cytokines (e.g., CXCL10, IL-6, IL-8), and extensive lung damage with thrombosis in the microvasculature (Chen et al., 2020; Wang et al., 2020; Zhou et al., 2020). While the cytokine storm occurrence in severe COVID-19 cases is evident and has been identified by lab measurements, we do not yet know exactly what propagates and triggers the storm (Gillot et al., 2021). Older people (smaller naive T cell pool for an effective adaptive response), men, and people with predisposing factors such as hypertension, diabetes, heart disease, or cancer are at increased risk of developing complications from SARS-CoV-2 infection than other individuals (Marshall et al., 2020).

In order to control the pandemic, major efforts have been made to produce and clinically validate new COVID-19 vaccines at an unprecedented speed. More than 13 billion COVID-19 vaccine doses have been administered worldwide (WHO, 2024), 50 vaccines were approved, and 242 are currently in clinical development or awaiting clinical validation (WHO, 2022b). Vaccination has undoubtedly been the most effective tool in the fight against COVID-19. Antiviral monoclonal antibodies can also be used in the treatment and prevention of COVID-19, especially in more fragile patients, including the immunocompromised and those who respond poorly to vaccines (Follmann et al., 2023). From December 2020 to March 2023, the WHO European Respiratory Surveillance Network estimated that vaccines reduced deaths by 57% and saved around 1.4 million lives (Network, 2024).

Diagnosis of SARS-CoV-2 infection

ACUTE

Nucleic acid amplification tests (NAATs)

On January 10, 2020, the first SARS-CoV-2 genetic sequence was uploaded to the Global Initiative on Sharing Avian Influenza Data (GISAID) platform (GISAID, 2024a). Rapidly, diagnostic companies and manufacturers developed NAATs to detect SARS-CoV-2 RNA in various clinical specimens (Bohn, Mancini, et al., 2020). NAATs are still considered the gold standard for SARS-CoV-2 identification in clinical specimens (Bohn, Lippi, et al., 2020). The most common NAAT type is the real-time reverse transcription polymerase chain reaction (rRT-PCR), which is used in both the Centers for Disease Control and Prevention (CDC)-developed assay and the WHO-endorsed assays (Bohn, Lippi, et al., 2020). URT and LRT specimens collected during the acute infection phase are deemed suitable for detecting SARS-CoV-2 with NAATs (Bohn, Lippi, et al., 2020). Saliva samples could be considered as an alternative when other samples cannot be collected from symptomatic patients, but only with validated

NAATs or laboratory-based antigen immunoassays (Butler-Laporte et al., 2021; G. Lippi, J. Favresse, et al., 2022). As for other measurands (Lippi et al., 2009; Plebani, 2006; Wauthier et al., 2022), important pre-analytical issues have been related to inadequate procedures for specimen collection, handling, transport and storage (especially using URT specimens) (Basso et al., 2020; Lippi, Simundic, et al., 2020). It is estimated that 20 to 30% of false negative results may occur with URT specimens, which is potentially due to sample collection issues (Bohn, Mancini, et al., 2020). Using LRT specimens could thus be more desirable for molecular testing, but this may not be clinically realistic due to the invasive nature of this type of sampling (Bohn, Mancini, et al., 2020). Therefore, the nasopharyngeal (NP) swab is still considered the gold standard sample matrix for detecting SARS-CoV-2 with molecular tests (Bohn, Lippi, et al., 2020). Interfering substances present in the specimens, sample contamination, and pipetting errors are additional important preanalytical issues (Bohn, Lippi, et al., 2020; Lippi, Simundic, et al., 2020).

The main gene targets employed by currently available NAATs include the *N*, *E*, *S*, *RNA-dependent RNA polymerase* (*RdRP*) and *open reading frame 1ab* (*ORF1ab*) genes (Bohn, Mancini, et al., 2020). To minimize the risk of false negative test results, it has been recommended to use at least two SARS-CoV-2 gene targets. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) guidelines also proposed a methodology for verification or validation of the analytical and clinical performance of SARS-CoV-2 assays (Bohn, Mancini, et al., 2020). It is also better to report the name and target genes used for the NAATs. Importantly, test positivity must be defined based on manufacturer's recommended cut-off, whilst a thoughtful clinical validation must be conducted when a different cutoff will be used.

Determination of infectiousness status represents an important need to identify patients requiring isolation. The proposed reference standard for establishing infectiousness is viral culture (*i.e.,* absence of viral culture generally implies absence of contagiousness). However, this technique is challenging to perform and requires high biosafety measures that preclude its use in clinical routine (Dinnes et al., 2022). Values of cycle threshold (Ct) of rRT-PCR assays have been proposed as a potential surrogate of viral culture results in clinical samples and for predicting illness severity (G. Lippi, J. Favresse, et al., 2022; Mancini et al., 2021). Compared to viral culture, Ct values are easier to estimate from amplification curves. However, the suitability of the Ct value to estimate contagiousness remained limited, since Ct results varies between analyzers and laboratories, and direct comparison of values obtained with different techniques must be avoided. The standardization of this measure remains therefore an unresolved issue to date (Buchta et al., 2021; G. Lippi, J. Favresse, et al., 2022; Loacker et al., 2022). An illustration of this lack of standardization is the range of Ct cut-offs values reported for contagiousness, which may vary from 24 to over 35

(Favresse, Gillot, Oliveira, et al., 2021). Furthermore, it also depends on the sample matrix, specimen collection and processing. Finally, a single test does not allow to identify whether patients with low viral load are at the early onset of symptoms or are instead recovering from their infection, thus being in the declining phase of viral load (Dinnes et al., 2022).

The clinical context of the patient is paramount for interpretation of SARS-CoV-2 NAAT results. In symptomatic subjects, the viral RNA can be detected at symptom onset and peaks within the first week. A NAAT can still be interpreted as positive several weeks after the onset of symptoms to subsequently becoming undetectable in most patients (Bohn, Mancini, et al., 2020). A negative NAAT therefore does not rule out SARS-CoV-2 infection when the test is performed too early or too late during the acute infection (Bohn, Lippi, et al., 2020). As well as for pre-analytical issues, analytical and post-analytical errors may also be associated with false negative test results.

In patients with signs and symptoms suggestive of SARS-CoV-2 infection (*i.e.,* high-risk patients), the diagnosis carried out with laboratory-based molecular assays should not be confirmed, due to the high positive and negative predictive values of these tests. If a negative result is obtained in a high-risk patient, a repeated test within 24-48h is recommended, using a different laboratory-based molecular assay if possible. In a patient with a low risk of infection (*i.e.,* hospital admission, contact tracing), the utilization of point-of-care (POC) molecular assays (Bohn, Mancini, et al., 2020; Padoan, Cosma, Aita, et al., 2022) has been reported as a valuable alternative to routine laboratory-based assays, since the diagnostic sensitivity and specificity of several of these tests is only marginally lower (*i.e.,* as high as 95% and 99%, respectively). These methods have low throughput but can provide timely results on a very short timeframe, allowing efficient patient triage compared to laboratory-based molecular assays with turnaround time (TAT) generally ranging between 4 to 12 hours (G. Lippi, J. Favresse, et al., 2022).

Since the identification of the initial strain in Wuhan, the wild-type (WT) variant, several mutations occurred in the SARS-CoV-2 genome, leading to appearance of different viral lineages (GISAID, 2024b). This is the typical consequence of a natural pressure to which all viruses are subjected. In this context, gene sequencing of SARS-CoV-2 is notably essential for monitoring of emerging lineages that may impact human health (G. Lippi, J. Favresse, et al., 2022). Five lineages have been designated as a variant of concern (VOC) by the WHO so far, namely the Alpha, Beta, Gamma, Delta and Omicron variants. First identified in November 2021, the Omicron lineage is to date the leading variant all over the world. This variant is characterized by a huge number of dominant mutations in the S protein, nearly half of which are located within the sequence of the RBD, thus conferring increased transmissibility and

considerable immune escape from acquired protection through COVID-19 vaccination or previous infection with other (different) SARS-CoV-2 variants. Currently, Omicron is largely dominant and several subvariants have emerged including BA.2, BA.2.12.1, BA.2.75.2, BA.4 and BA.5, BQ.1, XBB.1.5, BA.2.86, and JN.1. All these sublineages also demonstrated considerable escape to acquired immunity (Favresse, Gillot, et al., 2023; Lippi, Adeli, et al., 2021; Yang et al., 2024).

The choice of gene targets and primers used by manufacturers should be reviewed to ensure they considered robustness to at least the most common mutant strains and are targeted to highly conserved regions (Bohn, Mancini, et al., 2020; Buchta et al., 2022; Buchta et al., 2021). Each assay must hence be validated against newly emerged SARS-CoV-2 variants, to prevent the risk of generating false negative test results (Bohn, Lippi, et al., 2020; G. Lippi, J. Favresse, et al., 2022). The Omicron lineages BA.1, BA.1.1 and BA.3 are paradigmatic examples of variants that may generate specific test failures. The emergence of a 69–70del mutation in these variants causes a 6-nucleotide deletion (21765–21770) in the *S* gene of SARS-CoV-2, which results in deletion of two amino acids located between positions 69 (histidine) and 70 (valine) of the S protein, thus impairing the probe annealing in certain assays, ultimately leading to *S*-gene target failure (G. Lippi, J. Favresse, et al., 2022). More recently, an *in silico* evaluation found that the sublineages BA.4 and BA.5 may potentially result in false negative test results using four distinct laboratory-based molecular assays (Sharma et al., 2023).

Antigen testing

Compared to NAATs that detect SARS-CoV-2 RNA, antigen assays are aimed at identifying (and possibly measuring) the presence of viral antigens to indicate current viral infection (Bohn et al., 2021). Various collected specimens (*i.e.,* nasal, NP, saliva, blood, urine) can be used but NP specimens have been most widely validated and used (Bohn et al., 2021; Favresse, Bayart, David, Didembourg, et al., 2022; Ren et al., 2022; Veyrenche et al., 2022). Available assay methodology mainly includes rapid detection tests (RDTs) (*i.e.,* manual chromatographic immunoassays, also known as lateral flow assays (LFA)) used at the POC, as well as laboratory-based immunoassays (*i.e.,* automated immunoassays) (Bohn et al., 2021; Dinnes et al., 2022; Favresse, Gillot, Oliveira, et al., 2021; G. Lippi, B. M. Henry, & K. Adeli, 2022; Salvagno, Nocini, et al., 2021).

The viral N is the main target of the majority of antigen based RDTs (Dinnes et al., 2022). Compared to other SARS-CoV-2 antigens, the N antigen is the good choice for two main reasons (Bohn et al., 2021; G. Lippi, J. Favresse, et al., 2022). First, the protein is produced at higher levels compared to other viral SARS-CoV-2 proteins (*i.e.,* S protein), leading to higher assay sensitivity (Bohn et al., 2021). Second, the selective

pressure placed by the increasing number of seropositive people worldwide (either post-infection or post-vaccination) is responsible for boosting higher viral mutations in the *S* gene, encoding the mature S protein, so that the use of other viral antigens is at least theoretically more advisable (Bohn et al., 2021). The N antigen seems, however, less vulnerable to selective pressure to develop mutations and is not subjected to the risk of false positive test results in patients undergoing COVID-19 vaccination with vaccines encoding the S protein (G. Lippi, J. Favresse, et al., 2022). As with NAAT, it remains crucial to validate each immunoassay against newly emerged SARS-CoV-2 variants, to prevent false negative test results (Bayart et al., 2022; G. Lippi, J. Favresse, et al., 2022).

Proposed advantages for RDT in NP specimens include its widespread availability as decentralized testing, rapid TAT, patient stratification, overall lower cost, no use of specific equipment or highly trained staff, and preventative case identification. The time of positivization of self-performed RDTs was also reported to reflect the viral load in clinical samples (Salvagno, Henry, et al., 2023). Nevertheless, major concerns regarding analytical performance persist (Bohn et al., 2021). Based on available evidence, the sensitivity of RDTs is significantly lower compared to NAATs. Several factors should be considered when assessing RDTs performance: patient characteristics (clinical severity, type and time of onset of symptoms), viral load, and assay method (Bohn et al., 2021). A Cochrane systematic review has recently evaluated 49 different commercial immunoassays in 155 cohorts totaling 100,462 unique samples (Dinnes et al., 2022). Compared to NAAT, average sensitivity was higher in symptomatic (73.0%, 95% CI 69.3%-76.4%) compared to asymptomatic participants (54.7%, 95% CI 47.7%-61.6%). Average sensitivity was higher in the first week after symptom onset (80.9%, 95% CI 76.9%-84.4%) than in the second (53.8%, 95% CI 48.0%-59.6%). Average specificity was similarly high for symptomatic (99.1%) or asymptomatic (99.7%) participants. The sensitivity varied widely according to the different devices (from 34.3% to 91.3% in symptomatic and from 28.6% to 77.8% in asymptomatic participants) (Dinnes et al., 2022). The selection of the assay is hence of upmost importance. In this context, the WHO has set a minimum performance requirement compared to a reference NAAT of ≥80% diagnostic sensitivity and ≥97% diagnostic specificity, respectively (Bohn et al., 2021).

Recently, Kessler *et al.* found that an RDT correctly identified all rRT-PCR positive samples with Ct <25 and that inoculation of cell cultures of samples that were RDT-/rRT-PCR+ did not generate cytopathic effects, presuming the absence of contagiousness when RDT result is negative (Kessler et al., 2022). The population size was, however, low and some reports showed that RDT could miss specimens with Ct <25 (Favresse, Gillot, Oliveira, et al., 2021). Along with the limitations of using the Ct derived from rRT-PCR described above, using RDT for identification of contagious

patients should be interpreted with caution, especially in light of new and highly mutated SARS-CoV-2 variants. There is also a higher risk of pre-analytical errors that need to be taken into account, as well as the risk of misuse (Bohn et al., 2021; Favresse, Gillot, Oliveira, et al., 2021; Lippi, Henry, et al., 2023).

Compared to RDTs, laboratory-based assays represented a valuable alternative, with overall higher sensitivities (82.0-88.5%) and high specificities (93.0-99.5%), depending on studies, but still present lower performance compared to NAATs (**Figure 0.2**) (Favresse, Gillot, Oliveira, et al., 2021; Hartard et al., 2021; Horber et al., 2022; G. Lippi, J. Favresse, et al., 2022; G. Lippi, B. M. Henry, M. Montagnana, et al., 2022; Menchinelli et al., 2021). The sensitivity increased to 92.5-100% if considering only samples with higher viral loads (*i.e.,* Ct <25-30) (Hartard et al., 2021; Horber et al., 2022; G. Lippi, B. M. Henry, & K. Adeli, 2022; Menchinelli et al., 2021).

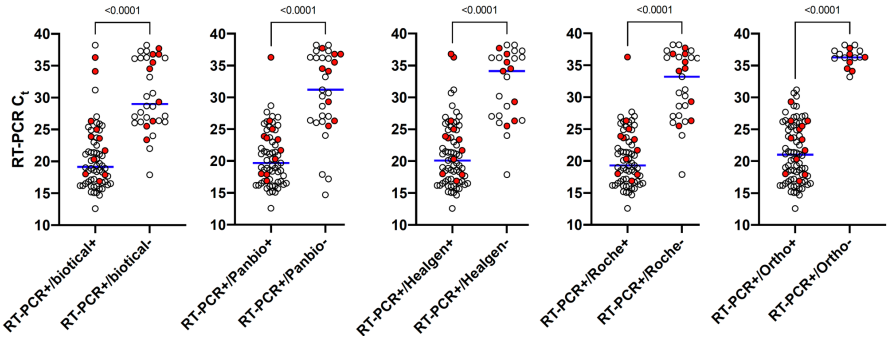

Figure 0.2: *Graphical representation of positive and negative antigen results according to RT-PCR Ct values. A significant difference in the Ct value is observed between the positive and negative tests for each antigen method. Importantly, only the automated antigen shows no overlap between Ct values obtained for positive and negative samples. Samples from asymptomatic subjects are highlighted in red (Favresse, Gillot, Oliveira, et al., 2021).*

Given the risk of missing true positive patients compared to NAATs, the place of antigen-based assays (especially RDT) should be considered carefully. In light of their lower sensitivity, current guidelines recommend performing a laboratory-based molecular assay in patients with high pre-test probability of SARS-CoV-2 infection (G. Lippi, J. Favresse, et al., 2022). When antigen-based tests are used, it is recommended to confirm negative test results by NAAT-based testing in high pre-test probability settings, since negative test result does not definitively exclude the presence of active infection (Bohn et al., 2021). In low or moderate pre-test probability settings, positive results obtained by RDT should be confirmed by NAAT-based testing (Bohn et al., 2021). These confirmatory processes can present some organizational challenges, but deserve to be considered for an appropriate patient management.

As earlier discussed, the optimal sample for diagnosing SARS-CoV-2 is an URT specimen, though the use of saliva has also been considered as a reliable option for some antigen immunoassays (G. Lippi, J. Favresse, et al., 2022). Ren *et al.* found 92% sensitivity and 100% specificity using an ECLIA (electrochemiluminescence immunoassay) targeting the N protein in saliva samples (Ren et al., 2022). Aita *et al.* also reported 90% sensitivity but lower specificity (92%) using another automated platform. The sensitivity rises to 100% when considering samples with Ct <30 (Aita et al., 2023). Although this matrix is interesting, especially in the ambulatory setting, it is important to consider the lower performance of RDTs using a saliva specimen.

The possibility of measuring SARS-CoV-2 antigens in blood has also been explored (Brasen et al., 2021; Favresse, Bayart, David, Didembourg, et al., 2022; Favresse, Bayart, David, Dogne, et al., 2022; Favresse, Bayart, David, Gillot, et al., 2022). Compared to NAAT, the clinical sensitivity ranged from 85.2% to 93.0% considering studies that included patients who developed symptoms up to a maximum of 2 weeks. The sensitivity increased to 94.2% to 100% with samples collected within the first days since symptom onset. However, clinical sensitivity significantly decreased after 2 weeks (43.2% to 74.5%) and after 4 weeks since symptom onset (from 0% to 34.2%). Given that the peak of the N antigen is typically reached after 7 days, as for the viral load in NP samples, and that a continuous decline is observed afterwards, the timing since symptoms is of paramount information for evaluation of antigenemia. The mean clinical specificity is 90.9% (Favresse, Bayart, David, Gillot, et al., 2022). Of note, the performance of the S antigen assay (sensitivities of 64% to 85.3%) in blood was lower compared to that of the N antigen assay (Favresse, Bayart, David, Didembourg, et al., 2022; Sigal et al., 2022). Higher concentrations of N and S antigens were observed in more severe patients (Favresse, Bayart, David, Didembourg, et al., 2022; Favresse, Bayart, David, Gillot, et al., 2022), as well as positive correlations with inflammatory biomarker levels (*i.e.,* CRP or IL-6) were found (Brasen et al., 2021). Moreover, compared to rRT-PCR (Favresse, Bayart, David, Dogne, et al., 2022), it may provide useful information about illness severity (**Figure 0.3**) (Favresse, Bayart, David, Didembourg, et al., 2022; Favresse, Bayart, David, Dogne, et al., 2022; Favresse, Bayart, David, Gillot, et al., 2022).

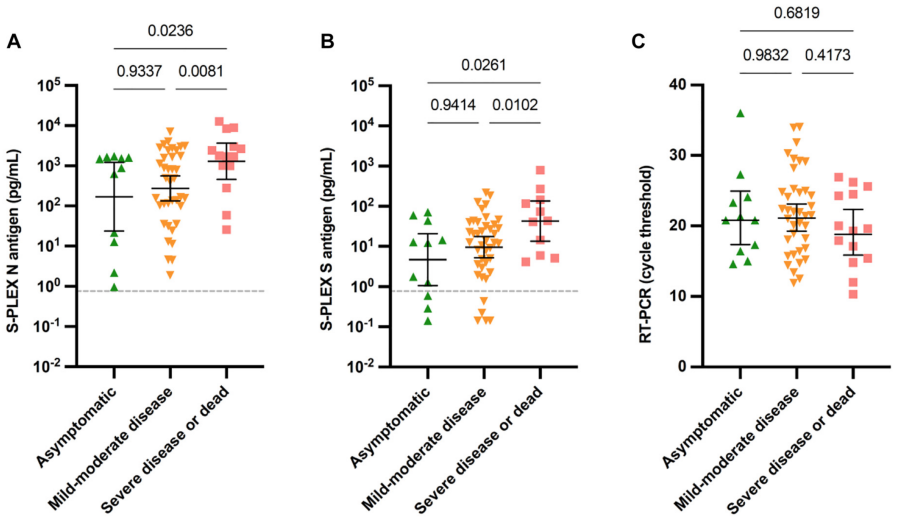

Figure 0.3: *Antigenemia and RT-PCR results according to the disease severity. Panels (A and B) refer to N- and S-antigens in serum, respectively. Panel (C) refers to Ct results obtained on NP samples. The grey dotted lines correspond to the positivity cut-off of antigen assays (Favresse, Bayart, David, Didembourg, et al., 2022).*

Finally, such measurements may facilitate patient triage to better optimize intensive care utilization in patients presenting early after symptom onset (Favresse, Bayart, David, Gillot, et al., 2022). The measurement of SARS-CoV-2 antigens in blood is, however, not widely used, though it displays better performance for predicting disease severity compared to NAAT-based testing.

RETROSPECTIVE

Humoral response or antibody testing

In response to SARS-CoV-2 infection, the humoral immunity will lead to generation of several types of Ig against the pathogen (especially IgA, IgG and IgM), which have a primary function to protect the body mucosae from virus penetration (*i.e.,* secretory IgG and dimeric IgA), as well as to neutralize the virus once it has colonized the body and/or entered the circulation (especially IgG) (Lippi, Adeli, et al., 2021). The measurement of antibodies is classically performed in serum or plasma obtained from a venipuncture, but the use of whole blood (Decru et al., 2020) or dried blood spots represent a valuable alternative (Galla et al., 2023; Weisser et al., 2021). The use of saliva has also been explored but requires further investigation (Martinez-Subiela et al., 2022).

Manufacturers have mostly focused on developing immunoassays against IgG or total antibodies (sum of all isotypes) rather than IgA and IgM (Bohn, Lippi, et al., 2020). The detection of IgG or total antibodies (*i.e.,* seroconversion) occurs approximately

within 7 to 14 days after symptom onset (Bohn, Lippi, et al., 2020; Favresse, Cadrobbi, et al., 2021; Favresse, Eucher, Elsen, Laffineur, et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020). The IgA response develops early, coinciding with that of IgM, peaks after 18 to 21 days, and appears to be even stronger and more persistent than IgM (Padoan, Sciacovelli, et al., 2020). Performance of IgM assays was found to be lower compared to IgG and total antibodies for detecting previously infected individuals (Dittadi et al., 2020; Mairesse et al., 2020; Padoan, Bonfante, Sciacovelli, et al., 2020; Tre-Hardy, Wilmet, et al., 2021). In convalescent patients, antibodies can still be measured in most after a long period of time (>8-10 months post-infection), with total antibodies being even more persistent compared to IgG (Dan et al., 2021; Favresse, Elsen, et al., 2021; Favresse, Eucher, et al., 2021; Favresse, Gillot, Di Chiaro, et al., 2021; Lau et al., 2021; Schaffner, Risch, Weber, et al., 2020). However, a slow decay is observed over time.

Available assays could target either the S protein, the RBD of the S1 of the S protein, or the N protein of SARS-CoV-2 (Bohn, Loh, et al., 2020). Antibodies against the N protein will only be generated in infected patients or in those receiving attenuated vaccines, while anti-S/RBD antibodies are generated in both infected and vaccinees. Some multiplex methods can measure several different targets within a run (Favresse, Brauner, et al., 2021; Gillot et al., 2020). Immunoassays can use different technologies (LFA, enzyme-linked immunosorbent assay (ELISA), CLIA), and can be quantitative, semi-quantitative or qualitative (Bernasconi et al., 2020; Haguet et al., 2021; Tollanes et al., 2020; Tollanes et al., 2021). Given the different types of assays, their fit for purpose properties needs to be validated, and the IFCC provides an *ad hoc* evaluation protocol (Bohn, Loh, et al., 2020). These evaluations are important since the performance of antibody assays are not equivalent, especially considering the use of rapid tests (Tollanes et al., 2020; Tollanes et al., 2021). The use of an orthogonal testing strategy has been for example proposed to avoid false positive results, especially in low prevalence settings (Bohn, Loh, et al., 2020; Favresse, Brauner, et al., 2021; Huyghe et al., 2020).

Compared to binding antibodies, NAbs represent the first line of response of adaptive immunity against SARS-CoV-2. They are of particular importance because inhibit the binding of the RBD at the surface of the S protein to the human ACE2 receptor, thus hampering host cell penetration (Favresse, Gillot, et al., 2023; Khoury et al., 2021; Lippi, Adeli, et al., 2021). NAbs represent the best correlate of immunity (Favresse, Gillot, et al., 2023; Feng et al., 2021; Khoury et al., 2021). It is important to highlight that current serology immunoassays available in the diagnostic market (*i.e.,* binding antibody assays) do not provide definitive information regarding patient immunity. For this analysis, a neutralization assay obtained in a cell culture system is needed to determine the presence of active antibodies and relative protection against future

infection (Bohn, Lippi, et al., 2020; Favresse, Gillot, et al., 2023; Lippi, Adeli, et al., 2021; Migueres et al., 2023). These latter can be measured using live virus neutralization test, pseudovirus neutralization test or by a surrogate assay (Favresse, Gillot, Di Chiaro, et al., 2021; Legros et al., 2021; Padoan, Bonfante, Sciacovelli, et al., 2020). Current methods used to measure NAbs present a low throughput, are time-consuming, need skillful operators, and require high levels of biosafety (especially for live virus neutralization assay). It would therefore be easier to use commercial assays that can be a surrogate of these reference methods (Favresse, Gillot, et al., 2023; Lippi, Adeli, et al., 2021). As for NAAT and antigen testing, there is a need for harmonization in serological assays, including specific concerns on quality controls (Dimech et al., 2023).

NAbs can theoretically also induce Fc-dependent effector functions that mediate antimicrobial functions independently of any neutralization effect. Without having any neutralization effect, some other antibodies (*i.e.,* non-neutralizing antibodies) can still engage immune effectors through their Fc domains. Accumulating evidence has linked these Fc-dependent antibody effector functions (*i.e.,* non-neutralizing function) to the outcome of a SARS-CoV-2 infection. These Fc-dependent antibody effector functions are not captured by binding and neutralization assays and their measurements is much more complicated as compared to binding and neutralizing antibodies. The assessment of “non-neutralizing antibodies” was outside the scope of our research (Zhang et al., 2023).

In the IFCC interim guidelines on the role of serology in COVID-19, several indications were formulated (Bohn, Loh, et al., 2020). First, it is obvious that compared to NAAT and antigen testing, the measurement of antibodies is not used for diagnosis of acute infection. Its measurement could, however, be useful as adjunct to molecular testing in patients presenting with suggestive clinical features (>14 days post symptom onset), but molecular/antigen testing for SARS-CoV-2 is negative, undetermined, or unavailable (Baron et al., 2020). It is also useful to serve as adjunct when persistently positive molecular tests occur in the absence of evidence of acute infection, such as late after resolving infection or to assist in the diagnostic workup of multisystem inflammatory syndrome in children (Bohn, Loh, et al., 2020). These guidelines were published before vaccination programs and were therefore not able to make a statement about the use of antibody testing in this context. The measurement of antibodies is also useful for seroprevalence study purposes and for developing risk prediction models (Bohn, Loh, et al., 2020). Indeed, more severe and non-immunocompromised patients tend to develop higher levels of anti-SARS-CoV-2 antibodies (Favresse, Eucher, et al., 2021; Favresse, Gillot, Di Chiaro, et al., 2021; Legros et al., 2021; Lippi, Horvath, et al., 2020). Other indications that deserve further validation may include identification of patients at higher risk to develop a

BKI. Indeed, lower levels of binding antibodies and NAbs in peri-infection (or during the pre-booster period) were observed in BK patients compared to those who did not develop infection (Barda et al., 2023; Bergwerk et al., 2021; Favresse, Dogne, et al., 2022).

It has been anticipated that anti-SARS-CoV-2 antibodies measurement may have a role in the vaccination campaign, especially in scenarios of vaccine scarcity or contexts of organizational complexity. These aspects will be discussed in a dedicated section of this thesis.

CHAPTER I: HUMORAL RESPONSE IN INFECTED INDIVIDUALS

I.I. Introduction

The first chapter will be dedicated to humoral response assessment in infected patients. Early in the pandemic, healthcare and laboratory professionals faced the release of many different commercial kits designed to measure binding antibodies. Hundreds of assays were released on the market without formal regulatory approval. A preliminary evaluation by the U.S. Food and Drug Administration (FDA) identified that out of 91 assays, only 27 were considered to have acceptable performance in terms of specificity and sensitivity (Hempel et al., 2024).

Antibody assays were manufactured under different formats (total antibodies, IgG, IgM, IgA), targeted different antigens (N, S, RBD), could be quantitative, semi-quantitative or qualitative, and could be performed using a laboratory analyzer or rapid LFA. Some kits were even designed to measure different antigens in one run (*i.e.,* multiplex kits). The demonstration of high analytical and clinical performance (sensitivity, specificity, negative predictive value, and positive predictive value) was important, notably for the diagnosis of past-infection or for seroprevalence studies. The humoral response was also described as a prognostic factor in patients. The inclusion of several samples from infected patients soon after RT-PCR positive results made it possible to determine the early kinetics of the humoral response. Additionally, the rigorous follow-up of patients with past infection also allowed to investigate the long-term dynamics of the humoral response. These commercial kits mostly measured the binding antibody response, but not the neutralizing capacity of antibodies. NAbs are of particular importance because they can prevent binding of the RBD of the S protein to the ACE2 receptor present on the surface of human cells, blocking the virus from entering host cells. They represent the best immunity correlate. The fact that the binding antibody response, especially against S or RBD, could be used to evaluate the neutralizing capacity of antibodies was quickly questioned. To fuel the discussion, we compared several binding antibody assays against NAbs. The latter antibodies can either be measured using a surrogate virus neutralization test (sVNT), a pseudovirus neutralization test (pVNT), or a plaque reduction neutralization test (PRNT). In our evaluations, we used a sVNT and a pVNT developed at UNamur. The pVNT was also developed to anticipate the arrival of vaccines and to test for the emergence of SARS-CoV-2 variants.

NAbs are only modestly correlated with commercial assay targeting S- or RBD-directed antibodies (Bayart, Douxfils, et al., 2021; Douxfils et al., 2021; Favresse, Gillot, et al., 2023; Favresse, Gillot, Di Chiaro, et al., 2021; Franchini et al., 2022; Legros et al., 2021; Meschi et al., 2021; Padoan, Bonfante, et al., 2021; Padoan, Bonfante, Sciacovelli, et al., 2020). Therefore, binding antibodies do not reliably reflect the presence of NAbs.

I.II. Early kinetics

I.II.1 Clinical Performance of the Elecsys Electrochemiluminescent Immunoassay for the Detection of SARS-CoV-2 Total Antibodies

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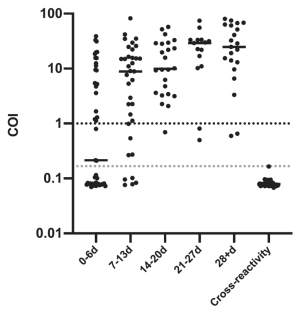
In the context of COVID-19, a wide range of serology immunoassays have been developed to complement the RT-PCR, with different SARS-CoV-2 antigen recognition and antibody specificity (Vashist, 2020). Serological testing is useful for the diagnosis, for characterization of the course of the disease, for identifying convalescent plasma donors as well as for epidemiological study, lockdown exit programs and COVID-19 vaccine development (Farnsworth & Anderson, 2020; Winter & Hegde, 2020). Due to the widespread dissemination of these new methods and the limited experience with these assays, it is crucial for laboratories to rigorously validate these methods before broad introduction into routine clinical practice. Independent validations are also needed to assure they are in line with the expected analytical and clinical performance (Farnsworth & Anderson, 2020; Kirkcaldy et al., 2020; Vashist, 2020; Winter & Hegde, 2020).

This study is the first to report the external validation of a new ECLIA test, the Elecsys anti-SARS-CoV-2 from Roche Diagnostics, allowing the detection of total antibodies (including IgG) specifically directed against SARS-CoV-2 N and performed on the cobas e801 module. The test result is given as a cut-off index (COI). According to the manufacturer, a result <1.0 is considered negative while a result ≥1.0 is considered positive. The within-run and between-run precision on 5 patient pools (COI mean of 0.081, 1.0, 8.7, 24, and 54) varied from 0.8% to 3.3%, and from 1.2% to 3.6%, respectively. Sample storage complied with the conditions listed in the package insert.

This retrospective study has been conducted from May 6 to 12, 2020 at the clinical biology laboratory of the Clinique Saint-Luc Bouge (SLBO, Namur, Belgium). Serum samples (n=140) obtained from 97 patients with a confirmed RT-PCR SARS-CoV-2 diagnosis were used to determine the clinical sensitivity of the assay. RT-PCR on respiratory samples (NP swab samples) was performed on the LightCycler 480 Instrument II using the LightMix Modular SARS-CoV-2 E-gene set (Roche Diagnostics). Serum samples were subdivided into different categories based on the number of days after a positive RT-PCR test as follows: 0-6 days: 45 sera; 7-13 days: 35 sera; 14-

20 days: 24 sera; 21-27 days: 15 sera; 28 days or more: 21 sera. Among the 60 samples evaluated 14 to ≥28 days after the RT-PCR positive detection, and according to the manufacturer's cut-off, the Elecsys anti-SARS-CoV-2 assay identified 55 true positive and 5 false negative samples. The sensitivity was 91.7% (95% CI: 81.6-97.2%). The cut-off provided by the ROC curve analyses (*i.e.,* >0.165) improved the performance of the test with a sensitivity of 100% (95% CI: 94.0-100%) (**Figure I.II.1**).

		Time from RT-PCR +					Time from symptom onset				
		0-6d	7-13d	14-20d	21-27d	≥28d	0-6d	7-13d	14-20d	21-27d	≥28d
	n	45	35	24	15	21	22	28	26	23	30
COI ≥1.0	True positive	21	26	23	13	19	3	20	23	20	29
	False negative	24	9	1	2	2	19	8	3	3	1
	Sensitivity (%)	46.7	74.3	95.8	86.7	90.5	13.6	71.4	88.5	87.0	96.7
	95% CI	31.7-62.1	56.7-87.5	78.9-99.9	59.5-98.3	69.6-98.8	2.9-34.9	51.3-86.8	69.9-97.6	66.4-97.2	82.8-99.9
COI >0.165	True positive	22	30	24	15	21	5	24	24	21	30
	False negative	23	5	0	0	0	17	4	2	2	0
	Sensitivity (%)	48.9	85.7	100	100	100	22.7	85.7	92.1	91.3	100
	95%CI	33.7-64.2	69.7-95.2	85.8-100	78.2-100	83.9-100	7.8-45.4	67.3-96.0	74.9-99.1	72.0-98.9	88.4-100
	Specificity (%)			100					100		
	95%CI*			95.4-100					95.4-100		



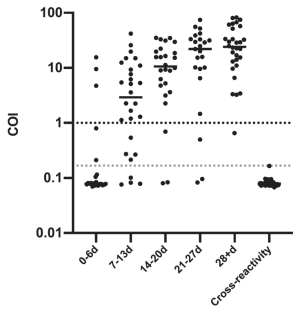


Figure I.II.1: *Clinical performance of the Elecsys anti-SARS-CoV-2 assay subdivided by time since RT-PCR positivity or the onset of symptoms. Cross-reactivity refers to the cross-reactivity test group. *Unaffected by the cutoff used (≥1.0 or >0.165). The dotted lines indicate the manufacturer's cutoff (in black) and the optimized cutoff (in gray).*

The sensitivity analysis was also performed considering the date of symptom~~s~~ onset. Among the 97 patients included, data about symptom~~s~~ onset was available for 92 patients and samples (n=129) were also subdivided into different categories according to the number of days after the onset of symptoms as follows: 0-6 days: 22 sera; 7-13 days: 28 sera; 14-20 days: 26 sera; 21-27 days: 23 sera; 28 days or more: 30 sera. Among the 79 samples evaluated 14 to ≥28 days after the onset of symptoms, and according to the manufacturer's cut-off, the Elecsys anti-SARS-CoV-2 assay identified 72 true positive and 7 false negative samples. The sensitivity was 91.1% (95% CI: 82.6-96.4%). The cut-off provided by the ROC curve analyses (*i.e.,* >0.165) improved the performance of the tests with a sensitivity of 95.1% (95% CI: 88.0-98.7%). Analyses of serum samples obtained 28 days or more after symptom~~s~~ onset provided a sensitivity of 96.7% (95% CI: 82.8-99.9%) and 100% (95% CI: 88.9-100%) with the manufacturer and the optimized cut-off, respectively (**Figure I.II.1.1**).

Considering samples obtained before 14 days (from RT-PCR + or symptom~~s~~ onset), sensitivities were not sufficient to be reliable in clinical practice (**Figure I.II.1.1**).

Non-SARS-CoV-2 sera (n=79) collected prior to the COVID-19 pandemic (between January 2019 and December 2019) with potential cross-reactivity were also analyzed. Samples included positive antinuclear antibodies (n=5), anti-thyroglobulin antibody (n=1), anti-treponema pallidum antibodies (n=2), antistreptolysin O (n=1), anti-TPO antibodies (n=4), chikungunya antibody (n=1), direct coombs (n=1), hepatitis B Ag (n=4), hepatitis C antibodies (n=7), hepatitis E antibodies (n=4), HIV antibodies (n=2), IgA chlamydia pneumoniae (n=1), IgG chlamydia trachomatis (n=1), IgG coxiella burneti (n=2), IgM borrelia (n=1), IgM coxiella burneti (n=1), IgM CMV (n=5), IgM EBV VCA (n=5), IgM mycoplasma pneumoniae (n=6), IgM Parvovirus B19 (n=7), IgM toxoplasma gondii (n=5), influenza antibodies (n=6), RAI (search for irregular agglutinins) (n=2), rheumatoid factor (n=5). The calculated specificity was 100% (95% CI: 95.44-100.0%). Using the ROC curve optimized cut-off (*i.e.,* >0.165) had no effect on the specificity performance (**Figure I.II.1.1**).

Thus, optimized cut-off showed excellent clinical performance from 14 days following RT-PCR positivity or since the onset of COVID-19 symptoms. Additional studies are needed to further confirm the optimal cut-off. Expert societies are also urged to provide guidance on when to perform serological investigations, e.g. since RT-PCR or symptom~~s~~ onset, since this is an important determinant of the true positivity rate.

I.II.2 Clinical Performance of Three Fully Automated Anti-SARS-CoV-2 Immunoassays Targeting the Nucleocapsid or Spike Proteins

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SUMMARY

Background: This study assesses the clinical performance of three anti-SARS-CoV-2 assays, namely EUROIMMUN anti-SARS-CoV-2 N (IgG) ELISA, Elecsys anti-SARS-CoV-2 N (total antibodies) assay, and LIAISON anti-SARS-CoV-2 S proteins S1 and S2 (IgG) assay.

Methods: One hundred and thirty-seven COVID-19 samples from 96 RT-PCR confirmed patients were used to perform the sensitivity analysis. Non-SARS-CoV-2 sera (n=141) with a potential cross-reaction to SARS-CoV-2 immunoassays were included in the specificity analysis.

Results: None of these tests demonstrated a sufficiently high clinical sensitivity to diagnose acute infection. Fourteen days since symptom onset, we did not reveal any significant difference between the three techniques in terms of sensitivities. However, the Elecsys performed better in terms of specificity.

Conclusion: All three anti-SARS-CoV-2 assays had equivalent sensitivities 14 days from symptom onset to diagnose past-COVID-19 infection. We also confirmed that anti-SARS-CoV-2 determination before day 14 is of less clinical interest.

Background

SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19), has led to significant morbidity and mortality (Fauci et al., 2020). The number of confirmed cases exceeds 7.8 million and the number of deaths worldwide stands at 431,541 deaths (WHO, 2020b).

The considered reference method for the diagnosis of SARS-CoV-2 infection is the RT-PCR) in respiratory samples (Vashist, 2020). However, the accuracy of the method depends on several factors including pre-analytical variables like sample type, collection, transport and storage (Lippi, Simundic, et al., 2020). The time since infection and the viral load are other factors affecting the sensitivity of the RT-PCR (Vashist, 2020). In addition, RT-PCR is not able to detect past infection (Winter & Hegde, 2020) and the throughput of RT-PCR is also limited because it requires a high

workload, skilled operators, expensive instrumentation, and crucial biosafety measures (Padoan, Cosma, et al., 2020). Access to RT-PCR tests remains limited in many countries worldwide while the virus is present in more than 150 countries.

The detection of anti-SARS-CoV-2 antibodies represents an additional method for the diagnosis of COVID-19, especially in patients who present late, with a low viral load (Farnsworth & Anderson, 2020). Detection of anti-SARS-CoV-2 antibodies is also useful to identify convalescent plasma donors and to screen the population to determine seroprevalence (Tre-Hardy, Blairon, et al., 2020; Zhao et al., 2020).

A wide range of serology immunoassays have therefore been developed to complement the RT-PCR, with different SARS-CoV-2 antigen targets and formats (Vashist, 2020). More than 100 manufacturers have notified that they are offering or plan to offer serological testing (FDA, 2020a). Due to the widespread dissemination of these methods and the limited experience with these new assays, it is essential for laboratories to independently validate these methods to assure they are in line with the expected analytical and clinical performance (Bohn, Lippi, et al., 2020; Diamandis et al., 2020; Farnsworth & Anderson, 2020; Kirkcaldy et al., 2020; Tre-Hardy, Wilmet, et al., 2020; Vashist, 2020; Winter & Hegde, 2020). This is also why some national authorities are planning broad validation campaigns to ensure they will offer the population with approved and controlled immunoassays which are cornerstone to fight this pandemic.

The aim of the present study is to assess and compare the clinical performance of three fully automated anti-SARS-CoV-2 immunoassays, namely: EUROIMMUN anti-SARS-CoV-2-N (IgG) ELISA, Elecsys anti-SARS-CoV-2-N (total antibodies) assay, and LIAISON anti-SARS-CoV-2 S proteins S1 and S2 (IgG) assay.

Material and methods

Study design

This retrospective study has been conducted from May 6 to 25, 2020 at the clinical biology laboratory of the Clinique Saint-Luc Bouge (SLBO, Namur, Belgium). A total of 137 serum samples were obtained from 96 COVID-19 patients confirmed positive to SARS-CoV-2 by RT-PCR. Antibody kinetics since the onset of symptoms was evaluated in the full cohort of patients. Non-SARS-CoV-2 sera (n=141) with a potential cross-reaction to SARS-CoV-2 immunoassays were included in the specificity analysis. Clinical performance were evaluated on three different platforms. Analytical performance is only reported for the newly available EUROIMMUN N assay as the analytical performance of the Elecsys anti-SARS-CoV-2-N (total antibodies) assay, and LIAISON anti-SARS-CoV-2 S proteins S1 and S2 (IgG) assay have already been reported elsewhere (Egger et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Tang et al., 2020; Tre-Hardy, Wilmet, et al., 2020).

Sample collection

Blood samples were collected from patients into serum-gel tubes (BD Vacutainer 8.5 mL tubes, Becton Dickinson, New Jersey, USA) or in lithium-heparin plasma tubes (BD Vacutainer 4.0 mL tubes) according to standardized operating procedures (SOP). The manufacturer recommendations authorize the use of these two matrices. Samples were centrifuged for 10 minutes at 1,885 × g (ACU Modular Pre Analytics, Roche Diagnostics). One hundred thirty-seven sera from 96 COVID-19 patients were collected from March 21 to May 25, 2020.

The study population displayed the following characteristics: 45 females and 51 males aged 24 to 93 years (mean age=63 years). Information on the days since the onset of symptoms was retrieved from medical records. Symptoms included fever, cough, fatigue, muscle aches, chest pain or pressure, difficulty breathing or shortness of breath, headache, sore throat, diarrhea, loss of taste, and loss of smell. Fever was the most frequent symptom (68.1%), followed by cough (60.4%), fatigue (58.2%), difficulty breathing (45.1%), and muscle aches (31.9%).

Non-SARS-CoV-2 sera with a potential cross-reaction to the SARS-CoV-2 immunoassay were collected before December 2019. Thirty-seven samples were kindly provided by the Department of Laboratory Medicine of Iris Hospitals South in Brussels. Samples have been stored in the laboratory serum biobank at -20°C. Frozen samples were thawed one hour at room temperature on the day of the analysis. Re-thawed samples were vortexed before the analysis.

Analytical procedures

Three anti-SARS-CoV-2 immunoassays were evaluated:

The anti-SARS-CoV-2-N ELISA (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) for the *in vitro* semiquantitative detection of IgG (also IgA and IgM, according to the insert kit of the manufacturer) to SARS-CoV-2 in human serum and plasma. All measurements were performed on the EUROIMMUN Analyzer I-2P. The result of a sample is given in the form of a ratio (extinction of patient sample/extinction of calibrator). According to the manufacturer, a ratio <0.80 is considered negative, a ratio ≥0.80 to <1.10 considered borderline, and a ratio ≥1.10 considered positive.

The Elecsys anti-SARS-CoV-2 N ECLIA (Roche Diagnostics, Basel, Switzerland) for the *in vitro* qualitative detection of total antibodies (including IgG) to SARS-CoV-2 in human serum and plasma. All measurements were performed on the cobas e801 module. The test result is given as a COI. According to the manufacturer, a result <1.00 is considered negative while a result ≥1.00 is considered positive [5].

The LIAISON SARS-CoV-2 S proteins S1/S2 assay (DiaSorin, Saluggia, Italy) for the *in vitro* quantitative detection of IgG to SARS-CoV-2 in human serum and plasma. All measurements were performed on the LIAISON-XL analyzer. The test result is given as AU/mL. According to the manufacturer, a result <12.0 is considered negative, a result ≥12.0 to <15.0 considered borderline, and a result ≥15.0 considered positive (Tre-Hardy, Wilmet, et al., 2020).

Only one calibration curve was done, and one batch of reagent was used for each of these platforms.

The RT-PCR for SARS-CoV-2 determination in respiratory samples (NP swab samples) was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV E-gene set.

Assessment of analytical performance (EUROIMMUN assay)

Precision

Precision was evaluated by using 2 pools of human and 2 internal quality controls provided by the manufacturer. Precision estimations were obtained by means of triplicates measurements of aliquots for a total of 5 consecutive days. Aliquots were stored at -20°C between analysis. Calculation was performed according to the Clinical and Laboratory Standards Institute (CLSI) EP15-A3 protocol.

Limit of blank, detection and quantification

The diluent provided by the manufacturer (diluent universal) was used as blank sample to determine the limit of blank (LOB), detection (LOD) and quantification (LOQ). The LOB has been determined by running the blank sample on three separate occasion to verify that the results are well <0.80. The LOD and LOQ have been determined by running 30 analyses of the blank sample using the following equations according the SH GTA 04 document – revision 1 of the COFRAC (COFRAC, 2020).

- LOD = mean of the 30 measurements + 3*standard deviation (SD)
- LOQ = mean of the 30 measurements + 10*SD

Linearity

Linearity was evaluated according to CLSI EP-06. A sample with a high total antibody level (*i.e.,* 9.52) was analyzed and diluted by a factor 2 on 5 consecutive dilutions. The manufacturer's diluent was used for the dilution. Observed values were compared to the expected ones and polynomial regression was calculated.

Carry-over evaluation

A sample with high IgG value (*i.e.,* 8.92) was run in triplicate (A1, A2, A3) and followed by a negative sample (*i.e.,* 0.10) also run in triplicate (B1, B2, B3). The carry-over

formula used is: (B1-B3)/(A3-B3) x 100. A carry-over below 1% is considered negligible.

Assessment of the clinical specificity

One hundred forty-one non-SARS-CoV-2 sera were analyzed for determining the cross-reactivity and established the specificity. Thirty-eight sera from COVID-19 negative healthy subjects and 103 sera from patients with a potential cross-reaction to the SARS-CoV-2 immunoassay were included in this study. Potential cross-reactive samples included positive antinuclear antibodies (n=5), anti-treponema pallidum antibodies (n=3), anti-thyroid peroxidase antibodies (n=3), antibodies RAI+ (search for irregular agglutinins) (n=5), chikungunya antibody (n=1), direct coombs (n=1), hepatitis B antigen (n=7), hepatitis C antibodies (n=7), hepatitis E antibodies (n=4), human immunodeficiency virus antibodies (n=2), IgA *Chlamydia pneumoniae* (n=1), IgM Borrelia + IgA *Helicobacter pylori* (n=1), IgM *Chlamydia pneumoniae* (n=1), IgG *Chlamydia trachomatis* (n=1), IgG *Coxiella burneti* (n=2), IgM *Coxiella burneti* (n=1), IgM cytomegalovirus (n=13), IgM Epstein-Barr virus viral capsid (n=5), IgM *Mycoplasma pneumoniae* (n=6), IgM parvovirus B19 (n=8), IgM *Toxoplasma gondii* (n=11), influenza A antibodies (n=4), influenza A and B (n=1), high level of total IgG (17.40 g/L) (normal range: 7.00-16.00 g/L) (n=1), both high levels of total IgM (5.26 g/L; normal range: 0.4-2.3 g/L) and total IgG (28.67 g/L) (n=1), rheumatoid factor (n=6), urinary infection with *Escherichia coli* (n=1), urinary infection with *Klebsiella oxytoca* (n=1). All these samples were collected before the COVID-19 pandemic and were stored at -20°C. The calculation of the specificity was stratified by excluding these cross-reactive samples from the pool of healthy subjects and combining the two cohorts.

Assessment of the clinical sensitivity

One hundred and thirty-seven sera obtained from 94 COVID-19 patients were analyzed to calculate the clinical sensitivity. Samples were subdivided according to the following different categories since symptom onset: 0-6 days: 23 sera; 7-13 days: 27 sera; 14-20 days: 24 sera; 21-27 days: 23 sera; 28 days or more: 40 sera. Clinical sensitivity for SARS-Cov-2 serological test depending on the onset of COVID-19 symptoms was carried out with the manufacturer's cut-off and with ROC curve adapted cut-offs.

Statistical analysis

Descriptive statistics were used to analyze the data. Sensitivity was defined as the proportion of correctly identified COVID-19 positive patients since symptom onset. Specificity was defined as the proportion of naïve patients or healthy volunteers classified as negative. The ROC AUC was calculated as the fraction of positive and negative determined according to the manufacturer's cut-off values for positive

results. Samples included for ROC curves analyses were sera obtained from at least two weeks after symptoms onset (n=87), sera selected to assess cross-reactivity (n=103) and sera from healthy volunteers (n=38). Data analysis was performed using GraphPad Prism software (version 8.2.1, California, USA) and MedCalc software (version 14.8.1, Ostend, Belgium). P value <0.05 was used as a significance level. Our study fulfilled the Ethical principles of the Declaration of Helsinki.

Results

Assessment of analytical performance (EUROIMMUN assay)

Repeatability and reproducibility results are summarized in **Supplementary materials**. Coefficients of variation (CV) are equal or lower to 7.6%. The LOB, detection and quantification were 0.033 ± 0.013, 0.072 and 0.164, respectively. For the linearity assessment, the regression equation was: $Y=3.3 + 1.7x - 0.12x^2$ with a coefficient of determination (R^2) of 0.99. Regarding the carry-over, the following ratios have been obtained for the different samples and the different runs: A1=8.92, A2=8.90, A3=9.19, B1=0.10, B2=0.11 and B3=0.10. The calculated carry-over was 0.0%.

Assessment of specificity

EUROIMMUN anti-SARS-CoV-2-N (IgG) ELISA

The calculated specificity was 96.5% (136/141) (95% CI: 91.9-98.8%) by using the manufacturer's cut-off (*i.e.,* ratio ≥0.80) and considering borderline results as false positive. The 5 false positive results were observed with 2 IgM CMV, one HIV antibodies, one hepatitis B Ag, and one in a healthy volunteer (respective ratios of 1.11, 1.35, 1.81, 0.82 and 0.96) using the manufacturer's cut-off. If considering borderline results as negative (n=2), the specificity increased to 97.9% (138/141) (95% CI: 93.9-99.6%). Using optimized cut-off (*i.e.,* ratio >0.40 COI), specificity was 94.3% (133/141) (95% CI: 89.1-97.5%). The calculated specificity was 97.4% (95% CI: 86.2-99.9%) and 96.1% (95% CI: 90.4-98.9%) for healthy volunteers and cross-reactive samples, respectively, by using the manufacturer's cut-off (**Table I.II.2.1**).

Elecsys anti-SARS-CoV-2-N (total antibodies) assay

The calculated specificity was 100% (141/141) (95% CI: 97.4-100%) by using the manufacturer's cut-off (*i.e.,* ≥1.00). Using optimized cut-off (*i.e.,* >0.165 COI) did not alter the specificity. The calculated specificity was 100% (95% CI: 90.8-100%) and 100% (95% CI: 96.5-100%) for healthy volunteers and cross-reactive samples, respectively, by using the manufacturer's cut-off (**Table I.II.2.1**).

LIAISON anti-SARS-CoV-2 spike proteins S1 and S2 (IgG) assay

The calculated specificity was 97.9% (138/141) (95% CI: 93.9-99.6%) by using the manufacturer's cut-off (*i.e.,* ≥12.0 AU/mL) and considering borderline results as false positive. The 3 false positive results were observed with one IgM *Toxoplasma gondii*, one IgM CMV and one with high level of total IgM (5.26 g/L) and high level of total IgG (28.67 g/L), with respective values of 32.0, 18.6 and 14.4 AU/mL) using the manufacturer's cut-off. If considering borderline results as negative (n=1), the specificity increased to 98.6% (139/141) (95% CI: 95.0-99.8%). Using optimized cut-off (ratio >3.94 AU/mL), specificity was 91.5% (129/141) (95% CI: 85.6-95.5%). The calculated specificity was 100% (95% CI: 90.8-100%) and 97.1% (95% CI: 91.7-99.4%) for healthy volunteers and cross-reactive samples, respectively, by using the manufacturer's cut-off (**Table I.II.2.1**).

Assessment of sensitivity

The calculated sensitivities classified according to different time categories since symptom onset are represented in **Table I.II.2.1**. Before 14 days since symptom onset, sensitivities (ranging from 70.4 to 85.2%) were not high enough to be reliably used in clinical practice, especially considering the LIAISON IgG assay.

EUROIMMUN anti-SARS-CoV-2-N (IgG) ELISA

After 2 weeks since symptom onset, the sensitivity was 90.8% (79/87) (95% CI: 82.7-96.0%) by using the cut-off provided by the manufacturer and considering borderline results (n=1) as positive. Using the optimized cut-off, the sensitivity (*i.e.,* ratio >0.40) was 95.4% (83/87) (95% CI: 86.6-98.7%) (**Figure I.II.2.1**). The sensitivity increased to 97.5% (39/40) (95% CI: 86.8-99.9%) from 28 days since symptom onset (**Table I.II.2.1**).

Elecsys anti-SARS-CoV-2-N (total antibodies) assay

After 2 weeks since symptom onset, the sensitivity was 92.0% (80/87) (95% CI: 84.1-96.7%) by using the cut-off provided by the manufacturer. Using the optimized cut-off, the sensitivity (*i.e.,* >0.165 COI) was 95.4% (83/87) (95% CI: 88.6-98.7%) (**Figure I.II.2.1**). The sensitivity increased to 100% (40/40) (95% CI: 91.2-100%) from 28 days since symptom onset (**Table I.II.2.1**).

LIAISON anti-SARS-CoV-2 spike proteins S1 and S2 (IgG) assay

After 2 weeks since symptom onset, the sensitivity was 88.5% (77/87) (95% CI: 79.9-94.4%) by using the cut-off provided by the manufacturer and considering borderline results (n=1) as positive. Using the optimized cut-off, the sensitivity (*i.e.,* >3.94 AU/mL) was 96.6% (84/87) (95% CI: 90.3-99.3%) (**Figure I.II.2.1**). The sensitivity increased to 97.5% (39/40) (95% CI: 86.8-99.9%) from 28 days since symptom onset (**Table I.II.2.1**).

			0-6d	7-13d	14-20d	21-27d	≥28d	Specificity (%) and 95% CI (combined, n=141)	Specificity (%) and 95% CI (HVs, n=38)	Specificity (%) and 95% CI (cross-reactivity, n=103)
		n	23	27	24	23	40			
EUROIMMUN, IgG*	Ratio ≥0.80	True positive	4	19	23	20	36			
		False negative	19	8	1	3	4	96.5 (91.9-98.8%)	97.4% (86.2-99.9%)	96.1% (90.4-98.9%)
		Sensitivity (%) and 95% CI	**17.4 (5.0-38.8%)**	**70.4 (49.8-86.3%)**	**95.8 (78.9-99.9%)**	**87.0 (66.4-97.2%)**	**90.0 (76.3-97.2%)**			
	Ratio >0.40	True positive	4	20	23	21	39			
		False negative	19	7	1	2	1	94.3 (89.1-97.5%)	NA	NA
		Sensitivity (%) and 95% CI	**17.4 (5.0-38.8%)**	**74.1 (53.7-88.9%)**	**95.8 (78.9-99.9%)**	**91.3 (72.0-98.9%)**	**97.5 (86.8-99.9%)**			
LIAISON, IgG	AU/mL ≥12.0	True positive	3	9	20	20	37			
		False negative	20	18	4	3	3	97.9 (93.9-99.6%)	100% (90.8-100%)	97.1% (91.7-99.4%)
		Sensitivity (%) and 95% CI	**13.0 (2.8-33.6%)**	**33.3 (16.5-54.0%)**	**83.3 (62.6-95.3%)**	**87.0 (66.4-97.2%)**	**92.5 (79.6-98.4%)**			
	AU/mL >3.94	True positive	6	15	23	22	39			
		False negative	17	12	1	1	1	91.5 (85.6-95.5%)	NA	NA
		Sensitivity (%) and 95% CI	**26.1 (10.2-48.4%)**	**55.6 (35.3-74.5%)**	**95.8 (78.9-99.9%)**	**95.7 (78.1-99.9%)**	**97.5 (86.8-99.9%)**			
Elecsys, total antibodies	COI ≥1.00	True positive	4	19	21	20	39			
		False negative	19	8	3	3	1	100 (97.4-100%)	100% (90.8-100%)	100% (96.5-100%)
		Sensitivity (%) and 95% CI	**17.4 (5.0-38.8%)**	**70.4 (49.8-86.3%)**	**87.5 (67.6-97.3%)**	**87.0 (66.4-97.2%)**	**97.5 (86.8-99.9%)**			
	COI >0.165	True positive	6	23	22	21	40			
		False negative	17	4	2	2	0	100 (97.4-100%)	NA	NA
		Sensitivity (%) and 95% CI	**26.1 (10.2-48.4%)**	**85.2 (66.3-95.8%)**	**91.7 (73.0-99.0%)**	**91.3 (72.0-98.9%)**	**100 (91.2-100%)**			

Table I.II.2.1: *Clinical performance of three anti-SARS-CoV-2 immunoassays since symptom onset with the manufacturer's cut-off and with optimized cut-offs. *The EUROIMMUN IgG assay is also sensitive to IgA and IgM, according to the insert kit of the manufacturer. HVs, healthy volunteers.*

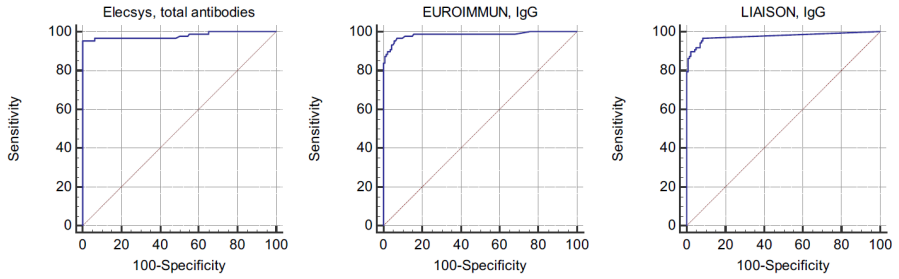

Figure I.II.2.1: *ROC curve analysis of three anti-SARS-CoV-2 immunoassays at more than 2 weeks after the symptom onset (n=87).*

Discussion

Serological testing is a useful strategy for the diagnosis, the characterization of the course of the disease, for identifying convalescent plasma donors as well as for epidemiological study, lockdown exit programs and COVID-19 vaccine development (Bohn, Lippi, et al., 2020; Farnsworth & Anderson, 2020; Long et al., 2020; Padoan, Cosma, et al., 2020; Winter & Hegde, 2020). To date, peer-reviewed data concerning the performance of SARS-CoV-2 immunoassays remained limited, but it is crucial for the society to be confident in the results of these assays. Therefore, independent validations of these methods before broad introduction into routine clinical practice is mandatory given the limited experience of the scientific community with these new assays (Bohn, Lippi, et al., 2020; Lippi, Salvagno, et al., 2020; Montesinos et al., 2020; Padoan, Cosma, et al., 2020; Tang et al., 2020; Tre-Hardy, Blairon, et al., 2020). We report here the external validation of the EUROIMMUN anti-SARS-CoV-2-N (IgG)

ELISA. Our results show satisfactory analytical performance. Repeatability and reproducibility studies determined on 2 different pools of sera from patients and 2 internal quality controls were ≤7.3% and ≤7.6%, respectively. The carry-over was negligible, and we found a LOQ of 0.164, which is lower than the optimized cut-off of 0.40 we found. Satisfactory analytical performance have also recently been reported for the Elecsys and LIAISON anti-SARS-CoV-2 assays and were not reassessed in this study (Egger et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Tang et al., 2020; Tre-Hardy, Wilmet, et al., 2020).

The Elecsys assay had a perfect specificity, considering both the manufacturer and the ROC curve adapted cut-off. The LIAISON assay had up to 3 false positive results and the EUROIMMUN assay had up to 5 false positive results. Tang *et al.* found a specificity of 98.7% on the Elecsys assay using 153 presumed negative specimens (Tang et al., 2020). Two false positive results from 2 patients with a negative RT-PCR results but with symptoms. Given that approximately 20% of the RT-PCR results might be falsely negative in COVID-19 patients (Bohn, Lippi, et al., 2020; Stowell & Guarner, 2020), the fact that Tang *et al.* considered these 2 patient results as false positive is questionable. For instance, Zhao *et al.* found that combining RT-PCR and antibody detection significantly improved the sensitivity of pathogenic diagnosis for COVID-19 (Zhao et al., 2020). In our study, only samples collected before the COVID-19 pandemic were included, excluding any confusion. Using a higher patient cohort of blood donors and intensive care unit (ICU) patients collected before the COVID-19 outbreak (n=456), Egger *et al.* only observed one false positive result on the Elecsys assay (Egger et al., 2020). Considering the LIAISON assay, Tré-Hardy *et al.* found a specificity of 100% and 99% using the manufacturer's cut-off or an adapted cut-off (*i.e.,* >6.1 AU/mL), respectively (Tre-Hardy, Wilmet, et al., 2020). We found lower specificities of 97.9% and 91.5% using the manufacturer's cut-off or our adapted cut-off (*i.e.,* >3.94 AU/mL). Plebani *et al.* found similar specificities of 96.8% and 88.9% using the manufacturer's cut-off or an adapted cut-off (*i.e.,* >6.2 AU/mL) (Plebani et al., 2020).

The higher specificity observed in the study of Tré-Hardy *et al.* (Tre-Hardy, Wilmet, et al., 2020) is probability due to the lower number of samples included (n=81) for the specificity calculation compared to our study (n=141) and the one of Plebani *et al.* (n=191) (Plebani et al., 2020). Interestingly, adapted cut-offs proposed on the LIAISON assay were all lower (>3.94, >6.1 AU/mL (Tre-Hardy, Wilmet, et al., 2020), >6.2 AU/mL (Plebani et al., 2020)) than the manufacturer's cut-off (*i.e.,* ≥12.0 AU/mL) using 3 independent cohorts of patients. The performance of these optimized cut-offs are not considered clinically different since there is an overlap between 95% CIs.

Current data suggest that seroconversion occurs approximately 7–14 days after symptom onset (Bohn, Lippi, et al., 2020; Padoan, Cosma, et al., 2020). While the

Elecsys and the EUROIMMUN assays detected more positive results earlier after onset of symptoms than the LIAISON assay, none of the assays demonstrated high enough clinical sensitivity to diagnose acute infection (*i.e.,* <14 days). From 14 days since symptom onset, sensitivities increased for all assays, especially using optimized cut-offs. Using manufacturer's cutoffs resulted in 8, 10, and 7 false negative specimens for the EUROIMMUN, LIAISON and Elecsys assays, respectively. Optimized cut-offs gave less false negative results (4, 3 and 4, respectively). Because of the overlapping of CIs at 95% between assays, we cannot conclude that one assay had a significantly higher true positivity rate.

Two studies having included less patients with symptoms since at least 14 days evaluated the performance of the Elecsys assay (Egger et al., 2020; Favresse, Eucher, Elsen, Laffineur, et al., 2020; Tang et al., 2020). Tang *et al.* found a sensitivity of 89.4% (n=47) (Tang et al., 2020) and Egger *et al.* a sensitivity of 100% (n=18). By using the manufacturer's cut-off, we found a somewhat similar sensitivity compared to Tang *et al.* (*i.e.,* 92.0%). However, they did not determine an optimized cut-off to increase the performance of the test. Fourteen days after RT-PCR positivity, Tré-Hardy *et al.* found a sensitivity of 91% and 100%, using the manufacturer's and an optimized cut-off on the LIAISON assay (Tre-Hardy, Wilmet, et al., 2020). It is important to note that the CI around 100% they found (92-100%) was consistent with our results (*i.e.,* 96.6% sensitivity; 95% CI: 90.3-99.3%). Plebani *et al.* published results in agreement with our finding with a sensitivity of 97.1% for the LIAISON assay (Plebani et al., 2020).

Conclusion

All three anti-SARS-CoV-2 assays had equivalent sensitivities 14 days from symptom onset to diagnose past-COVID-19 infection. We also confirmed that anti-SARS-CoV-2 determination before day 14 is of less clinical interest. However, the Elecsys assay had the higher specificity compared to the EUROIMMUN and the LIAISON assays. Further studies specifically designed to evaluate long-term evolution of antibody response are also needed.

I.II.3 An Original Multiplex Method to Assess Five Different SARS-CoV-2 Antibodies

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SUMMARY

Background: Accurate SARS-CoV-2 serological assays are urgently needed to help diagnose infection, determine past exposure of populations and assess the response to future vaccines. The study aims at assessing the performance of the multiplex D-tek COVIDOT 5 IgG assay for the detection of SARS-CoV-2 IgG antibodies (N, S1+S2, S1, S2 and RBD).

Methods: Sensitivity and dynamic trend to seropositivity were evaluated in 218 samples obtained from 46 rRT-PCR confirmed COVID-19 patients. Non-SARS-CoV-2 sera (n=118) collected before the COVID-19 pandemic with a potential cross-reaction to the SARS-CoV-2 immunoassay were included in the specificity analysis.

Results: A gradual dynamic trend since symptom onset was observed for all IgG antibodies. Sensitivities before day 14 were suboptimal. At ≥21 days, sensitivities reached 100% [93.4-100%] for N, S1+S2, S2 and RBD-directed IgG and 96.3% [87.3-99.6%] for S1-directed IgG. In 42 out of 46 patients (91.3%), all 5 antibodies were detected at ≥14 days. The four remaining patients had between 2 and 4 positive antibodies at their respective maximal follow-up period. The specificity was 100 % for S1+S2, S2 and RBD, 98.3% for N and 92.4% [86.0-96.5%] for S1-directed IgG. The combined use of antigens increases the early sensitivity whilst enforcing high specificity.

Conclusion: Sensitivities at ≥21 days and specificities were excellent, especially for N, S1+S2, S2 and RBD-directed IgG. Caution is however required when interpreting single S1-directed reactivities. Using a multiplex assay complies with the orthogonal testing algorithm of the CDC and allows a better and critical interpretation of the serological status of a patient.

Background

Th SARS-CoV-2 is responsible for the ongoing pandemic. As of the 15th of November, it has led to more than 53 million confirmed cases and to more than 1.3 millions deaths (WHO, 2020c).

Currently, the gold standard method for the diagnosis of COVID-19 is detection of SARS-CoV-2 RNA in NP samples through rRT-PCR, targets of which may include a combination of *N, E, RdRp, orf1a* and *orf1b* genes (Vashist, 2020). The detection of anti-SARS-CoV-2 antibodies represents an additional method for the diagnosis of COVID-19, especially in patients who present late, with a low viral load (Farnsworth & Anderson, 2020). The combination of rRT-PCR and antibody detections significantly improved the sensitivity of pathogenic diagnosis for COVID-19 (Zhao et al., 2020).

A wide range of serology immunoassays have therefore been developed to complement the rRT-PCR, with different SARS-CoV-2 antigen targets and formats (Jaaskelainen et al., 2020; Theel et al., 2020; Van Elslande, Decru, et al., 2020; Vashist, 2020). The main SARS-CoV-2 antigens used are the N and the S protein (S) (W. Liu et al., 2020; McAndrews et al., 2020; Premkumar et al., 2020; Van Elslande, Decru, et al., 2020). The N participates in RNA package and virus particle release. The transmembrane S glycoprotein comprises two functional subunits responsible for binding to the host cell receptor (N-terminal S1) and fusion of the viral and cellular membranes (C-terminal S2) (Lan et al., 2020; Walls et al., 2020). The RBD is located at the C-terminal region of the S1. Recombinant RBD has been shown to be sufficient to bind ACE2 (cell entry receptor) (Lan et al., 2020). Assays using the RBD have also been developed (GeurtsvanKessel et al., 2020; Horber et al., 2020).

The performance of these assays varied because of the choice of the antigen for a particular target, the nature and structure of the target itself (purified versus recombinant, full-length versus truncated, eukaryotic versus prokaryotic expression system), or the disparity of the patients cohorts (Farnsworth & Anderson, 2020; Lippi & Plebani, 2020; Vashist, 2020; Winter & Hegde, 2020). Furthermore, little is known about how antibody profiles across SARS-CoV-2 antigen specificities evolve early following infection and track differentially with disease trajectory. There is also a need for improvement of current serology immunoassays for detecting infection early after the symptom onset (Hachim et al., 2020).

The aim of this study is to report the performance of the multiplex COVIDOT 5 IgG assay for the detection of SARS-CoV-2 total antibodies.

Material and methods

Study design

This retrospective study has been conducted from June 4 to July 10, 2020, at the clinical biology laboratory of the University Hospital of Tivoli (CHU Tivoli, La Louvière, Belgium). Sera collected before the COVID-19 outbreak (between January and November 2019) were included in the specificity analysis (n=118). Case serum samples (n=218) with a confirmed rRT-PCR SARS-CoV-2 diagnosis were included in the sensitivity analysis and were obtained from 46 patients. Information on the days

since the onset of symptoms was collected from the medical records. Only patients with at least two longitudinal sera samples and with a follow-up of at least 14 days since symptoms onset were included.

Sample collection

Blood samples were collected from patients into serum-gel tubes (BD SST II Advance, Becton Dickinson, New Jersey, USA) or lithium-heparin plasma tubes (BD Vacutainer) according to SOP and manufacturer recommendations. Samples were centrifuged for 10 minutes at 1,740 × g (Sigma 3 – 16KL). Sera were stored in the laboratory serum biobank at -20°C from collection date. Frozen samples were thawed one hour at room temperature on the day of the analysis. Re-thawed samples were vortexed before the analysis.

Analytical procedures

The BlueDiver COVIDOT 5 IgG (D-tek sa, Mons, Belgium), a commercially available CE-marked enzyme immunoassay has been assessed for the *in vitro* semi-quantitative detection, in human sera or plasma, of IgG antibodies against SARS-COV-2 antigens. The assay is automated and performed on the BlueDiver Instrument. The BlueDiver COVIDOT 5 IgG is composed of 24 ready-to-use reagent cartridges and 24 multiplex strip tests that allow the simultaneous detection of antibodies targeting the 5 principal epitopes of the COVID-19: (1) the N protein, (2) the S protein (ectodomain, S1+S2), (3) the S1 of the S protein, (4) the S2 of the S protein and (5) the RBD of the S1. The test strips are made of a plastic backing covered with nitrocellulose on which the antigens are coated. A schematic representation of a COVIDOT-5 IgG test strip is presented in **Supplementary materials**. During the automated test procedure, the BlueDiver Instrument sequentially incubates the strips in the wells of ready-to-use reagent cartridges. The strips are incubated with diluted patients' sera (10µl of sample is required and diluted according to the instrument sequence using the diluent buffer provided in the kit). Enzyme activity, if present, leads to the development of purple dots on the membrane pads. The intensity of the coloration is directly proportional to the amount of antibody present in the sample. The Dr DOT software measures the color intensity by converting into Dr Dot AU (numeric values ranging from 0 [negative result] to 100 [high positive result]). Each strip contains the above-mentioned antigens plus 2 built-in controls (positive and negative). For each individual antigen, a result <5 AU is considered non-reactive (or negative), a result between 5 and 10 is considered doubtful, and a result >10 is considered reactive (or positive). A doubtful result was considered positive in our evaluation. The test is positive if at least 1 antibody is positive, whatever the antibody. The CV obtained with low and high positive samples ranged from 1.8% to 9.9%.

The rRT-PCR for SARS-CoV-2 determination in respiratory samples (NP swab samples) was performed with the GeneFinder COVID-19 Plus RealAmpl kit (Osang Healthcare Co., Ltd) or with the Xpert Xpress SARS-CoV-2 kit (Cepheid). A dipstick immunochromatographic test (COVID-19 Ag Respi-Strip, Coris BioConcept, Gembloux, Belgium) designed to detect SARS-CoV-2 antigen in NP secretions was also used for the inclusion of two patients. This RDT has been shown to be 100% specific compared to rRT-PCR (Scohy et al., 2020).

Clinical specificity

Non-SARS-CoV-2 sera (n=118) with a potential cross-reaction to the SARS-CoV-2 immunoassay were analyzed. Samples included positive antinuclear antibodies (n=6), hepatitis B Ag (n=13), hepatitis C antibodies (n=6), IgM cytomegalovirus (n=6), IgM Epstein-Barr virus viral capsid (n=14), IgM *Mycoplasma pneumoniae* (n=19), IgM polyclonal activation (n=1), IgM *Toxoplasma gondii* (n=4), IgG monoclonal components (n=2), rheumatoid factor (n=4), pregnant women (n=6), random sera (n=11) and dialyzed patients (n=26) were also included for the specificity calculation.

Clinical sensitivity

Sera of 46 patients obtained at different time points since the onset of COVID-19 symptoms were used to calculate the clinical sensitivity. The demographic data of this population are presented in **Table I.II.3.1** (**Table I.II.3.1**). From these 46 patients, a total of 218 serum samples were available. minimal follow-up of at least 14 days was required (Long et al., 2020; Zhao et al., 2020). Samples were subdivided according to the following categories: 0-6 days: 43 sera; 7-13 days: 59 sera; 14-20 days: 62 sera; ≥21 days: 54 sera. The maximal follow-up time was 53 days since symptom onset.

Demography	
Age (mean [min-max])	63.7 [38.0-93.0]
Males	n=28
Females	n=18
Delay between symptoms and PCR (median [min-max])	5 [0-14]
Number of blood sampling per patient (median [min-max])	4 [2-10]
Hospitalized (non ICU)	n=25
Hospitalized (ICU)	n=21

Table I.II.3.1: *Demographic data of the COVID-19 population included.*

Dynamic trend to seropositivity

The average dynamic trend to seropositivity was evaluated using all serum samples since symptoms onset. Samples were subdivided according to following categories:

0-6 days: 43 sera; 7-9 days: 24 sera; 10-12 days: 33 sera; 13-15 days: 42 sera; 16-21 days: 37 sera; >21 days: 39 sera.

Statistical analysis

Descriptive statistics were used to analyze the data. Sensitivity was defined as the proportion of correctly identified COVID-19 positive patients initially positive by rRT-PCR SARS-CoV-2 determination in respiratory samples and with COVID-19 symptoms. Specificity was defined as the proportion of naïve patients classified as negative. A ROC curve analysis was performed to determine the possibility of defining adapted cut-offs to improve clinical performance (den Hartog et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Plebani et al., 2020). Samples included for ROC curves analyses were sera obtained from at least 14 days since symptom onset (n=116) and sera from the specificity study (n=118) (**Supplementary materials**). A heatmap was used to visualize the evolution of the number of positive antibodies (from 0 to 5 different antibodies) since the onset of symptoms for each patient. Inter-rater agreement (Cohen's kappa) was also evaluated. Data analysis was performed using GraphPad Prism software (version 9.0.0, California, USA) and MedCalc software (version 14.8.1, Ostend, Belgium). The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the CHU Tivoli (approval number 1351).

Results

Clinical specificity

Using the manufacturer's cut-off (*i.e.,* AU >5), the specificity [95% CI] for the 5 different IgG antibodies varied between 92.4% [86.0-96.5%] and 100% [96.9-100%]. When antigens were considered separately, a specificity of 100% was observed for S2, S1+S2 and RBD-directed IgG and of 98.3% for N-directed IgG. The lowest specificity was observed with the S1-directed IgG (9 false positive results out of the 118 samples: 92.4% specificity). When all antigens were considered together, the cumulative specificity for the 5 antibodies was 90.7% [83.4-95.3%] or 98.3% [94.0-99.8%] if the contribution of S1-directed IgG was excluded. Using ROC curve adapted cut-offs increased the specificity for N (AU >7) and S1+S2-directed antibodies (AU >12) from 97.5% to 98.3% and from 98.3% to 100%, respectively (**Table I.II.3.2**). ROC curve adapted cut-offs were equal to the manufacturer's lower cut-off for other antigens (*i.e.,* AU >5). Noteworthy is the fact that all false positive samples were positive to only one antigen (**Figure I.II.3.1**).

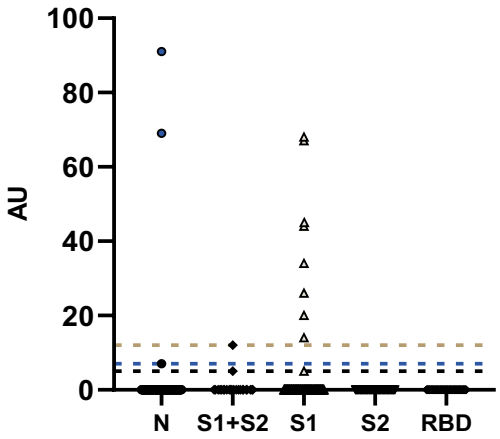


Figure I.II.3.1: *Cross-reactivity of the multiplex COVIDOT 5 IgG assay to non-SARS-CoV-2 sera (n=118). Manufacturer's threshold is represented with a black dotted line (AU=5). The blue dotted line corresponded to the ROC curve adapted cut-off for N-directed IgG and blue points are false positive results (patients having hepatitis C antibodies or IgM Toxoplasma gondii). The brown dotted line corresponded to the ROC curve adapted cut-off for S1+S1-directed IgG. Beige points are false positive results for S1-directed antibodies (patients under dialysis (n=3), or positives for IgM Toxoplasma gondii, hepatitis B Ag (n=2), IgM Mycoplasma pneumoniae (n=2) or antinuclear antibodies). Black points are considered true negative results.*

Clinical sensitivity

The sensitivity [95% CI] of each antibody during the first week since symptom onset (0-6 days) was low (<50%) and ranged from 11.6% [3.9-25.1%] for RBD-directed IgG to 37.9% [15.3-43.7%] for N-directed IgG. The sensitivity increased during the second week (7-13 days) to achieve at least 50% except for S1-directed IgG (40.7% [28.1-54.3%]). During the third week (14-20 days), the sensitivity of S1-directed IgG was still the lowest (71.0% (58.1-81.8%) while other antibodies had a sensitivity ranging from 83.9% [72.3-92.0%] for RBD to 91.9% [82.2-97.3%] for N-directed IgG. From 21 days since symptom onset, sensitivities reached 100% [93.4-100%] for all antibodies except for S1-directed IgG (96.3% [87.3-99.6%]) (**Table I.II.3.2**). Interestingly, a substantial between-individual variation was observed in the antibody response generated following SARS-CoV-2 infection, and patients exhibited various antibody signatures over the first 3 weeks after symptoms onset **(Figure I.II.3.2)**.

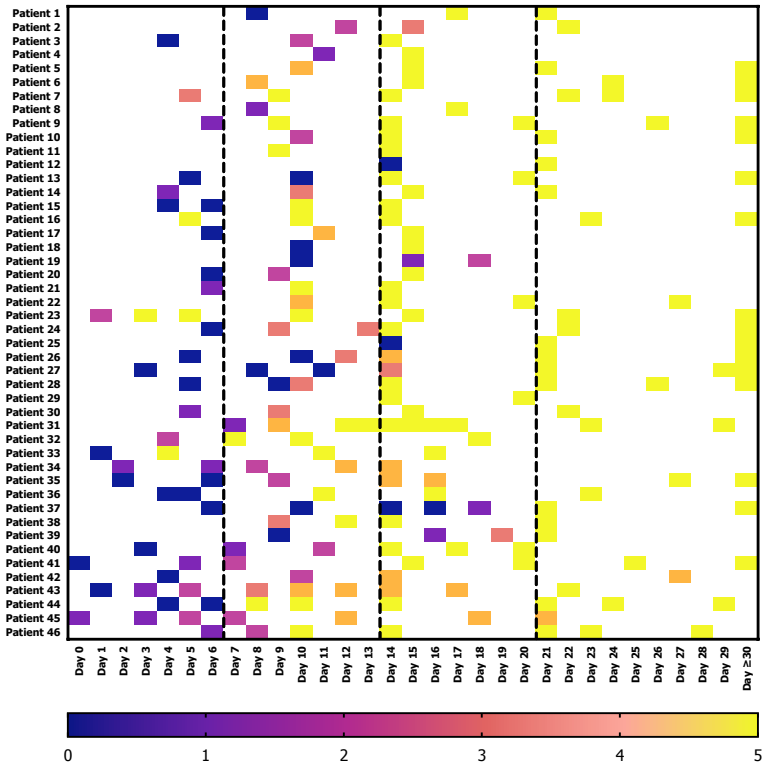


Figure I.II.3.2: *Heatmap of the dynamic trend to seropositivity for each patient.*

If considering that at least 1 antibody, whichever one out of 5, was sufficient to attest positivity, the sensitivity increased significantly, compared to any particular monoplex interpretation, to 46.5% [31.2-62.4%], 83.1% [71.0-91.6%] and 93.6% [84.3-98.1%] at weeks 1, 2 and 3, respectively. Excluding the particular anti-S1 reactivity, if at least one antibody out of N, S1+S2, S2 or RBD-directed IgG was required for positivity, the sensitivity was 37.2% [23.0-53.4%], 81.4% [69.1-90.3%] and 93.6% [84.3-98.1%] at weeks 1, 2 and 3, respectively. However, the 100% sensitivity was not reached significantly earlier with the multiplex algorithm for interpretation (at least 1 antibody positive) as compared to any antibody considered separately, since all antibodies (except S1-directed IgG) were positive in all tested samples from 3 weeks after symptoms (**Table I.II.3.2**). Using ROC curve adapted cut-offs did not have any impact on sensitivities from 14 days since symptom onset. The inter-rater agreement between the different antibodies varied from 0.48 (S1 versus S2; moderate agreement) to 0.82 (S1+S2 versus S2; almost perfect agreement) (**Table I.II.3.3**).

	n	N	S1+S2	S1	S2	RBD	≥1 antibody	≥1 antibody*
Sensitivity [95% CI]	218	75.2% [69.0-80.8]	69.3% [62.7-75.3]	58.3% [51.4-64.9]	75.7% [69.4-81.2]	65.6% [58.9-71.9]	83.0% [77.4-87.8]	77.9% [71.9-83.3]
Day 0-6	43	26.1% [14.3-41.1]	16.3% [6.8-30.7]	16.3% [6.8-30.7]	27.9% [15.3-43.7]	11.6% [3.9-25.1]	46.5% [31.2-62.4]	37.2% [21.0-50.9]
Day 7-13	59	67.8% [54.4-79.4]	52.5% [39.1-67.7]	40.7% [28.1-54.3]	74.6% [61.6-85.0]	54.2% [40.8-67.3]	83.1% [71.0-91.6]	81.4% [69.1-90.3]
Day 14-20	62	91.9% [82.2-97.3]	85.5% [74.2-93.1]	71.0% [58.1-81.8]	88.7% [78.1-95.3]	83.9% [72.3-92.0]	93.6% [84.3-98.1]	93.6% [84.3-98.1]
Day ≥21	54	100% [93.4-100]	100% [93.4-100]	100% [87.3-99.6]	100% [93.4-100]	100% [93.4-100]	100% [93.4-100]	100% [93.4-100]
Specificity [95% CI]	118	98.3% [94.0-99.8]	100% [96.9-100]	92.4% [86.0-96.5]	100% [96.9-100]	100% [96.9-100]	90.7% [83.4-95.3]	98.3% [94.0-99.8]

Table I.II.3.2: *Overall diagnostic performance of the different IgG assays taken separately or combined (with or without S1 antigen). ROC curve adapted cut-offs have been used for N and S1+S2-directed IgG (>7 and >12, respectively). The cut-off of 5 AU was used for S1, S2 and RDB-directed IgG. *Excluding the contribution of S1 antibodies.*

	S1+S2	S1	S2	RBD
N	0.71 [0.60-0.81]	0.50 [0.39-0.60]	0.68 [0.57-0.79]	0.70 [0.59-0.80]
S1+S2		0.63 [0.51-0.73]	0.82 [0.73-0.90]	0.81 [0.73-0.90]
S1			0.48 [0.37-0.58]	0.65 [0.56-0.75]
S2				0.65 [0.54-0.76]

Table I.II.3.3: *Inter-rater agreement (Cohen's kappa) between the different assays in COVID-19 patients (218 samples for sensitivity ([95% CI]).*

Dynamic trend to seropositivity

A gradual increase in antibody titers (AU) and positivity rates (%) since symptom onset was observed for all IgG antibodies. In 42 out of 46 patients (91.3%), the full spectrum of all 5 antibodies was detected at ≥14 days. Higher titers were observed throughout the antibody kinetics for N-directed IgG while lower titers were observed for S1-directed IgG (**Figure I.II.3.3**). A delayed increase in positivity rates was also observed in particular for S1-directed IgG (**Figure I.II.3.4**).

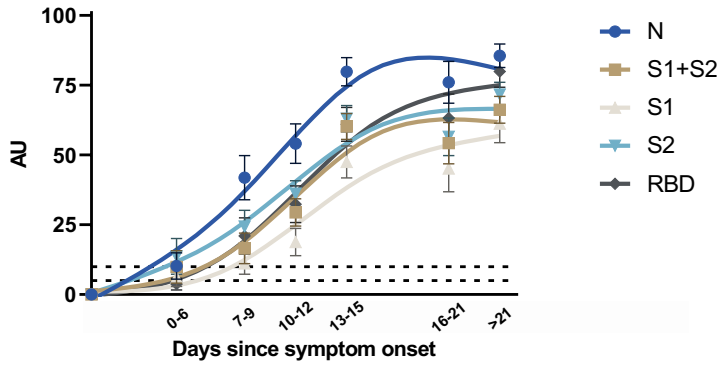
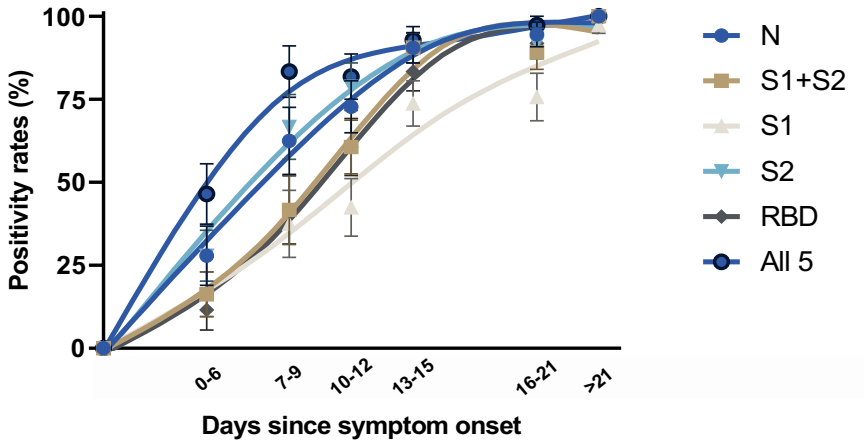


Figure I.II.3.3: *Dynamic trend of absolute signal for each IgG in 218 samples from 46 patients. Manufacturer's thresholds are represented with dotted lines (5 and 10 AU).*



	Days since symptom onset	0-6	7-9	10-12	13-15	16-21	≥21
	n	**43**	**24**	**33**	**42**	**37**	**39**
N	n positive (>7 AU)	12	15	24	38	35	39
	Positivity rate (%)	27.9	62.5	72.7	90.5	94.6	100
S1+S2	n positive (>12 AU)	7	10	20	38	33	39
	Positivity rate (%)	16.3	41.7	60.6	90.5	89.2	100
S1	n positive (≥5 AU)	7	9	14	31	28	38
	Positivity rate (%)	16.3	37.5	42.4	73.8	75.7	97.4
S2	n positive (≥5 AU)	12	16	26	38	34	39
	Positivity rate (%)	27.9	66.7	78.8	90.5	91.9	100
RBD	n positive (≥5 AU)	5	10	20	35	34	39
	Positivity rate (%)	11.6	41.7	60.6	83.3	91.9	100
≥ 1 antibody	n positive	20	20	27	39	36	39
	Positivity rate (%)	46.5	83.3	81.8	92.9	97.3	100

Figure I.II.3.4: *Dynamic trend to seropositivity for each IgG in 218 samples from 46 patients.*

Discussion

This study is the first to report the performance of the multiplex COVIDOT 5 IgG assay for the detection of 5 different SARS-CoV-2 IgG antibodies. Sensitivities at ≥21 days since symptom onset were excellent. However, sensitivities in the early phase of symptom onset were still too low to be used in clinical practice to confirm COVID-19 on the sole basis of serology testing, as confirmed in other studies (Bohn, Lippi, et al., 2020; Horber et al., 2020; Van Elslande, Decru, et al., 2020).

There is accumulative data about the clinical performance of SARS-CoV-2 immunoassays (Horber et al., 2020; Padoan, Cosma, et al., 2020; Pfluger et al., 2020; Plebani et al., 2020; Van Elslande, Decru, et al., 2020). These evaluations focused on the evaluation of mono-antigenic assays or, to a lesser extent, bi-antigenic assays. Currently, commercial serological assays are using the N antigen (Roche, Abbott, Euroimmun) (Favresse, Eucher, Elsen, Laffineur, et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Jaaskelainen et al., 2020; Plebani et al., 2020; Van Elslande, Decru, et al., 2020), the S1 antigen (Euroimmun) (Jaaskelainen et al., 2020; Tre-Hardy, Wilmet, et al., 2020), the S1+S2 antigen (DiaSorin) (Jaaskelainen et al., 2020; Plebani et al., 2020; Tre-Hardy, Wilmet, et al., 2020), the RBD antigen (Siemens, Wantai) (GeurtsvanKessel et al., 2020; Horber et al., 2020; Z. L. Liu et al., 2020) or the N and S antigens combined (iFlash, Maglumi, Mikrogen) (Plebani et al., 2020; Van Elslande, Decru, et al., 2020). Correlation between assays, using the same antigens or not, are often suboptimal and highlights a lack of harmonization for the detection of anti-SARS-CoV-2 antibodies (Fill Malfertheiner et al., 2020; Horber et al., 2020; Mairesse et al., 2020; Pfluger et al., 2020; Van Elslande, Decru, et al., 2020). Overall, serological assays are mostly using N- or S-proteins (Premkumar et al., 2020; Van Elslande, Decru, et al., 2020). However, the RBD antigen represents a promising antigen because it is poorly conserved between SARS-CoVs and other pathogenic human CoVs (Premkumar et al., 2020), and is therefore less likely to cross-react in serological antibody tests. Furthermore, a strong correlation between levels of RBD binding antibodies and SARS-CoV-2 NAbs in patients has been found (Premkumar et al., 2020).

So far, only very few studies have explored the possibility of using multiplex methods for SARS-CoV-2 antibodies (Ayouba et al., 2020; den Hartog et al., 2020; Johnson et al., 2020; van Tol et al., 2020) and different technologies have been used, namely Luminex-based assay (N and S antigens) (Ayouba et al., 2020), protein micro-array assay (N and S antigens) (van Tol et al., 2020), bead-based immune assay (S1, RBD and N antigens) (den Hartog et al., 2020), and solid-phase chemiluminescent assay (trimeric S, S1, RBD and N antigens) (Johnson et al., 2020). Noteworthy, the COVIDOT is the only multiplex assay that also target the S2 antigen.

There are several advantages and perspectives of using a multiplex method for the detection of anti-SARS-CoV-2 antibodies. We identified 4 different situations where it can be useful.

Advantage #1: Improving clinical sensitivity

In our cohort, 42 patients out of 46 (91.3%) developed antibodies for the 5 antigens ≥14 days since symptom onset. Four patients developed ≤4 antibodies at their respective maximal follow-up periods. The first patient had only 2 different antibodies (N and RBD-directed IgG). The second and the third had N, S2, S1+S2, and RBD-directed IgG and the last had N, S1, S2, and S1+S2-directed IgG (**Figure I.II.3.4**). If these patients had been analyzed with a mono-antigenic assay (against S1 or RBD), this would have classified these patients as negative. Patients who did not develop such antibodies have been described elsewhere (Fill Malfertheiner et al., 2020; Z. L. Liu et al., 2020).

The patient with only 2 antibodies 18 days since symptom onset is not likely to be a false positive result because no false positive has been observed for RBD in our specificity evaluation and because an increase in N titers has been observed between day 15 (AU=51) and 18 (AU=86). It is possible that patients only presented N and RBD and not S1-directed IgG. Additionally, the cumulative detection of multiple antibodies significantly improved the sensitivity in the early phase since symptom onset (**Table I.II.3.2**).

Advantage #2: Improving clinical specificity

Multiplex methods increase the overall specificity in a testing workflow (Ayouba et al., 2020; den Hartog et al., 2020), which is in line with the orthogonal testing algorithm proposed by the CDC (CDC, 2020). The CDC algorithm has been used for large serological studies, and false positive results could have been identified (Fischer et al., 2020; Havers et al., 2020; Stringhini et al., 2020). A recent study also found that orthogonal test strategies improved the clinical specificity because false positive results across 5 different platforms were assay-specific (Pfluger et al., 2020). Other reports also described assay-specific false positive results (Soleimani et al., 2020; Theel et al., 2020; Tre-Hardy, Wilmet, et al., 2020). Having 5 different targets in the same run and on the same platform might therefore be ideal to identify real false positive patients (Jaaskelainen et al., 2020; Van Elslande, Decru, et al., 2020). In our study, no pre-COVID-19 samples cross-reacted simultaneously with 2 different targets (**Figure I.II.3.1**) while positive patients mostly had the 5 different antibodies 14 days since symptom onset (**Figure I.II.3.2 and I.II.3.4**). Multiplex assays could therefore dispense the use of other analyses to confirm each positive patient and is relevant for seroprevalence studies. This approach might also improve the TAT and decrease overall costs in a routine testing workflow.

Advantage #3: Vaccination

Multiplex methods could face the future widespread of vaccines by measuring the vaccine response. Because most vaccines will use the S protein or S-domains as immunogen (Lee & McGeer, 2020), assays targeting the N-protein would therefore not be a good candidate to evaluate the vaccine response (Levinson, 2020). Having only assays targeting the S-protein might, however, be misleading in some situations. If a patient develops COVID-19-related symptoms following the vaccination, an assay with multiple targets might differentiate neo-COVID-19 infection from side effects due to the vaccination (*i.e.,* flu-like syndrome). The rise of N-directed antibodies, in addition to S-directed antibodies, following natural SARS-CoV-2 infection, might be usefully tested is this situation.

Advantage #4: Prediction of disease outcome

Recently, Atyeo *et al.* found that convalescent individuals developed a response mainly focused towards the S protein, whereas deceased individuals developed a response mainly focused on the N protein (Atyeo et al., 2020). RBD-specific responses were also more present in deceased individuals. The utilization of a multiplex method paves the way to define an antibody signature that differentiates disease trajectory and outcome. The clinical significance of variable antibody signatures, *i.e.,* the presence or the absence of multiple subtypes of antibodies against the different antigens of the virus, remains to be determined.

Our study has some limitations. Because of the retrospective design of our study, the number of samples per patient was not harmonized and the follow-up of some patients was longer compared to others. Nevertheless, we decided to only include patients with a minimal follow-up period of 14 days. We were also not able to compare the COVIDOT 5 IgG multiplex assay to mono-specific assays and to correlate our result with a neutralization assay. We also further highlight the need to evaluate the long-term kinetics of the different antibody responses. In this context, a multiplex method might also be of interest.

Conclusion

This study is the first to report the clinical performance of a multiplex assay for the simultaneous detection of 5 different SARS-CoV-2 antibodies. Sensitivities at ≥21 days or more since symptom onset were 100% for N, S1+2, S2 and RBD-directed IgG with specificities ranging from 98.3% to 100%. The specificity of S1-directed IgG was however moderate (92.4%) and may require some caution when interpreting single S1-directed reactivities. Having multiple antigen targets in one assay complies with the orthogonal testing algorithm of the CDC and allows a better and critical interpretation of the serological status of a patient. Research perspectives are also

promising especially in the field of vaccination or in predicting the disease trajectory and outcome.

I.II.4 Clinical Performance of the Panbio Assay for the Detection of SARS-CoV-2 IgM and IgG in COVID-19 Patients

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SUMMARY

Following the SARS-CoV-2 pandemic, numerous serological tests have been developed, including rapid diagnostic tests. This study aims at assessing the clinical performance of the Panbio IgG/IgM COVID-19 test (Abbott), a rapid LFA for the qualitative detection of IgG and IgM against SARS-CoV-2. One hundred and thirty-eight samples from 95 COVID-19 patients with a positive SARS-CoV-2 RT-PCR were analyzed to assess the clinical sensitivity. Seventy-six pre-COVID-19 samples were used to evaluate the clinical specificity. Two independent and blinded raters determined visually the presence or absence of the IgG, IgM and control lines for each test after 10 and 20 minutes. The sensitivity obtained with samples collected more than 14 days after the onset of symptoms was 95.2% for IgG. IgM were less frequently detected (highest sensitivity of 20.5%). The specificities obtained were 98.7% and 100% and for IgG and IgM respectively. In addition, the sensitivity of the assay was better when the reading was performed at 20 minutes than at 10 minutes, whereas the specificity was unchanged. The Panbio COVID-19 IgG/IgM rapid test presents high sensitivities for IgG 14 days since symptom onset but a low sensitivity for IgM. The specificity was excellent for both IgG and IgM.

Introduction

Rapid tests are designed for use where a preliminary screening test result is required and are especially useful in resource-limited countries or for broad screening campaign where access to blood sampling may be difficult or not obligatory. However, these tests have to be of high quality, user-friendly for use in resource-poor settings, quick and easy to perform and they have to require little or no additional equipment. In the context of COVID-19, all the above-mentioned criteria are of importance as serological tests may be useful for the diagnosis, for the characterization of the course of the disease, for identifying convalescent plasma donors directly on site, for lockdown exit programs, for epidemiological study and for the assessment of COVID-19 vaccine response (Winter & Hegde, 2020). Due to their widespread dissemination and the limited experience with these assays, it is crucial for laboratories to rigorously validate these methods before broad introduction into

routine clinical practice. This study aims at evaluating the clinical performance of the Panbio COVID-19 IgM/IgG rapid test (Abbott, Chicago, United States) in a population of COVID-19 patients.

Materials and methods

Sample collection

This study was conducted from June 16, 2020, to June 24, 2020. Blood samples were collected from patients into serum-gel tubes (BD Vacutainer 8.5 mL tubes, Becton Dickinson, New Jersey, USA) or lithium heparin plasma tubes (BD Vacutainer 4.0 mL tubes) according to SOP and manufacturer recommendations. Samples were centrifuged for 10 minutes at 1,885 to 2,500 × g (ACU Modular Pre Analytics, Roche Diagnostics). A total of 214 samples were collected from April, 2019 to May 25, 2020, and stored in the laboratory biobank at -20°C. Pre-COVID-19 samples (n=76) were all collected before March 2020, the start of the pandemic in Belgium. One hundred and thirty-eight samples from 95 COVID-19 patients were collected between March 21, 2020, and May 25, 2020. Frozen samples were thawed at room temperature. The study fulfilled the ethical principles of the Declaration of Helsinki.

Analytical procedures

The Panbio IgG/IgM COVID-19 rapid test (Abbott) is a rapid LFA for the qualitative detection of IgG and IgM directed against SARS-CoV-2 in human whole blood, serum or plasma specimens. The Panbio test was performed according to the manufacturer's instruction for use. Briefly, 10 µL of sample were applied into the specimen well and then two drops of buffer were applied. Raters determined visually the presence or absence of the IgG, IgM and control lines for each test 10 and 20 minutes after the addition of the buffer. As recommended by the manufacturer, even a slight colored striped was considered positive.

The RT-PCR for SARS-CoV-2 determination in respiratory samples (NP swab samples) was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV *E*-gene set.

Assessment of the clinical sensitivity

Samples (n=138) obtained from 95 patients with a confirmed RT-PCR SARS-CoV-2 diagnosis were assessed to determine the clinical sensitivity of the assay. Sensitivity was defined as the proportion of correctly identified COVID-19 positive patients since symptom onset. Antibody kinetics was evaluated using all samples dividing in different categories based on the number of days after the symptom onset, as follows: 0-2 days (n=15); 3-5 days (n=6); 6-8 days (n=14); 9-11 days (n=9): 12-14 days (n=11): 15-17 days (n=13); 18-21 days (n=13); 22-25 days (n=15); 26-31 days (n=13); 32-40 days (n=12) and more than 40 days (n=17).

Assessment of the clinical specificity

Non-SARS-CoV-2 samples (n=76) collected prior to the COVID-19 pandemic (between April and June 2019) with potential cross-reactions (n=38) were also analyzed to assess the specificity. Samples included positive antinuclear antibodies (n=4), anti-thyroglobulin antibody (n=1), anti-*Treponema pallidum* antibodies (n=1), anti-TPO antibodies (n=3), direct coombs (n=1), hepatitis B Ag (n=3), IgA *Chlamydia pneumoniae* (n=1), IgG *Chlamydia trachomatis* (n=1), IgM *Borrelia burgdorferi* (n=1), IgM Cytomegalovirus (n=4), IgM *Mycoplasma pneumoniae* (n=1), IgM Parvovirus B19 (n=1), IgM *Toxoplasma gondii* (n=6), IgG polyclonal activation (n=1), IgM and IgG polyclonal activation (n=1), search for irregular agglutinins (n=5), rheumatoid factor (n=1), urinary infection with *Escherichia coli* (n=1), urinary infection with *Klebsiella oxytoca* (n=1), and samples from 38 healthy volunteers were included for the specificity calculation. Specificity was defined as the proportion of naïve patients classified as negative.

Evaluation of reading conditions

Two independent and blinded raters determined visually the presence or absence of the IgG, IgM and control lines for each test after 10 and 20 minutes. In case of discrepancies, a third blinded and independent rater checked the presence or absence of the lines. Consensus results between all raters were used. The intra-rater (10 minutes versus 20 minutes) and the inter-rater (rater 1 versus rater 2) concordances were determined.

Statistical analysis

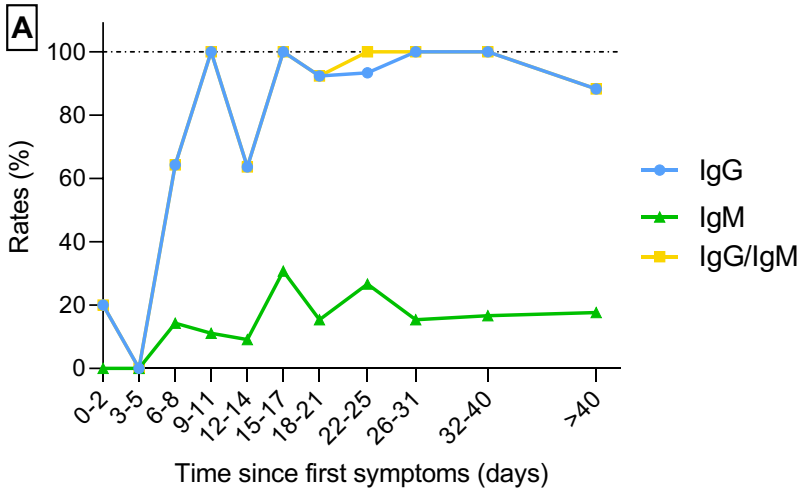
Data analysis was performed using GraphPad Prism software (version 8.2.1, California, USA) and MedCalc software (version 14.8.1, Ostend, Belgium). CIs for sensitivity and specificity were "exact" Clopper-Pearson CIs. The Cohen's kappa coefficient was used to assess the intra and inter-rater concordance.

Results

Clinical performance

All the tests (n=214) were valid (*i.e.,* the control line was visible). Kinetics of the sensitivity of the Panbio assay to detect IgG and IgM since the onset of the first symptoms is described in the **Figure 1 I.II.4.1** (**Figure I.II.4.1**). After 14 days since symptom onset, the Panbio assay detected IgG in 95.2% (95% CI 88.1-98.7%). Before 14 days since first symptoms, sensitivities were not high enough to be reliably used in clinical practice (50.9%, 95% CI 37.1-64.7%).

A



B

Days since first symptoms (number of samples)	0-2 (15)	3-5 (6)	6-8 (14)	9-11 (9)	12-14 (11)	15-17 (13)	18-21 (13)	22-25 (15)	26-31 (13)	32-40 (12)	>40 (17)
IgG positive (n)	3	0	9	9	7	13	12	14	13	12	15
IgG sensitivity (%)	20.0	0.0	64.3	100.0	63.6	100.0	92.3	93.3	100.0	100.0	88.2
IgG sensitivity 95% CI (%)	4.3 - 48.1	0.0 - 45.9	35.1 - 87.2	66.4 - 100.0	30.8 - 89.1	75.3 - 100.0	64.0 - 99.8	68.1 - 99.8	75.3 - 100.0	73.5 - 100.0	63.6 - 98.5
IgM positive (n)	0	0	2	1	1	4	2	4	2	2	3
IgM sensitivity (%)	0.0	0.0	14.3	11.1	9.1	30.8	15.4	26.7	15.4	16.7	17.7
IgM sensitivity 95% CI (%)	0.0 - 21.8	0.0 - 45.9	1.8 - 42.8	0.3 - 48.3	0.2 - 41.3	9.1 - 61.4	1.9 - 45.5	7.8 - 55.1	1.9 - 45.4	2.1 - 48.4	3.8 - 43.4
IgG/IgM positive (n)	3	0	9	9	7	13	12	15	13	12	15
IgG/IgM sensitivity (%)	20.0	0.0	64.3	100.0	63.6	100.0	92.3	100.0	100.0	100.0	88.2
IgG/IgM sensitivity 95% CI (%)	4.3 - 48.0	0.0 - 45.9	35.1 - 87.2	66.4 - 100.0	30.8 - 89.0	75.3 - 100.0	63.8 - 99.8	78.2 - 100.0	75.3 - 100.0	73.5 - 100.0	63.6 - 98.5

Figure I.II.4.1: *Kinetics of the sensitivity of the Panbio assay since the onset of symptoms. A. Kinetics of the sensitivity of the Panbio assay since the onset of first symptoms to detect IgG (blue dots), IgM (green triangle), and IgG and/or IgM (yellow squares). The result of each test was determined visually after 20 minutes by two independent and blinded operators. B. Sensitivities of the Panbio assay for IgG, IgM, and IgG and/or IgM since the onset of first symptoms.*

IgM were less frequently detected by the Panbio assay, with sensitivities of 7.3% (95% CI 2.0-17.6%) and 20.5% (95% CI 12.4-30.8%) for samples the first 14 days and for those obtained more than 14 days since symptom onset respectively. The highest sensitivity for IgM obtained in a particular category based on the number of days after the symptom onset was 30.8% (95% CI 9.1-61.4%) (**Figure I.II.4.1**).

Only one sample was positive for IgM and negative for IgG. This sample was collected 22 days after the first symptoms. The sensitivity of the Panbio assay to detect IgM and/or IgG within the first 14 days since symptom onset was unchanged compared to the sensitivity to detect IgG (50.9%; 95% CI 37.1-64.7%). After 14 days since symptom onset, the Panbio assay detected IgG and/or IgM in 96.4% (95% CI 89.8-99.3%) of samples.

Among the 76 samples collected before the COVID-19 pandemic, only one sample from a healthy volunteer gave a false positive result with IgG. Samples with potential

cross-reaction gave no false positive result. The specificity was 98.7% (95% CI 92.9-100.0%) and 100% for IgG and IgM respectively.

Evaluation of reading conditions

The inter-rater variability was excellent when the tests were read at 10 minutes and 20 minutes for both IgG (Cohen's kappa coefficient at 10 minutes and 20 minutes were 0.972 and 0.991 respectively) and IgM (Cohen's kappa coefficient at 10 minutes and 20 minutes were 0.945 and 0.974). In addition, the sensitivity of the assay was better when the reading was performed at 20 minutes than at 10 minutes (**Table I.II.4.1**), whereas the specificity was unchanged. Cohen's kappa coefficients for the different time of reading were lower for IgM than IgG, indicating that the time of reading influence more IgM results than IgG (**Table I.II.4.1**). The positive lines (IgM and IgG) read at 10 minutes were always positive at 20 minutes.

	A. Number of samples read positive for IgG/total number of samples		
	Reading after 10 min	**Reading after 20 min**	
Rater 1	105/138 (76.1%)	106/138 (76.8%)	0.991
Rater 2	106/138 (76.8%)	107/138 (77.5%)	0.991
κ coefficient between raters	0.972	0.991	
	B. Number of samples read positive for IgM/total number of samples		
	Reading after 10 min	**Reading after 20 min**	
Rater 1	21/138 (15.2%)	22/138 (15.9%)	0.922
Rater 2	19/138 (13.8%)	21/138 (15.2%)	0.945
κ coefficient between raters	0.945	0.974	

Table I.II.4.1: *Evaluation of the impact of the rater and the time of reading on the IgG (A) and IgM (B) test results.*

Discussion

The detection of anti-SARS-CoV-2 antibodies represents an additional method for the diagnosis of COVID-19 which may significantly improve the sensitivity of pathogenic diagnosis for COVID-19 when combined with RT-PCR (Pan et al., 2020). A wide range of assays have been developed including ELISA, CLIA, ECLIA and rapid tests (Favresse, Eucher, Elsen, Laffineur, et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Jaaskelainen et al., 2020; Mairesse et al., 2020; Montesinos et al., 2020). The main advantage of rapid diagnostic tests is that they do not require specific equipment and are easy to use. Furthermore, these tests are rapid, and they can be easily implemented in a low-resource laboratory.

The WHO encourages laboratories to perform independent assay validation, in particular regarding the clinical utilization of rapid device (WHO, 2020a). Based on the conclusions of the study of the Frederick National Laboratory for Cancer

Research, a Federally Funded Research and Development Center sponsored by the National Cancer Institute, the FDA concluded that a list of 65 serological assays should not be distributed (FDA, 2020b). External validations of these tests are therefore paramount, and a plenty of data are arriving in the literature (Bernasconi et al., 2020; Favresse, Eucher, Elsen, Laffineur, et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Jaaskelainen et al., 2020; Kohmer et al., 2020; Mairesse et al., 2020; Montesinos et al., 2020; Ong et al., 2020; Van Elslande, Houben, et al., 2020; Wu et al., 2020). Given the leading position of Abbott for COVID-19 testing, independent external validation of their assays is mandatory to ensure the performance are in line with their claims.

In our evaluation, the sensitivity obtained for all samples collected more than 14 days after the onset of symptoms was 95.2% for IgG. The Panbio assay showed weak sensitivity for IgM (**Figure I.II.4.1**). The specificities obtained were 98.7% and 100% and for IgG and IgM respectively. In the instructions for use, Abbott Diagnostics mentioned a sensitivity and a specificity of 95.8% and 94.0%, respectively. In the manufacturer's study, 48 samples of RT-PCR confirmed patients and 50 pre-COVID-19 samples were analyzed. Taken apart, IgG had a sensitivity and a specificity of 95.8% and 100% and IgM a sensitivity and a specificity of 56.3% and 94%. Our results are in agreement with these claims and we even obtained a better specificity for IgM although the sensitivity was lower than claimed. However, in the information provided by the manufacturer, the details of the studied populations were lacking, *i.e.,* timing between symptom onset or since RT-PCR positivity and the blood sampling as well as the characteristics of samples included for specificity calculation.

As observed on other assays and platforms, *i.e.,* LFA, ELISA, CLIA, ECLIA (Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Tang et al., 2020; Van Elslande, Houben, et al., 2020), we found that sensitivities before 14 days since symptom onset were not sufficient to be reliably used in clinical practice. We therefore recommend obtaining a control or confirmatory sample after 14 days to increase the detection rate of possible past-COVID-19 infection.

Comparing the clinical performance of these rapid tests is hazardous. Indeed, the design of studies vary widely across studies, *i.e.,* number of positive and negative samples, the definition of negative samples, number of days since symptoms or since RT-PCR positivity, comparison to a neutralization test. Some studies included only a very limited number of patients (Kohmer et al., 2020), included control samples collected during the pandemic period (Bernasconi et al., 2020; Montesinos et al., 2020), defined different categories since symptom onset (*i.e.,* < or >7 days (Ong et al., 2020), 0-6, 7-13, 14-25 days (Van Elslande, Houben, et al., 2020), or 5-9, 10-18 days (Kohmer et al., 2020)), or different categories since RT-PCR positivity (Kohmer et al., 2020). Moreover, as with other rapid LFA (Favresse, Gillot, Oliveira, et al., 2021),

we showed that the result may depend on the reader and on the timing of reading (20 minutes better than 10 minutes). The utilization of an automated reader may be useful to decrease the inter-individual variation, especially when the colored stripe appears very thin.

Conclusions

The Panbio COVID-19 IgM/IgG rapid test presents high sensitivities for IgG 14 days since symptom onset but a very low sensitivity for IgM. The specificity was excellent for both IgG and IgM. Further investigations designed to evaluate the clinical performance of Panbio over a longer period of time is needed to further consider its use in seroprevalence studies.

I.III. Long-term kinetics

I.III.1 Long-term Kinetics of Anti-SARS-CoV-2 Antibodies in a Cohort of 197 Hospitalized and Non-hospitalized COVID-19 Patients

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The quite recent emergence of the SARS-CoV-2 pandemic precludes long-term investigations of the immunologic response towards this new pathogen. Depending on the pathogen, serological persistence has been shown to last for months to years, as for SARS-CoV or other human CoV (Huang et al., 2020). Antibody responses to SARS-CoV-2 can be detected in most infected individuals 14 days after the symptom onset (Bohn, Loh, et al., 2020; Favresse, Cadrobbi, et al., 2021; Favresse, Eucher, Elsen, Laffineur, et al., 2020). Recent reports are inconsistent regarding the persistence of antibodies directed against SARS-CoV-2 (Ibarrondo et al., 2020; Wajnberg et al., 2020). These differences may be explained by multiple reasons but are more probably related to methodological issues than real different immunogenic effects. The aim of this study was to evaluate the long-term kinetics of anti-SARS-CoV-2 antibodies in a population of RT-PCR confirmed positive SARS-CoV-2 subjects and to describe the kinetics of antibodies in hospitalized patients compared to the one of non-hospitalized patients, including asymptomatic individuals.

A total of 197 patients with a confirmed SARS-CoV-2 RT-PCR were retrospectively included from March 21 to October 27, 2020. Demographics of patient participants are present in **Supplementary materials**. A total of 314 serum samples was analyzed for the detection of anti-SARS-CoV-2 antibodies. The WHO clinical progression scale was used to categorize patients according to disease severity (score 1=asymptomatic, non-hospitalized; score 2-3=mild disease, non-hospitalized; score >3=moderate-severe disease, hospitalized) (Characterisation & Management of, 2020). Information of symptom onset was gathered in clinical files of patients and/or by contacting the medical practitioners. Blood samples were collected into serum-gel or in lithium-heparin plasma tubes (BD Vacutainer tubes, Becton Dickinson, New Jersey, USA) according to SOP. Samples were centrifuged for 10 minutes at 1,885 × g (ACU Modular Pre-Analytics, Roche Diagnostics). The Elecsys anti-SARS-CoV-2 N ECLIA (Cobas e801, Roche Diagnostics, Basel, Switzerland) for the *in vitro* qualitative detection of total antibodies (IgG, IgM and IgA) to SARS-CoV-2 was used. The test result is given as a COI. According to the manufacturer, a result <1.0 is considered negative while a result ≥1.0 is considered positive. An optimized cut-off of 0.165 was

also considered based on our previous validation (Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020) which has been confirmed by a recent study performed by the National SARS-CoV-2 Serology Assay Evaluation Group (*i.e.,* 0.128) (National, 2020). The specificity of the test was excellent in several independent evaluations (99.8-100%) (Egger et al., 2020; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Jahrsdorfer et al., 2021; National, 2020). The RT-PCR for SARS-CoV-2 determination in respiratory samples (NP swab samples) was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV E-gene set.

Samples were subdivided according to the following categories since symptom onset, 0-1 week: 44 sera; 1-2 weeks: 30 sera; 2-4 weeks: 60 sera; 4-6 weeks: 18 sera; 6-11 weeks days: 47 sera; 11-17 weeks: 57 sera; 17-26 weeks: 34 sera and 26-32 weeks: 24 sera. The antibody kinetics was determined separately for hospitalized and non-hospitalized patients. In asymptomatic patients, the day of RT-PCR positivity was used instead of the day of symptom onset. A kinetics for patients who had at least 3 blood samplings with a last collection time at more than 7 weeks since symptom onset was also evaluated separately (10 non-hospitalized and 11 hospitalized patients).

Descriptive statistics were used to analyze the data. The mean COI results (and 95% CI) were plotted against the different timeframes. Sensitivity was defined as the proportion of correctly identified COVID-19 positive patients initially positive by RT-PCR SARS-CoV-2 determination. Smoothing splines with four knots were used to estimate the time kinetics curve in hospitalized (WHO score >3) and non-hospitalized patients (WHO score 2-3). Dunn's multiple comparisons test was used to assess potential differences between sampling timings. Data analysis was performed using GraphPad Prism software (version 9.0.0, California, USA). P value <0.05 was used as a significance level. The study fulfilled the Ethical principles of the Declaration of Helsinki.

In symptomatic patients, a gradual increase in antibody titers up to the last timepoint was observed (**Figure I.III.1.1**). We confirm that sampling before 2 weeks does not permit to identify previous or ongoing infection due to insufficient sensitivity. However, within the first week, the positivity trend was higher in hospitalized patients (*i.e.,* 50%) compared to non-hospitalized patients (*i.e.,* 20%), an observation already made by Long *et al.* (Long et al., 2020) and by Gillot *et al.* (Gillot et al., 2020). From 4 to 6 weeks, excellent sensitivities were observed (**Table I.III.1.1**, **Figure I.III.1.1**). Individual results for hospitalized patients were largely above the manufacturer's cut-off. In non-hospitalized patients, one asymptomatic subject did not developed antibodies against the N (**Figure I.III.1.1a**).

	Weeks since symptoms	0-1	1-2	2-4	4-6	6-11	11-17	17-26	26-32
Non-hospitalized WHO score 2-3 (+1)	n	14 (+6)	6 (+4)	23 (+2)	13 (+0)	31 (+7)	31 (+8)	24 (+4)	17 (+1)
	Mean (95% CI)	1.0 (-0.4-2.4)	7.7 (0-15.5)	22.4 (14.8-30.0)	41.4 (28.7-54.1)	49.1 (33.4-64.7)	70.0 (51.5-88.5)	80.0 (60.4-99.5)	84.8 (44.5-125.3)
	Sensitivity (%) (95% CI)	20.0 (5.7-43.7)	90.0 (55.5-99.9)	96.0 (79.7-99.9)	100 (75.3-100)	100 (90.8-100)	97.4 (86.4-99.9)	100 (87.7-100)	100 (81.5-100)
Hospitalized WHO score >3	n	24	20	35	5	9	18	6	6
	Mean (95% CI)	6.7 (-1.6-14.9)	9.0 (3.3-14.6)	22.1 (14.4-30.0)	24.0 (-11.8-59.9)	68.5 (46.0-90.9)	84.7 (71.7-97.7)	117.6 (70.5-164.7)	131.2 (80.9-181.4)
	Sensitivity (%) (95% CI)	50.0 (29.2-70.1)	85.0 (62.1-96.8)	94.3 (80.8-99.3)	100 (47.8-100)	100 (66.4-100)	100 (81.5-100)	100 (54.1-100)	100 (54.1-100)

Table I.III.1.1: *Anti-SARS-CoV-2 titers (mean COI and 95% CI) from symptom onset in hospitalized and non-hospitalized COVID-19 patients. Numbers between brackets correspond to asymptomatic patients (WHO score 1). The cut-off used to calculate sensitivity was 0.165.*

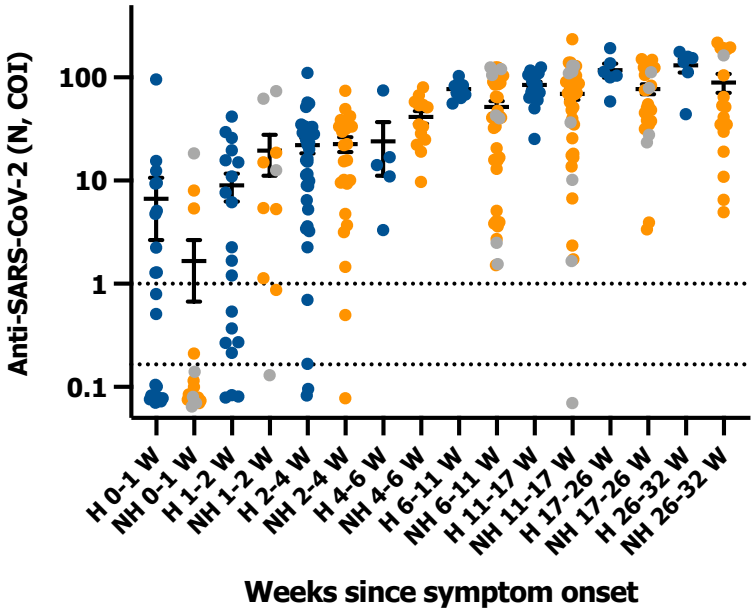

Figure I.III.1.1a: *Anti-SARS-CoV-2 titers (mean COI and 95% CI) from symptom onset in hospitalized (blue points) and non-hospitalized (orange points) COVID-19 patients (timeframe in weeks). Grey points correspond to asymptomatic patients who had a positive RT-PCR.*

A trend towards higher antibody titers in hospitalized patients was also observed from weeks 6 to 11. The difference was higher if considering weeks 17 to 32 (**Figure I.III.1.1b**). Other studies also reported higher levels of antibodies in patients with more severe disease (Figueiredo-Campos et al., 2020; Gudbjartsson et al., 2020; Long et al., 2020; Seow et al., 2020). Of the 21 patients for which at least three

independent blood samples were available for a minimal follow-up period of 7 weeks, a decrease in antibody titer was observed for 4 non-hospitalized patients out 10 (40%). In hospitalized patients, the titer gradually increased to reach a plateau without any decrease (n=11; 100%) (**Supplementary materials**). Nevertheless, the association was not found to be significant in our study (p>0.05).

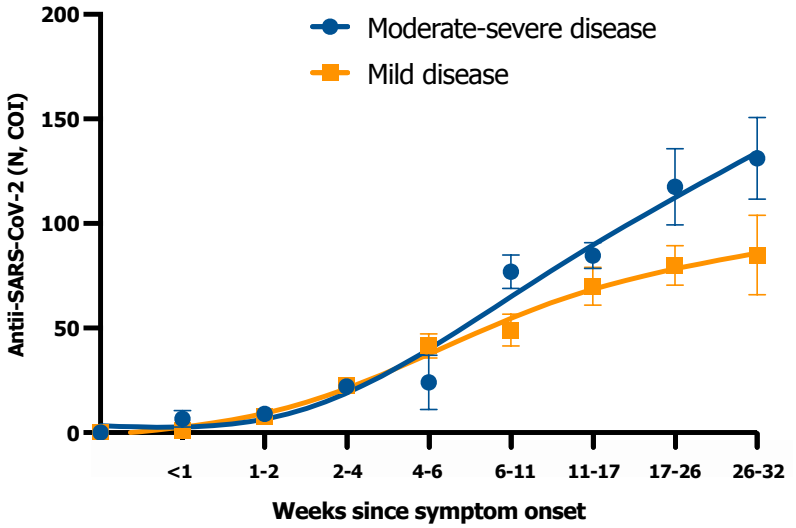

Figure I.III.1.1b: *Long-term kinetics of anti-SARS-CoV-2 in hospitalized (blue points) and non-hospitalized (orange points) COVID-19 patients (timeframe in weeks). Smoothing splines with four knots were used to estimate the time kinetics curve (mean standard ± error of the mean). Asymptomatic patients were excluded from the analysis.*

Importantly, the antibody kinetics may vary according to the type of assay considered. Recent studies are in line with the current results and also found a sustained antibody response against the N antigen using the Roche total antibody assay, on a lower follow-up period (*i.e.,* 3 to 4 months) (Gudbjartsson et al., 2020; Muecksch et al., 2020). A sustained antibody response against the RBD antigen, as assessed by the Wantai and the Siemens total antibody assays, was also observed up to 4 months (Gudbjartsson et al., 2020; Muecksch et al., 2020). A decrease in anti-RDB IgG and anti-S IgG levels was similarly observed over a period of up to 5 months in recent reports (Figueiredo-Campos et al., 2020; Gudbjartsson et al., 2020; Ibarrondo et al., 2020; Isho et al., 2020; Iyer et al., 2020). A significant decrease in sensitivity was also found using the Abbott assay (N IgG), in studies with up to 4 months of follow-up (Gudbjartsson et al., 2020; Muecksch et al., 2020).

The sustained antibody response as measured with total antibody assays (N and RBD) compared to IgG assays may be due to the additional response of non-IgG antibody isotypes. The reasons for the differences in assay performance over time for assays targeting the same antigen remain however unclear (Muecksch et al., 2020).

Whether the antibodies measured with commercial assays have a neutralizing capacity is paramount to indicate the potential level of protective immunity against SARS-CoV-2 infection. Antibody titers generated with available assays correlated differently with NAb titers (Brigger et al., 2021; Muecksch et al., 2020; Padoan, Bonfante, Pagliari, et al., 2020). The Roche assay was the weaker predictor of neutralizing capacity (r=0.56, p=0.0001) compared to the Abbott assay (N IgG) (r=0.69, p<0.0001), Siemens assay (RDB total antibodies) (r=0.74, p<0.001), and the S1/S2-based DiaSorin assay (S1/S2 IgG) (r=0.84, p<0.0001) (Muecksch et al., 2020). Jahrsdörfer *et al.* and Padoan *et al.* confirm that the weaker correlation was observed using the Roche assay (Jahrsdorfer et al., 2021; Padoan, Bonfante, Pagliari, et al., 2020), and McAndrews *et al.* found that 86% of individuals positive for RBD-directed antibodies exhibited neutralizing capacity, whereas only 76% of positive N-directed antibodies exhibited neutralizing capacity (McAndrews et al., 2020). The fact that anti-N assays have the lowest neutralizing capacity could be expected, as NAbs are directed to the S protein responsible for enabling cell entry. Indeed, a strong correlation between levels of anti-RBD or anti-S antibodies and neutralizing capacity has been found in independent evaluations (Figueiredo-Campos et al., 2020; Ibarrondo et al., 2020; Iyer et al., 2020; Premkumar et al., 2020; Wajnberg et al., 2020). Neutralizing capacity remained robust from 1 to 5 months in several studies (Brigger et al., 2021; Figueiredo-Campos et al., 2020; Iyer et al., 2020), although modest declines at 3 to 5 months were observed by Wajnberg *et al.* and *Isho* et al. (Isho et al., 2020; Wajnberg et al., 2020). Other studies however observed a significant decrease of 2 to 4-fold, in neutralizing capacity up to 3 months (Crawford et al., 2021; Muecksch et al., 2020; Prevost et al., 2020; Seow et al., 2020; Wang et al., 2021).

It is important to keep in mind that some patients may develop anti-S or anti-RBD antibodies but may not have detectable NAbs. These are only correlation studies which are not related to direct measures of neutralizing capacity (Muecksch et al., 2020). The fact that NAbs constitute a major protective mechanism against SARS-CoV-2 infection deserves that further investigation are done in this area to assess to long-term inhibition capacity of SARS-CoV-2 antibodies (Muecksch et al., 2020; National, 2020; Wajnberg et al., 2020). The contribution of B cells and T cells to immunity to SARS-CoV-2 should also be more explored and it seems important to remind that previous exposure to SARS-CoV-2 might not guarantee total immunity in all cases since reinfection with SARS-CoV-2 have been described (Tillett et al., 2021; To, Hung, Ip, et al., 2021).

In conclusion, we found a gradual increase in anti-N total antibody titers for up to 32 weeks since symptom onset. Even if some non-hospitalized patients showed a slight tendency towards a decrease of their antibody titer, this study found that detection

rates were similar in hospitalized or non-hospitalized patients after one week from symptom onset and last at least 7.5 months. Although the majority of asymptomatic patients (95%) developed a sustained antibody response, one patient did not developed antibodies 11 weeks after the RT-PCR positivity supporting the claim that caution is advised when interpreting anti-SARS-CoV-2 antibodies in asymptomatic subjects.

I.III.2 Persistence of Anti-SARS-CoV-2 Antibodies Depends on the Analytical Kit: A Report for Up to 10 Months after Infection

Microorganisms. 2021. 9(3)

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SUMMARY

Background: Several studies have described the long-term kinetics of anti-SARS-CoV-2 antibodies but long-term follow-up data, *i.e.,* >6 months, are still sparse. Additionally, the literature is inconsistent regarding the waning effect of the serological response. The aim of this study was to explore the temporal dynamic changes of the immune response after SARS-CoV-2 infection in hospitalized and non-hospitalized symptomatic patients over a period of 10 months.

Methods: Six different analytical kits for SARS-CoV-2 antibody detection were used. Positivity rates, inter-assay agreement and kinetic models were determined.

Results: A high inter-individual and an inter-methodology variability was observed. Assays targeting total antibodies presented higher positivity rates and reached the highest positivity rates sooner compared to assays directed against IgG. The inter-assay agreement was also higher between these assays. The stratification by disease severity showed a much-elevated serological response in hospitalized versus non-hospitalized patients in all assays.

Conclusion: In this 10-month follow-up study, serological assays showed a clinically significant difference to detect past SARS-CoV-2 infection with total antibody assays presenting the highest positivity rates. The waning effect reported in several studies should be interpreted with caution because it could depend on the assay considered.

Introduction

Currently, the revelation of SARS-CoV-2 RNA through RT-PCR from NP swab samples is considered the gold standard method for the diagnosis of acute SARS-CoV-2 infection. Less invasive salivary samples have also been reported as an alternative to NP swab samples (Butler-Laporte et al., 2021). The targeted genes for RT-PCR detection may include a combination of *N*, *E*, *RdRp*, *orf1a*, and *orf1b* genes.

The detection of anti-SARS-CoV-2 antibodies serves as an adjunct to molecular testing for the diagnosis of COVID-19 especially in patients who present late with a

low viral load. Serological testing has been successfully used to evaluate seroprevalence, to identify convalescent plasma donors, to monitor herd immunity and for risk predictions (Bohn, Loh, et al., 2020; Farnsworth & Anderson, 2020). Antibody assessment and monitoring are also likely to play a key role in the context of the global vaccination strategy (Lippi, Sciacovelli, et al., 2021).

Compared to commercial immunoassays, only neutralization activity assays reliably measure the actual protective immunity of antibodies (Padoan, Bonfante, Pagliari, et al., 2020). However, neutralization activity assays are only reserved to specialized laboratories and require high workload, skillful operators, expensive installations, crucial biosafety measures and have, to date, a low throughput. Therefore, the use of fully automated immunoassays that have a well-demonstrated correlation with neutralization activity should be considered in the routine clinical setting (Lippi, Sciacovelli, et al., 2021).

Current serological assays use different SARS-CoV-2 antigen targets (*i.e.,* N, S proteins (S) and the RBD) and formats (*i.e.,* IgG, IgA, IgM or total antibodies). Most assays possess a unique target but multiplex assays have also been developed and validated (Favresse, Brauner, et al., 2021; Gillot et al., 2020). The N participates in RNA packages and the release of virus particles while the transmembrane S glycoprotein comprises two functional subunits responsible for binding to the host cell receptor (N-terminal S1) and for the fusion of the viral and cellular membranes (C-terminal S2) (Lan et al., 2020). The RBD is located at the C-terminal region of the S1 (Walls et al., 2020). The RBD interacts with human cells that express ACE2 and induces the entry of the virus.

The antibody response to SARS-CoV-2 infection has been shown to be directed against multiple antigens of the virus including different epitopes of the S protein. Antibodies targeting the RBD in the C-terminal region of the S1 have been considered neutralizing (Ju et al., 2020). Other antibodies target the N or nonstructural proteins and their detection can be used as markers of recent infection (Favresse, Brauner, et al., 2021; Gillot et al., 2020).

Reports evaluating the antibody persistence inconsistently mention a waning effect of the serological response (Duysburgh et al., 2020; Hartley et al., 2020; Patel et al., 2020). Based on these observations, a few authors have claimed that cross-sectional seroprevalence studies to evaluate population immunity may underestimate rates of prior infections (Patel et al., 2020). A recent report also suggests that changing the vaccine policy to give to previously infected individuals only one dose of the vaccine would not negatively impact their antibody response and may consequently free up many vaccine doses (Florian Krammer et al., 2021). Therefore, the divergent opinions regarding the antibody persistence warrant further investigations to ensure an accurate and reliable evaluation of serological status of each individual because,

depending on the vaccinal strategy that will be applied in the next coming weeks, this could represent the saving of up to 100 million vaccine doses worldwide (WHO, 2021).

The aim of this study was therefore to explore the temporal dynamic changes of immune response after SARS-CoV-2 infection in hospitalized and non-hospitalized symptomatic patients for a period up to 10 months using different analytical kits for SARS-CoV-2 antibody detection. This will permit the investigation of, and provide more insight into, the understanding of this possible waning effect.

Material and Methods

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of Saint-Luc Bouge (Bouge, Belgium, approval number B0392020000005).

Patients and samples collection

This study was conducted at the clinical biology laboratory of the Clinique Saint-Luc (Bouge, Namur, Belgium). A total of 201 samples from 84 patients with a confirmed SARS-CoV-2 RT-PCR were retrospectively included from March 26, 2020 to January 6, 2021. Information on the days since the onset of symptoms was collected from the medical records. When data about symptoms were not available (n=15), the day of diagnosis (*i.e.,* RT-PCR result) was used instead. Different time intervals were also created to calculate the rate of positive samples (*i.e.,* 0-15; 15-40; 41-100; 101-150; 151-200; 201-235; and 236-300 days). Blood samples were collected into serum-gel tubes (BD SST II Advance, Becton Dickinson, New Jersey, USA) according to SOP and manufacturer recommendations. Samples were centrifuged for 10 min at 1,740 x g on a Sigma 3-16KL centrifuge. Sera were stored in the laboratory serum biobank at −20 °C from the collection date. Frozen samples were thawed during 1 h at room temperature on the day of the analysis. Re-thawed samples were vortexed before the analysis.

Analytical procedures

Six commercial immunoassays were used to evaluate the long-term kinetics of antibodies. The characteristics of these assays are presented in **Table I.III.2.1** (**Table I.III.2.1**). Each patient's sample was analyzed on the six different assays. The results rendered below the LOQ of the assay were rounded to the LOQ of each assay to allow quantitative calculations and data processing.

No	Manufacturer	Platform	Technology	Antigenic target	Antibody format	Positivity cut-off	Units
1	Roche Diagnostics	Cobas 801	ECLIA	RBD	Total antibodies	≥0.8	U/mL
2	Roche Diagnostics	Cobas 801	ECLIA	N	Total antibodies	≥1.0	COI
3	DiaSorin	Liaison XL	CLIA	S1+S2	IgG antibodies	≥15	AU/mL
4	Ortho Clinical Diagnostics	VITROS 3600	CLIA	S1	IgG antibodies	≥1.0	S/C
5	Ortho Clinical Diagnostics	VITROS 3600	CLIA	S1	Total antibodies	≥1.0	S/C
6	Thermo Fisher Scientific	Phadia 250	ELiA	S1	IgG antibodies	>10	U/mL

Table I.III.2.1: *Characteristics of the six assays used in this study.*

The RT-PCR for SARS-CoV-2 determination in respiratory samples (NP swab samples) was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV *E*-gene set.

Statistical analyses

Descriptive statistics were used to analyze the data. A Mann-Whitney test was used to compare the different groups. The positivity rates were calculated as the proportion of SARS-CoV-2 positive samples by serological tests initially categorized as positive by RT-PCR. A non-linear regression model with log-transformed data was used to compute the antibody kinetics since symptom onset (or diagnosis) using the following equation:

$$[(AUC * disappearance\ rate * appearance\ rate) / (appearance\ rate - disappearance\ rate)] * [Exp(-disappearance\ rate * days\ since\ symptom\ onset) - Exp(-appearance\ rate * days\ since\ symptom\ onset)]$$

A survival analyze was also performed to estimate the cumulative probability of positive samples since symptom onset (or diagnosis) using a log-rank Mantel-Cox test comparison. Inter-rater agreements, *i.e.,* agreement and Cohen's kappa, and correlation studies were also determined. A p value <0.05 was used as a significance level. Data analysis was performed using GraphPad Prism (version 9.0.1, California, CA, USA), MedCalc (version 14.8.1, Ostend, Belgium) and JMP software (version 15.2.1, Cary, NC, USA).

Results

Population characteristics

Among these 84 individuals, 44 were females (median age=46 years; min-max: 24–95 years) and 40 were males (median age=61 years; min-max: 24–88 years). Multiple

sequential sera were available for 55 patients and 17 required hospitalization (*i.e.,* categorized as severe patients). Hospitalized patients were elder (median age=74 years) compared to non-hospitalized patients (median age=46 years; p=0.0007). The median time between the symptom onset and the RT-PCR was three days (inter-quartile range (IQR): 1–8 days).

Kinetics of positivity rates

In samples collected early since symptom onset (*i.e.,* <15 days), positivity rates were low. The Roche N total antibody assay had the highest positivity rate in this period (*i.e.,* 69.2%) while the DiaSorin S1+S2 IgG and the Phadia S1 IgG assays showed the lowest positivity rates (*i.e.,* 38.5%). At the second timepoint, the highest positivity rates were observed for the Roche N and the Ortho S1 total assays (*i.e.,* 96.3% and 100%, respectively). A gradual increase in positivity rates toward a plateau was observed for both the Roche RBD and the Ortho S1 total assays with the latter reaching the plateau earlier than the Roche RBD total antibody assay. The highest positivity rates for the other assays, were observed at the fourth timepoint (*i.e.,* for the Phadia S1 IgG and the Ortho S1 IgG assays) and at the fifth timepoint (*i.e.,* for the Roche N total antibody and the DiaSorin S1+S2 IgG assays) (**Table I.III.2.2**). Overall, the total assays presented higher positivity rates and reached their highest positivity rates sooner compared to IgG assays.

	DSO (or diagnosis)	**0-15**	**16-40**	**41-100**	**101-150**	**151-200**	**201-235**	**236-300**
	Timepoint no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	n	**13**	**27**	**29**	**33**	**35**	**29**	**35**
Roche RBD total Ab (U/mL)	Median	3.7	16.9	127	169	254	165	295
	Interquartile range	0.4-110	2.4-170	24.6-411	68.9-429	84.1-591	64.4-687	87.6-541
	Positivity rate (%)	61.5	77.8	96.6*	100*	100*	96.6*	100*
Roche N total Ab (COI)	Median	6.4	19.1	49.6	63.9	63.9	45.3	28.9
	Interquartile range	0.1-26.4	6.0-29.8	14.5-84.6	34.9-114	20.6-126	13.9-74.0	11.4-66.0
	Positivity rate (%)	69.2	96.3	93.1	97.0	100*	93.1	94.3
DiaSorin S1+S2 IgG (AU/mL)	Median	5.4	36.6	64.2	58.6	75.5	64.2	66.7
	Interquartile range	3.8-78.5	11.0-99.1	21.8-161	34.2-120	36.1-104	24.0-123	23.6-101

	Positivity rate (%)	38.5	70.4	82.8	90.9	97.1*	86.2	91.4
Ortho S1 IgG (S/C)	Median	0.2	6.8	9.6	8.5	9.9	6.0	7.6
	Interquartile range	0.01-11.6	2.8-13.9	3.1-14.7	5.6-13.3	3.8-12.9	2.6-12.6	2.1-12.2
	Positivity rate (%)	46.2	77.8	79.3	97.0*	94.3	86.2	94.3
Ortho S1 total Ab (S/C)	Median	18.9	60.1	138	228	308	219	341
	Interquartile range	0.18-113	10.9-164	24.8-339	115-457	130-500	111-469	150-515
	Positivity rate (%)	61.4	100*	96.6*	100*	100*	96.6*	100*
Phadia S1 IgG (U/mL)	Median	3.8	20.0	31.0	25.0	21.0	15.0	15.0
	Interquartile range	0.7-148	11.0-152	10.5-134	12.0-64.0	5.3-45.0	4.9-37.5	4.7-28.0
	Positivity rate (%)	38.5	77.8	75.9	81.8*	68.6	55.2	60.0

Table I.III.2.2: *Positivity rates according to different time points using six different assays. * Represents maximal positivity rates observed. DSO = days since symptom onset.*

Kinetics models of serological response

Figure I.III.2.1 represents the level of antibody response by days after symptom onset according to severity (**Figure I.III.2.1**). Depending on the assay and/or the population considered, a rapid increase in antibody titers was observed followed by a plateau phase or a decrease phase. After 300 days, hospitalized patients had overall a higher maximal response peak and a more persistence antibody response (e.g., the Roche N total antibody and the DiaSorin S1+S2 IgG assays). The maximal concentration (C_{max}) was consistently higher in hospitalized patients than in non-hospitalized. **Figure I.III.2.2** represents the cumulative probability of positive samples after 14 days until the last follow-up point, *i.e.,* 300 days (**Figure I.III.2.2**). Of this selected cohort of 188 samples collected after the fourteenth day, 2, 8, 10, 21, 25, and 57 samples were reported negative for the Ortho S1 total antibody, the Roche RBD total antibody, the Roche N total antibody, the Ortho S1 IgG, the DiaSorin S1+S2 IgG and the Phadia S1 IgG, respectively. Assays targeting total antibodies, *i.e.,* Ortho S1 total antibody, Roche RBD total antibody and Roche N total antibody, had the highest cumulative probability of positive samples at the latest follow-up period compared to IgG assays (p value <0.0001). The Ortho S1 total antibody assay was the only test that did not statistically differ from the 100% of cumulative positive probability, *i.e.,* "all positive" on the figure at the latest follow-up point (p=0.1573). The Phadia S1 IgG was the only

test having a cumulative probability of positive samples below 50%. The median survival was 239 days with this test. Between the different assays, the Roche RBD total antibody and the Ortho S1 total antibody showed the highest agreement, Cohen's kappa index and correlation coefficient (r). An agreement of at least 95% was only reached for total assays.

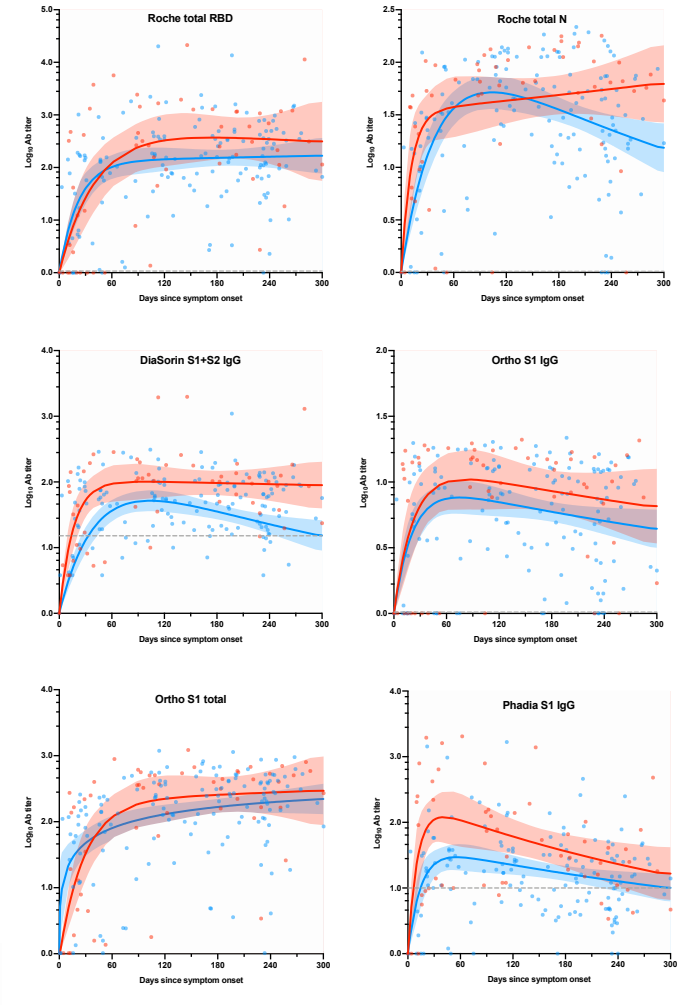


Figure I.III.2.1: *Level of antibody response by days after symptom onset according to severity. Depending on the assay and/the population considered, a rapid increase in antibody titers was observed followed by a plateau phase or a decrease phase. Blue curves (and 95% CI) and points represent non-hospitalized patients. Red curves (and 95% CI) and points represent hospitalized patients. The dotted grey line corresponds to the manufacturer's cut-off for positivity.*

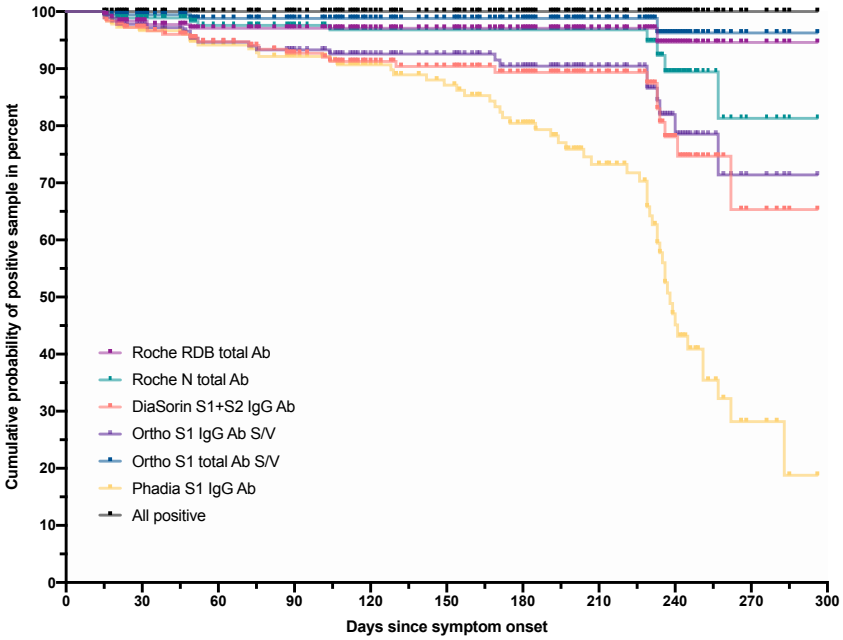


Figure I.III.2.2: *The cumulative probability of positive samples after 14 days until the last follow-up point, i.e., 300 days, using six different commercial assays.*

Discussion

In this study, 201 sera samples from 84 RT-PCR confirmed COVID-19 patients with a 10-month follow-up period since symptom onset were included. Each patient's sample was analyzed on six commercial assays. As previously reported, the serological kinetics showed a high degree of heterogenicity that was patient-dependent but we also reported that these differences were also assay-dependent (**Figure I.III.2.1**) (Dan et al., 2021; Lau et al., 2021). The performance of these assays up to 15 days since symptom onset was particularly low because of the natural dynamics of the production of Ig (Bohn, Loh, et al., 2020; Favresse, Brauner, et al., 2021; Gillot et al., 2020) Assays targeting total antibodies presented higher positivity rates and reached their highest positivity rates sooner than IgG assays. The inter-assay agreement was also higher between these total assays. The stratification by disease severity, expressed in this study by the patient's hospitalization status, showed a higher serological response in severe cases which is consistent with our previous observations (Favresse, Brauner, et al., 2021; Figueiredo-Campos et al., 2020; Gillot et al., 2020; Gudbjartsson et al., 2020; Lau et al., 2021; Seow et al., 2020). The Phadia S1 IgG assay has a low performance to detect past SARS-CoV-2 infection compared to other assays. The manufacturer could probably consider redefining the cut-off, as has already been done for other assays, in order to improve the sensitivity (Favresse, Cadrobbi, et al., 2021; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020).

However, in this study, only the cut-offs of the manufacturers were used so that there was no advantage for one method over another. Interestingly, the evaluation of the kinetic models demonstrated that assays targeting total antibodies consistently showed an increase of antibody titer, at least in hospitalized patients (**Figure I.III.2.1, left panel**). The same tendency was also observed in non-hospitalized patients except for the test that targeted antibodies directed against the N. On the other hand, assays targeting antibodies directed against the S1 showed a slight decrease in antibody titers, except for the DiaSorin S1+S2 IgG in hospitalized patients (**Figure I.III.2.1, right panel**). The drop in cumulative probability of positive samples is consistently highest for the Phadia S1 IgG compared to all other tests. The Ortho S1 total antibody assay performed better than the Roche N total antibody assay but was not statistically different from the Roche RBD total antibody assay. The Roche RBD and N total antibody assays also performed better than all IgG assays. No statistically significant differences were observed between the DiaSorin S1+S2 IgG and the Ortho S1 IgG.

Multiple studies have evaluated the long-term kinetics of anti-SARS CoV-2 antibodies using various assays. A sustained antibody response against the N antigen using the Roche N total antibody assay was found in several studies, *i.e.,* between three and seven and a half months (Favresse, Elsen, et al., 2021; Gudbjartsson et al., 2020; Muecksch et al., 2020). A maintained antibody response against the RBD antigen, as assessed by the Wantai and the Siemens total antibody assays, was also observed up to four months (Gudbjartsson et al., 2020; Muecksch et al., 2020). A decrease in anti-RDB IgG and anti-S IgG levels was similarly observed over a period of up to five months in recent reports(Ibarrondo et al., 2020; Isho et al., 2020; Iyer et al., 2020) and a significant decrease in sensitivity was also found in studies with up to five months of follow up with the Abbott assay, which was directed against N IgG (Gudbjartsson et al., 2020; Muecksch et al., 2020; Sun et al., 2020). The YHLO assay, which detects both anti-N and anti-S IgG, showed high sensitivities from five weeks to three months after symptom onset (K. Li et al., 2020). Wajnberg *et al.* found stable antibody titers over a period of at least three months and only modest declines at the five-month time point (Wajnberg et al., 2020).

In our study, the sustained antibody response as observed with total antibody assays (N and RBD) compared to IgG assays may be due to the additional response of non-IgG antibody isotypes. However, the reasons for the differences in assay performance over time for assays targeting the same antigen remain unclear (Muecksch et al., 2020). The nature and structure of the target itself (for example, purified versus recombinant, full-length versus truncated, eukaryotic versus prokaryotic expression system) as well as the protocol definition for determining the cut-off may, at least in part, affect the variability between assays (Favresse, Brauner, et al., 2021).

Whether the antibodies measured with commercial assays have a neutralizing capacity is paramount for indicating the potential level of protective immunity against SARS-CoV-2 infection. Recently, Padoan *et al.* found that the Ortho S1 IgG (R^2adj=0.544) and DiaSorin S1+S2 IgG (R^2adj=0.402) assays were more correlated to neutralization activity compared to the Ortho S1 total antibody (R^2adj=0.117) and Roche N total antibody (R^2adj=0.046) assays (Padoan, Bonfante, Pagliari, et al., 2020). The fact that anti-N assays showed a low correlation with the neutralizing capacity was expected as NAbs are directed against the S protein that is responsible for enabling the entry of the virus into the cells that express ACE-2. A strong correlation between the levels of anti-RBD or anti-S antibodies and the neutralizing capacity has been found in several reports (Figueiredo-Campos et al., 2020; Ibarrondo et al., 2020; Ju et al., 2020; Premkumar et al., 2020; Wajnberg et al., 2020). The neutralizing capacity was found to be maintained from one to five months (Brigger et al., 2021; Figueiredo-Campos et al., 2020; Iyer et al., 2020). However, although modest declines have been observed at three to five months (Isho et al., 2020; Wajnberg et al., 2020), a few studies have pointed out a significant decrease of two to four-fold in neutralizing activity up to three months (Crawford et al., 2021; Muecksch et al., 2020; Prevost et al., 2020; Seow et al., 2020; Wang et al., 2021).

Data with a longer follow-up, *i.e.,* ≥6 months, are however still sparse in the literature. Recently, Dan *et al.* found a slight decreasing but stable antibody response (anti-S IgG, anti-RBD and anti-N using ELISAs) in a population of 188 COVID-19 patients, representing a total of 254 samples, with a maximal follow-up of 8 months post-symptom onset. Forty-three samples were collected at ≥ 6 months after the initial infection (Dan et al., 2021). Positivity rates at six to eight months were 90% (36/40 samples) for anti-S IgG, 88% (35/40 samples) for anti-RBD IgG and 80% (32/40 samples) for anti-N IgG. The positivity rate of patients with positive NAbs was 90% (36/40) (Dan et al., 2021). In a population of 293 patients, Lau *et al.* also observed a trend towards lower antibody titers and neutralizing activity after seven months since illness but with a positivity rate of almost 100% after 30 days using an anti-RBD IgG ELISA assay (Lau et al., 2021). A correlation of 0.53 was found between the ELISA assay and the neutralizing activity. They also found a stronger antibody response in severe patients compared to mildly infected patients (Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020). Ripperger *et al.* found that anti-RBD, anti-S2 and NAbs remained detectable through five to seven months after illness (Ripperger et al., 2020).

In a population of 25 COVID-19 patients with a maximal follow-up of 8 months, Hartley *et al.* observed that anti-N and anti-RBD IgG were found in each 24/25 and 25/25 patients while NAbs was detected in 22/25 patients. They noted a decline in neutralization titers and antibody levels over time (Hartley et al., 2020). Nevertheless, they noted the persistence of SARS-CoV-2-specific B-memory cells,

which could represent a more robust surrogate of long-lived humoral immune responses compared to antibodies (Florian Krammer et al., 2021).

It is important to remember that a few patients may develop specific antibodies but may not have detectable NAbs. These are only correlation studies that are not related to direct measures of neutralizing activity (Muecksch et al., 2020). The fact that NAbs constitute a major protective mechanism against SARS-CoV-2 infection deserves further investigations (Lau et al., 2021; Muecksch et al., 2020; Wajnberg et al., 2020). A few differences between various neutralization assays, e.g., pseudo-particle neutralization, microneutralization, fluorescent focus reduction assays, microneutralization assays, plaque reduction neutralization tests, also exist with microneutralization tests found to be less sensitive than plaque reduction neutralization assays (Lau et al., 2021; Riepler et al., 2020).

The cellular measurements of the immune response have been proposed to be reliable markers for the maintenance of immunity following natural infection or vaccination (Hartley et al., 2020; Rodda et al., 2021; Sokal et al., 2021). Such approaches should be explored more. Even if previous exposure to SARS-CoV-2, either by true infection or by exposure to a vaccine, significantly decreases the risk of further positive RT-PCR tests,(Baden et al., 2021; Logunov et al., 2021; Lumley et al., 2021; Polack et al., 2020; Voysey et al., 2021) total immunity might not be guaranteed in all individuals because reinfection with SARS-CoV-2 exist (Baden et al., 2021; Logunov et al., 2021; Polack et al., 2020; Tillett et al., 2021; To et al., 2020; Voysey et al., 2021).

Conclusion

This study shows that assays are not equal for detecting past SARS-CoV-2 infection or investigating seroprevalence in samples for up to 10 months since symptom onset. Assays targeting the total antibody response have the highest positivity rates and perform better than tests targeting only IgG. The waning effect reported in several studies should be interpreted with caution because it may mostly depend on the assay considered. Even if previous exposure to SARS-CoV-2 decreases the risk of subsequent SARS-CoV-2 positivity, total immunity might not be guaranteed in all individuals. Further studies are required to correlate the seropositivity after such a long period post-infection with appropriate serological neutralization assays.

I.IV. Binding versus neutralizing antibodies

I.IV.1 Identification of SARS-CoV-2 Neutralizing Antibody with Pseudotyped Virus-based Test on HEK-293T hACE2 Cells

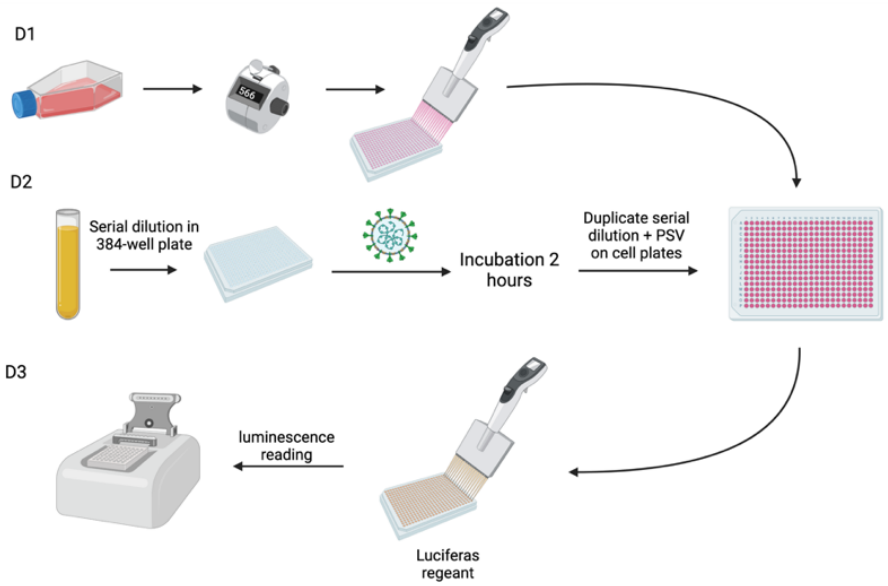
Bio-protocol. 2022. 12(7)

Constant Gillot, Julien Favresse, Vincent Maloteau, Jean-Michel Dogné, Jonathan Douxfils

SUMMARY

NAbs are of particular importance because they can prevent binding of the RBD of the S protein to the ACE2 receptor present at the surface of human cells, preventing virus entry into the host cells. The gold standard method for detection of NAbs is the PRNT. Based on the measurement of cell lysis due to viral infection, this test is able to detect antibodies that prevent cell infection. This technique requires the use of live pathogens, *i.e.,* SARS-CoV-2 in this case, and must be done in a biosafety level 3 (BSL3) laboratory. In addition, it requires expensive installations, skillful and meticulous staff, and a high workload, which prevents its wide implementation even in research laboratories. A SARS-CoV-2 pseudovirus will express the S protein responsible for cell entrance, but will not express the pathogenic genetic material of the virus, making them less dangerous for laboratory staff and the environment.

Graphical abstract (D = day)



Introduction

The gold standard method for detection of NAbs is the PRNT (Perera et al., 2020). Based on the measurement of cell lysis due to viral infection, this test is able to detect antibodies that prevent the cell infection (Lau et al., 2021; Muruato et al., 2020). Such technique requires the use of live pathogens, *i.e.*, SARS-CoV-2 in this case, and must be done in a BSL3 laboratory. In addition, it requires expensive installations, skillful and meticulous staff, and a high workload, which prevent its wide implementation even in research laboratories (Lee et al., 2021; Muruato et al., 2020). Such facilities are not widely available, and only very specialized institutions can offer access to BSL3 laboratories and trained staff. Quite similar neutralization techniques based on pseudoviral particles (called pVNT) have been developed, and can be performed in BSL2 laboratories, allowing higher throughput (Nie et al., 2020a). A SARS-CoV-2 pseudovirus will express the S protein responsible for cell entrance, but will not express the pathogenic genetic material of the virus, making them less dangerous (Nie et al., 2020a, 2020b).

Materials and Reagents

1. Sterile white 384-well µClear flat bottom cell culture plate with lid (Greiner Bio-One, Kremsmünster, Austria, catalog number: 781098)
2. Sterile 384-well flat bottom assay plate with lid (Corning, NY, USA, catalog number: 3701)
3. Pipette tip 200 µL (Thermo Fisher Scientific, Waltham, MA, USA, catalog number: AM12650)
4. Eppendorf tube (Sigma Aldrich, Saint-Louis, MO, USA, catalog number: T2795)
5. 50 mL reagent reservoir sterile polystyrene (Merck, Overijse, Belgium, catalog number: CLS4870)
6. HEK-293T hACE2 (Invivogen, San Diego, CA, USA, catalog number: HKB-hACE2)
7. SARS-CoV-2 Pseudoviral Particles (E-enzyme, Gaithersburg, MD, USA, catalog number: SCV2-PsV-001)
8. Dulbecco's Modified Eagle Medium (DMEM), with L-glutamine and glucose (Lonza, Bâle, Switzerland, catalog number: LO BE12-604F)
9. FireFly Luciferase kit (E-enzyme, Gaithersburg, MD, USA, catalog number: CA-L165-10)
10. Tryptan blue (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA, catalog number: T10282)

Equipment

1. Spectramax 3 iD (Molecular Devices, LLC, CA, USA)
2. Laminar flow hood (Thermo Fisher Scientific, Waltham, MA, USA, MSC Advantage 1.8 catalog number: 51025413)
3. Electronic multichannel 5–125 µL pipette (Brand, Transferpette -12 electronic, catalog number: 705453)
4. Monochannel 5–50 µL pipette (Socorex, Ecubens, Switzerland, catalog number: 825.0050)
5. Centrifuge 5702 (Eppendorf, Hamburg, Deutschland, catalog number: 5702000320)
6. Neubauer counting slide (Hecht Assistant, Altnau, Switzeland, catalog number: 40441)
7. Julobo ED Water bath (Sigma Aldrich, Saint-Louis, MO, USA, catalog number: Z615498)

Software

1. GraphPad Prism software (version 9.1.0, San Diego, CA, USA)

Procedure

A. Cell inoculation on a 384-well plate

To determine the quantity of cell suspension necessary, a calculation of this type must be made:

1. A volume of 15 µL of cell suspension within ± 8.5 x 10^3 cells are seeded in each well of the 384-wells plate. The quantity of cells suspension to prepare is 15 µL x 384 x X, where X is the number of plates to prepare.
2. To prepare this cell suspension, after counting and centrifugation, add the volume required to have 566 cells per µL.
3. To prepare the counting slide, the slat is stuck with water to the slide.
    a. In an Eppendorf, add 50 µL of cell solution to put in the wells and 50 µL of trypan blue. Mix.
    b. Place 10 µL of this mix in each part of the counting slide. Under a microscope, the living cells inside the squares are counted. Living cells appear transparent and dead cells appear blue.

c. To calculate the number of cells per milliliter, the following formula must be used, where n is the number of cells counted using a Neubauer counting slide.

$$\frac{Cells}{ml} = (n \times 10\,000)/(\frac{4 \times 1}{2})$$

4. The new cell suspension is maintained in a scotch bottle with constant agitation at moderate power. The 384-well plates are then filled with the cell suspension at a volume of 15 µL per well, with an **electronic multichannel micropipette**.

5. The plates are then annotated with "cell type—# of passages—operator's initials" and incubated in a calibrated oven for cell culture **at 37°C during 24 h**.

B. Serum dilutions

1. Heat inactivates the serums in a water bath **at 56°C for 30 min**.

2. Twenty-six sera can be diluted on a 384-well plate. Dilutions are made in line, and start at a 1:2 dilution, up to a 1:5120 dilution. If further dilutions are required, a second 384-well plate should be used.

3. Before making the serum dilutions, each well must be filled with 30 µL of dilution medium (DMEM + 10% HyClone FetalClone Serum) using **an electronic multichannel micropipette,** except for columns 2 and 12 which are filled with 50 µL.

4. Add 10 µL of serum in the first well, using a monochannel pipette 5–50 µL. Serial dilution of the sera can then be started, and proceeds as follows:

   - Flush 15 times in the aliquot.
   - Take a volume of 30 µL and place it in the first well.
   - Flush 15 times in the first well.
   - Change tips to collect liquid from the first to the second well.
   - Repeat the previous steps until the end of the serial dilution.

   In the last well, 30 µL must be removed, so that all wells contain the same volume.

5. A **cell control** (CC) and a **viral check** (VC) must be performed. The cell control (CC) is an assay in which **cells are incubated with culture medium**. The viral check consists of **the incubation of viruses without any sera**, in step C.1, 17.9 µL of SARS-CoV-2-PP must be incubated with 7.1 µL of culture medium.

6. Centrifugate at 161 × *g* during **5 min**.

C. Interaction between antibodies and Pseudoviral Particles

1. Dilute the pseudovirus three times in culture medium, to obtain the necessary volume for the analysis.
2. In each well of a 384-well plate, add 17.9 µL of diluted SARS-CoV-2-Pseudoviral Particles with an **electronic multichannel micropipette,** and 7.1 µL of dilution serums previously performed with a **manual multichannel micropipette (5 µL–50 µL)**. Each sample is carried out in duplicate. For one dilution serum, two tests are carried out.
3. The plates are then annotated with "operator's initial" and incubated in a calibrated oven for cell culture **at 37°C during 2 h**.

D. Inoculation of the virus on cells

1. First, start by emptying the culture medium from the 384-well plate containing the cells. Once this is done, transfer 17.5 µL of each column from the plate containing serum dilutions and viral particles to the cells plate with a **manual multichannel micropipette (5 µL–50 µL)**. Repeat the procedure, changing tips between each serum.
2. Add 7.5 µL of DMEM + 10% FC into each well.
3. Let incubate for **42 h at 37°C**.

E. Signal Measurement

1. Remove the supernatant and add 20 µL of eEnzyme's luciferase assay reagent into each well with an **electronic multichannel micropipette**.
2. Read in a luminescence plate reader. There must be a proportional relationship between luminescence and dilutions, the higher the dilutions, the higher the signal. Indeed, the luciferase enables the detection of infected cells, the more there is of antibody, the less the cells will be infected.

Data analysis

Based on the relative light units (RLU) values from each sample, a percentage of inhibition can be calculated. The following formula must be applied to each dilution for each sample:

$$Relative\ inhibition = \frac{RLU\ sample\ X - RLU\ negative\ control}{RLU\ viral\ control - RLU\ cell\ control}$$

The different percentages of inhibition are used to plot the evolution of the relative inhibition as a function of the serum dilution. By intrapolation of the sigmoid curves

obtained, it is possible to determine the dilution at which 50% inhibition is achieved, called the IC_{50}. The results obtained via statistical software give us the logarithm of the dilution in comparison to the 1:10 dilution, considered our initial condition. This logarithm is then transformed into a numerical dilution within the range achieved. A sample is considered negative if the IC_{50} value of this sample is below the 1:20 dilution. An example of the expected results is shown in **Figure I.IV.1.1** (**Figure I.IV.1.1**).

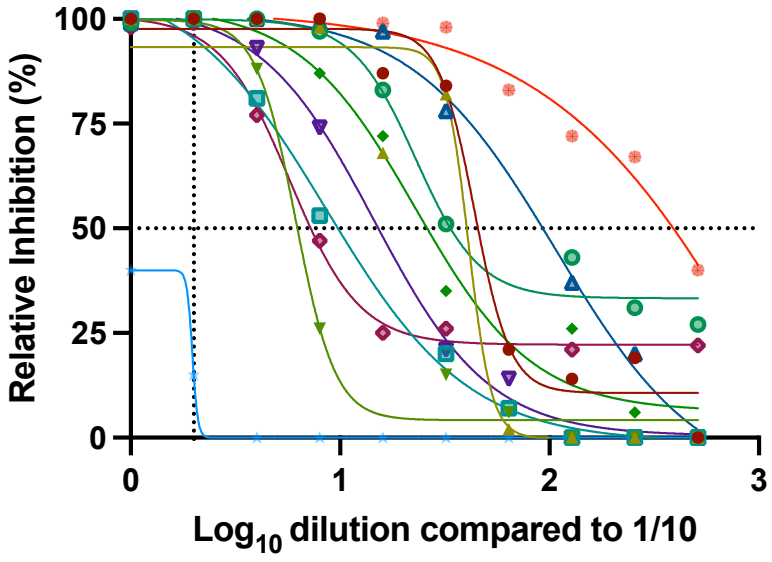


Figure I.IV.1.1: *Percentage of relative inhibition as a function of the log_{10} of the dilution compared to the 1:10 dilution.*

I.IV.2 NEUTRALIZING ANTIBODIES IN COVID-19 PATIENTS

Viruses. 2021. 13(7) PART I

Julien Favresse, Constant Gillot, Laura Di Chiaro, Christine Eucher, Marc Elsen, Sandrine Van Eeckhoudt, Clara David, Laure Morimont, Jean-Michel Dogné, Jonathan Douxfils

SUMMARY

Background: The evaluation of the neutralizing capacity of anti-SARS-CoV-2 antibodies is important because they represent real protective immunity. In this study we aimed to measure NAbs in COVID-19 patients.

Methods: One-hundred and fifty long-term samples from 75 COVID-19 patients were analyzed with a sVNT and compared to six different SARS-CoV-2 serology assays.

Results: The agreement between the sVNT and pVNT results was found to be excellent (*i.e.,* 97.2%). In COVID-19 patients, a stronger response was observed in moderate–severe versus mild patients (p=0.0006). A slow decay in NAbs was noted in samples for up to 300 days after diagnosis, especially in moderate–severe patients (r=−0.35, p=0.03). Moreover, the six binding assays were significantly correlated to NAbs (p<0.0001). The highest r was observed with the Phadia S IgG assay (r=0.89).

Conclusion: A stronger neutralizing capacity in moderate–severe versus mild COVID-19 patients, in which a slow decay with time was observed. Significant correlations between NAbs and antibody titers as assessed by six binding assays were found, with higher r toward IgG assays targeting the S protein.

Introduction

The revelation of SARS-CoV-2 RNA through a RT-PCR from NP swab samples is considered the gold standard method for the diagnosis of acute SARS-CoV-2 infection. Nevertheless, individuals with positive RT-PCR results represent only a limited fraction of all infections, given the limited availability and the brief time window in which RT-PCR testing presents the highest sensitivity (Gudbjartsson et al., 2020; R. Li et al., 2020).

The detection of specific antibodies following SARS-CoV-2 infection allows for the evaluation of the seroprevalence, the identification of convalescent plasma donors, the monitoring of herd immunity, the generation of risk prediction models, and is also likely to play a key role in the context of the global vaccination strategy (Bohn, Loh, et al., 2020; Joyner et al., 2021). Anti-SARS-CoV-2 NAbs are of particular importance because these are the antibodies which inhibit the binding of the RBD of

the surface S protein to the human ACE2 receptor. The complex formed between the virus S protein and the human ACE2 is responsible for the virus entry into hosts cells and the inhibition of the formation of this complex may thereby prevent infection and reduce disease severity (Premkumar et al., 2020; Shang et al., 2020).

Compared to SARS-CoV-2 antibody assays, which measured all the antibodies that are able to recognize the S protein, only assays measuring NAbs reliably measure the real protective immunity of antibodies (Padoan, Bonfante, Pagliari, et al., 2020). The current gold standard method to measure NAbs is the conventional virus neutralization test, which requires a BSL3 laboratory to manipulate the live pathogen. These tests are reserved for very specialized laboratories and further require a high workload, skillful operators, and expensive installations, and they have a low throughput (Lippi, Sciacovelli, et al., 2021; Tan et al., 2020). The use of a SARS-CoV-2 sVNT based on antibody-mediated blockage of the interaction between the ACE2 receptor protein and the RBD has been found to be an attractive alternative (Perera et al., 2021; Tan et al., 2020; Valcourt et al., 2021).

In this study, we investigated neutralizing capacity by means of an sVNT in previous COVID-19 patients. The specificity of the sVNT and its agreement with six SARS-CoV-2 antibody tests were also determined. A subset of samples was also tested with a pVNT.

Materials and Methods

COVID-19 patients and vaccinated recipients

Demographic data for the group of patients are presented in **Table I.IV.2.1 (Table I.IV.2.1)**. One-hundred and fifty samples from 75 patients with a confirmed SARS-CoV-2 RT-PCR were retrospectively included from 26 March 2020 to 6 January 2021. Among them, 39 were females (median age=45; min–max: 24–95 years) and 36 were males (median age=62; min–max: 24–88 years). Multiple sequential sera were available for 41 patients. Seventeen patients required hospitalization and were categorized as moderate–severe patients, according to the WHO categorization (Marshall et al., 2020). Information on the days since the onset of symptoms was collected from medical records and was available for 63 patients. When data about symptoms were not available (*n*=12), the day of diagnosis (*i.e.,* the RT-PCR result) was used instead. The median time since diagnosis was 169 days (range, 11–296) and 139 days (range, 10–290) in mild and moderate–severe COVID-19 patients, respectively (p=0.39).

Additionally, 250 samples collected before January 2020 were assessed to evaluate the clinical specificity of the sVNT.

Demography	
Previous COVID-19 patients (n)	***75***
Females (*n* (%))	**39 (52%)**
Age (median (min–max))	45 (21–95)
Males (*n* (%))	**36 (48%)**
Age (mean (min–max))	62 (24–88)
Moderate–severe (*n* (%))	**17 (22.7%)**
Time since diagnosis (median, (range))	169 (11–266)
Mild (*n* (%))	**58 (77.3%)**
Time since diagnosis (median, (range))	139 (10–290)
Total number of samples	**150**

Table I.IV.2.1: *Demographic data for the past-COVID-19 patients. The difference between the total number of samples and the number of patients/subjects is explained by multiple timepoints for blood sampling.*

Sample collection

Blood samples were collected in serum-gel tubes (BD SST II Advance, Becton Dickinson, NJ, USA) and centrifuged for 10 min at 1740*g on a Sigma 3-16KL centrifuge. Sera were stored in the laboratory serum biobank at −20 °C from the collection date. Frozen samples were thawed for 1 h at room temperature on the day of the analysis. Re-thawed samples were vortexed before the analysis. All samples were collected at the Clinique Saint-Luc (Bouge, Namur, Belgium). The study protocol was in accordance with the Declaration of Helsinki. All vaccinated participants provided informed consent prior to the collection of data and specimens (EudraCT registration number: 2020-006149-21).

Analytical procedures

Neutralizing capacity was estimated by performing an sVNT. The iFlash-2019-nCoV NAbs assay is a one-step competitive paramagnetic particle CLIA for the quantitative determination of 2019-nCoV NAbs in human serum and plasma. The assay detects NAbs that block the binding of RBD and ACE2. First, NAbs (if present) react with the RBD antigen coated on paramagnetic microparticles to form a complex. Second, the acridinium-ester-labeled ACE2 conjugate is added to competitively bind to the RBD-coated particles, which have not been neutralized by the NAbs (if present) from the sample, and these form another reaction mixture. Under a magnetic field, magnetic particles are adsorbed to the wall of the reaction tube, and unbound materials are washed away by the wash buffer. The resulting chemiluminescent reaction is measured in RLU, with an inverse relationship between the amount of NAbs and the RLU value detected. According to the manufacturer, it shows excellent positive (98.5%) and negative percentage agreement (96.1%) with the conventional PRNT. A result <10.0 AU/mL is considered negative and a result ≥10.0 AU/mL is considered

positive (according to the manufacturer's information). The sVNTs were performed on an iFlash1800 automated magnetic CLIA analyzer from Shenzhen YHLO Biotech Co., Ltd. Internal quality controls (negative and positive) and 6 sera from COVID-19 patients at various NAbs titers were analyzed 10 times in a row to calculate the within-run precision of the assay. The positive internal quality control was also analyzed for a period of 15 days to calculate the between-run precision.

A total of 71 random samples (*i.e.,* 23 pre-pandemic and 48 past-COVID-19 patient samples) were also assessed by means of a pVNT. Details about the method are presented in **Supplementary materials**. A sample is considered negative if the half maximal inhibitory concentration (IC_{50}) value of this sample is below the dilution 1/20.

All samples from the first group, which was composed of COVID-19 patients, were also analyzed on 6 commercial immunoassays, namely: the Roche N total antibody assay (positivity cut-off=1.0 COI), the Roche RBD total antibody assay (positivity cut-off=0.8 U/mL), the DiaSorin S1/S2 IgG assay (positivity cut-off=15 AU/mL), the Ortho S1 IgG assay (positivity cut-off=1.0 S/V (sample signal/threshold value)), the Ortho S1 total antibody assay (positivity cut-off=1.0 S/V), and the Phadia S1 IgG assay (positivity cut-off=10 U/L), as described elsewhere (Favresse, Eucher, et al., 2021). The Roche N total assay was also used to determine the serological status of vaccinated participants before vaccine injection.

RT-PCR for SARS-CoV-2 determination in NP swab samples was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV *E*-gene set.

Statistical analysis

Descriptive statistics were used to analyze the data. Sensitivity was defined as the proportion of correctly identified COVID-19 positive patients who were initially positive, according to an RT-PCR SARS-CoV-2 determination in NP swab samples. Specificity was defined as the proportion of pre-pandemic samples classified as negative. A Mann–Whitney test was used to assess potential differences in median time since diagnosis in mild versus moderate–severe COVID-19 patients. A linear regression model was implemented to evaluate the long-term kinetics of NAbs in past-COVID-19 patients. A simple linear regression and Pearson correlations were computed to assess the potential association between NAb titers and antibody titers obtained using 6 non-neutralizing commercial methods. Inter-rater agreements were also determined. The CV ((SD/mean) × 100 (%)) of the quantitative titers were used to determine the repeatability and intermediate precision of the assay. A $p<0.05$ was used as a significance level. Data analysis was performed using GraphPad Prism software (version 9.1.0, San Diego, CA, USA).

Results

Clinical specificity and precision of the assay

Considering the cohort of 250 pre-pandemic samples, only one sample was above the positivity threshold of 10 AU/mL (*i.e.,* 15.7 AU/mL), leading to a specificity of 99.6% (CI 95%: 97.8%–99.9%). The mean of the NAb titers was 3.0 AU/mL (CI 95%: 2.7–3.2 AU/mL) (**Figure I.IV.2.1**). The within-run CV ranged from 4.1% to 15.0% for NAb titers, ranging from 11.2 to 802.2 AU/mL. A higher CV was observed using the negative quality control (45.6% at a concentration of 4.4 AU/mL (min-max, 1.8–8.8 AU/mL) (**Table I.IV.2.2**). The between-run CV using the positive internal quality control was 10.0%.

	Neg. Control	Pos. Control	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F
YHLO NAb Assay	6.36	54.3	10.8	42.0	266.9	576.2	727.6	783.8
	3.85	50.7	10.6	42.2	263.3	634.9	799.8	827.1
	1.84	55.3	14.7	45.6	278.0	667.8	837.1	867.4
	4.02	51.8	11.3	40.4	286.6	856.4	854.9	789.7
	3.28	60.6	10.2	41.2	287.3	863.0	739.4	814.3
	2.33	52.5	9.89	39.3	280.2	832.4	726.9	770.7
	4.18	53.2	10.5	41.3	250.2	609.5	796.7	820.7
	8.83	53.2	11.1	43.8	269.7	827.6	787.4	785.5
	5.03	56.8	13.0	42.3	292.2	765.3	799.4	811.1
	4.43	53.0	9.81	42.2	271.0	823.8	753.6	751.5
Mean	**4.42**	**54.1**	**11.2**	**42.0**	**274.5**	**745.7**	**782.3**	**802.2**
SD	**2.01**	**2.83**	**1.54**	**1.74**	**12.8**	**111.7**	**44.5**	**32.9**
CV (%)	**45.6**	**5.23**	**13.8**	**4.14**	**4.67**	**15.0**	**5.69**	**4.11**

Table I.IV.2.2: *Precision of the sVNT using controls and patient samples. All materials were analyzed 10 times in a row.*

sVNT versus pVNT

Over the 71 samples tested in pVNT and sVNT, the agreement between the two methods was 97.2%. One sample was considered positive by pVNT but negative by sVNT, and one sample was considered negative by pVNT but positive by sVNT. These were the only two discordant results out of 71 samples, and they were close to the positivity cut-off of the sVNT (*i.e.,* 9.6 and 10.1 AU/mL, respectively).

Neutralizing antibodies in COVID-19 patients

Figure I.IV.2.1 represents the NAb titers obtained in past-COVID-19 patients. The mean NAb titer in moderate–severe patients was significantly higher compared to mild patients (125 versus 33.9 AU/mL, p=0.0006) (**Figure I.IV.2.1**). All moderate–severe patients had positive NAbs (39/39) and 80.2% of mild patients were positive (89/111).

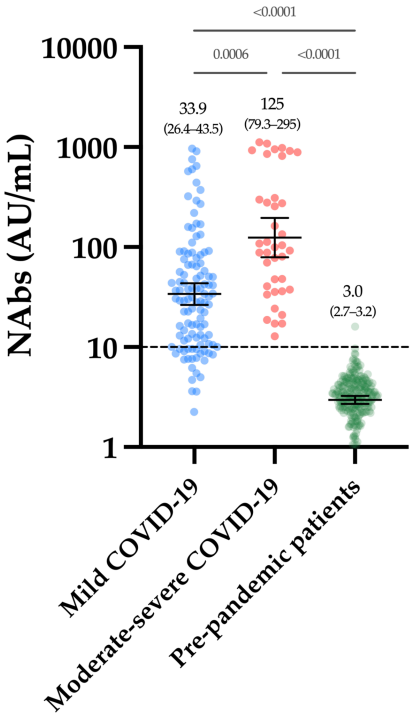


Figure I.IV.2.1: *NAb titers obtained in the first group of COVID-19 patients and in the pre-pandemic cohort. The black dotted line corresponds to the positivity threshold of 10 AU/mL.*

Considering only samples obtained ≥14 days since diagnosis, a weak but significant decay in NAb titers was observed over time in moderate–severe COVID-19 patients ($r=-0.35$, $p=0.03$). The apparent slow decrease observed in mild COVID-19 patients was not statistically significant ($r=-0.14$, $p=0.14$) (**Figure I.IV.2.2**).

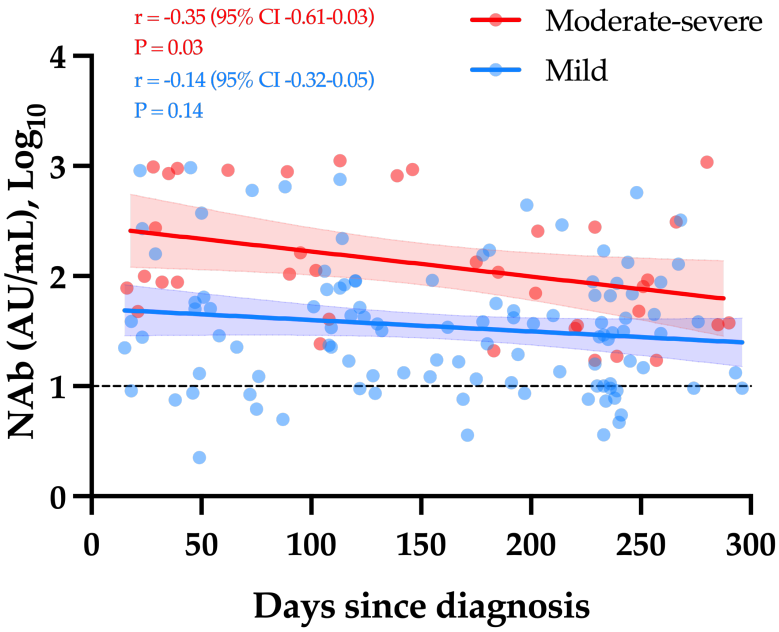


Figure I.IV.2.2: *The kinetics of NAbs in moderate–severe versus mild COVID-19 (group 1). The black dotted line corresponds to the positivity threshold of 10 AU/mL.*

The correlations between NAbs and SARS-CoV-2 antibody assays is presented in **Figure I.IV.2.3** (**Figure I.IV.2.3**). The six assays were significantly correlated to NAbs ($p<0.0001$). The highest r was observed with the Phadia S IgG assay ($r=0.89$) and the lowest one was observed on the Roche N assay ($r=0.46$). Except the Roche S total and Ortho IgG assays, higher correlations were obtained for IgG assays and weaker correlations for total assays (**Figure I.IV.2.3**). The agreement between methods was good and ranged from 82.7% to 88.0%.

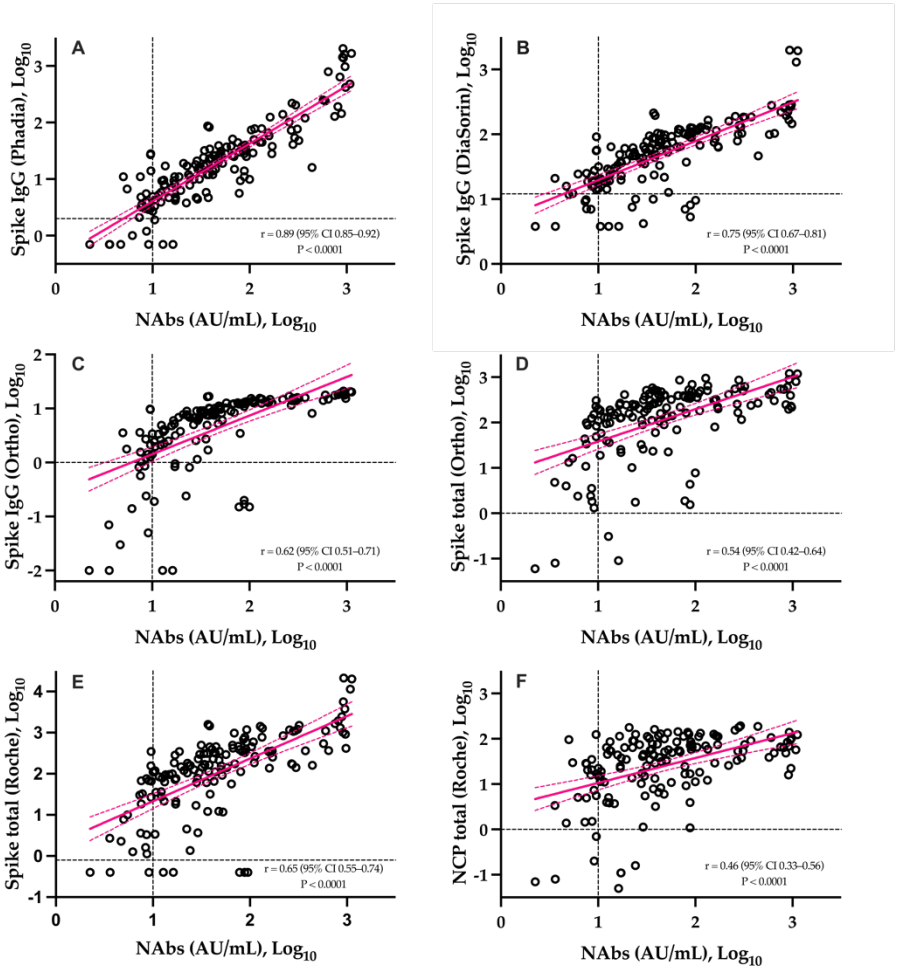


Figure I.IV.2.3: *Head-to-head comparison of the sVNT to six different SARS-CoV-2 antibody assays. Black dotted lines correspond to the positivity threshold of each assay. A: Phadia IgG S assay; B: DiaSorin IgG S assay; C: Ortho IgG S assay; D: Ortho total antibody S assay; E: Roche total antibody S assay; F: Roche total antibody N assay.*

Discussion

In this study, we evaluated the neutralizing capacity in COVID-19 patients. For that purpose, an sVNT was used. The method was based on antibody-mediated blockage of the interaction between the ACE2 receptor protein and the RBD. Since some reports demonstrated that some non-RBD targeting antibodies could possess neutralizing capacity (Chi et al., 2020; Suryadevara et al., 2021), the agreement of the sVNT with pVNT was evaluated using a subset of our cohort of COVID-19 patients. An excellent agreement of 97.2% was found and is consistent with the manufacturer's data. We also found that the specificity of the sVNT using a panel of 250 pre-pandemic samples was excellent (*i.e.,* 99.6%) using the manufacturer's cut-

off of 10.0 AU/mL. A potential cut-off refinement using a ROC curve analysis did not reveal the usefulness of an optimized cut-off, as already performed for some serological assays (Favresse, Brauner, et al., 2021; Favresse, Cadrobbi, et al., 2021; Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020; Gillot et al., 2020; Mairesse et al., 2020; Plebani et al., 2020; Tre-Hardy, Wilmet, et al., 2021). The excellent specificity observed in our study was in line with that claimed by the manufacturer (*i.e.,* 99.3%) using 270 samples from healthy volunteers who had no COVID-19 infection history and no vaccination history (manufacturer's information). The precision of the assay was also good (**Table I.IV.2.2**).

As observed in previous reports (Lau et al., 2021), a stronger neutralizing activity was identified in moderate–severe compared to mild COVID-19 patients (**Figure I.IV.2.1**).

The slow decay in NAbs with time was also consistent with some reports (Crawford et al., 2021; Isho et al., 2020; Lau et al., 2021; Muecksch et al., 2020; Prevost et al., 2020; Seow et al., 2020; Wajnberg et al., 2020; Wang et al., 2021), especially considering mild–moderate patients. A stronger SARS-CoV-2 antibody response in severe patients was also reported (Favresse, Eucher, et al., 2021). Compared to SARS-CoV-2 antibody assays, only neutralization activity assays reliably measure the real protective immunity of generated antibodies. There is also a high demand for the neutralization tests in specific clinical and industrial settings (e.g., for identification purposes with convalescent plasma or to support the development of vaccines). However, the conventional virus neutralization test requires live pathogens and is reserved for very specialized laboratories, requiring a high workload, skillful operators, specific and expensive facilities, and a BSL3 laboratory, and on top of that, they have a low result throughput (Lippi, Sciacovelli, et al., 2021; Tan et al., 2020). The use of automated and quantitative assays with a short turn-around time that have a well-documented correlation with the neutralizing activity should be preferred (Lippi, Sciacovelli, et al., 2021; Padoan, Bonfante, Pagliari, et al., 2020; Tang & Farnsworth, 2021). In our study, we observed that the Phadia S1 IgG assay had the highest correlation compared to sVNT (r=0.89) (**Figure I.IV.2.3**). The second, better correlated assay was the DiaSorin S1/S2 assay (r=0.75). This is in line with the findings of Legros et al., who showed a correlation of 0.71 using a microneutralization assay (Legros et al., 2021). The Ortho S1 IgG assay had a higher correlation compared to the Ortho S1 total assay, as observed in a study by Padoan et al. (Padoan, Bonfante, Pagliari, et al., 2020). Considering anti-N antibodies, the Roche total assay presented the lowest correlation with the results of the sVNT (r=0.46). Patel et al. obtained similar conclusions when comparing the Roche N total assay to neutralizing activity (r=0.40) (Patel et al., 2021). We therefore confirm that the strongest correlations are observed using anti-S or anti-RBD assays (Figueiredo-Campos et al., 2020; Ibarrondo et al., 2020; McAndrews et al., 2020; Premkumar et

al., 2020; Wajnberg et al., 2020) and our study highlights that correlations were especially high with the IgG assay. The fact that anti-N assays had a low correlation with the neutralizing activity was expected, as NAbs are directed against the S protein (Favresse, Elsen, et al., 2021). Nevertheless, it is important to keep in mind that a few patients may develop specific antibodies, *i.e.,* antibodies detected by conventional serological assays, which do not translate into a detectable neutralizing activity. We therefore think that the assessment of the neutralizing activity using an sVNT on an automated platform (without the disagreement of the gold standard technique) might be valuable.

In conclusion, we found a stronger neutralizing capacity in moderate–severe versus mild COVID-19 patients, in which a slow decay with time was observed. Significant correlations between NAbs and antibody titers as assessed by six binding assays were found, with higher r toward IgG assays targeting the S protein.

CHAPTER II: FIRST AND SECOND DOSES OF VACCINE

II.I. Introduction

Safe and effective COVID-19 vaccines were urgently needed to limit the escalation of cases and deaths, especially among the elderly, in individuals with preexisting medical conditions, and in front-line HCWs. Major efforts have been made to develop, produce and clinically validate new COVID-19 vaccines at an unprecedented speed (Golob et al., 2021; Polack et al., 2020; Walsh et al., 2020).

Several vaccines were rapidly available including those developed by BioNTech and Pfizer (BNT162b2, mRNA encapsulated in lipid coat), Moderna (mRNA-1273, mRNA encapsulated in lipid coat), AstraZeneca-Oxford (ChAdOx1 nCoV-19/AXD1222, Chimpanzee adenovirus), and Janssen Pharmaceutica (Ad26.COV2.S, Human adenovirus serotype 26). The vaccines from BioNTech/Pfizer and Moderna deliver mRNA coding for a SARS-CoV-2 S protein. Vaccines from AstraZeneca-Oxford and Janssen Pharmaceutica use a modified adenovirus with an inserted sequence encoding the S protein. The overall efficacy against symptomatic disease was around 95% 14 days after the second dose for mRNA vaccines and between 66% and 76% >14 days after the second dose for adenovirus-based vaccines. The efficacy against severe infection was 100% for all vaccines except for Ad26.COV2.S (*i.e.,* 85%) (Golob et al., 2021).

In this second chapter, we present the first series of data of the CRO-VAX HCP study. This was a multicenter, prospective, and interventional study that was designed to assess the antibody response in a population of healthcare professionals having received the two initial doses of the BNT162b2 mRNA COVID-19 vaccine. Two-hundred and thirty-one volunteers from three medical centers in Belgium were enrolled. Participants received the first vaccine dose between January 18, and February 17, 2021. The second dose was administered 21 days after the first one. All volunteers underwent a blood test within 2 days prior to the first vaccine dose. Thereafter, regular blood samples were obtained up to 6 months after vaccine injection (Favresse, Bayart, Mullier, Dogne, et al., 2021).

The complete kinetics of the humoral response (early increase, peak, and potential decrease) could therefore be studied. The humoral response was assessed using binding antibodies and NAbs, through sVNT or pVNT. At that time of the pandemic, the WT variant was predominant.

II.II. Early humoral response (BNT162b2)

II.II.1 Early Antibody Response in Healthcare Professionals after Two Doses of SARS-CoV-2 mRNA Vaccine (BNT162b2)

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SUMMARY

Background: Data on the immune response after two doses of BNT162b2 are so far limited. Previously infected individuals were excluded from pivotal clinical trials and the optimal dose regimen in this population has not been clearly studied. The CRO-VAX HCP study aims to investigate the early antibody response in a population of healthcare professionals having received two doses of the BNT162b2 mRNA COVID-19 vaccine.

Methods: The CRO-VAX HCP study is a multicenter, prospective, interventional study conducted in several sites in Belgium. The study included 231 healthcare professional volunteers who received the two-dose regimen of the BNT162b2 mRNA COVID-19 vaccine. Of these, 73 were previously infected by SARS-CoV-2 and 158 were uninfected and seronegative. In the first group, blood samples were collected at baseline and after 2, 4, 7, 10, 14, 21, and 28 days. In the second group, samples were obtained at baseline and after 14 and 28 days. Antibodies against the SARS-CoV-2 N and the RBD of the S1 of the S protein were measured in all individuals at different time points.

Results: In uninfected individuals, 95.5% (95% CI 91.0-98.2%) developed anti-S antibodies after 14 days and a 24.9-fold rise (95% CI 21.4-28.9%) in antibody titer was observed after the second dose. In previously infected individuals, peak antibody response was reached after 7 days (*i.e.,* 6,347 U/mL) and the second dose did not lead to significantly higher antibody titers (*i.e.,* 8,856 to 11,911 U/mL). Antibody titers were higher in previously infected individuals.

Conclusions: This study supports the concept that a single dose of BNT162b2 would be sufficient in previously infected individuals.

Introduction

The efficacy and safety of the two-dose regimen BNT162b2 mRNA COVID-19 vaccine (Pfizer-BioNTech, Mainz, Germany) has been proved and led in late December to its approval by several regulatory authorities (Amit et al., 2021; Polack et al., 2020;

Walsh et al., 2020). Nevertheless, data on the immune response after two doses of BNT162b2 are so far limited (F. Krammer et al., 2021; Manisty et al., 2021; Prendecki et al., 2021; Saadat et al., 2021). Additionally, individuals who had previous clinical or microbiological diagnosis of COVID-19 were excluded from pivotal clinical trials (Polack et al., 2020; Prendecki et al., 2021; Walsh et al., 2020), precluding the evaluation of the vaccine response in this particular subpopulation.

Methods

The CRO-VAX HCP study is a multicenter, prospective, and interventional study designed to assess the antibody response in a population of healthcare professionals having received two doses of the BNT162b2 mRNA COVID-19 vaccine. Two-hundred and thirty-one volunteers from 3 medical centers in Belgium were enrolled. All participants provided informed consent prior to collection of data and specimen. The study was approved by the ethical committees of the 3 medical centers (approval number: 2020-006149-21). Participants received the first vaccine dose from January 18, 2021, to February 17, 2021. The second dose was administered 21 days after the first one. All volunteers underwent a blood drawn within 2 days before the first vaccine dose. Volunteers were then included in two follow-up protocols in a 1:2 ratio. In the first group, samples were collected at baseline and after 2, 4, 7, 10, 14, 21, and 28 days while in the second group, samples were obtained at baseline and after 14 and 28 days.

Antibodies against the SARS-CoV-2 N (anti-N; Elecsys Anti-SARS-CoV-2 N qualitative ECLIA, Roche Diagnostics, Machelen, Belgium) and the RBD of the S1 of the S protein (anti-S; Elecsys anti-SARS-CoV-2 S quantitative ECLIA, Roche Diagnostics) were measured at each time point in all serum samples.

Statistical analysis was performed with GraphPad Prism 9.0.1 (GraphPad Software). Antibody titers between groups were tested using a Dunn's multiple comparisons test, with $p<0.05$ considered significant.

Results:

In our cohort, 73.6% (n=170) were females (mean age=42.6 years; range, 23-66 years) and 26.4% (n=61) were males (mean age=42.8 years; range, 23-64 years). Sixty-five persons had a previous positive RT-PCR diagnosis (mean days since RT-PCR=99; range, 34-337). Among these, 63 persons had symptoms while only 2 were asymptomatic, none requiring hospitalization. Eight additional participants with positive anti-N antibodies at baseline but without evidence of clinical or microbiological diagnosis of COVID-19 in the past were recategorized as previous COVID-19 positive patients (detailed information of the population is presented in **Supplementary materials**).

In uninfected, seronegative individuals, the rate of seroconversion after the first dose was 55.6% (95% CI 41.4-69.1%) and 95.5% (95% CI 91.0-98.2%) at day 10 and 14, respectively (**Figure II.II.1.1**). Among individuals included in the first group, none had positive anti-S antibodies before day 4 and only one participant seroconverted at day 7 (1.8%; 95% CI 0.1-9.4%). From day 21, all participants had detectable anti-S antibodies (100%; 95% CI 93.3-100%). At day 28 and following the second vaccine dose, a 24.9-fold (95% CI 21.4-28.9) increase was observed compared to day 21.

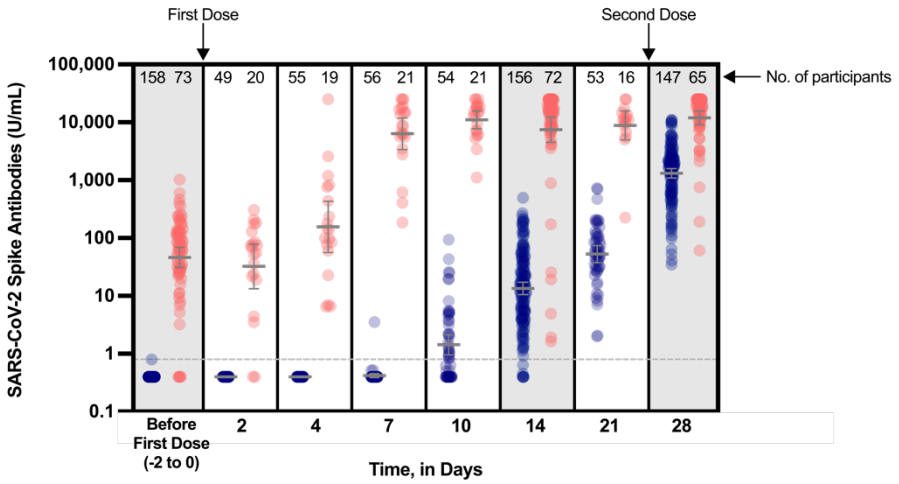

Figure II.II.1.1: *Evolution of SARS-CoV-2 S antibodies (U/mL) in individuals with previous SARS-CoV-2 infection (red points) and in seronegative persons without declared history of infection (blue points). Blood samplings before the first vaccine dose were obtained maximum 2 days before. Geometric means with 95% CIs are shown, if applicable. The grey dotted line corresponds to the positivity cut-off (i.e., 0.8 U/mL) of the Elecsys anti-SARS-CoV-2 S quantitative ECLIA. An automatic dilution of 1/100 at >250 U/mL was performed by the analyzer to extend the measurement domain up to 25,000 U/mL. Forty-two samples were rounded to 25,000 U/mL out of 1,038 (4%). Results <0.4 U/mL (limit of quantification) were rounded to 0.4. Up to day 4, blood samplings performed one day earlier or later compared to the expected blood times collection were allowed. From day 7, two days were allowed. Individuals with incomplete samplings were not excluded from the analysis.*

In individuals with a previous clinical or microbiological diagnosis of COVID-19, no change in anti-S titers was observed up to day 4. Only 5 samples from 3 previously participants with a previous molecular diagnosis of SARS-CoV-2 infection but who were seronegative at inclusion turned seropositive after 4 days. At day 7, a significant 139.9-fold (95% CI 110.8-172.1) increase in anti-S titers was observed. Following the second dose, a 262.4-fold (95% CI 228.1-294.4) increase from baseline was observed. Nevertheless, mean titers at days 14 (*i.e.,* 7,437 U/mL), 21 (*i.e.,* 8,856 U/mL) and 28 (*i.e.,* 11,911 U/mL) were non-significantly different from those at day 7 (6,347 U/mL) (p>0.99). Anti-N titers remained unchanged over the 28 days (**Supplementary materials**).

Considering each time point separately, anti-S titers of previously infected individuals were always statistically higher compared to uninfected individuals (**Supplementary materials**). At day 7, anti-S titers from previously infected individuals (*i.e.,* 6,347 U/mL) were non-significantly different from titers detected after the second dose of BNT162b2 in previously uninfected individuals (*i.e.,* 1,312 U/mL). From 14 days after the first dose of BNT162b2 anti-S titers of uninfected individuals (from 13.5 to 52.7 U/mL) were similar to anti-S titers of individuals with a previous clinical or microbiological diagnosis of COVID-19 at baseline (45.4 U/mL) ($p>0.99$). After the second dose, anti-S titers of uninfected individuals (*i.e.,* 1,312 U/mL) were statistically higher compared to baseline levels of previously infected individuals (*i.e.,* 45.4 U/mL; $p<0.0001$).

Discussion

In this study, we report a stronger humoral response in individuals with previous SARS-CoV-2 infection after the first dose of BNT162b2, supporting the concept that this first dose would act as a boost of a previous immunization, as also observed by others (F. Krammer et al., 2021; Manisty et al., 2021; Prendecki et al., 2021; Saadat et al., 2021). This is further supported by the non-significant increase in antibody titers reported after the second dose compared to antibody titers already observed 7 days after the first dose. Evaluation of the pre-vaccinal serological status could therefore be proposed as a strategy to identify patients who will only require the booster dose (Manisty et al., 2021). Pan-Ig assays should be preferred in this context to ensure maximal sensitivity to previous SARS-CoV-2 immunization (Favresse, Eucher, et al., 2021). Further studies are however needed to determine whether a booster dose in previously infected patients or if a delayed administration of the second dose in uninfected persons could provide sufficient and effective long-term protection.

Our study has some limitations. The findings should be completed by the assessment of the neutralizing capacity of the anti-S antibodies and by investigation of the cellular immune response. Importantly, while the conclusions of this study are of interest to support the concept of a single booster dose strategy in previously infected individuals, the efficacy of this dose regimen should be confirmed in a sufficiently powered study evaluating clinical outcomes.

This study (EudraCT registration number: 2020-006149-21) has a planned follow-up of two years. We would therefore be able to determine the long-term kinetics of the humoral response in both uninfected and previously infected participants.

II.II.2 Neutralizing Antibodies in Vaccine Recipients After Two Doses of BNT162b2

Viruses. 2021. 13(7) PART II

Julien Favresse, Constant Gillot, Laura Di Chiaro, Christine Eucher, Marc Elsen, Sandrine Van Eeckhoudt, Clara David, Laure Morimont, Jean-Michel Dogné, Jonathan Douxfils

SUMMARY

Background: The evaluation of the neutralizing capacity of anti-SARS-CoV-2 antibodies is important because they represent real protective immunity. In this study we aimed to measure and compare NAbs in COVID-19 patients and in vaccinated individuals.

Methods: Sequential samples obtained from 90 individuals who had received the complete dose regimen of BNT162b2 were analyzed with a sVNT and compared to the results obtained in an unvaccinated population.

Results: In the vaccinated population, 83.3% of COVID-19-naive individuals had positive NAbs 14 days after the first dose and all were positive 7 days after the second dose, *i.e.,* at day 28. In previously infected individuals, all were already positive for NAbs at day 14. At each time point, a stronger response was observed for previously infected individuals ($p<0.05$). The NAb response remained stable for up to 56 days in all participants.

Conclusion: Vaccinated participants had significantly higher NAb titers compared to COVID patients. In previously infected vaccine recipients, one dose might be sufficient to generate sufficient NAbs.

Introduction

The revelation of SARS-CoV-2 RNA through RT-PCR from NP swab samples is considered the gold standard method for the diagnosis of acute SARS-CoV-2 infection. Nevertheless, individuals with positive RT-PCR results represent only a limited fraction of all infections, given the limited availability and the brief time window in which RT-PCR testing presents the highest sensitivity (Gudbjartsson et al., 2020; R. Li et al., 2020).

The detection of specific antibodies following SARS-CoV-2 infection allows for the evaluation of the seroprevalence, the identification of convalescent plasma donors, the monitoring of herd immunity, the generation of risk prediction models, and is also likely to play a key role in the context of the global vaccination strategy (Bohn,

Loh, et al., 2020; Joyner et al., 2021). Anti-SARS-CoV-2 NAbs are of particular importance because these are the antibodies which inhibit the binding of the RBD of the surface S protein to the human ACE2 receptor. The complex formed between the virus S protein and the human ACE2 is responsible for the virus entry into hosts cells and the inhibition of the formation of this complex may thereby prevent infection and reduce disease severity (Premkumar et al., 2020; Shang et al., 2020).

Compared yo SARS-CoV-2 antibody assays, which measured all the antibodies that are able to recognize the S protein, only assays measuring NAbs reliably measure the real protective immunity of antibodies (Padoan, Bonfante, Pagliari, et al., 2020). The current gold standard method to measure NAbs is the conventional virus neutralization test, which requires a BSL3 laboratory to manipulate the live pathogen. These tests are reserved for very specialized laboratories and further require a high workload, skillful operators, and expensive installations, and they have a low throughput (Lippi, Sciacovelli, et al., 2021; Tan et al., 2020). The use of a SARS-CoV-2 sVNT based on antibody-mediated blockage of the interaction between the ACE2 receptor protein and the RBD has been found to be an attractive alternative (Perera et al., 2021; Tan et al., 2020; Valcourt et al., 2021).

In this study, we investigated neutralizing capacity by means of an sVNT in (1) previous COVID-19 patients and (2) volunteers vaccinated with BNT162b2.

Materials and Methods

COVID-19 patients and vaccinated recipients

Demographic data for the two groups are presented in **Table II.II.2.1** (**Table II.II.2.1**). In the COVID-19 patient group, 150 samples from 75 patients with a confirmed SARS-CoV-2 RT-PCR were retrospectively included from 26 March 2020 to 6 January 2021. Among them, 39 were females (median age=45; min–max: 24–95 years) and 36 were males (median age=62; min–max: 24–88 years). Multiple sequential sera were available for 41 patients. Seventeen patients required hospitalization and were categorized as moderate–severe patients, according to the WHO categorization (Marshall et al., 2020). Information on the days since the onset of symptoms was collected from medical records and was available for 63 patients. When data about symptoms were not available (*n*=12), the day of diagnosis (*i.e.,* the RT-PCR result) was used instead. The median time since diagnosis was 169 days (range, 11–296) and 139 days (range, 10–290) in mild and moderate–severe COVID-19 patients, respectively (p=0.39).

In the second group, 90 healthcare volunteers who were scheduled to receive the complete dose regimen of the BNT162b2 mRNA COVID-19 vaccine were prospectively enrolled. Among them, 71.1% (*n*=64) were females (median age=44 years; range, 25–64 years) and 28.9% (*n*=26) were males (median age=48 years;

range, 25–63 years). Thirty persons had a previous positive RT-PCR diagnosis (median days since RT-PCR=158; range, 46–337). Among these, 29 persons were classified as mild cases and had positive anti-N antibodies, whereas only one was asymptomatic (positive RT-PCR diagnosis and no anti-N antibodies detected). Participants received the first vaccine dose from 25 January 2021 to 16 February 2021. The second dose was administered 21 days after the first one. All volunteers had blood drawn within 2 days before the first vaccine dose and additional blood samples were then collected after 14, 21, 28, 42, and 56 days.

Additionally, 250 samples collected before January 2020 were assessed to evaluate the clinical specificity of the sVNT.

Demography	
Group 1: Previous COVID-19 patients (n)	***75***
Females (*n* (%))	**39 (52%)**
Age (median (min–max))	45 (21–95)
Males (*n* (%))	**36 (48%)**
Age (mean (min–max))	62 (24–88)
Moderate–severe (*n* (%))	**17 (22.7%)**
Time since diagnosis (median, (range))	169 (11–266)
Mild (*n* (%))	**58 (77.3%)**
Time since diagnosis (median, (range))	139 (10–290)
Total number of samples	**150**
Group 2: BNT162b2 vaccine recipients (n)	***90***
Females (*n* (%))	**64 (71.1%)**
Age (mean (min–max))	44 (25–64)
Males (*n* (%))	**26 (28.9%)**
Age (mean (min–max))	48 (25–63)
Patients with a previous RT-PCR + (*n* (%))	**30 (33.3%)**
Time since diagnosis (median, (range))	158 (46–337)
Moderate–severe (*n* (%))	0 (0.0%)
Mild (*n* (%))	29 (96.7%)
Asymptomatic (*n* (%))	1 (3.3%)
Total number of samples	**550**

Table II.II.2.1: *Demographic data for (1) the past-COVID-19 group and (2) the vaccinated group. The difference between the total number of samples and the number of patients/subjects is explained by multiple timepoints for blood sampling.*

Sample collection

Blood samples were collected in serum-gel tubes (BD SST II Advance, Becton Dickinson, NJ, USA) and centrifuged for 10 min at 1740*g on a Sigma 3-16KL centrifuge. Sera were stored in the laboratory serum biobank at −20 °C from the collection date. Frozen samples were thawed for 1 h at room temperature on the day of the analysis. Re-thawed samples were vortexed before the analysis. All samples were collected at the Clinique Saint-Luc (Bouge, Namur, Belgium). The study protocol was in accordance with the Declaration of Helsinki. All vaccinated participants provided informed consent prior to the collection of data and specimens (EudraCT registration number: 2020-006149-21).

Analytical procedures

Neutralizing capacity was estimated by performing an sVNT. The iFlash-2019-nCoV NAbs assay is a one-step competitive paramagnetic particle CLIA for the quantitative

determination of 2019-nCoV NAbs in human serum and plasma. The assay detects NAbs that block the binding of RBD and ACE2. First, NAbs (if present) react with the RBD antigen coated on paramagnetic microparticles to form a complex. Second, the acridinium-ester-labeled ACE2 conjugate is added to competitively bind to the RBD-coated particles, which have not been neutralized by the NAbs (if present) from the sample, and these form another reaction mixture. Under a magnetic field, magnetic particles are adsorbed to the wall of the reaction tube, and unbound materials are washed away by the wash buffer. The resulting chemiluminescent reaction is measured in RLU, with an inverse relationship between the amount of NAbs and the RLU value detected. According to the manufacturer, it shows excellent positive (98.5%) and negative percentage agreement (96.1%) with the conventional PRNT. A result <10.0 AU/mL is considered negative and a result ≥10.0 AU/mL is considered positive (according to the manufacturer's information). The sVNTs were performed on an iFlash1800 automated magnetic CLIA analyzer from Shenzhen YHLO Biotech Co., Ltd.

A total of 71 random samples (*i.e.,* 23 pre-pandemic and 48 past-COVID-19 patient samples) were also assessed by means of a pVNT. Details about the method are presented in **Supplementary Materials**. A sample is considered negative if the IC50 value of this sample is below the dilution 1/20.

The Roche N total assay was also used to determine the serological status of vaccinated participants before vaccine injection.

In group 1 and in previously infected individuals from group 2, RT-PCR for SARS-CoV-2 determination in NP swab samples was performed on the LightCycler 480 Instrument II (Roche Diagnostics) using the LightMix Modular SARS-CoV *E*-gene set.

Statistical analysis

Descriptive statistics were used to analyze the data. NAb titers among the two vaccinated groups at different time points were tested using an ANOVA multiple comparisons test. A $p<0.05$ was used as a significance level. Data analysis was performed using GraphPad Prism software (version 9.1.0, San Diego, CA, USA).

Results

Neutralizing antibodies in vaccinated volunteers

The **Figure II.II.2.1** represents the evolution of NAbs in a group of 90 vaccinated individuals (**Figure II.II.2.1**). In uninfected, seronegative individuals (*n*=60/90), none had detectable anti-N antibodies nor NAbs at baseline. At day 14, the rate of seroconversion after the first dose was 83.3% (*n*=50/60) with a 5.1-fold increase of NAb titers. Seven days after the administration of the second dose, a 114.3-fold increase was observed from baseline and all individuals had NAb titers above the positivity threshold. At days 42 and 56, the mean titers were not statistically different from those obtained at day 28 (p>0.99) (**Figure II.II.2.1**).

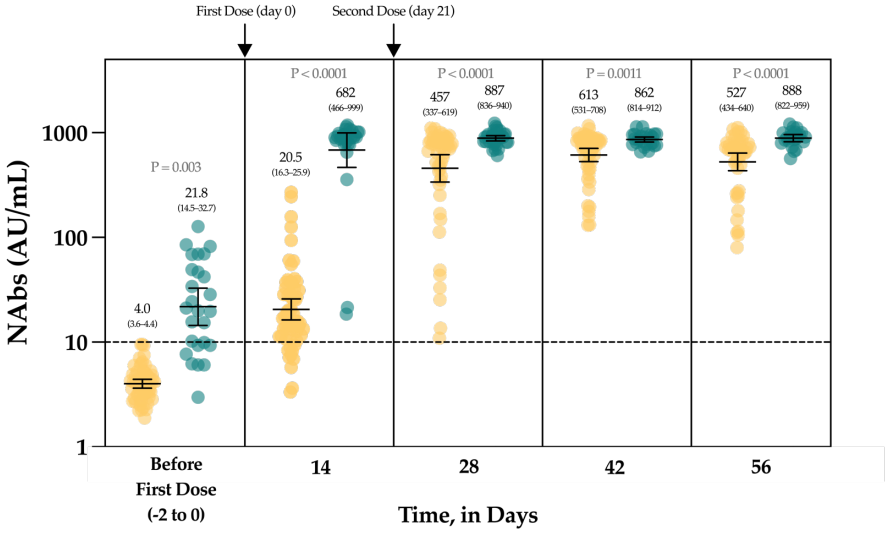

Figure II.II.2.1: *The evolution of NAbs in a group of 90 vaccinated participants. Uninfected individuals are represented in yellow and previously infected individuals are represented in green turquoise. The black dotted line corresponds to the positivity threshold of 10 AU/mL.*

In individuals with a previous SARS-CoV-2 infection, 26.7% (*n*=8/30) had negative NAbs at baseline and all individuals had positive anti-N results. At day 14, a significant 31.3-fold increase in NAbs was observed, with all individuals becoming positive. Compared to the NAb titers observed at day 14, the second dose administration had no significant impact on the NAb titers until up to day 56 (p>0.99) (1.3-fold increase) (**Figure II.II.2.1**).

Considering each time point separately, NAbs were always statistically higher in previously infected individuals compared to uninfected individuals (**Figure II.II.2.1**). The mean NAb titers of previously infected individuals at baseline were not different from those observed in uninfected individuals 14 days after the first dose

administration (p>0.99). NAbs titers in previously infected individuals at day 14 were not different from titers obtained in uninfected individuals at days 28 and 56 (p>0.05).

All vaccinated participants had significantly higher NAb titers after the complete dose regimen of the BNT162b2 vaccine compared to our cohort of COVID-19 patients (**Figure II.II.2.2**).

Discussion

In this study, we evaluated the neutralizing capacity in two groups of COVID-19 patients and healthcare professionals who had received the complete dose regimen of the BNT162b2 vaccine. For that purpose, an sVNT was used. The method was based on antibody-mediated blockage of the interaction between the ACE2 receptor protein and the RBD.

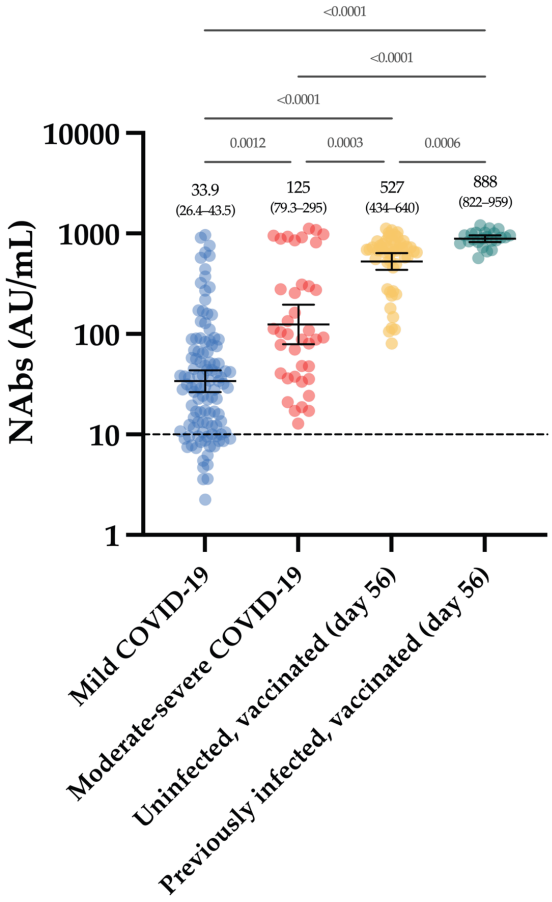

Figure II.II.2.2: *NAb titers obtained in the first group (moderate–severe and mild COVID-19), compared to those obtained in the group of vaccinated participants, at day 56. The black dotted line corresponds to the positivity threshold of 10 AU/mL.*

In the group of vaccinated individuals from the CRO-VAX HCP study (Favresse, Bayart, Mullier, Dogne, et al., 2021), we evaluated the neutralizing response in a cohort of 90 volunteers, of which 60 were uninfected and 30 were previously infected by SARS-CoV-2, having received the complete dose regimen of the BNT162b2 vaccine. NAbs were measured at baseline, *i.e.,* just before the administration of the first dose, and at 14, 28, 42 and 56 days. So far, few reports have investigated the neutralizing response in vaccinated subjects (Anichini et al., 2021; Ebinger et al., 2021; Lustig, Nemet, et al., 2021; Prendecki et al., 2021; Saadat et al., 2021; Terpos et al., 2021) and they mainly included few participants, only investigating the effect of the first dose [42,43,45], or did not include previously infected individuals (Terpos et al., 2021). In our study, a significant increase in NAb titers was seen after the first dose (*i.e.,* a 5.1- and a 31.1-fold increase in uninfected and previously infected individuals, respectively) in all participants (**Figure II.II.2.1**). Interestingly, the neutralizing capacity was similar when comparing previously infected individuals at baseline and naive individuals after the first dose, an observation that is similar to that of Manisty et al. using the Roche RBD total assay (Manisty et al., 2021). After the second dose, a significant increase in NAb titers was only observed in uninfected individuals (*i.e.,* a 22.3-fold increase between day 14 and 28). Afterwards, the peak of the neutralizing capacity seems to have been reached at day 42 (*i.e.,* 613 AU/mL) and a slight but non-significant decrease was observed at day 56 (527 AU/mL), which could be explained by the natural clearance of antibodies via excretion or mostly via catabolism (Lobo et al., 2004). Terpos et al. obtained similar findings using the cPass™ sVNT from GenScript (Terpos et al., 2021). All participants were considered positive 7 days after the second dose. In previously infected individuals, NAb titers at days 28 to 56, *i.e.,* 7 and 35 days after the second dose, were not significantly different from those at day 14 after the first dose (**Figure II.II.2.1**). The non-significant differences between the neutralizing capacity after the first dose and after the second dose support the concept only one dose might be sufficient to generate a complete NAb response in individuals with a previous SARS-CoV-2 infection (**Figure II.II.2.4**). Using an sVNT, Ebiger et al. also noticed a similar response after the second dose in previously infected individuals, but the number of participants who had received the second dose was low (*n*=11) and they were followed up for a maximum of 28–42 days (Ebinger et al., 2021). Evaluation of the pre-vaccinal serological status could therefore be proposed as a strategy to identify patients who will only require the booster dose (Manisty et al., 2021). In this context, pan-Ig assays should be preferred due to their higher sensitivity observed in long-term studies (up to 1 year post-infection) (Favresse & Douxfils, 2021; Favresse, Eucher, et al., 2021) compared to Nabs, which were negative in eight out of 30 (73.3%) previously infected individuals in our cohort (median days since RT-PCR=158) (**Figure II.II.2.1**). The NAb titer after the first dose in previously infected

individuals was not significantly different from the NAb titers of uninfected individuals after the two-dose regimen ($p>0.05$), even if lower mean titers were reported (**Figure II.II.2.1**). This finding is inconsistent with the recent data of Anchini et al., who reported significantly higher NAb titers in previously infected individuals after the first dose compared to the uninfected individuals who had received two doses (Anichini et al., 2021).

Our study (EudraCT registration number: 2020-006149-21) has a planned follow-up of two years. We will therefore be able to determine the long-term kinetics of the humoral response in both uninfected and previously infected participants.

In conclusion, vaccinated participants had significantly higher NAb titers after the complete dose regimen of the BNT162b2 vaccine compared to our cohort of COVID-19 patients. In light of these data, we can hypothesize that only one dose of the BNT162b2 vaccine might be sufficient in previously infected individuals to generate sufficient NAb titers to confer a sufficient serological immunity.

II.III. Three-month follow-up (BNT162b2)

II.III.1 Antibody Titers Decline 3-month Post-Vaccination With BNT162b2

Emerging Microbes and Infections. 2021. 10(1)

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SUMMARY

Background: Several studies reported on the humoral response in subjects having received the BNT162b2 mRNA COVID-19 vaccine. However, data on the kinetics of antibodies 3 months post-vaccination are currently lacking and are important to drive the future vaccination strategy.

Methods: The CRO-VAX HCP study is an ongoing multicenter, prospective, and interventional study designed to assess the antibody response in a population of healthcare professionals who had received two doses of the BNT162b2 mRNA COVID-19 vaccine. Two-hundred individuals underwent a blood drawn within 2 days before the first vaccine dose. One-hundred and forty-two persons (71%) were categorized as seronegative at baseline while 58 (29%) were seropositive. Samples were then collected after 14, 28, 42, 56, and 90 days. Antibodies against the SARS-CoV-2 N and the RBD of the S1 of the S protein were measured in all individuals at different time points.

Results: Using a one-compartment kinetics model, the time to maximum concentration (T_{max}) was estimated at 36 ± 3 days after the first dose and the estimated half-live ($T_{1/2}$) of antibodies was 55 days (95% CI: 37-107 days) in seronegative participants. In seropositive participants, the T_{max} was estimated at 24 ± 4 days and the estimated $T_{1/2}$ was 80 days (95% CI: 46-303 days). The antibody response was higher in seropositive compared to seronegative participants.

Conclusion: In both seropositive and seronegative subjects, a significant antibody decline was observed at 3 months compared to the peak response. Nevertheless, the humoral response remained robust in all participants.

Introduction

The efficacy and safety of the two-dose regimen of BNT162b2 mRNA COVID-19 vaccine (Pfizer-BioNTech, Mainz, Germany) has been proved and led to its approval

by several regulatory authorities in late December 2020 (Polack et al., 2020). Several studies have reported on the humoral response in vaccinated subjects but the results were only available after one administration of the vaccine and if the response after the second dose was evaluated, the follow-up of the participants was limited, *i.e.,* below 3 months (Ebinger et al., 2021; Favresse, Bayart, Mullier, Dogne, et al., 2021; Padoan, Dall'Olmo, et al., 2021; Salvagno, Henry, et al., 2021; Tre-Hardy, Cupaiolo, et al., 2021) Therefore, a longer follow-up period is needed to assess the antibody kinetics in individuals after a two-dose regimen of BNT162b2. These data are important, especially since the question about a third dose has been raised by the pharmaceutical industries which will led to important societal, logistical and economic consequences.

Material and methods

The CRO-VAX HCP study is an ongoing multicenter, prospective and interventional study designed to assess the antibody response in a population of healthcare professionals having received two doses of the BNT162b2 mRNA COVID-19 vaccine (Comirnaty), as previously described in details (Favresse, Bayart, Mullier, Dogne, et al., 2021). All participants provided informed consent prior to collection of data and specimen. The study was approved by a central ethical committee (approval number: 2020-006149-21). Participants received the first vaccine dose from January 18, 2021, to February 17, 2021. The second dose was administered 21 days after the first one. All volunteers underwent a blood drawn within 2 days before the first vaccine dose. Samples were then collected after 14, 28, 42, 56, and 90 days. Blood samplings performed earlier or later compared to the expected blood times collection were allowed (10% variation; *i.e.,* 90 days ± 4.5 days). In this interim report, data from a total of 200 participants were available after three months.

Antibodies against the SARS-CoV-2 N (anti-N; Elecsys Anti-SARS-CoV-2 N total qualitative ECLIA, Roche Diagnostics, Machelen, Belgium) and the RBD of the S1 of the S protein (Elecsys anti-SARS-CoV-2 S total quantitative ECLIA, Roche Diagnostics) were measured at each time point. Results above 0.8 U/mL (manufacturer's cut-off) or 0.165 COI (cut-off index; as found previously(Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020)) for anti-S and anti-N antibodies were considered positives.

Means and 95% CIs were used to describe the data. The between group difference of antibody titers were tested using a Tukey multiple comparison test. A multiple testing correction was applied in the multiple group comparison. A one-compartment modeling was used to describe the kinetics of the antibody response in seropositive and seronegative subjects, assuming a steady decay rate over time. The $T_{1/2}$ was obtained from the one-compartment modeling which permitted the calculation of the elimination rate of the antibody response. Statistical analyses were

performed using GraphPad Prism 9.0.1 (GraphPad Software) and JMP Pro 16.0.0 (SAS Institute Inc., South Carolina, United States). p<0.05 was considered significant.

Results

In this cohort, 77.5% (n=155) were females (mean age=43 years; range, 23-66 years) and 22.5% (n=45) were males (mean age=41 years; range, 24-64 years). One-hundred and forty-two persons (71%) were categorized as seronegative at baseline while 58 (29%) were seropositive (*i.e.,* subjects having levels of anti-N and anti-S antibodies at baseline above the positivity cut-off). Anti-N antibodies remained stable in seropositive participants (**Figure II.III.1.1**). In the seronegative group, no participant developed anti-N antibodies. None of the previously infected participants required hospitalization at the time of SARS-CoV-2 infection.

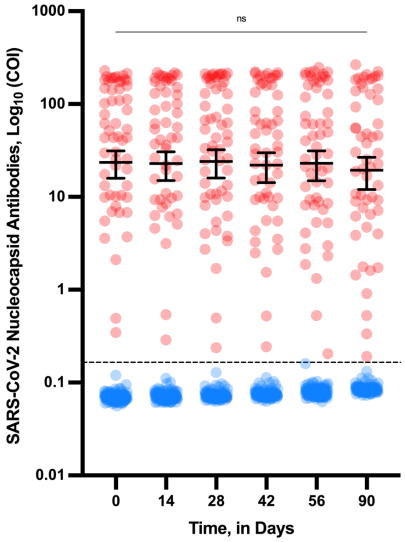

Figure II.III.1.1: *Evolution of SARS-CoV-2 N antibodies (COI) in seronegative (blue) and seropositive individuals (red) according to the time since the first vaccine dose administration. Means with 95% CIIs (log_{10}) are shown. The black dotted line corresponds to the positivity cut-off (i.e., 0.165 COI). ns = non-significant differences between timepoints (p<0.05).*

In seronegative individuals, the rate of seroconversion 14 days after the first dose was 95.7% (**Figure II.III.1.2a**). From day 28 to day 90, all participants had detectable anti-S antibodies. The maximal antibody response was reached between days 28 and 42 (2,204 versus 1,863; p=0.20), with a 48.8 to 57.7-fold increase compared to day 14 (*i.e.,* 38.2 U/mL). Afterward, a continuous decrease was observed at days 56 (*i.e.,* 1,517 U/mL) and 90 (*i.e.,* 1,262 U/mL) (**Supplementary materials**, **Figure II.III.1.2a**). In seropositive individuals, the maximal antibody response was reached between days 14 and 42 (from 15,540 to 16,935; p>0.05), which represents a mean 122.1-fold increase compared to baseline (*i.e.,* 132 U/mL). Afterward, a continuous decrease

was observed at days 56 (*i.e.,* 13,315 U/mL) and 90 (*i.e.,* 8,919 U/mL) (**Supplementary materials**, **Figure II.III.1.2a**). All participants still had detectable anti-S antibodies up to day 90. Considering each time point separately, anti-S titers of seropositive individuals were always statistically higher compared to seronegative individuals (p<0.0001) (**Supplementary materials**). Importantly, the inter-individual variability was important in each group.

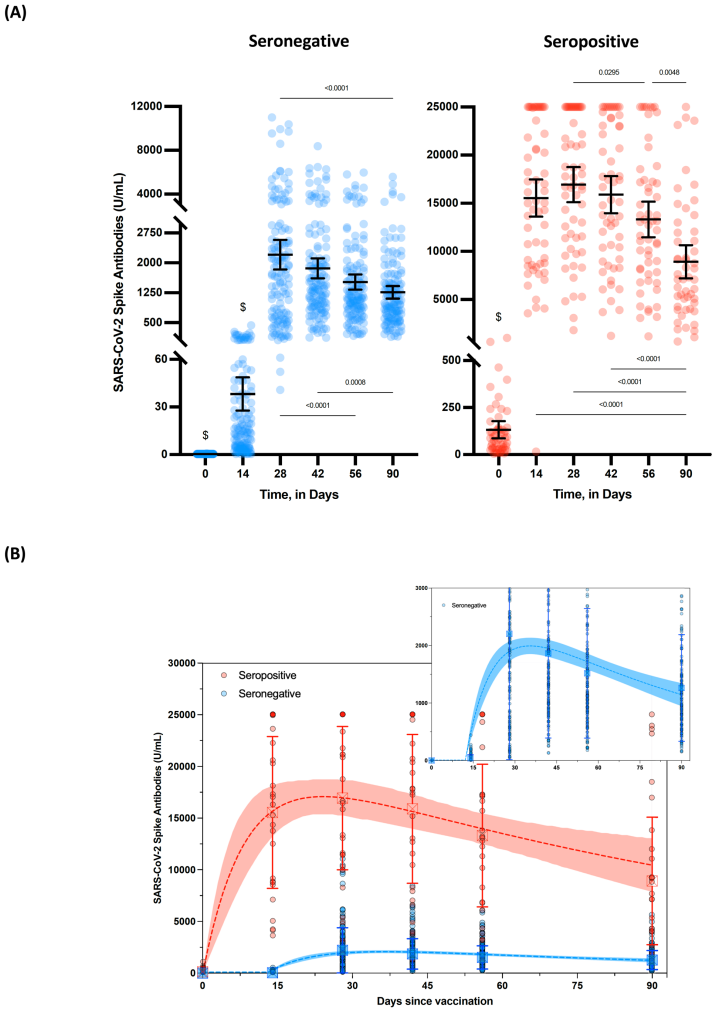

Figure II.III.1.2: *Evolution of SARS-CoV-2 S antibodies (U/mL) in seronegative (blue) and seropositive individuals (red) according to the time since the first vaccine dose administration.* ***(A)*** *Means with 95% CIs are shown. An automatic dilution of 1/100 at >250 U/mL was performed by the analyzer to extend the measurement domain up to 25,000 U/mL. Forty-six samples were rounded to 25,000 U/mL out of 1,195 (3.8%). Results <0.4 U/mL (limit of quantification) were rounded to 0.4.* $^{\$}$ *= statistically different from all other groups (i.e., p<0.0001).* ***(B)*** *Kinetic models of the humoral response based on a one-compartment model. A zoom of the seronegative population is presented in the right-upper part of the figure. Means with one SD are shown.*

The estimated $T_{1/2}$ of antibodies observed from data collected until 90 days post-vaccination for seronegative participants was 55 days (95% CI: 37-107 days) as calculated by the one-compartmental model. The T_{max} was estimated at 36 ± 3 days. In seropositive participants, the estimated $T_{1/2}$ of antibodies after 90 days was 80 days (95% CI, 46-303 days) and the T_{max} was 24 ± 4 days (**Figure II.III.1.2b**).

Discussion

In this study, we report a significant antibody decline 3 months post-vaccination in both seronegative and seropositive individuals who received two doses of the BNT162b2 vaccine. The highest mean antibody titer was observed between days 14 and 42 for seropositive participants and between 28 and 42 days for seronegative participants (**Supplementary materials**, **Figure II.III.1.2**). Based on the one-compartment model, the T_{max} was estimated at 24 ± 4 days in seropositive versus 36 ± 3 days in seronegative participants (**Figure II.III.1.2b**). Previous studies also found an earlier maximal response in seropositive individuals (Favresse, Bayart, Mullier, Dogne, et al., 2021; Salvagno, Henry, et al., 2021).

At 3 months, a mean antibody decrease of 37.9% and 44.7% in seronegative and seropositive individuals was identified from the highest mean antibody response (**Supplementary materials**, **Figure II.III.1.2**). Nevertheless, it is important to notice that all participants still had a robust antibody response at 3 months. Moreover, the vaccination with BNT162b2 elicited much higher antibody titers at 3 months compared to the titers collected in serum from convalescent patients using the same assay (*i.e.,* Roche Elecsys anti-S pan-Ig assay) (Favresse, Eucher, et al., 2021; Schaffner, Risch, Aeschbacher, et al., 2020). Using the $T_{1/2}$ derived from the kinetics model, we could predict a drop below the positivity threshold (*i.e.,* 0.8 U/mL for anti-S) after 554 days for seronegative and after 1,184 days for seropositive individuals. These predictions remain to date speculative and will need to be confirmed by subsequent sampling times but this could help to design vaccination strategies (Rubin, 2021). The aim is to keep sufficient antibody levels to protect vaccinated subjects against WT SARS-CoV-2 but also the related variants, which have all demonstrated some forms of immunity escape (Rubin, 2021). It has also been demonstrated that NAbs is a better CoP against infection than global serological testing and this may also serve in the future as a biomarker to ensure a proper protection at the patient's level (Khoury et al., 2021). A high correlation ($r > 0.86$, $p < 0.001$) between the anti-S assay from Roche Diagnostics and a sVNT was found (L'Huillier et al., 2021). However, a limitation of this study included the lack of measurement of neutralizing capacity measurement. Data about the contribution of the cellular immune response are also missing.

Data about the long-term antibody kinetics in vaccinated subjects are still scarce. In a population of 33 healthy adults having received the Moderna mRNA-1273 vaccine

and followed up to day 209, the estimated $T_{1/2}$ was 52 days (95% CI: 46-58 days) using an exponential decay model (Doria-Rose et al., 2021). In a cohort of 188 unvaccinated COVID-19 patients (mostly not hospitalized: 174/188) who were followed for up to 8 months, the antibody $T_{1/2}$ was 83 days (95% CI: 62-126 days) (Dan et al., 2021).

All these results were consistent with the results obtained in this study in both seronegative (*i.e.,* 55 days, 95% CI: 37-107 days) and seropositive participants (*i.e.,* 80 days, 95% CI: 46-303 days). Because the 95% CI are overlapping, we cannot conclude that $T_{1/2}$ are different between seropositive and seronegative participants. This study (EudraCT registration number: 2020-006149-21) has a planned follow-up of two years, with the next blood sampling campaign planned in July 2021. This will permit to further refine the kinetics model and to provide better estimate of the antibody response in both seropositive and seronegative individuals.

II.IV. Six-month follow-up (BNT162b2)

II.IV.1 Waning of IgG, Total and Neutralizing Antibodies 6 months Post-Vaccination With BNT162b2 in Healthcare Workers

Vaccines. 2021. 9(10)

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SUMMARY

Background: Several studies reported on the early humoral response in individuals having received the BNT162b2 COVID-19 vaccine. However, data about the long-term duration of antibodies are still scarse and are important to design the vaccination strategy.

Methods: 231 healthcare professionals received the two doses regimen of BNT162b2. Of these, 158 were seronegative and 73 were seropositive at baseline. Samples were collected at several timepoints. The NAbs and binding antibodies against the N and the S protein of SARS-CoV-2 were measured. The serological response was modeled using a one-compartment kinetics model.

Results: At day 180, a significant antibody decline was observed in seronegative (-55.4% with total antibody assay; -89.6% with IgG assay) and seropositive individuals (-74.8% with total antibody assay; -79.4% with IgG assay). The estimated $T_{1/2}$ of IgG from the peak humoral response was 21 days (95% CI: 13-65) in seronegative and 53 days (95% CI: 40-79) in seropositive individuals. The estimated $T_{1/2}$ of total antibodies was longer and ranged from 68 days (95% CI: 54-90) to 114 days (95% CI: 87-167) in seropositive and seronegative individuals, respectively. The decline of NAbs was more pronounced (-98.6%) and around 45% of the subjects tested were negative at day 180.

Conclusion: In seropositive and seronegative subjects, a highly significant antibody decline was observed at 6 months compared to the peak response with an important proportion with non-detectable level of NAbs. Whether this decrease correlates with an equivalent drop in the clinical effectiveness against the virus would require appropriate clinical studies.

Introduction

The efficacy and safety of the two-dose regimen of BNT162b2 mRNA COVID-19 vaccine (Pfizer-BioNTech, Mainz, Germany) has been proved and led to an emergency use authorization (EUA) delivered on the 11th of December 2020 (Polack et al., 2020). On the 23rd of August 2021, the BNT162b2 vaccine became the first COVID-19 vaccine approved by the FDA (FDA, 2021). Real-world data on the BNT162b2 also confirmed the high effectiveness of this vaccine in reducing laboratory-confirmed infection and viral load in infected individuals, as well as reducing COVID-19 hospitalization and death. (Amit et al., 2021; Dagan et al., 2021; Levine-Tiefenbrun et al., 2021; Shamier et al., 2021)

Around the world, massive vaccination campaigns started in early 2021 and data about the immunological response emerged in the literature to document the evolution of humoral response in subjects vaccinated against SARS-CoV-2 (Braeye et al., 2021; Ebinger et al., 2021; Favresse, Bayart, Mullier, Dogne, et al., 2021; Favresse, Gillot, Di Chiaro, et al., 2021; Hirotsu et al., 2021; Padoan, Dall'Olmo, et al., 2021; Salvagno, Henry, et al., 2021; Tre-Hardy, Cupaiolo, et al., 2021). In the majority of seronegative subjects, antibody response was positive two weeks after the first dose and the peak response was observed around 28 days (Favresse, Bayart, Mullier, Dogne, et al., 2021; Lustig, Sapir, et al., 2021). The antibody titers were also higher in previously infected individuals compared to seronegative subjects and recent studies found a decline in antibody titers at 3 months (Cocomazzi et al., 2021; Favresse, Bayart, Mullier, Dogne, et al., 2021; Favresse, Bayart, Mullier, Elsen, et al., 2021; Salvagno et al., 2022c). This antibody decline needs to be well monitored because it may provide important information to support the decision-making on the potential use of a booster dose. However, longer-term kinetic data of the humoral response after the two-dose regimen of BNT162b2 are still scarce in the literature.

The CRO-VAX HCP study is an ongoing multicenter, prospective and interventional study designed to assess the antibody response in a population of healthcare professionals (HCPs) having received two doses of the BNT162b2 mRNA COVID-19 vaccine. We report hereby an interim analysis on the data obtained on the humoral response after a 6-month follow-up.

Material and methods

Study design and participants

The CRO-VAX HCP study is an ongoing multicenter, prospective, and interventional study designed to assess the antibody response in a population of HCPs having received two doses of the BNT162b2 mRNA COVID-19 vaccine (Comirnaty), as described in **Supplementary materials**. The study was approved by a central ethical committee (approval number: 2020-006149-21) and a total of 231 participants were

enrolled in the study. All participants provided informed consent prior to data and specimen collections. Participants received the first vaccine dose from January 18, 2021, to February 17, 2021. The second dose was administered 21 days after the first one. All volunteers underwent blood drawn within 2 days before the first vaccine dose. Samples were then collected after 14, 28, 42, 56, 90 and 180 days following the first dose. Blood samplings performed earlier or later than the expected blood collection times were allowed with a maximal allowed percentage of 10% (*i.e.,* 180 days ± 18 days). Subjects having levels of anti-N and anti-S antibodies at baseline above the positivity cut-offs of the assays were considered seropositive while the others are considered COVID-19 naïve and are classified as seronegative.

Analytical procedures

Antibodies against the RBD of the S1 of the S protein were measured at each time point by two different analytical methods: the Elecsys assay that measured SARS-CoV-2 total antibodies (combination of IgG, IgM and IgA) (Roche Diagnostics, Machelen, Belgium) with a positivity cut-off of 0.8 U/mL and the Architect assay that measured SARS-CoV-2 IgG (Abbott, Wavre, Belgium) with a positivity cut-off of 50 AU/mL. Total antibodies against the SARS-CoV-2 N (anti-N; Roche Diagnostics, Machelen, Belgium) were also measured. Results above 0.165 COI (Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020) were considered positive. For the total antibody assay, alternative cut-offs (*i.e.,* 15 U/mL and 133 U/mL)(Resman Rus et al., 2021) were also investigated.

NAbs were assessed on a subset of 60 subjects at different time points (0, 28, 90 and 180 days) using a pVNT as described elsewhere (Douxfils et al., 2021; Nie et al., 2020b). For this latter assay, samples were considered negative if the IC_{50} value was below the dilution of 1/20.

Statistical analysis

Means and 95% CIs were used for data description. The between-group differences of antibody titers were tested using a Tukey multiple comparison test. A multiple testing correction was applied in the multiple group comparison. For kinetic modeling, a one compartment modeling was used to describe the kinetics of the antibody response in seropositive and seronegative subjects, assuming a steady decay rate over time. The $T_{1/2}$ was obtained from the one compartment modeling which permitted the calculation of the elimination rate of the antibodies. The mean time needed to cross the various thresholds of interest defined above was also determined based on this model. Correlation studies (IgG, total antibodies and NAbs) were evaluated with a Spearman's rank correlation test. A Cohen's kappa agreement test was also performed between assays. Statistical analyses were performed using GraphPad Prism 9.0.1 (GraphPad Software, San Diego, California USA,

www.graphpad.com) and JMP Pro 16.0.0 (SAS Institute Inc., South Carolina, United States). $p<0.05$ was considered significant.

Results

Demographic data

Among the participants, 170 (73.6%) were females (mean age=43 years; range, 23-66 years) and 61 (26.4%) were male (mean age=43 years; range, 23-64 years). One hundred and fifty-eight subjects (68.3%) were COVID-19 naïve and were categorized as seronegative at baseline while 73 (31.6%) were seropositive. All demographic data of the population are presented in **Supplementary materials**.

Anti-N Antibodies

Among the cohort, anti-N antibodies remained stable in seropositive participants up to 6 months compared to pre-vaccinal titers ($p>0.05$) (**Supplementary materials**). At the individual level, 3 participants (1.3%) had a significant increase in their anti-N antibody levels. The first subject was seronegative before vaccination and had a positive RT-PCR 93 days since the first vaccine dose. The B.1.1.7 variant was identified by sequencing. The subject had a close contact with an infected patient and was asymptomatic. The second subject was seropositive at baseline with a documented positive RT-PCR in April 2020 and reported a positive RT-PCR 71 days after the first dose. The subject developed minor symptoms. No sequencing was available. The third subject was seronegative before vaccination and reports a positive RT-PCR carried out in the context of persisting flu-like symptoms, only 2 days after the first dose.

IgG and total antibodies

In seronegative individuals, the maximal antibody response was reached at day 28 with a mean total antibody titer of 2,204 U/mL (95% CI: 1,833 – 2,575 U/mL) and a mean IgG titer of 18,785 AU/mL (95% CI: 16,020 – 21,549 AU/mL). A continuous decrease was observed up to day 180 with an observed mean total antibodies titer of 998 U/mL (95% CI: 848 –1,148) and an observed mean IgG titer of 1,949 AU/mL (95% CI: 1,565 – 2,332) which represent a decrease of 54.7% and 89.6%, respectively (**Table II.IV.1.1**, **Figure II.IV.1.1**). In seropositive individuals, the maximal antibody response was reached at day 28 and day 42 for total and IgG antibodies, respectively. The mean total antibodies titer was 16,935 U/mL (95% CI: 15,112 – 18,759) and the mean IgG titer was 30,678 AU/mL (95% CI: 26,600 – 34,755). A continuous decline was also observed between day 28 or day 42 and day 180 with a total antibody titer of 4,270 U/mL (95% CI: 3,324 – 5,215) which represent a decrease of 74.8% and 79.4%, respectively (**Table II.IV.1.1**, **Figure II.IV.1.1**). All participants still had

detectable anti-S antibodies 6 months after the first vaccine dose (*i.e.,* total antibodies titer ≥ 0.8 U/mL and IgG titer ≥ 50 AU/mL).

SARS-CoV-2 total antibodies				
	Seronegative	**Seropositive**	**Ratio +/-**	**p**
Before first dose	0.40 (0.39-0.41) U/mL	132.0 (86.1-177.6) U/mL	330	<0.0001
14 days	38.2 (27.7-48.6) U/mL	15,540 (13,606-17,473) U/mL	406	<0.0001
28 days	2,204 (1,883-2,575) U/mL	16,935 (15,112-18,759) U/mL	7.7	<0.0001
42 days	1,863 (1,613-2,113) U/mL	15,896 (13,968-17,824) U/mL	8.5	<0.0001
56 days	1,517 (1,326-1,708) U/mL	13,315 (11,464-15,165) U/mL	8.8	<0.0001
90 days	1,262 (1,104-1,420) U/mL	8,919 (7,201-10,637) U/mL	7.1	<0.0001
180 days	998 (848-1,148) U/mL	4,270 (3,324-5,215) U/mL	4.3	<0.0001
SARS-CoV-2 IgG antibodies				
	Seronegative	**Seropositive**	**Ratio +/-**	**p**
Before first dose	21.2 (20.8-21.6) AU/mL	556.6 (385.3-727.9) AU/mL	26.3	<0.0001
14 days	679.9 (548.7-811.2) AU/mL	27,753 (23,226-32,239) AU/mL	40.8	<0.0001
28 days	18,785 (16,020-21,549) AU/mL	29,845 (25,484-34,206) AU/mL	1.6	<0.0001
42 days	17,507 (15,685-19,328) AU/mL	30,678 (26,600-34,755) AU/mL	1.8	<0.0001
56 days	12,862 (11,441-14,284) AU/mL	22,115 (19,174-25,056) AU/mL	1.7	<0.0001
90 days	6,050 (5,371-6,729) AU/mL	14,509 (12,477-16,541) AU/mL	2.4	<0.0001
180 days	1,949 (1,565-2,332) AU/mL	6,333 (5,072-7,593) AU/mL	3.2	0.342
Pseudovirus Neutralization Test†				
	Seronegative	**Seropositive**	**Ratio +/-**	p
Before first dose	11.9 (10.0-13.8) dilution^{-1}	43.8 (29.0-58.5) dilution^{-1}	3.7	<0.0001
28 days	1,955 (1,287-2,622) dilution^{-1}	2,091 (981-3,202) dilution^{-1}	1.1	0.823
90 days	127.6 (84.3-170.9) dilution^{-1}	163.1 (83.5-243) dilution^{-1}	1.3	0.390
180 days	26.1 (20.1-32.1) dilution^{-1}	30.5 (18.2-42.7) dilution^{-1}	1.2	0.463

Table II.IV.1.1: *Evolution of SARS-CoV-2 S antibodies (U/mL) in seronegative and seropositive persons using the Roche Elecsys, the Abbott Architect assays and the pseudovirus neutralizing test. Means with 95% CIs are reported. The between group difference of antibody titers were tested using a Tukey multiple comparison test. A multiple testing correction was applied in the multiple group comparison. p<0.05 was considered significant. †pVNT have only been performed in 60 subjects.*

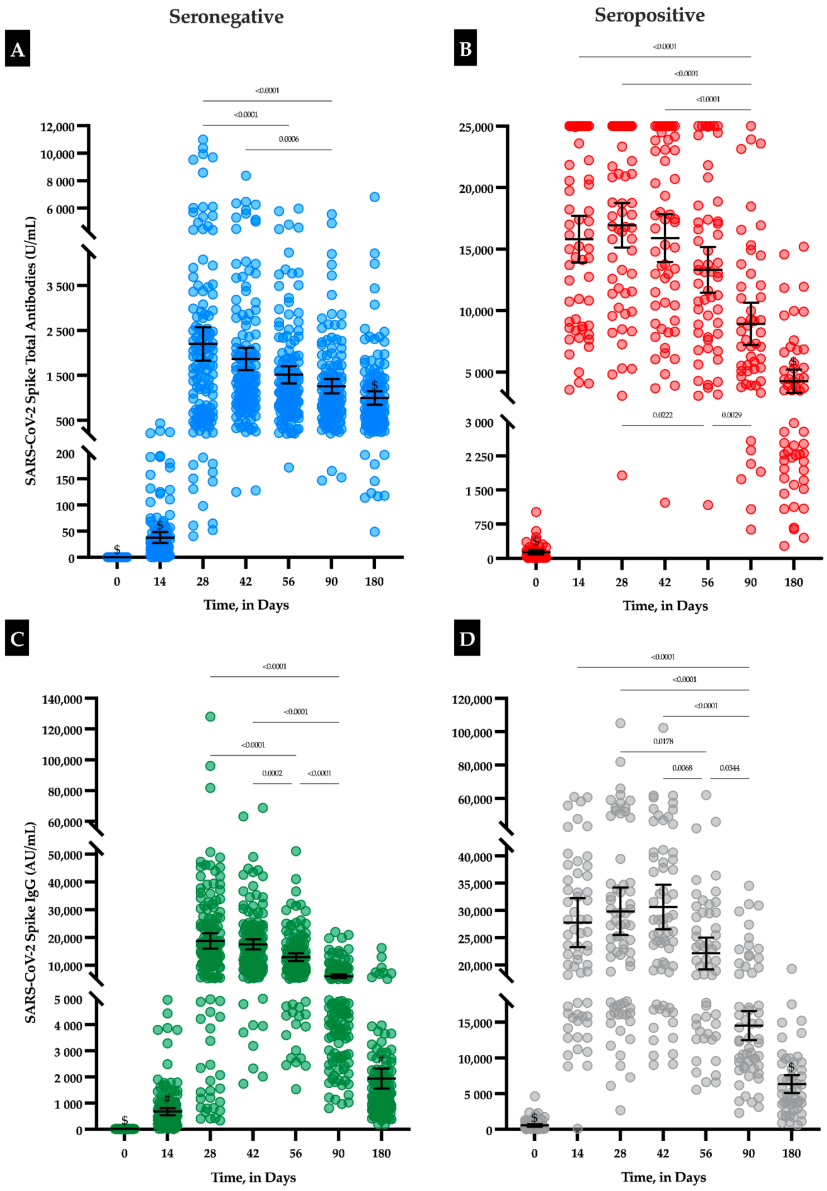

Figure II.IV.1.1*: Evolution of SARS-CoV-2 S antibodies (U/mL) in seronegative (A and C for total antibodies and IgG, respectively) and seropositive individuals (B and D for total antibodies and IgG, respectively) according to the time since the first vaccine dose administration. Means with 95% CIs are shown. [A and B] Using the total antibody assay, an automatic dilution of 1/100 at >250 U/mL was performed by the analyzer to extend the measurement domain up to 25,000 U/mL. Forty-six samples were rounded to 25,000 U/mL out of 1,337 (3.4%). Results <0.4 U/mL (limit of quantification) were rounded to 0.4. [C and D] Using the IgG assay, an automatic dilution of 1/4 at >40,000 AU/mL was manually performed to extent the measurement domain to 160,000 AU/mL. Results <21 AU/mL (limit of quantification) were rounded to 21. $ = statistically different from all other groups (i.e., p<0.0001). # = statistically different from all other groups (i.e., p<0.0001) except between time points 14 and 180.*

Considering each time points separately, anti-S titers of seropositive individuals were always statistically higher compared to seronegative individuals (p<0.0001), except for IgG at day 180 time point (**Table II.IV.1.1**). The difference of titers between seronegative and seropositive individuals was higher when measuring total antibodies compared to IgG, but the difference tends to decrease over time (**Table II.IV.1.1**, **Figure II.IV.1.1**).

Using the kinetics model, the T_{max} in seronegative subjects for total antibodies and IgG was comparable with 36.3 days (95% CI: 30.2 – 42.5) versus 34.5 days (95% CI: 31.7 – 37.2). The estimated $T_{1/2}$ for total antibodies was w) obtained for IgG. In seropositive subjects, the T_{max} for total antibodies and IgG were also comparable (23.3 days (95% CI: 18.7 – 28.0) versus 25.0 days (95% CI: 20.4 – 29.9), and was shorter compared to seronegative. The estimated $T_{1/2}$ for total antibodies was 68 days (95% CI: 54 – 90) and slightly longer compared to IgG (*i.e.,* 53 days, 95% CI: 40 – 79) (**Figure II.IV.1.2**).

According to the model, a mean time of 229 days (95% CI: 134 – 277) in seronegative and 529 days (95% CI: 283 – 623) in seropositive would be needed to cross the threshold of 50 AU/mL for the IgG assay. For the total antibody assay, a mean time of 830 days (95% CI: 508 – 1,000) in seronegative and 718 days (95% CI: 425 – 826) in seropositive would be needed to cross the threshold of 15 U/mL which was defined by the manufacturer as a cut-off for detection of inhibitory effects. Using the threshold of 133 U/mL (Resman Rus et al., 2021), the mean time needed would be 470 days (95% CI: 341–585) and 507 days (95% CI: 359–591) in seronegative and seropositive subjects, respectively.

Among the 1,443 samples analyzed on both assays, IgG and total anti-S antibodies showed an almost perfect agreement (*i.e.,* Cohen's kappa=0.97) with a Spearman's r of 0.892 (95% CI: 0.881–0.902; p<0.0001) **(Supplementary materials**

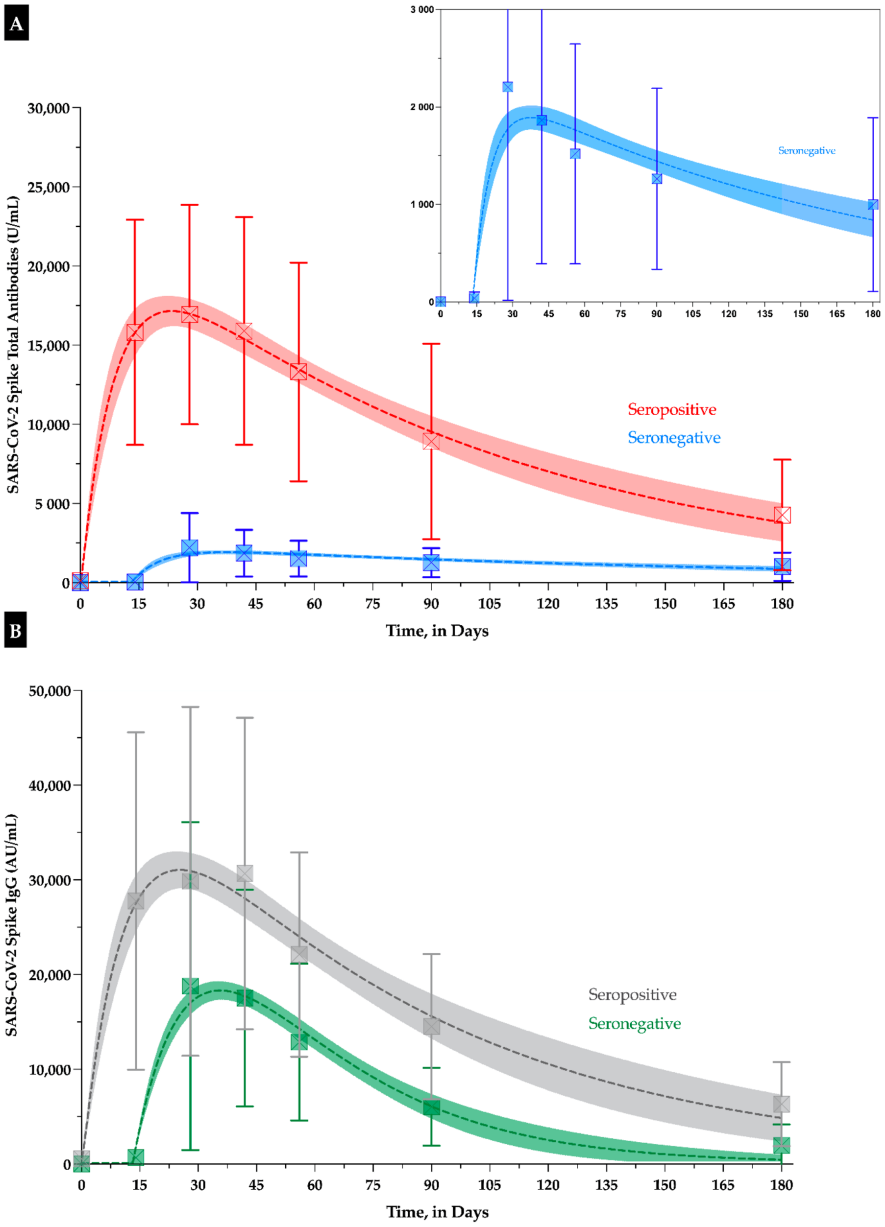

Figure II.IV.1.2: *Kinetic modelisation of (A) total antibodies and (B) IgG serological response. A zoom of the seronegative population is presented in the right-upper part of the figure A. Means plus/minus SD are shown at the different timepoints. The magnitude of the response depends on the analytical kit and the difference between COVID-19 naïve and seropositive individuals is less marked with IgG than with total antibodies.*

Neutralizing antibodies

In the 42 seronegative subjects included in this subgroup analysis, NAbs increased from a mean dilution factor of 11.9 (95% CI: 9.96 – 13.8) at day 0 to 1,955 (95% CI: 1,287 – 2,622) at day 28, which represents an increase of 99.4% ($p<0.0001$) with all subjects having detectable NAbs at day 28. At day 90 and day 180, the mean dilution factors decreased to 127.6 (95% CI: 84.3 – 170.9) and 26.1 (95% CI: 20.1 – 32.1), which represents, respectively, a significant decrease of 93.5% and 98.7% compared to day 28. At these time points, the positivity rates dropped at 95.2% and 45.0% (**Figure II.IV.1.3**), respectively. In the 18 seropositive subjects, 72.2% of the subjects had detectable NAbs at baseline. At day 28, the mean dilution factor increased from 43.8 to 2,091 which represents an increase of 97.9% ($p<0.001$). At day 90 and day 180, the mean dilution factors were 163.1 (95% CI: 83.5 – 242.6) and 30.5 (95% CI: 18.2 – 42.7) which represents a significant decrease of 92.2% and 98.5%, respectively. All subjects had detectable levels of NAbs at day 28 and day 90 but the positivity rate decreases to 44.4% at day 180. Considering each time points separately, NAbs of seropositive individuals were not statistically different compared to seronegative individuals ($p>0.9998$). The kinetic model found that the estimated $T_{1/2}$ of NAbs in this subgroup was 16 days (95% CI: 9 to 59 days) and that the time to reach the negativity cut-off was 135 days (95% CI: 55–179 days). A significant correlation with total antibodies was found ($r=0.63$, $p<0.0001$) (**Supplementary materials**) but the strength of agreement was moderate with the manufacturer's cut-off (Cohen's kappa=0.60). The use of alternative cut-off (15 and 133 U/mL) did not increase the agreement (0.58 and 0.54, respectively). For the IgG assay, we observed a better correlation ($r=0.78$, <0.0001), with still a moderate strength of agreement (Cohen's kappa=0.66) (**Supplementary materials**). No alternative cut-off could enhance the observed agreement.

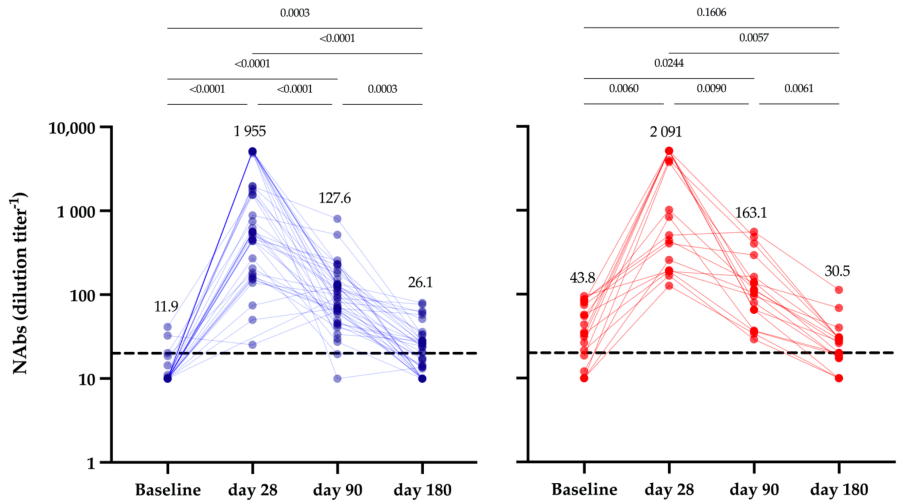

Figure II.IV.1.3: *Evolution of SARS-CoV-2 NAbs in seronegative (blue, n=42) and seropositive individuals (red, n=18) at baseline and 1 month, 3 months and 6 months after the first vaccine shot.*

Discussion

In this study, compared to the peak antibody response, a significant antibody decline 6 months post-vaccination with BNT162b2 COVID-19 vaccine was reported. The decline was highly significant for total antibodies, IgG and NAbs in both seronegative and seropositive participants.

In COVID-19 naïve subjects, the decline of total antibodies at 6 months was lower (*i.e.,* -54.7%) than the decline of IgG (*i.e.,* -89.6%) or NAbs (-98.7%) while in seropositive participants, the decline of total antibodies and IgG at 6 months was quite similar (-74.8% versus -79.4%) and lower than the decline of NAbs (-98.7%). The distinct kinetics observed for the total antibody assays compared to IgG may be explained by the additional response of non-IgG antibody isotypes, which may persist several months post-vaccination (Favresse, Eucher, et al., 2021; Knies et al., 2021). On the other hand, in subjects who had already developed an immune response due to exposition to SARS-CoV-2 or occurrence of COVID-19 disease, the serological response following a *de novo* exposure to the antigen is mainly dominated by the IgG response while the IgM response is reduced, or even absent. This may explain why the total and IgG antibodies declines are closer in the seropositive subgroup (Erdman et al., 1993; To, Hung, Chan, et al., 2021). Interestingly, in seropositive subjects, the anti-N antibodies level did not decline over at least day 180 (Favresse, Elsen, et al., 2021). Some groups have suggested that anti-N antibodies confer additional immunity in seropositive subjects but this is subject to debate in the literature (Perkmann et al., 2021). Besides, in our subgroup analysis, we did not identify a

difference in terms of NAbs titers between seropositive and seronegative subjects (**Table II.IV.1.1**).

Data about the long-term antibody kinetics in BNT162b2 recipients are still scarce. Israel *et al.* just found that the mean SARS-CoV-2 IgG antibody titer (Abbott Architect) after BNT162b2 vaccination decreased by 93.7% at 6 months (*i.e.,* 765 AU/mL) compared to the highest mean antibody response (*i.e.,* 12,153 AU/mL) (Israel et al., 2021). These data are confirmed by our study, especially in the seronegative cohort. The higher mean titers observed in our study may be related to the lower age of our cohort compared to the one of Israel *et al.* (42.0 versus 56.5 years), which has been reported as a confounding factor for antibody response (Bayart, Morimont, et al., 2021; Tober-Lau et al., 2021). Moreover, the antibody decline was also higher compared to convalescent patients where a drop of 4 to 7% every month has been reported compared to drop observed in our vaccinated subjects (Egbert et al., 2021; Israel et al., 2021). Finally, the proportion of vaccinees below the threshold of 50 AU/mL was higher (*i.e.,* 16.1% at 6 months) compared to the one in convalescent subjects (*i.e.,* 10.8% at 9 months) (Israel et al., 2021). In our study, no participant had IgG or total antibody titers below the manufacturers' threshold. In contrast to Israel *et al.*, we also enrolled subjects categorized as seropositive before the first vaccine injection and observed an IgG decrease of 79.4% in this group (**Figure II.IV.1.1**). As reported elsewhere, the vaccination with BNT162b2 elicited much higher total antibodies and IgG titers compared to ones obtained in convalescent patients (Favresse, Eucher, et al., 2021; Israel et al., 2021). In our model, the T_{max} for total antibodies and IgG was reached at a similar timeframe in the two cohorts. The model also predicts a drop of IgG below the positivity threshold (*i.e.,* 50 AU/mL) after 229 days (95% CI: 134 – 277 days) for seronegative and after 529 days (95% CI: 283 – 623 days) for seropositive individuals. Regarding total antibodies, depending on the cut-off used, these times range from 470 (95% CI: 341 – 585 days) to 830 days (95% CI: 508 – 1,000 days) in seronegative and from 507 (95% CI: 359 – 591 days) to 718 days (95% CI: 42 – 826) in seropositive subjects. These predictions need to be confirmed by subsequent sampling times to refine the reliability of the model. However, these data could already support the different government and competent authorities in the decisions that will need to be taken for the next steps of the vaccination strategy (Rubin, 2021). These observations are in line with the statement of the CEO of Pfizer who declared that a third vaccine dose would be likely needed 12 months following the first shot (FDA, 2021). If assuming that the antibody decline is constant over time, our kinetic model predicts a decrease below the positivity threshold between 229 and 830 days, depending on the assay used (total versus IgG antibodies) and the serological status of the subject before vaccination. The aim will be to keep an effective humoral response to protect vaccinated subjects against the WT SARS-CoV-2, but more importantly, against VOC. It is therefore important to have reliable

models to predict when the drop will be too high to maintain a sufficient humoral response. In addition, as some VOC have demonstrated an immune escape, it is important to realize that our models may even be optimistic since higher NAb titers may be needed to provide a similar degree of protection than the one reported during the clinical development of these vaccines (Rubin, 2021).

More and more data support the concept that NAbs correlate with protection against infection and it has been suggested that it may serve in the future as a biomarker that ensure a proper protection at the individual's level (Khoury et al., 2021). Earle *et al.* observed a relatively good correlation between the neutralizing (r_s=0.79) or binding antibodies (r_s=0.93) and VE (Earle et al., 2021). In addition, in a study on 1,497 healthcare professionals having received the two-dose regimen of the BNT162b2 vaccine, Bergwerk *et al.* identified lower NAbs and IgG titers in the 39 subjects who developed a SARS-CoV-2 infection despite a full-vaccination scheme. This supports the concept that NAbs, or assays that correlate with NAbs, may be an appropriate indicator of the protection at the individual level (Bergwerk et al., 2021).

The automated assays used in the present study (*i.e.,* Roche Elecsys and Abbott Architect) do not specially measure NAbs but a significant correlation between the anti-S assay from Roche Diagnostics (r=0.63, p<0.0001) or Abbott (r=0.78 ; p<0.0001) and pVNT was found (**Supplementary materials**) (Jung et al., 2021; L'Huillier et al., 2021). Nevertheless, these correlations were weak and do not reflect the percentage of subjects with NAbs under the positivity threshold at 6 months, *i.e.,* 55% with the pVNT assay versus 0% with both total and IgG antibodies. Several other studies reported the same conclusions with various serological assays available on the market (Dolscheid-Pommerich et al., 2021; Ferrari et al., 2021; Jung et al., 2021; Lustig, Sapir, et al., 2021). These discrepancies seem to suggest that the manufacturers' cut-off is not adequate to reflect the neutralizing capacity and should be significantly increased to reach a better agreement with NAbs, although improvements seem difficult to reach (**Supplementary materials**). Indeed, some patients presenting high levels of total or IgG antibodies at 6 months no longer have NAbs at the same time point. This highlights the fact that we are probably still far from reaching a substitution of pVNT assays by the surrogate IgG or total antibodies assays currently on the market. Thus, the clinical implications of the waning in NAbs we observed is not yet clear on a clinical point of view and the establishment of thresholds associated to protection are still needed, but the link between low NAbs titer and BKI may not be excluded and justify the application of appropriate vaccination strategies, especially in frail patients (Bergwerk et al., 2021; Hacisuleyman et al., 2021; Shrotri et al., 2021; Stephenson, 2021).

Despite the call of the WHO to temporary halt the administration of COVID vaccine boosters (NatureEditorial, 2021), some countries already decided to administer such

booster dose. In Israel and France, a third dose of the BNT162b2 vaccine is given to people over 50 or 65 and to other vulnerable persons (HAS, 2021). In Germany, UK and USA, boosters are planned to be administered to certain groups of persons. In an editorial published in *Nature on* the 17th of August 2021, authors agreed with the WHO to call a temporary halt to COVID vaccine boosters. Indeed, at the time of the editorial, 58% of people in high-income countries had received at least one vaccine dose compared to 1.3% in low-income countries. In some particular cases (*i.e.,* immunosuppressant drugs), boosters might be warranted but there is still little evidence that these additional shots are needed to protect the fully vaccinated people (NatureEditorial, 2021).

Finally, besides the humoral response, there is still few data concerning the cell-mediated immunity responses, and in which extent this protection contributes to the long-term efficacy of the vaccines. Indeed, there is increasing evidence that an early and coordinated T cell response is associated with less severe COVID-19 and provide longer-term protection, even against VOC (Bonifacius et al., 2021; Sattler et al., 2020; Tan et al., 2021).

Conclusion

We found a highly significant decrease in NAbs, IgG and total antibodies in both seropositive and seronegative subjects, 6 months after the administration of the first dose of BNT162b2. The decline of NAbs was more pronounced and around 45% of the subjects tested are negative at 6 months. Further studies are needed to elucidate the relationship between the decline of the humoral response and the clinical efficacy of the vaccine. Moreover, with various kinetics observed, our results also raise the question of which antibody types will be the most clinically relevant to assess the humoral decline since none of those reported in this study seems to show a sufficient correlation and agreement with the neutralizing capacity. This study has a planned follow-up of two years, with the next blood sampling campaign planned in January 2022. This will permit to further refine the kinetics model and to provide better estimate of the antibody response in both seropositive and seronegative individuals.

CHAPTER III: FIRST VACCINE BOOSTER

III.I. Introduction

Six months after administration of the first two doses of the BioNTech/Pfizer vaccine, a highly significant decrease in NAbs, IgG, and total antibodies was observed. The humoral response decrease was also associated with a proportional reduction in VE against symptomatic disease as reported in the literature (Bayart, Douxfils, et al., 2021; GeurtsvanKessel et al., 2022; Infantino et al., 2022; Padoan, Cosma, Bonfante, et al., 2022; Padoan, Cosma, Della Rocca, et al., 2022; Salvagno et al., 2022a, 2022b). Moreover, the emergence and surge of SARS-CoV-2 variants such as Omicron, which presented a considerable escape to acquired immunity, was also responsible for the lower VE.

Administration of a third dose was therefore expected to boost NAb levels and CRO-VAX HCP study participants who received this homologous booster were asked to be followed up in a similar manner as after the first and second vaccine doses. Binding antibodies were measured at each timepoint, as well as NAbs with an updated pVNT for the Omicron BA.1 variant (Favresse, Gillot, et al., 2023).

Omicron's surge was responsible for a high number of vaccinated patients who still developed a BKI, including a significant proportion of CRO-VAX HCP participants. We therefore tried to evaluate whether the humoral response in the peri-infection period could help identify participants at risk of developing a BKI. This was performed both for the BNT162b2 and the mRNA-1273 boosters (Gillot et al., 2023; Gillot et al., 2024).

Based on VE data obtained from clinical trials and on our own generated results, we also aimed at confirming the role of NAbs as the best CoP against symptomatic disease in the Omicron era (Favresse, Gillot, et al., 2023).

III.II. Six-month follow-up (BNT162b2)

III.II.1 Vaccine-Induced Binding and Neutralizing Antibodies Against Omicron 6 Months After a Homologous BNT162b2 Booster

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SUMMARY

Background: Evidence about the long-term persistence of the booster-mediated immunity against Omicron is mandatory for pandemic management and deployment of vaccination strategies.

Methods: A total of 155 healthcare professionals (104 COVID-19 naive and 51 with a history of SARS-COV-2 infection) received a homologous BNT162b2 booster. Binding antibodies against the S protein and NAbs against Omicron were measured at several time points before and up to 6 months after the booster. Geometric mean titers (GMT) of measured antibodies were correlated to VE against symptomatic disease.

Results: Compared to the highest response, a significant 10.2 and 11.5-fold decrease in neutralizing titers was observed after 6 months in participants with and without history of SARS-CoV-2 infection. A corresponding 2.5 and 2.9-fold decrease in binding antibodies was observed. The estimated $T_{1/2}$ of NAbs in participants with and without history of SARS-CoV-2 infection was 42 (95% CI, 25–137) and 36 days (95% CI, 25–65). Estimated $T_{1/2}$ were longer for binding antibodies: 168 (95% CI, 116–303) and 139 days (95% CI, 113–180), respectively. Both binding and NAbs were strongly correlated to VE (r=0.83 and 0.89). However, binding antibodies and NAbs were modestly correlated, and a high proportion of subjects (36.7%) with high binding antibody titers (*i.e.,* >8,434 BAU/mL) did not have neutralizing activity.

Conclusion: A considerable decay of the humoral response was observed 6 months after the booster, and was strongly correlated with VE. Our study also shows that commercial assays available in clinical laboratories might require adaptation to better predict neutralization in the Omicron era.

Introduction

Early efficacy trials and real-world data on the BNT162b2 mRNA vaccine confirmed its high effectiveness in reducing laboratory-confirmed infection, COVID-19

hospitalization and death (Dagan et al., 2021; Levine-Tiefenbrun et al., 2021; Polack et al., 2020; Thomas et al., 2021). Nevertheless, a gradual decline in VE over time has been observed within the first months after the initial two-dose regimen (Chemaitelly et al., 2021; Patalon et al., 2022; Tartof et al., 2021; Thomas et al., 2021). This waned efficacy was consistent with the decrease of NAbs observed by multiple independent studies (Bayart, Douxfils, et al., 2021; Levin et al., 2021; Padoan, Cosma, Bonfante, et al., 2022) supporting NAbs as a strong correlate of COVID-19 protection (Cromer et al., 2021; Earle et al., 2021; Feng et al., 2021; Khoury et al., 2021).

Moreover, since the beginning of the pandemic, several mutations occurred in the SARS-CoV-2 genome leading the different lineages of the virus (GISAID, 2024b). Five of these lineages have been designated as VOC by the WHO, namely the Alpha, Beta, Gamma, Delta and Omicron variants (WHO, 2022b). Discovered in November 2021, the Omicron lineage is to date the leading variant over the world (GISAID, 2024b). This variant is characterized by 32 dominant mutations in the S protein, 15 of which are located in the RBD conferring an increased transmissibility and a considerable immune escape from acquired protection through SARS-CoV-2 vaccination or a previous infection (Catry et al., 2022; Gupta et al., 2022; Lu et al., 2021; Lyke et al., 2022; Muik et al., 2022; Nemet et al., 2022; Planas et al., 2022; X. Wang et al., 2022). Currently, Omicron is largely dominant and several subvariants have emerged including BA.2, BA.2.12.1, BA.4 and BA.5 (GISAID, 2024b). All these subvariants have also demonstrated a considerable escape to acquired immunity (Cao et al., 2022; Hachmann et al., 2022; Lyke et al., 2022).

The current BNT162b2 vaccine, which has been elaborated on the sequence of the WT SARS-CoV-2 (Krammer, 2020), has been shown to be less effective against Omicron compared to other VOC (Andrews et al., 2022; Hansen et al., 2021; Powell et al., 2022; Suah et al., 2022) and the VE also waned over time to reach zero to 22.3% 6 months after the second BNT162b2 dose (Altarawneh et al., 2022; Andrews et al., 2022; Chemaitelly et al., 2022; Chemaitelly et al., 2021; Patalon et al., 2022). With the decreased efficacy of vaccines over time and the emergence of highly transmissible SARS-CoV-2 variants that escape neutralization, many countries have deployed third doses of COVID-19 vaccines.

The administration of a homologous BNT162b2 booster dose increased the VE to 58.9% (IQR: 52.7-63.3%) within 2 to 4 weeks (Abu-Raddad et al., 2022; Altarawneh et al., 2022; Andrews et al., 2022; Chemaitelly et al., 2022; Patalon et al., 2022). This increase was consistent with the rise of binding (median fold increase=26.8; IQR: 13.6-51.7) (Eliakim-Raz et al., 2021; Romero-Ibarguengoitia et al., 2022; Salvagno & Lippi, 2022) and NAbs (median fold increase=27.3; IQR: 10.2-52.7) (Cheng et al., 2022; Lassauniere et al., 2022; Muik et al., 2022; Nemet et al., 2022; Schmidt et al., 2022; van Gils et al., 2022). However, a waning of protection against symptomatic

diseases was rapidly observed 8 to 14 weeks after the booster (median VE=37.9%; IQR: 24.6-45.1%) (Andrews et al., 2022; Chemaitelly et al., 2022; Patalon et al., 2022). Although protection against severe COVID-19 remains higher, the CDC reported that after receiving both 2 and 3 doses, the VE was lower during the Omicron-predominant than during the Delta-predominant period at all time points evaluated (Ferdinands et al., 2022).

Evidence about the long-term persistence of the booster-mediated immunity against Omicron is crucial knowledge for pandemic response. The aim of this study was to evaluate 6-months humoral response in a cohort of HCW who received the homologous BNT162b2 booster.

Material and methods

Study design and participants

The CRO-VAX HCP study is a Belgian multicenter, prospective, and interventional study that was designed to assess the antibody response in a population of HCW from 18 to 65 years of age having received two doses of the BNT162b2 mRNA COVID-19 vaccine (Comirnaty, Pfizer-BioNTech) (Bayart, Douxfils, et al., 2021; Favresse, Bayart, Mullier, Dogne, et al., 2021; Favresse, Bayart, Mullier, Elsen, et al., 2021). The study was approved by a central ethical committee (approval number: 2020-006149-21) and a total of 231 participants were initially enrolled. Participants received the first vaccine dose between 18 January and 17 February 2021. The second dose was then administered 21 days after the first one. Thereafter, participants were proposed to receive a homologous booster that was administered between 8 November 2021 and 31 January 2022. A total of 155 volunteers (67.1%) agreed to receive the booster and to pursue the study. Blood was collected at 7 different time points for the evaluation of the booster-induced immunity, *i.e.,* maximum 2 days before the booster injection and after 7, 14, 28, 56, 90 and 180 days (*i.e.,* 6 months). Blood samplings performed earlier or later than the expected blood collection times were allowed with a maximal allowed percentage of 10% (*i.e.,* 180 days=18 days). Volunteers who missed a blood sampling were not excluded from the analysis. Subjects having positive antibodies against the SARS-CoV-2 N antigen before the booster were considered seropositive (*i.e.,* history of SARS-CoV-2 infection) while the others were COVID-19 naive and classified as seronegative. Anti-N were also used to document the development of a BKI during the study follow-up.

Analytical Procedures

Neutralizing antibodies

A pVNT was used to assess the neutralization potency of BNT162b2-elicited antibodies against the Omicron BA.1 variant. Pseudoviruses were from E-enzyme

(Gaithersburg, MD, USA). SARS-CoV-2 Pseudoviral Particles are replication-deficient Maloneymurine leukemia virus (MLV or MuLV) pseudotyped with the SARS-CoV-2 S protein carrying the Omicron B1.1.529 genotype. They also contain the ORF for firefly luciferase as a reporter. Briefly, HEK293T hACE2 cells were seeded at the density of 8500 cells/well in a white 384-well cell culture plate. The sera used are heat-inactivated by a water bath at 54°C for 30 minutes and then serially diluted in a culture medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). Thereafter, samples are mixed in a 1:4 ratio with pseudovirus and incubated for 2 hours at 37°C. This mixture is added to the cells and incubated for 48 hours at 37°C. The reading is done by adding a reagent to measure the activity of luciferase which is proportional to the cells infected by the pseudovirus. Raw data obtained in RLU are normalized to the positive control where cells are incubated with pseudovirus in the absence of serum. The antibody titer is determined as the dilution of serum at which 50% of the infectivity is inhibited (IC_{50}) as determined by a non-linear sigmoid regression model. A sample with a titer of less than 1/20 is considered negative (Douxfils et al., 2021; Gillot et al., 2022).

Binding antibodies

Binding antibodies against the RBD of the S1 of the SARS-CoV-2 S protein were measured by the Elecsys Anti-SARS-CoV-2 S assay that measured total antibodies (Roche Diagnostics, Basel, Switzerland) with a positivity cut-off of 0.8 BAU/mL. An automatic dilution of 1/100 at >250 BAU/mL was performed by the analyzer to extend the measurement domain up to 25,000 BAU/mL. Additionally, total antibodies against the SARS-CoV-2 N (Roche Diagnostics) were measured using the Elecsys Anti-SARS-CoV-2 assay. Results above 0.165 COI were considered positive (Favresse, Eucher, Elsen, Tre-Hardy, et al., 2020).

Statistical Analyses

Median and IQR were used to present demographic data and GMT $\pm$ and 95% CIs for binding antibodies and NAbs. The between-group differences were tested using a Tukey multiple comparison test with a multiple testing correction.

The kinetic models for binding antibodies and NAbs were calculated using the following equation and using non-log transformed data:

$$\frac{(a * b)}{[(a - b * basal\ response) * Exp^{(-Days\ since\ vaccination * c)}] + [b * Exp^{(Days\ since\ vaccination * d)}]}$$

Where "*a"* stands for the *maximal antibody response*, "*b"* stands for *the baseline response*, "c" for the *antibody production rate* and "*d"* for the *antibody elimination rate*.

The elimination rate was obtained from the model which permitted the calculation of the $T_{1/2}$. The T_{max} and the mean time needed to cross the positivity threshold were also determined based on this model. In each patient, time points corresponding to the BKI were removed from kinetics to avoid rebound response bias.

Pearson's correlation was performed for the comparison between binding antibodies and neutralization titer. A Cohen's kappa agreement test was also calculated, and a ROC curve analysis was performed to identify the best cut-off to predict the neutralizing of the Omicron BA.1 variant (>1/20) using the binding antibody assay.

Spearman's rank correlation was performed for the comparison between log-transformed geometric means of binding antibodies or NAbs and reported VE expressed in percentage against symptomatic disease. The VE was retrieved from the literature for the Omicron lineage only (Abu-Raddad et al., 2022; Altarawneh et al., 2022; Andrews et al., 2022; Buchan et al., 2022; Hansen et al., 2021; Patalon et al., 2022; Suah et al., 2022). Furthermore, only VE that concerned the homologous BNT162b2 booster administered to adults were included. The timings of blood collections were matched with those of published VE.

Statistical analyses were performed using GraphPad Prism 9.4.0 (GraphPad Software, San Diego, CA, USA), JMP Pro 16.0.0 (JMP, version 16.0.0. SAS Institute Inc., Cary, NC, USA), and MedCalc Software (version 14.8.1, Ostend, Belgium). $p<0.05$ was considered significant.

Results

Demographic Data

A total of 155 HCW were included in the study. Among the participants, 112 (72.3%) were female (median age=45 years; IQR: 36–54 years) and 39 (27.7%) were male (median age=41 years; range, 29–57 years). Age was the same among gender (p=0.27). A total of 104 subjects (67.1%) were COVID-19 naive before booster administration while 51 (32.9%) had a previous history of infection. Age was not significantly different between groups (p=0.36). The median time between first and third vaccine dose was 305 days (IQR: 294–310 days) and the median follow-up time was 489 days (IQR: 475–498 days) since first dose. A total of 75 participants (48.4%) developed a BKI after the booster as evidenced by the new development or the rising of antibodies against the N.

Neutralizing antibodies against the Omicron BA.1 variant

In participants with no history of SARS-CoV-2 infection, the highest measured neutralizing capacity was reached at day 28 with a GMT of 221 (95% CI: 175–277), representing a 15.5-fold increase from baseline (*i.e.,* 14.3; 95% CI: 12.1–16.8). A continuous decrease was then observed up to day 180 with an observed GMT of 19.3

(95% CI: 15.1–24.6), which represents a 11.5-fold decrease. At 6 months, the mean neutralizing titer was not significantly different from baseline (**Table III.II.1.1**, **Figure III.II.1.1**).

		Never infected (n=104)		History of infection (n=51)		
		GMT (95% CI)	% pos. samples	*GMT (95% CI)*	% pos. samples	p
$pVNT_{50}$ titer (dilution^{-1})	Before booster	*14.3 (12.1-16.8)*	16.0	*17.1 (12.8-22.7)*	29.4	*>0.99*
	7 days	*42.4 (29.6-60.8)*	59.6	*47.5 (26.7-84.3)*	74.0	*>0.99*
	14 days	*177 (122-266)*	92.3	*168 (104-269)*	100	*0.91*
	28 days	*221 (175-277)*	100	*264 (186-373)*	100	*>0.99*
	56 days	*125 (94.0-165)*	92.7	*170 (105-275)*	100	*0.99*
	90 days	*33.3 (25.8-42.9)*	71.4	*53.9 (34.4-84.4)*	80.0	*>0.99*
	180 days	*19.3 (15.1-24.6)*	37.2	*26.0 (18.3-36.8)*	63.2	*>0.99*
Binding antibodies (BAU/mL)	Before booster	*480 (407-566)*	0.0	*1,999 (1,590-2,512)*	6.1	*<0.0001*
	7 days	*14,879 (12,056-18,364)*	86.6	*15,842 (12,618-19,891)*	91.9	*>0.99*
	14 days	*18,834 (17,295-20,509)*	92.0	*17,461 (15,028-20,288)*	97.1	*0.99*
	28 days	*17,386 (15,834-19,090)*	93.4	*15,271 (13,241-17,613)*	90.2	*0.85*
	56 days	*14,463 (13,002-16,088)*	81.0	*12,123 (9,724-15,113)*	68.8	*>0.99*
	90 days	*11,505 (9,915-13,351)*	73.4	*9,610 (7,017-13,160)*	62.5	*>0.99*
	180 days	*6,508 (5,080-8,338)*	38.6	*6,868 (4,461-10,573)*	52.6	*>0.99*

Table III.II.1.1: *Fifty percent relative inhibition pseudovirus-neutralization titers and binding antibodies titers of sera from vaccine recipients, collected before and after the homologous BNT162b2 booster. The percentage of positive sera according to the assay considered are also represented. Positive cut-offs were >20 dilution titer^{-1} and >8,434 BAU/mL for NAbs and binding antibodies, respectively. The p expresses the statistical difference between GMT of seronegative and seropositive persons.*

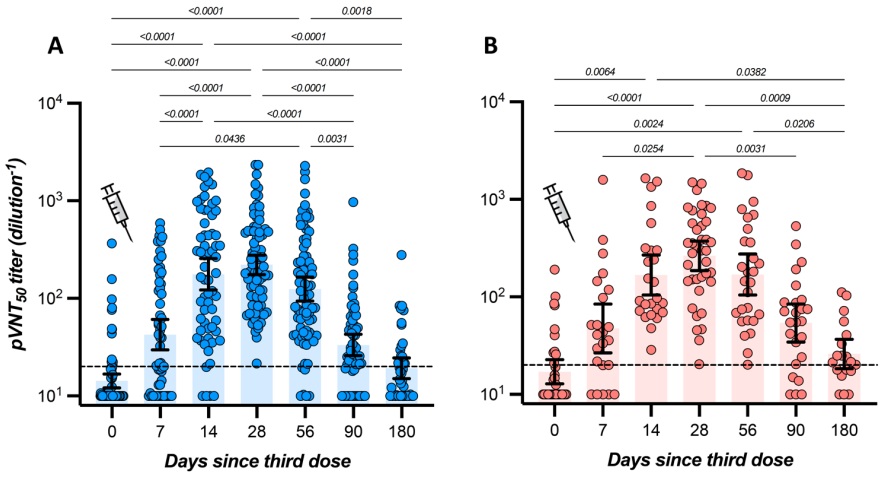

Figure III.II.1.1: *Fifty percent relative inhibition pseudovirus-neutralization titers of sera from vaccine recipients, collected before and after the homologous BNT162b2 booster, with a 6-month follow-up. The*

SARS-CoV-2-S pseudovirus bears the Omicron BA.1 variant S protein. The positivity cut-off corresponds to a dilution titer of 1/20. The blue color corresponds to individuals who were never infected (A) and the red color to individuals who have a history of SARS-CoV-2 infection (B). Geometric means and 95% CI are represented.

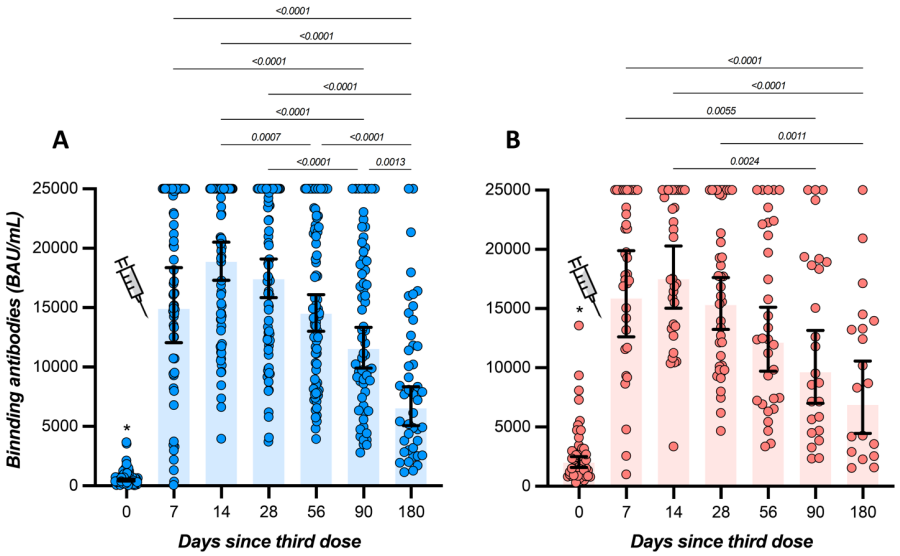

Figure III.II.1.2: *Binding antibodies of sera from vaccine recipients, collected before and after the homologous BNT162b2 booster, with a 6-month follow-up. The positivity cut-off is 0.8 BAU/mL. The blue color corresponds to individuals who were never infected (A) and the red color to individuals who have a history of SARS-CoV-2 infection (B). Geometric means and 95% CI are represented. * = The time point at baseline (or "zero") was significantly lower compared to other time points.*

In participants with history of SARS-CoV-2 infection, the highest neutralizing capacity was also reached at day 28 with a GMT of 264 (95% CI: 186–373), corresponding to a 15.4-fold increase from baseline (*i.e.,* 17.1; 95% CI: 12.8–22.7). As observed in COVID-19 naive individuals, a continuous decline was observed up to day 180 with a GMT of 26.0 (95% CI: 18.3–36.8), which represents a 10.2-fold decrease. The mean titer at 6 months was comparable to baseline (**Table III.II.1.1**, **Figure III.II.1.1**). For each time point, no significant differences were observed in individuals with or without history of SARS-CoV-2 infection (p>0.05) (**Table III.II.1.1**). The proportion of detectable Omicron-specific NAbs was low at baseline (16.0% and 29.4% for participants without or with history of SARS-CoV-2 infection, respectively) and progressively increased to achieve 100% at day 28 for COVID-19 naive individuals and 100% between days 14 and 56 for past-COVID-19 subjects. Afterward, the seroprevalence progressively decreased to achieve 37.2% and 63.2% after 6 months in individuals without or with previous history of SARS-CoV-2 infection (**Figure III.II.1.1**). The estimated $T_{1/2}$ of NAbs for COVID-19 naive participants was 36 days (95% CI: 25–65 days). The T_{max} was estimated at 18 days (95% CI: 14–22 days). In previously infected subjects, the estimated $T_{1/2}$ of NAbs was 42 days (95% CI, 25–137

days) and the T_{max} was reached at 24 days (95% CI: 15–32 days). Estimations for $T_{1/2}$ and T_{max} were not significantly different between groups. According to the model, a mean time of 182 days (95% CI: 118–234) in COVID-19 naive participants and 214 days (95% CI: 110–297) in previously infected subjects would be needed to cross the dilution titer threshold of 1/20 (**Figure III.II.1.3a**).

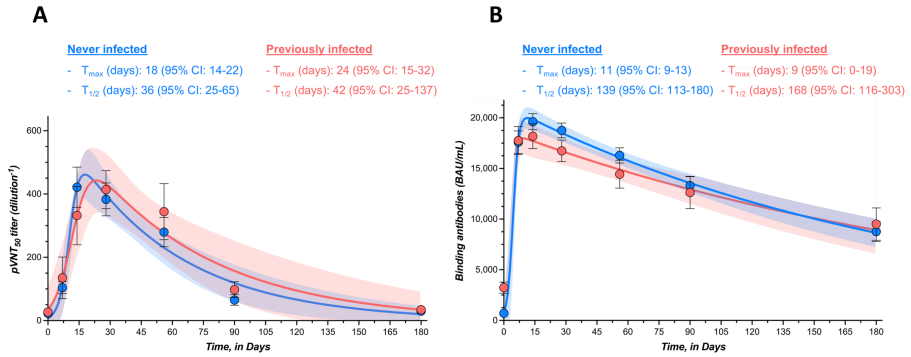

Figure III.II.1.3: *Kinetics models of (A) NAbs against Omicron and (B) binding antibodies after the homologous BNT162b2 booster. Means plus/minus SD are shown at the different time points. The blue color corresponds to individuals who were never infected and the red color to individuals who were previously infected with the SARS-CoV-2.*

Binding antibodies

In participants with no history of SARS-CoV-2 infection, the highest measured binding antibody response was reached at day 14 with a GMT of 18,834 BAU/mL (95% CI: 17,295–20,509), representing a 39.2-fold rise from baseline (*i.e.,* 480 BAU/mL; 95% CI: 407–566). A continuous decrease was then observed up to day 180 with an observed GMT of 6,508 BAU/mL (95% CI: 5,080–8,338), which represents a 2.9-fold decrease compared to day 14. Levels of binding antibodies at 6 months were higher compared to baseline (**Table III.II.1.1**, **Figure III.II.1.2**).

In participants who were previously infected, the highest binding antibody response was reached at day 14 with a GMT of 17,461 BAU/mL (95% CI: 15,028–20,288), corresponding to a 8.7-fold increase from baseline (*i.e.,* 1,999 BAU/mL; 95% CI: 1,590–2,512). A continuous decline was observed up to day 180 with a GMT of 6,868 BAU/mL (95% CI: 4,461–10,573), which represents a 2.5-fold decrease in binding antibody titers at 6 months. Six-month titers were higher compared to baseline titers (**Table III.II.1.1**, **Figure III.II.1.2**). Except at baseline (*i.e.,* just before the administration of the booster), no significant differences were observed in individuals with or without history of SARS-CoV-2 infection (p>0.05) (**Table III.II.1.1**). All participants still had detectable positive binding antibodies 6 months after the booster (*i.e.,* >0.8 BAU/mL). The estimated $T_{1/2}$ of binding antibodies for COVID-19 naive participants was 139 days (95% CI: 113–180 days) and the T_{max} was reached at

11 days (95% CI: 9–13 days). In previously infected subjects, the estimated $T_{1/2}$ of binding antibodies was 168 days (95% CI, 116–303 days) and the T_{max} was reached at 9 days (95% CI: 0–19 days). Estimations for $T_{1/2}$ and T_{max} were not significantly different between groups. According to the model, a mean time of 186 days (95% CI: 155–223) in COVID-19 naive participants and 194 days (95% CI: 142–283) in previously infected subjects would be needed to cross the threshold of 8,434 BAU/mL (**Figure III.II.1.3b**). This threshold represents the binding antibody titer needed to ensure a neutralizing activity of 1/20 (**Figure III.II.1.4b**).

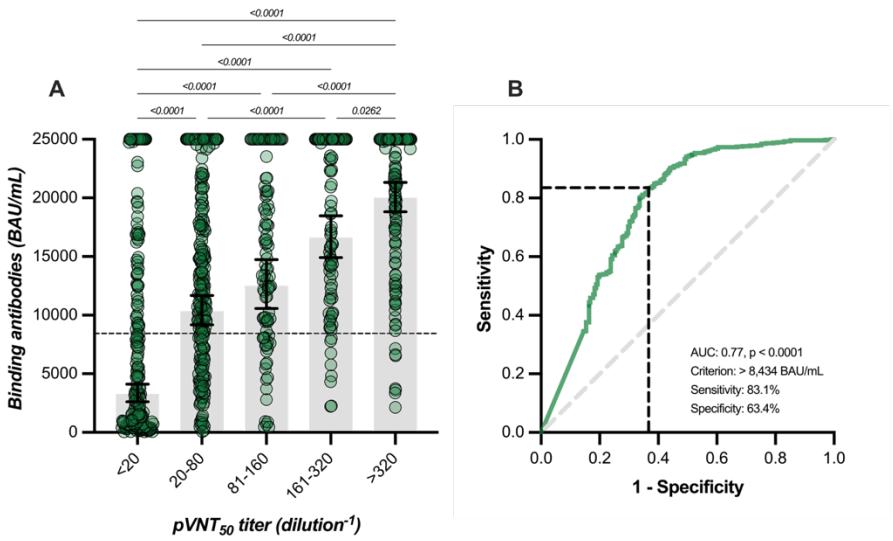

Figure III.II.1.4: *(A) Binding antibodies according to rank categories of NAbs against the Omicron BA.1 variant. Geometric means and 95% CI are represented. (B) ROC curve analysis between binding antibodies (continuous variable) and NAbs (i.e., >1/20 as the classification variable). The >8,434 criterion (BAU/mL) corresponds to the best Youden index calculated.*

Binding antibodies versus neutralizing antibodies and correlation to vaccine efficacy

A significant correlation between binding antibodies and neutralizing titers was found (r=0.51, 95% CI: 0.46–0.56, p<0.0001) but the strength of agreement was null using the manufacturer's cut-off of 0.8 BAU/mL since all results for binding antibodies were positive. Furthermore, there was a proportional and significant increase in binding antibodies according to categories of neutralizing titers. GMT for binding antibodies corresponding to neutralizing titer categories <20, 20–80, 81–160, 161–320, and >320 were 3,286, 10,351, 12,481, 16,588, and 20,036 BAU/mL (**Figure III.II.1.4a**). Based on the ROC curve analyses, an alternative cut-off of 8,434 BAU/mL for binding antibodies was identified to predict the neutralization of the Omicron BA.1 variant with a calculated sensitivity and specificity of 83.1% and 63.4%,

respectively (AUC=0.77, p<0.0001) (**Figure III.II.1.4b**). Therefore, there was a high proportion of sera (*i.e.,* 36.6%) without NAbs that had high titers of binding antibodies. Using this adapted cut-off induced a Cohen's kappa of 0.45 (95% CI: 0.38–0.51) that corresponds to a moderate agreement. The GMT of binding and NAbs obtained in our study correlated strongly with the VE (%) from symptomatic infection (r=0.83 (95% CI: 0.63–0.93), p<0.0001 and r=0.89 (95% CI: 0.72–0.95), p<0.001, for binding antibodies and NAbs, respectively), with the respective equations: "y = 0.01890*x + 3.251" and "y = 0.02106*x + 1.085" (**Figure III.II.1.5**).

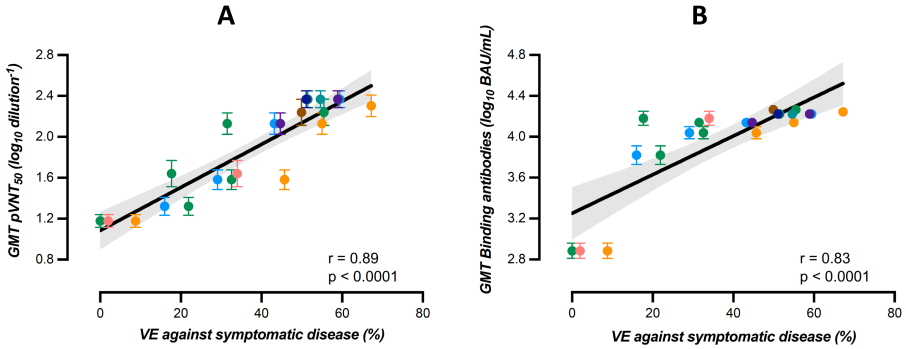

Figure III.II.1.5: *GMT (±95% CI) of (A) NAbs and (B) binding antibodies against the VE against symptomatic disease (%). GMT from individuals with and without previous SARS-CoV-2 infection were merged. VE (%) were gathered from the literature. Each color corresponds to a single study.*

Discussion

Although homologous boosting with BNT162b2 vaccine elicited high titers of binding antibodies and NAbs to Omicron BA.1 in the first weeks following vaccine administration, the response waned substantially within 6 months. The rapid increase in the humoral response was observed regardless of previous SARS-CoV-2 history. After 6 months, the decay of NAbs was higher compared to binding antibodies (11.5 and 10.2-fold decrease versus 2.9 and 2.5-fold-decrease in COVID-19 naive and in previously infected subjects, respectively). Accordingly, binding antibodies presented a significantly higher $T_{1/2}$ (139-168 days) as compared to NAbs (36-42 days). Nevertheless, the mean time to reach the neutralization cut-off was similar, *i.e.,* 186 to 194 days versus 182 to 214 days, for binding and NAbs, respectively. The proportion of participants who was considered negative for binding antibodies and NAbs was also related.

Interestingly, the global humoral response was not different in participants with a history of SARS-CoV-2 infection compared to COVID-19 naive participants. This feature was not seen after the second dose for binding antibodies (Bayart, Douxfils, et al., 2021; Bayart, Morimont, et al., 2021). This is somewhat in contradiction with the study of Alatarwneh *et al.* who found that vaccination enhanced protection

among persons who had had a previous SARS-CoV-2 infection. Hybrid immunity resulting from previous infection and recent booster vaccination conferred the strongest protection in this study (Altarawneh et al., 2022).

Very few studies documented the long-term kinetics of antibodies following the booster administration. After a follow-up period of 3 months, Lyke *et al.* found a 3.5-fold decrease in NAbs against Omicron in a population of 50 participants having received the BNT162b2 booster. The observed decay was quite similar as in our study at 3 months (*i.e.,* 5.5-fold decrease) (Lyke et al., 2022). Munro *et al.* found a considerable decay of IgG titers 7 months after the homologous BNT162b2 booster in a small population of 31 COVID-19 naive subjects (26,982 at 28 days and 3,761 U/mL at 7 months: 7.2-fold decrease) (Munro et al., 2022). NAbs were not evaluated. Regev-Yochay *et al.* also identified a decrease of IgG titers 4-5 months after the homologous booster administration (2,102 at 28 days and 383 BAU/mL at 4-5 months resulting in a 5.5-fold decrease). A parallel decay in NAbs was also observed (2,629 at 28 days and 480 BAU/mL at 4-5 months resulting in a 5.5-fold decrease). Only COVID-19 naive participants were included (n=154) (Regev-Yochay et al., 2022). The waning of the humoral response observed in our study and in the literature for the post-booster period (Munro et al., 2022; Regev-Yochay et al., 2022) was consistent with the one observed after the second dose of BNT162b2 (Bayart, Douxfils, et al., 2021; Levin et al., 2021). The distinct kinetics observed for IgG and total antibodies may be explained by the additional response of non-IgG antibody isotypes, which may persist several months after vaccination.

The waning of antibodies over time after the BNT162 booster was proportional to the decrease of VE identified in the literature (Abu-Raddad et al., 2022; Andrews et al., 2022; Buchan et al., 2022; Chemaitelly et al., 2022; Ferdinands et al., 2022; Hansen et al., 2021; Patalon et al., 2022; Suah et al., 2022) and we found a stronger correlation between GMT of NAbs (r=0.89) compared to GMT of binding antibodies (r=0.83), yet the difference was not significantly different. Previous studies focusing on primary vaccine schemes and mostly on the WT virus identified that SARS-CoV-2 antibodies are a strong CoP (Cromer et al., 2021; Earle et al., 2021; Feng et al., 2021; Khoury et al., 2021). Lower levels of binding antibodies and NAbs during the peri-infection period were described in BK patients in comparison to control patients (*i.e.,* patients who did not develop infection), supporting the role of antibodies in protecting against infection (Bergwerk et al., 2021; Favresse, Dogne, et al., 2022). Our results therefore reinforce the conclusions of these preliminary studies and show that these also applied to Omicron after the booster administration.

Although a waning of binding and NAbs was observed as well as similar correlations between mean titers and VE, the two methods used to measure these antibodies were not commutable. Indeed, NAbs, that represent a first layer of adaptive

immunity against COVID-19, were only modestly correlated (r=0.51) against the commercial assay used. This latter was therefore not adapted to predict the presence of NAbs. The refining of the cut-off for binding antibodies at 8,434 BAU/mL allowed us to improve the prediction of NAbs, but the performance remained moderate since there is still a significant proportion of samples with high binding antibody titers that do not correspond to neutralizing activity against Omicron (**Figure III.II.1.3**). Therefore, diagnostic companies should need to rethink their current commercial assays (*i.e.,* modification of antigen and epitopes) to design assays capable of predicting neutralizing activity against emerging and highly mutated SARS-CoV-2 variants (Lippi, Adeli, et al., 2021). This would also avoid any misinterpretation (*i.e.,* high protection in case of high binding antibody titers) (Lippi, Adeli, et al., 2021). Methods used to measure NAbs present a low throughput, are time-consuming, need skillful operators, and require high levels of biosafety (especially for live virus neutralization assay) (Lippi, Adeli, et al., 2021). It would therefore be easier to use commercial assays that can be a surrogate of these reference methods.

In our study, we evaluated the vaccine-induced neutralizing activity against Omicron BA.1. We were not able at that time to evaluate the neutralizing activity against Omicron sublineages BA.2, BA.2.12.1, BA.3 or BA.4/5. Lyke *et al.* identified similar neutralizing titers 29 days after the Moderna mRNA−1273 booster between BA.1, BA.2 and BA.3, while a modest decline was observed for BA.2.12.1 (1.5-fold) and BA.4/5 (2.5-fold) in a total of 16 subjects (Lyke et al., 2022). Accordingly, Hachmann *et al.* found no difference between BA.1 and BA.2, but significant lower titers for BA.2.12.1 (2.2-fold) and BA.4/5 (3.3-fold), 14 days after the BNT162b2 homologous booster in 27 participants (Hachmann et al., 2022). Cao *et al.* confirmed the same mean titers of NAbs in 50 participants between BA.1 and BA.2 28 days after the CoronaVac homologous booster, and lower neutralizing activity compared to BA.1 for BA.2.12.1 (1.2-fold) and BA.4/5 (1.6-fold) (Cao et al., 2022). However, Bowen *et al.* found similar neutralizing titers between BA.1, BA.2, BA.2.12.1 and B4/5, ±30 days after mRNA−1273/BNT162b2 booster in 13 participants (Bowen et al., 2022). A strong increase after the booster was also observed for Omicron sublineages (Bowen et al., 2022; Hachmann et al., 2022; Lyke et al., 2022). Our results 6 months after the booster might therefore be overestimated compared to BA.2.12.1 and B4/5 sublineages.

The administration of a fourth dose is currently under discussion (Calderon-Margalit et al., 2022) and some interim recommendations have been formulated (ECDC/EMA, 2022; WHO, 2022a). Considering the waning of VE over time and the considerable immune escape of new emerging variants, the fourth dose seems unavoidable to restore a sufficient level of NAbs. The efficacy of a fourth dose (or second booster) against symptomatic disease, hospitalization and severe COVID-19 has already been

proved (Bar-On, Goldberg, Mandel, et al., 2022; Gazit et al., 2022; Magen et al., 2022). The protection against confirmed infection, however, started to wane from 4 weeks since the fourth dose (Gazit et al., 2022; Magen et al., 2022). Magen *et al.* estimated a VE of 61% (95% CI: 58-64%) against symptomatic disease 14-30 days after the fourth dose, which is consistent with the VE found 14-30 days after the third dose (57%; 95% CI: 51-64%) (Abu-Raddad et al., 2022; Altarawneh et al., 2022; Andrews et al., 2022; Chemaitelly et al., 2022; Patalon et al., 2022). The vaccine-induced antibody titers after the fourth dose have been evaluated in few studies (Munro et al., 2022; Regev-Yochay et al., 2022). Two studies have enrolled 31 and 154 individuals and found a 7.8 and 11.4-fold rise in IgG 14 days after the fourth BNT162b2 dose (Munro et al., 2022; Regev-Yochay et al., 2022). A consistent 10.7-fold increase in NAbs against Omicron was observed (Regev-Yochay et al., 2022). This increase in NAbs was similar to the one reported in our study 14 days after the third BNT162b2 dose (9.8 to 12.4-fold increase). It was concluded that the maximal immunogenicity of mRNA vaccines that was achieved after three doses was similar compared to antibody levels generated after the fourth dose (Munro et al., 2022; Regev-Yochay et al., 2022). Taken all together, VE and antibody levels after the second booster were consistent with the ones after the first booster if considering the same time intervals since injection. Our linear model that could predict the level of VE according to neutralizing titers might therefore also be applicable for the fourth dose, but this deserves further validation.

The efficacy of the current formulation of the vaccine is at most around 65% against symptomatic disease 4 weeks after the administration of the first booster. On 25 June 2022, Pfizer and BioNTech announced that they are working on a Omicron-adapted mRNA vaccine. On 31 August 2022, the FDA amended the emergency use authorization of the Moderna COVID-19 Vaccine and the Pfizer-BioNTech COVID-19 Vaccine to authorize bivalent formulations of the vaccines. This bivalent vaccine, also called "updated booster", contains two mRNA components of the virus (*i.e.,* one original strain and one in common between the BA.4 and BA.5 lineages). This adapted version is expected to boost the protection against Omicron.

Conclusion

A rapid and significant increase in booster-induced antibodies was observed from 7 days after the homologous BNT162b2 booster. Thereafter, a considerable antibody waning was noticed within 6 months, which was strongly correlated to VE data available in the literature. The impact of previous SARS-CoV-2 infection on the humoral response was non-significant after a first complete cycle of vaccination. Binding and NAbs against Omicron followed a similar kinetics of decay but were only modestly correlated. A substantial increase of cut-off for binding antibodies was needed to increase the prediction of a neutralizing activity. Nevertheless, there was

still a considerable number of participants with high binding antibody titers that did not present any neutralizing capacity. Commercial assays available in clinical laboratories might therefore require adaptation to better predict NAbs which represent the best CoP. Our kinetic models might also be useful to determine the timing of fourth dose administration.

III.III. Prediction of breakthrough infection cases (BNT162b2)

III.III.1 Peri-Infection Titers of Neutralizing and Binding Antibodies as a Predictor of COVID-19 Breakthrough Infections in Vaccinated Healthcare Professionals: Importance of the Timing

Clinical Chemistry and Laboratory Medicine. 2023. 61(9)

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SUMMARY

Background: The BNT162b2 mRNA vaccine is highly effective in reducing COVID-19 infection, hospitalization and death. However, many subjects developed a BKI despite a full vaccination scheme. Since the waned efficacy of mRNA vaccines is correlated with the decrease of antibodies occurring over time, we aimed at evaluating whether lower levels of antibodies were associated with an increased risk of BKI in a cohort of BK subjects who received three vaccine doses.

Methods: Total binding antibodies against the RBD of the S1 (Roche Diagnostics, Machelen, Belgium) and NAbs using the Omicron B.1.1.529 variant pseudovirus were measured. Based on individual kinetic curves, the antibody titer of each subject was interpolated just before the BKI and compared to a matched-control group that did not develop a BKI.

Results: Lower levels of total binding antibodies and NAbs were observed compared to the control group (6.900 [95% CI; 5.101-9.470] versus 11.395 BAU/mL [8.627-15.050] [p=0.0301] and 26.6 [18.0-39.3] versus 59.5 dilution titer^{-1} [32.3-110] [p=0.0042], respectively). The difference between BK and control subjects was mostly observed for NAbs before three months after the homologous booster administration (46.5 [18.2-119] versus 381 [285-509] [p=0.0156]). Considering the measurement of total binding antibodies before 3 months, there was no significant difference (p=0.4375).

Conclusion: Our results showed that subjects who developed a BKI had lower levels of NAbs and total binding antibodies compared to controls. The difference was mostly noticeable considering NAbs, especially for infections occurring before 3 months after the booster administration.

Introduction

The BNT162b2 mRNA vaccine is highly effective in reducing laboratory-confirmed infection, COVID-19 hospitalization and death (Dagan et al., 2021; Francis et al., 2022; Levine-Tiefenbrun et al., 2021; Lin et al., 2022; Polack et al., 2020; Thomas et al., 2021).

Nevertheless, a gradual decline in VE over time has been observed within the first months after the initial two-dose and three-dose regimens (Arbel et al., 2022; Chemaitelly et al., 2021; Mizrahi et al., 2021; Tartof et al., 2021). Moreover, the VE of mRNA vaccines further decreased with the emergence of VOC, including the appearance of the Omicron variant on November 2021 (Favresse, Gillot, et al., 2023; Kurhade et al., 2022). Many subjects who received two or three vaccine doses therefore developed a BKI despite a full vaccination scheme (S. T. Tan et al., 2023). This also led to severe infections, especially in frail patients, and predictors of this lack or reduced VE should be detected in order to adapt vaccination and/or protection strategies in these subjects (Grewal et al., 2022). There is therefore an interest in identifying vaccinated subjects at higher risk of developing an infection.

Since the waned efficacy of mRNA vaccines is correlated with the decrease of antibodies occurring over time (Cromer et al., 2022; Earle et al., 2021; Favresse, Douxfils, et al., 2022; Favresse, Gillot, et al., 2023; Feng et al., 2021; Gilbert et al., 2022; Khoury et al., 2021; Levin et al., 2021), a lower level of antibodies was hypothesized to be associated with a higher risk of BK infection (Asamoah-Boaheng et al., 2022; Bergwerk et al., 2021). The aim of this study was to evaluate whether BK cases occurring after the administration of the homologous booster presented lower antibody levels as compared to a matched-control group without BK infection. For that purpose, binding antibodies against the RBD and NAbs against the Omicron BA.1 variant were measured.

Material and methods

Study design and participants

The CRO-VAX study is a Belgian prospective, interventional, multicenter study that was designed to assess the antibody response in a population of HCW from 18 to 65 years of age having received three doses of the BNT162b2 mRNA COVID-19 vaccine (Comirnaty, Pfizer-BioNTech). The study was approved by a central ethical committee (approval number: 2020-006149-21) (Bayart, Douxfils, et al., 2021; Favresse, Bayart, Mullier, Dogne, et al., 2021; Favresse, Bayart, Mullier, Elsen, et al., 2021; Favresse, Gillot, et al., 2023; Favresse, Gillot, Di Chiaro, et al., 2021). The homologous booster was administered between 8 November 2021 and 31 January 2022 and blood samples were collected at 7 different time points, *i.e.,* just before the booster injection and after 7, 14, 28, 56, 90, and 180 days.

BK cases were defined as individuals who had a positive rRT-PCR result during the study, along with the development of anti-N antibodies in participants who were never infected or significant increase in anti-N in participants with a history of previous infection. The delay since vaccine administration and positive rRT-PCR was known for each BK case. The BK group was compared to a matched-control group. Controls received the three BNT162b2 vaccine doses and did not develop a BK infection, as confirmed by the absence of anti-N antibodies rise. They were matched on sex, age, timing (*i.e.,* corresponding to time of infection of the matched-BK), and on type of antibody decrease kinetics.

Analytical procedure

Neutralizing antibodies

A pVNT was used to assess the neutralization potency of BNT162b2-elicited antibodies against the Omicron BA.1 variant. The antibody titer is determined as the dilution of serum at which 50% of the infectivity is inhibited (IC_{50}) as determined by a non-linear sigmoid regression model. A sample with a titer of less than 1/20 was considered negative. More details about the method have been described elsewhere (Douxfils et al., 2021; Gillot et al., 2022).

Binding antibodies

Binding antibodies against the RBD of the S1 of the SARS-CoV-2 S protein were measured by the Elecsys Anti-SARS-CoV-2 S assay that measured total antibodies (Roche Diagnostics, Machelen, Belgium) with a positivity cut-off of 0.8 BAU/mL. An automatic dilution of 1/100 at >250 BAU/mL was performed by the analyzer to extend the measurement domain up to 25,000 BAU/mL. Additionally, total antibodies against the SARS-CoV-2 N were measured using the Elecsys Anti-SARS-CoV-2 assay (Roche Diagnostics). Results above 0.165 cut-off index were considered positive. Binding antibodies were analyzed on a cobas e 801 analytical unit (Roche Diagnostics).

Statistical analysis

Median and IQR were used for demographic data while GMT and 95% CIs were used to present binding antibodies and NAbs. The between-group difference was evaluated using a Wilcoxon matched-pairs signed rank test. In order to evaluate the antibody level just before the infection in BK cases, a kinetic model for each participant was computed to interpolate the most precise antibody level just before the infection (*i.e.,* 10 days before the positive rRT-PCR). Based on the antibody decrease pattern of each participant, a simple linear regression or a non-linear regression (*i.e.,* one-phase decay) was modeled to permit the retrieve of the expected antibody level at the corresponding timepoint. The matched-control of

each BK case, having the same sex and age, was also selected to have a similar antibody kinetic model (simple linear or non-linear regression). The antibody level of each control was also interpolated to match the exact same timing of infection as the BK case. Ratio of cases to controls have also been computed to document on the difference between these groups and their respective measurand at key timepoints. Data analysis was performed using GraphPad Prism 9.4.1 (San Diego, CA, USA) with $p<0.05$ significant level.

Results

Twenty-four patients who developed a BK infection after the booster were identified. Most developed mild symptoms (88%) while few were asymptomatic (12%). Among these, 6 (25%) were men and 18 (75%) were women. The median age of the BK group was 43.0 years (IQR: 37.0 – 52.8; min – max: 23 – 63 years). The median BK infection time after the booster administration was 106 days (IQR: 66.0 – 132 days, min-max: 46.0 – 156 days). Seven participants (29.2%) developed a BK infection before day 90 and 17 (70.8%) after 90 days. The anti-N level before the infection was 0.31 (95% CI: 0.03–0.59) and after the infection was 47.94 (95% CI: 18.07 – 77.81). The control group was also composed of 24 individuals (6 men and 18 women). The median age of 44.0 years (IQR: 38.3–53.0; min–max: 25–61 years) was not significantly different compared to the BK group (p=0.51). Each BK case was matched against a control individual who had the same sex, age, and timing since the booster administration (**Supplementary materials**).

In the BK cases, we observed a GMT of NAbs of 26.6 (95% CI=18.0–39.3) that was significantly lower compared to the control group considering all data (59.5; 95% CI=32.3–110; p=0.0042). The difference was more pronounced if considering the participants who developed a BK infection before day 90 (46.5 (95% CI=18.2–119) versus 381 (95% CI=285-509); p=0.0156). After 90 days, the difference was no longer significant (21.1 (95% CI=14.0–31.9) versus 27.7 (95% CI=17.2–44.6); p=0.3028) (**Figure III.III.1.1**). The kinetics of NAbs also differed from the two groups (**Figure III.III.1.2**).

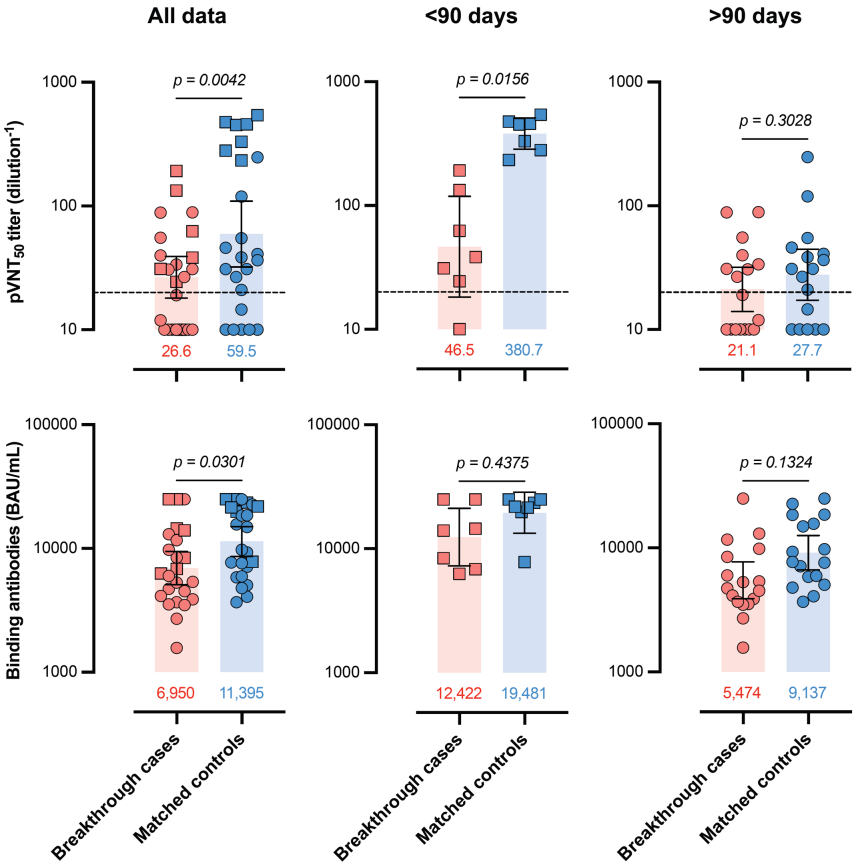

Figure III.III.1.1: *NAbs and binding antibody titers among BK cases and matched-controls. Geometric means and 95% CI are represented. BK cases are represented in red and controls in blue. Samples collected before 90 days since the booster administration are represented with a "square" and samples collected after 90 days with a "dot". The positivity cut-off for NAbs corresponds to a dilution titer of 1/20.*

The difference between BK and controls was less obvious considering the measurement of binding antibodies. A significant difference was only found considering all data (6.950 (95% CI=5.101–9.470) versus 11.395 (95% CI=8.627–15.050)) (p=0.0301). If considering the participants who developed a BK infection before or after 90 days, no significant difference was identified (**Figure III.III.1.1**). Regarding the kinetics, there is a tendency for higher titers in controls, although 95% intervals of the regression models were overlapping (**Figure III.III.1.2**).

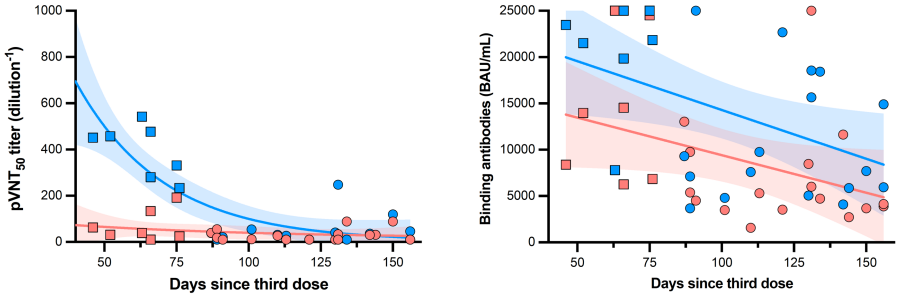

Figure III.III.1.2: *Kinetics of NAbs and binding antibody titers among BK cases and matched-controls. The kinetics are represented with its 95% CI. BK cases are represented in red and controls in blue. Samples collected before 90 days since the booster administration are represented with a "square" and samples collected after 90 days with a "dot".*

Ratio of cases to controls were lower considering the NAb titers of all participants as compared to binding IgG (0.45 (95% CI=0.24–0.84) versus 0.61 (95% CI=0.42–0.89)) but this difference was non-significant (p=0.64). The difference was however significant for the samples collected <90 days after the booster (0.12 (95% CI=0.04–0.36) versus 0.64 (95% CI=0.30–1.38); p=0.02). After 90 days, ratios increased (0.76 (95% CI=0.40–1.47) versus 0.60 (95% CI=0.37–0.97) and were non-significantly different (p=0.37).

Discussion

In our study, we found that BK cases presented lower levels of NAbs and binding antibodies as compared to matched-control individuals who did not develop a BK infection. The difference was mostly noticeable considering NAbs, especially in subjects who developed a BK infection before 90 days.

Since the levels of antibodies is correlated to the VE, some previous studies already hypothesized lower levels of antibodies in peri-infection BK samples compared to controls (Asamoah-Boaheng et al., 2022).

In the study of Bergwerk *et al.*, lower levels of NAbs and binding IgG (Beckman Coulter) were observed in a cohort of 22 Alpha BK cases compared to a matched-control group (192.8 versus 533.7 AU and 11.2 versus 21.8 AU, respectively). Antibodies were measured the day of the BK diagnosis and the median time of BK since the second BNT162b2 vaccine dose was 36 days. Torres *et al.* found similar conclusions with the detection of binding antibodies at 3 months being associated with a lower risk of Delta BK (Torres, Bellido-Blasco, et al., 2022). Our group also published about significantly lower binding total antibody levels in 16 Omicron BK cases as compared to controls (Favresse, Dogne, et al., 2022). All cases developed the infection less than 90 days after the homologous BNT162b2 booster (Favresse, Dogne, et al., 2022). Two other studies concluded about the absence of difference

(Torres, Giménez, et al., 2022; Yang et al., 2022). The first included 33 Omicron BK cases and measured NAbs and total binding antibodies (Roche Diagnostics) at around the time of infection (Torres, Giménez, et al., 2022). The second included around 50 Delta BK and measured binding IgG levels (Yang et al., 2022). Interestingly, the median time of BK infection in both studies was 105 days and 10-24 weeks after vaccination with BNT162b2. These results are consistent with ours showing that the difference between BK cases and controls was no more observed for late BK infection (*i.e.,* >90 days). We also showed that the proportion of cases to ratio was lower considering the measurement of NAbs compared to binding antibodies, mostly for samples collected >90 days (0.12 versus 0.64). This observation is similar to that of Bergwerk *et al.* (0.35 versus 0.65) (Bergwerk et al., 2021).

As the NAbs correlate with the level of protection against re-infection, our results suggests that about 3 months (90 days) after the booster, the effectiveness of this protection strongly decreases. The probability of having a BK infection after 90 days would therefore depend more on the prevalence of the disease, on the variant in circulation and on the application of sanitary measures by the population.

In addition to be a predictor of the risk of BK infection, increased levels of antibodies measured in serum collected within 7 days of symptom onset or diagnosis of Omicron infected vaccinated subjects were associated with a decrease in the occurrence of fever, hypoxia, CRP elevation, and lymphopenia (Kim et al., 2022). Patients with higher antibody levels also had lower viral loads obtained by RT-PCR than those with lower antibody levels (Bergwerk et al., 2021; Kim et al., 2022).

The study of Brada *et al.* focused on the pre-vaccination levels of binding IgG as a predictor of subsequent BK infection. They found that binding IgG levels over 700 BAU/mL (SARS-CoV-2 IgG II Quant (Abbott, IL, USA)) was associated with a 35% reduced risk of infection in the six months following vaccination. In our study, BK did not have lower NAbs or binding antibody levels compared to the matched-control group before the administration of the booster dose (data not known) (Barda et al., 2023).

In conclusion, our results showed that subjects who developed an Omicron BK infection had lower levels of NAbs and binding antibodies. The difference against control individuals who did not develop a BK infection was mostly noticeable considering NAbs, especially for BK infection occurring within 3 months after the booster administration.

III.IV. Prediction of breakthrough infection cases (mRNA-1273)

III.IV.1 Assessment of the neutralizing antibody response in Omicron breakthrough cases in healthcare workers who received the homologous booster of Moderna mRNA-1273.

Virology, 2024 ahead of print.

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Introduction

Various vaccines against SARS-CoV-2 were developed during the pandemic, including those from Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) (Li et al., 2022). These mRNA vaccines have been accepted by the FDA and by the EMA between December 2020 and January 2021 (Baden et al., 2021; Polack et al., 2020). These vaccines were developed to protect against severe SARS-CoV-2 infection. However, their efficacy waned over time, especially with the emergence of new variants that escape immunity (Pilishvili et al., 2021; Rosenberg et al., 2022). This decrease of VE has been correlated to the levels of NAbs, which may differ according to the vaccine, to the delay since the last administrated dose and to the emergence of new variants and subvariants (e.g., BA.1, BA.5, XBB.1.5 or more recently BA.2.86 and JN.1). In previous investigations, we described cases of BKI in individuals who had received the homologous booster dose of BNT162b2 (Favresse, Dogne, et al., 2022). We demonstrated that the risk of BKI was significantly increased in participants presenting lower levels of antibodies in the peri-infection period (Cromer et al., 2022; Feng et al., 2021; Gillot et al., 2023).

The aim of the present study is to confirm that the prediction model for infection obtained with our previous data could be replicated using another cohort of patients who have received the mRNA-1273 booster. We evaluate the levels of binding antibodies against the RBD of the S protein and the levels of NAbs in individuals who developed a BKI after having received three doses of mRNA-1273. We compared their serological response to the one of a matched-control group that did not develop a BKI.

Materials & Methods

Study design and sample collection

The study was conducted at the Iris Sud Hospital (HIS-IZZ, Brussels, Belgium). This prospective study was designed to evaluate the kinetics of the humoral response following COVID-19 vaccination over a 2-year period in a population of HCW. Participants received three doses of mRNA-1273 vaccine (Spikevax, Moderna). The study was in accordance with the Declaration of Helsinki and International Conference for Harmonization for good practice and has been approved by the Ethical Committee of HIS-IZZ (ethical agreement number: CEHIS/2021-007). The booster dose was administered between the 5 October 2021 and the 29 December 2021.

A total of 51 participants were monitored after injection of the booster dose. Samples were collected at baseline and after 14, 90 and 180 days. BKI cases were defined as individuals having a positive RT-PCR confirmed by the subsequent development of anti-N antibodies in subjects without a history of SARS-CoV-2 infection or by a significant increase of anti-N antibodies in previously infected subjects. The BKI group was compared to a 1:1 matched-control group of the same sex and age who did not develop a BKI.

Analytical Procedure

Neutralizing antibodies

A pVNT using an Omicron BA.1.1.529 pseudovirus and an Omicron BA.5 variant (eEnzyme, Gaithersburg, MD, USA) was used to quantify the NAbs response following the administration of the third dose of mRNA-1273. A result below a pVNT titer dilution of 20 was considered negative. The technique has been described in details elsewhere (Gillot et al., 2022).

Binding antibodies

The Elecsys anti-SARS-CoV-2 S assay (Roche Diagnostics, Machelen, Belgium) was used to quantify the total anti-SARS-CoV-2 RBD antibodies response. The positive cut-off provided by the manufacturer was 0.8 BAU/mL, and an automatic dilution of 1/100 at >250 BAU/mL was performed to extend the quantification domain to 25,000 BAU/mL. This test was performed on a cobas e 801 analytical unit (Roche Diagnostics, Machelen, Belgium).

Statistical Analysis

To compare binding antibody and NAb titers between groups, the antibody titer of each individual was interpolated based on their respective kinetic curves extracted from the whole population after having excluded the timepoints corresponding to

the BKI. Population kinetics was modeled according to the following non-linear one-compartmental model:

$$\frac{(a * b)}{((a - b) * Exp\begin{pmatrix} -days\ since\ vaccination * c) + \\ b * Exp(days\ since\ vaccination * d) \end{pmatrix}}$$

where a = Y_{max}, b = Y_0, c = antibody production and d = antibody elimination.

For individuals who developed a BKI, the titer of binding antibodies and NAbs were estimated at the last sampling time before the date of the positive PCR. Antibody titers in controls were obtained according to the same procedure. GMT and 95% CI were used to present the data. Median and IQR were used for the description of the population. A Shapiro-Wilk normality test was performed to assess whether the data distribution was normal. Ratio of cases to controls have been computed to document on the difference between the two groups and their respective measurand at key timepoints. Differences between groups were computed using a parametric t-test. Data analysis was performed using GraphPad Prism 9.3.1 (San Diego, CA, USA) and JMP Pro 16.0.0 (JMP, Version 16.0.0 SAS institute Inc., Cary, NC, USA, 1989-2021). Significant level was set at a p of 0.05.

Results

Among the 51 individuals, 18 (35%) developed a BKI after the administration of the booster dose of mRNA-1273. Among the BKI cases, 5 (28%) were men and 13 (72%) were women. The median age of the BKI cases was 50.2 years (IQR: 45.8–57.5). Eleven participants (61%) developed a BKI before day 90 and 7 (39%) after 90 days. The matched-controls group was composed of 18 individuals, *i.e.,* 5 men and 13 women. The median age was 51.2 (IQR: 42.1–57.1) and did not differ from the BKI group (p=0.62).

The GMT of NAbs against the BA.1 variant in the BKI was 278.1 (95% CI: 168.1–324.1). This titer was significantly lower compared to the matched-control group when considering all data (GMT=477.4; 95% CI: 316.2–541.0; p=0.0057). Results were similar for the BA.5 variant with a GMT of 152.0 (95% CI: 76.9–172.9) for the BKI compared to 262.0 (95% CI: 171.3 – 301.8) in the control group (p=0.0043). When considering only samples collected >90 days post-vaccination, the difference between the two groups was no longer significant for both BA.1 or BA.5 variants (p=0.1620 and 0.0727, respectively) (**Figure III.IV.1.1a and III.IV.1.1b**). For binding antibodies, the GMT were significantly different with a titer of 12,155 BAU/mL (95% CI: 9361–13,916) for the BKI and a titer of 14,360 (95% CI: 12,341–15,805) for

controls (p=0.0142). As with NAbs, no significant difference was observed after 90 days following the booster injection (p=0.3741) (**Figure III.IV.1.1c**).

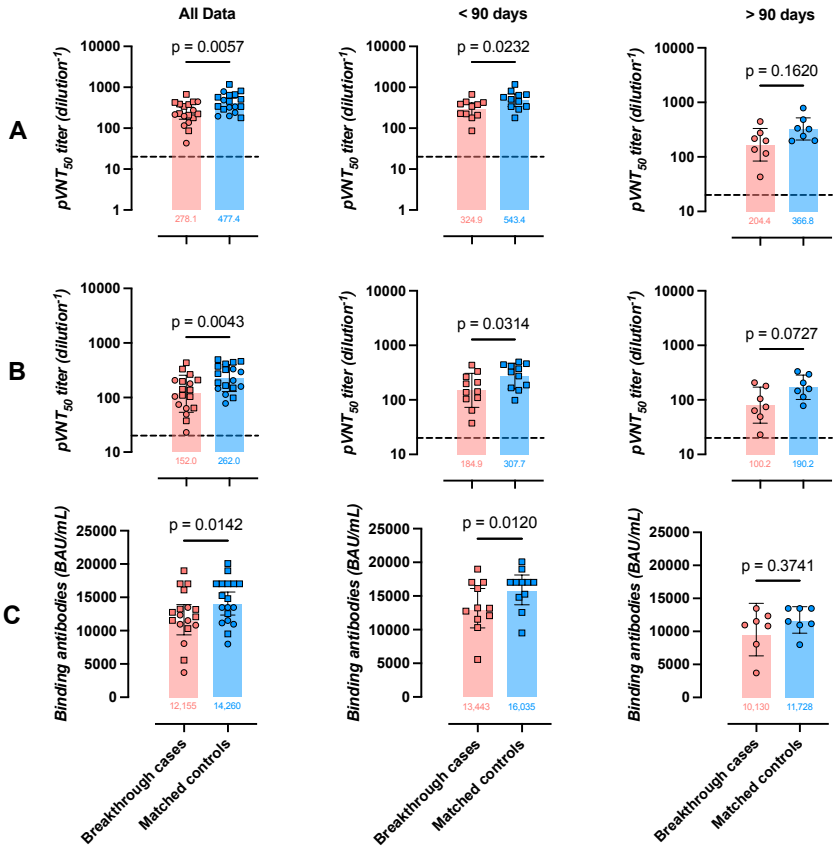

Figure III.IV.1.1: *NAb titers against Omicron BA.1 (A), BA.5 (B) and binding antibody (C) titers among BKI cases and matched-controls. GMT and 95% CI are represented. BKI are represented in red and controls in blue. Samples collected before 90 days since the booster administration are represented with a "square" and samples collected after 90 days with a "dot". The positivity cut-off for NAbs corresponds to a dilution titer of 1/20.*

When comparing the case-to-control ratios for binding antibody titers and BA.1 NAb titers, we observed a lower ratio for NAbs (0.56 (95% CI: 0.39–0.81) versus 0.82 (95% CI: 0.68–0.98); p=0.0048). The difference was also significant (p=0.0042) if considering only the data collected before day 90 (0.59 (95% CI: 0.42–0.83) versus 0.82 (95% CI: 0.70–0.95)). After 90 days following booster administration, the difference was no more significant (0.51 (95% CI: 0.19–1.37) versus 0.82 (95% CI: 0.50–1.35); p=0.1477). The same observations applied for the Omicron BA.5 variant with significant differences considering all data for NAbs and binding antibodies (0.52 (95% CI: 0.36–0.74) versus 0.82 (95% CI: 0.68–0.98); p=0.0011) and before day 90

(0.55 (95% CI: 0.36–0.82) versus 0.82 (95% CI: 0.70–0.95); p=0.0033) but no more significant after 90 days (0.47 (95% CI: 0.19–1.13) versus 0.82 (95% CI: 0.50–1.35); p=0.0601).

Discussion

In this study, we reported that individuals who developed a BKI following the administration of the mRNA-1273 booster dose presented lower binding antibody and NAb titers against Omicron BA.1 and BA.5 before the episode as compared to a matched-control group that did not experience a BKI. This observation was only valid for the whole dataset and for data collected before day 90.

Using a cohort of individuals having received the BNT162b2 booster, we previously showed a significant difference between BKI cases and controls before day 90 for NAb titers against Omicron BA.1 with GMT of 46.5 (95% CI=18.2–119.0) versus 381.0 (95% CI=285.0–509.0) (p=0.0156). The difference was also significant considering the whole dataset (26.6 (95% CI=18.0–39.3) versus 59.5 (95% CI=32.3–110.0); p=0.0042) (Gillot et al., 2023). In the same study and concerning the measurement of binding antibodies, there was a significant difference only considering all data (p=0.0301), but not for data collected before 90 days (p=0.4375) (Gillot et al., 2023). These results are therefore quite consistent with the results we have observed with the mRNA-1273 booster dose. Abu-Raddad *et al.* found a reduction of SARS-CoV-2 infection by Omicron BA.1 variant of 49.3% for the BNT162b2 and of 47.3% for the mRNA-1273 compared to a non-vaccinated population (p>0.05) (Abu-Raddad et al., 2022). On the other hand, Wang *et al.* reported differences between recipients of mRNA-1273 compared to BNT162b2, with a significant reduction in the risk of BKI using the mRNA-1273 (L. Wang et al., 2022). Cong *et al.* also observed a lower incidence of BKI with the mRNA-1273 as compared to the BNT162b2 vaccine with an incidence rate ratio of 1.66 (95% CI: 1.17–2.35) (C. Liu et al., 2022). The fact that these observations are related to a more pronounced humoral response using the mRNA-1273 booster still deserved further investigations.

In our study, we also showed significant lower case-to-control ratios for NAbs compared to binding antibodies for samples collected before 90 days. This observation was consistent with other reports having used the BNT162b2 vaccine, including our previous findings and those of Bergwerk *et al*. which reported a case-to-control ratio of 0.36 (95% CI: 0.16–0.79) considering NAbs and a ratio of 0.51 (95% CI: 0.28–0.34) considering binding antibodies. (Bergwerk et al., 2021; Gillot et al., 2023) Considering that there is no difference in case-to-control ratio after 90 days between binding antibody and NAb titers strengthens the role of NAbs as a predictor early (within 3 months) BKI compared to the measurement of binding antibodies (Earle et al., 2021).

We observed a high rate of BKI (61%) within 90 days following the booster suggesting a potential immune escape of the Omicron variant (either BA.1 or BA.5) against the mRNA-1273 vaccination, even in the early weeks post-booster dose. This higher rate of BKI before 90 days can be explained by the epidemiological context at that time. In our study, the sampling period runs from 5 October 2021 to 30 June 2022, covering an outbreak in the epidemic in February 2022 (approximately 33 to 118 days post booster administration). From week 01 of 2022 to week 04, there is an 225.7% increase in COVID-19 cases which can explain our observation (Sciensano, 2024). Almendro-Vasquez *et al.* reported 12 BKI within 1 month after the booster administration and 5 after 3-6 months in a cohort of 77 individuals (Almendro-Vázquez et al., 2022).

A limitation of the study is that a blood sampling just before the BKI was not available. Consequently, we used the sample of the last sampling before the BKI episode and interpolate the level of antibodies at the time of BKI using a kinetic model derived from the whole population.

In conclusion, our study found that individuals receiving the mRNA-1273 booster and who developed a BKI presented lower levels of binding antibodies and NAbs before the infection as compared to a matched-control group. These results confirmed those already observed for the BNT162b2 booster. Differences were mostly noticeable considering the measurement of NAbs, reinforcing its role in the prediction of BKI.

CHAPTER IV: BIVALENT VACCINE BOOSTER

IV.I. Introduction

The levels of both NAbs and binding antibodies rapidly increased after the administration of the first booster dose (Favresse, Gillot, et al., 2023; Padoan, Cosma, Della Rocca, et al., 2022; Salvagno et al., 2022a). Importantly, this increase in NAbs in the weeks after booster administration was correlated with VE but a decrease of both NAbs, binding antibodies (and VE) was also noticed after 12 weeks (Favresse, Gillot, et al., 2023; Salvagno et al., 2022c). The appearance of variants also induced a significant decrease in VE which was largely explained by immune escape mechanisms.

Aware of the new circulating variants, vaccine manufacturers modified the vaccine formulation to incorporate a modified sequence in addition to the WT sequence (*i.e.,* bivalent booster) in order to boost VE by reducing this immune escape phenomenon.

On August 31, 2022, the FDA authorized the Moderna and Pfizer–BioNTech bivalent vaccines for emergency use as a single booster dose administered at least 2 months after primary or booster vaccination. Each vaccine contained equal amounts of mRNA encoding the S protein from the ancestral (D614G) and the BA.4/5 strains (Lin, Xu, Gu, Zeng, Wheeler, et al., 2023). Booster doses with bivalent variant-adapted vaccines aimed to provide broader protection against circulating and emerging variants (Wang et al., 2023). In Europe, the European Medicines Agency (EMA) made a positive recommendation regarding the use of the BA.1 bivalent adapted vaccine on September 1, 2022. On September 12, 2022, the recommendation was extended to the BA.4/5 bivalent vaccine. Preliminary evaluations showed that the VE of the bivalent boosters was higher than that of the first and second monovalent (*i.e.,* D614G strain) boosters against hospitalization or death (Andersson et al., 2023; Arbel et al., 2022; Huiberts et al., 2023; Lin, Xu, Gu, Zeng, Wheeler, et al., 2023). The VE of the BA.1 (riltozinameran) and the BA.4/5 (famtozinameran) bivalent boosters had a similar VE during the BA.4/5 predominance period (Andersson et al., 2023).

CRO-VAX HCP study participants who accepted to pursue the study underwent blood tests before and after the bivalent booster. At that time, the proposed bivalent booster contained either BA.1 or BA.4/5. The multiple variants that have circulated led us to adapt several times our pVNT technique. In addition to NAbs against BA.1, we were therefore able to measure NAbs against BA.5, XBB.1.5, BA.86.2, FL.1.5.1, and JN.1.

The list of the different variants identified in Belgium is presented below (Sciensano, 2024).

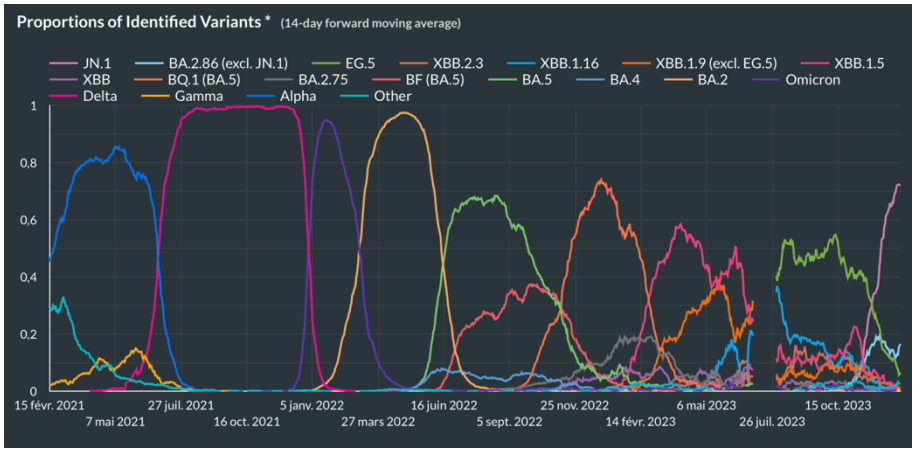

Table IV.I.1: *Proportions of identified variants in Belgium between February 15, 2021 and January 1, 2024. Each color corresponds to a specific variant.*

In our evaluation, D614G, Delta, BA.1, BA.5, XBB.1.5, FL.1.5.1, BA.2.86 and JN.1 variants were studied.

In addition to studying the humoral response, bivalent booster administration also allowed us to explore the cellular response, through the use of an IGRA. The impact of this booster on cellular immunity was evaluated as well as its long-term kinetics (Favresse, Cabo, et al., 2023; Favresse, Gillot, Closset, et al., 2024).

IV.II One-month follow-up (adapted BNT162b2)

IV.II.1 Cellular Immunity Against SARS-CoV-2 is Predominantly Boosted in Vaccinated Individuals With no History of Infection

Journal of Infection. 2023. 87(2)

Julien Favresse, Julien Cabo, Jonathan Douxfils

Despite a substantial reduction in humoral immunity, COVID-19 vaccines still show robust protection against severe COVID-19 disease, even against highly mutated variants (Moss, 2022; Wherry & Barouch, 2022). Accumulating evidence suggests that T cell response plays a key role in the protection against severe disease (*i.e.,* hospitalization and death) (Ledford, 2022; Giuseppe Lippi et al., 2022; Wherry & Barouch, 2022). Two recent papers published in Journal of Infection (Bonnet et al., 2022; Pighi et al., 2023) found that the cellular immunity as assessed with an IGRA declined progressively 6 to 12 months after full vaccination with various COVID-19 vaccines, especially in those with no history of SARS-CoV-2 infection. In the present study, we would like to confirm these findings and to show the impact of the second booster administration on the cellular immunity; a feature not explored in the two above-mentioned studies.

On September 2022, 54 participants of the CRO-VAX HCP study (Favresse, Gillot, et al., 2023) received the second and bivalent adapted BNT162b2 booster. Forty were females (median age=51.0 years; IQR=43.3–58.8) and 14 were males (median age=52.5 years; IQR=43.8–59.8). Age was not different between females and males (p=0.60, Man-Whitney test). Most of the participants (45/54; 83.3%) had a history of SARS-CoV-2 infection. Blood was collected in lithium heparin and serum separator tubes (BD Vacutainer, Becton Dickinson, New Jersey, USA) just before and 28 days after the booster administration. The study was approved by a central ethical committee (CHU UCL Namur, Yvoir, Belgium; approval number: 2020-006149-21). Total antibodies against the N (Roche Diagnostics) were measured using the Elecsys Anti-SARS-CoV-2 assay. Results above 1.0 COI were considered positive and indicates a previous SARS-CoV-2 infection. Moreover, the T cell-mediated immune response was assessed using the cobas IGRA SARS-COV-2 Tubes and the Elecsys IGRA SARS-CoV-2 assay (Roche Diagnostics). The test measures the release of IFNγ from T cells in response to an *in vitro* SARS-CoV-2 stimulation in whole blood samples which have been formerly in contact with SARS-CoV-2 coated antigens (Salvagno, Pighi, et al., 2023). Median and IQR were used to present the data. A Mann-Whitney test was used to assess the impact of the second booster on cellular immunity. A multiple comparison test was used to evaluate the effect of anti-N levels on the cellular

immunity. Results were categorized as <1.0 COI, 1.0 to 10.0 COI and >10.0 COI. A Spearman correlation was also performed for the comparison between anti-N and IFNγ. Statistical analyses were performed using GraphPad Prism 9.5.1 (GraphPad Software, Massachusetts, USA). p<0.05 was considered statistically significant.

Before the second booster administration, we found a significant and positive correlation between anti-N and IFNγ (r=0.39 (95% CI=0.11–0.61), p=0.005). Individuals with negative anti-N had significantly lower levels of IFNγ as compared to individuals with high anti-N, *i.e.,* >10.0 COI (INFγ level of 0.18 versus 1.00 IU/mL, p=0.007). These data are consistent with those published by Bonnet *et al.* and Pighi *et al.* (Bonnet et al., 2022; Pighi et al., 2023). One month after the bivalent booster administration, a significant increase in IFNγ was only observed for individuals with no history of SARS-CoV-2 infection (from 0.18 to 0.51 IU/mL, fold-increase=2.85, p=0.04). Mean fold increase 28 days after the bivalent booster in individuals with positive anti-N were close to 1 (*i.e.,* 1.09 and 1.02) (**Table IV.II.1.1 and Figure IV.II.1.1**). Additionally, the correlation between anti-N and IFNγ was no longer significant after the second booster administration (r=0.14 (-0.14-0.40), p=0.30).

Anti-N (COI)	Before booster	After booster	Fold-increase	P value
<1 (n=9)	0.18 IU/mL 95% CI: 0.08–0.90	0.51 IU/mL 95% CI: 0.33–1.87	2.85	0.04 (*)
1–10 (n=21)	0.63 IU/mL 95% CI: 0.36–0.73	0.69 IU/mL 95% CI: 0.40–1.6	1.09	0.22 (ns)
>10 (n=24)	1.00 IU/mL 95% CI: 0.48–2.30	1.02 IU/mL 95% CI: 0.60–1.93	1.02	0.97 (ns)

Table IV.II.1.1: *INFγ levels before and after the bivalent booster in subjects with low (<1.0 COI), intermediate (1-10 COI) and high (>10 COI) anti-N antibodies.*

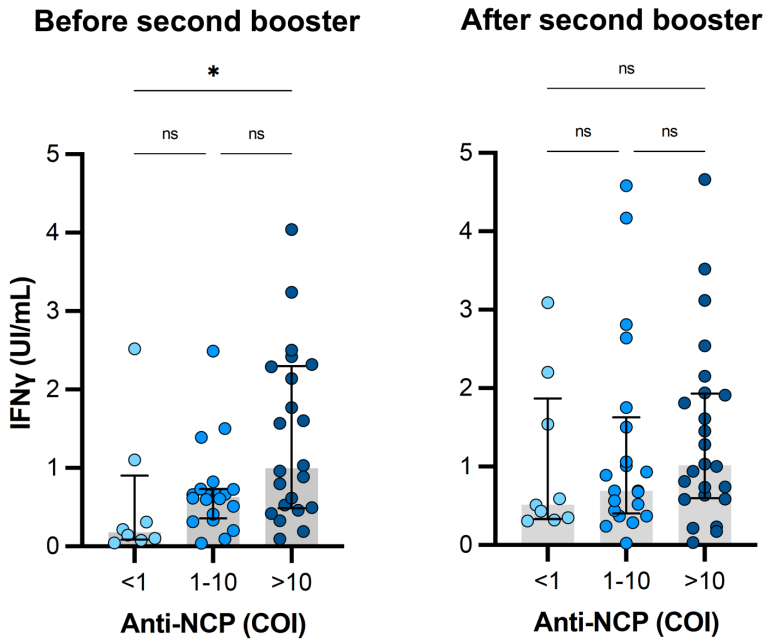

Figure IV.II.1.1: *Comparison of INFγ levels before and after the bivalent booster in subjects with low (<1.0 COI), intermediate (1-10 COI) and high (>10 COI) anti-N antibodies. Results were only statistically different before booster administration between subjects with low and high anti-N antibodies.*

Based on these findings, we confirm that individuals with no history of SARS-CoV-2 infection presented a reduced cellular immunity but were those who were more susceptible to benefit from a second booster in terms of cellular immunity. These findings need to be confirmed in other studies with a larger population.

IV.III. Six-month follow-up (adapted BNT162b2)

IV.III.1 Durability of Humoral and Cellular Immunity Six Months After the BNT162b2 Bivalent Booster

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SUMMARY

Background: Studies about the duration of the humoral and cellular response following the bivalent booster administration are still scarce. We aimed at assessing the humoral and cellular response in a cohort of HCW who received this booster.

Methods: Blood samples were collected before the administration of the bivalent booster from Pfizer-BioNTech and after 14, 28, 90, and 180 days. NAbs against either the D614G strain, the Delta variant, the BA.5 variant, or the XBB.1.5 subvariant were measured. The cellular response was assessed by measurement of the release of IFNγ from T cells in response to an *in vitro* SARS-CoV-2 stimulation.

Results: A substantial waning of NAbs was observed after 6 months (23.1-fold decrease), especially considering the XBB.1.5 subvariant. The estimated $T_{1/2}$ of NAbs was 16.1 days (95% CI=10.2–38.4 days). Although most participants still present a robust cellular response after 6 months (*i.e.,* 95%), a significant decrease was also observed compared to the peak response (0.95 versus 0.41 UI/L, p=0.0083).

Conclusion: A significant waning of the humoral and cellular response was observed after 6 months. These data can also help competent national authorities in their recommendation regarding the administration of an additional booster.

Introduction

NAbs against Omicron variants and subvariants, which represents a strong CoP from SARS-CoV-2 infection, have been shown to significantly increase after bivalent booster administrations (Carr et al., 2023; Khoury et al., 2021; Nilles et al., 2023; Seekircher et al., 2023). Accumulating evidence suggests that T cell response, *i.e.,* helper CD4+ and cytotoxic CD8+ T cells, plays a key role in the protection against severe disease (Wherry & Barouch, 2022). In contrast to NAbs, T cells are more resilient against highly mutated emerging variants, with >80% of epitopes conserved among T cells (Tarke et al., 2022; Wherry & Barouch, 2022). Currently, the long-term kinetics of the humoral and cellular immunity has been poorly explored.

Material and Methods

In this study, we present the 6-month humoral and cellular results from the participants of the multicenter and prospective CRO-VAX HCP study who received the bivalent booster (ethical approval number: 2020-006149-21). Thirty-six were females (median age=51.0 years; IQR=43.0–58.8) and fifteen were males (median age=51.0 years; IQR=35.0–59.0). Ages were non-significantly different between females and males (p=0.88). The majority of the participants (45/51; 88.2%) had a history of SARS-CoV-2 (**Appendix online**).

A pseudovirus-neutralization test was used to assess the neutralization potency of vaccines-elicited antibodies against either the D614G strain, the Delta variant, the BA.5 Omicron variant, or the XBB.1.5 Omicron subvariant. The antibody titer is determined as the dilution of serum at which 50% of the infectivity is inhibited (IC_{50}) as determined by a nonlinear sigmoid regression model (**Appendix online**). NAbs against BA.5 were measured at each time point but NAbs against the D614G strain, the Delta variant and the XBB.1.5 Omicron subvariant were only measured at 6 months in a subset of 30 participants randomly selected.

Total antibodies against the NC were measured using the Elecsys Anti-SARS-CoV-2 assay (**Appendix online**).

The T cell-mediated immune response was assessed using the cobas IGRA SARS-COV-2 Tubes and the Elecsys IGRA SARS-CoV-2 assay. The test measures the release of IFNγ from T cells in response to an *in vitro* SARS-CoV-2 stimulation in whole blood samples which have been formerly in contact with the SARS-CoV-2 coated antigens. More than 180 different SARS-CoV-2 antigens (structural (S, membrane and N) and non-structural) are coated on the antigen tube, enabling a substantial coverage of commonly occurring HLA subtypes for stimulation of both CD4+ and CD8+ T cells. The assay is therefore robust to detect different variants (**Appendix online**).

The detailed statistical analysis process is presented in the **Appendix online**.

Results

The highest measured neutralizing capacity against the BA.5 variant was reached at day 28 with a GMT of 1,095 (95% CI=903.4–1,327), representing a significant 7.0-fold increase from baseline (*i.e.,* 157; 95% CI=112–219, p<0.0001). A substantial decrease was then observed up to 180 days with an observed GMT of 47.4 (95% CI=36.6–61.6, p<0.0001), which represents a 23.1-fold decrease. The neutralizing capacity at 180 days was significantly lower compared to baseline (p=0.0004). The proportion of detectable NAbs (*i.e.,* <1:20) was 93.6%, 100%, 100%, 98.0%, and 85.4% at baseline and 14, 28, 90, and 180 days after the administration of the vaccine (**Figure IV.III.1.1a**). The fold change in the neutralizing capacity against BA.5 was similar

between participants who received the BA.1 or the BA.4/5 booster (p>0.05) (**Appendix online**). The estimated $T_{1/2}$ of NAbs was 16.1 days (95% CI=10.2–38.4 days). According to the model, a mean time of 137 days (95% CI=76–170) would be needed to cross the dilution titer threshold of 1:20 (**Figure IV.III.1.1b**).

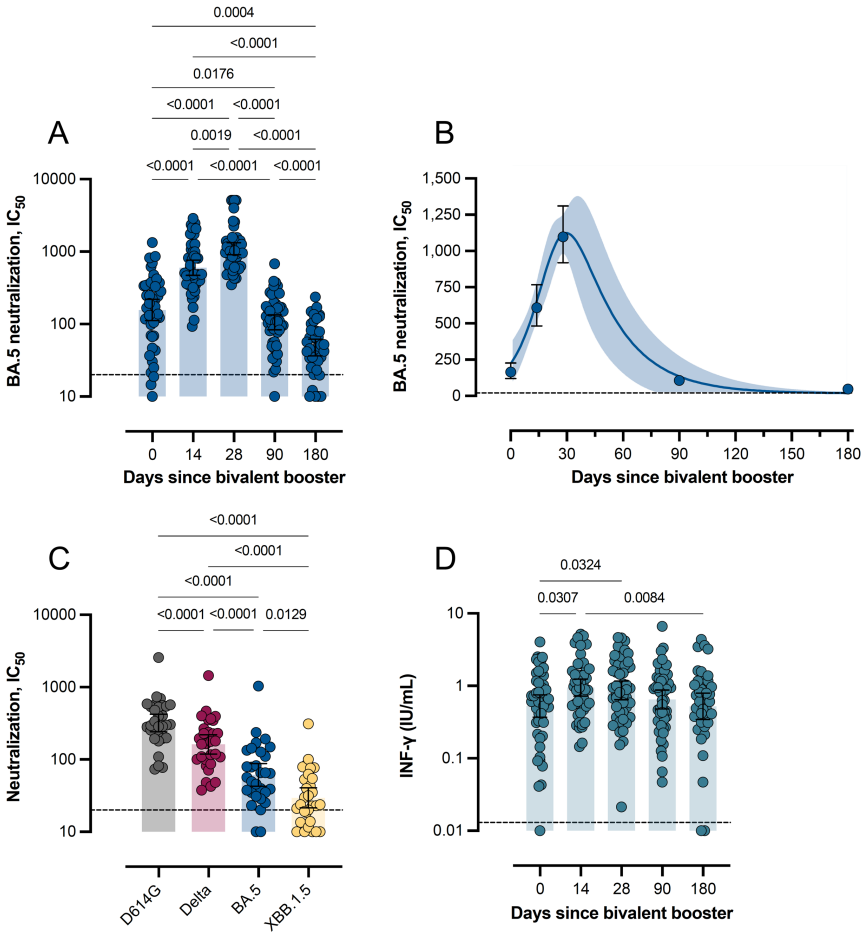

Figure IV.III.1.1: *(A) Evolution of NAbs against the BA.5 Omicron variant before and after the bivalent booster with a 6-month follow-up in a population of 51 healthy volunteers. GMT was 157 (95% CI: 112–219), 598 (470–761), 1,095 (903–1,327), 106 (83.4–134), and 47.4 (36.6–61.6) at baseline and after 14, 28, 90, and 180 days. (B) Kinetic models of the neutralizing capacity against the BA.5 Omicron variant. (C) Comparison of the neutralizing capacity against the D614G strain, the Delta and BA.5 Omicron variants, and the XBB.1.5 Omicron subvariant in a population of 30 healthy volunteers 6 months after having received the bivalent booster. GMT was 319 (95% CI: 241–423), 162 (119–220), 61.4 (42.7–88.2), and 29.5 (21.4–40.6) for the D614G strain, the Delta variant, the BA.5 Omicron variant, and the XBB.1.5 Omicron subvariant. The dotted line represents the positivity cut-offs for NAbs (dilution titer of 1:20). (D) Evolution of the cellular response by means of the measurement of IFNγ. GMT was 0.53 UI/mL (95% CI: 0.37–0.75), 0.95 (0.72–1.24), 0.87 (0.65–1.17), 0.65 (0.48–0.87), and 0.52 (0.34–0.79) at baseline and after 14, 28, 90, and 180 days. The positivity cut-off for IFNγ was 0.013 IU/mL. Geometric means and 95% CIs are represented. Only p values <0.05 were graphically represented.*

At 6 months, NAbs against the Delta variant, the BA.5 Omicron variant and the XBB.1.5 Omicron subvariant were 1.97, 5.20, and 10.81 lower compared to the D614G strain. The proportion of detectable NAbs was 100%, 100%, 91.3%, and 66.6%, respectively (**Figure IV.III.1.1c**).

The highest T cell response was observed after 14 days with a GMT of 0.95 UI/mL (95% CI=0.72–1.24), representing a significant 1.97-fold increase from baseline (*i.e.,* 0.48; 95% CI=0.30–0.77, p=0.0306). A significant decrease was then observed up to 180 days with an observed GMT of 0.41 (95% CI=0.21–0.82, p=0.0083), representing a 2.28-fold reduction compared to day 14 and a 1.17-fold decrease from baseline. The IFNγ responses at 90 and 180 days were not different from baseline (p=0.91 and 0.95). The proportion of detectable levels of IFNγ was 98%, 100%, 100%, 100%, and 95% at baseline, 14, 28, 90, and 180 days (**Figure IV.III.1.1d**). The fold change in the IFNγ response against BA.5 was similar between participants who received the BA.1 or the BA.4/5 booster (**Appendix online**).

Eleven participants (21.6%) developed a BKI between 90 and 180 days; which is consistent with the drop of NAbs. The infection was associated with a significant rise in BA.5 NAbs(fold increase of 2.55, p=0.0039). The impact on the IFNγ release was not significant in these patients (p=0.4961) (**Appendix online**).

Discussion and Conclusion

The increase of NAbs following the administration of the bivalent booster we documented in our study (*i.e.,* 7.0-fold increase) was consistent with the conclusions of other studies (Carr et al., 2023; N. H. Tan et al., 2023). As for the humoral response following the first two doses of BNT162b2 and the homologous boosters (Bayart, Douxfils, et al., 2021; Favresse, Gillot, et al., 2023; Kato et al., 2022; Uwamino et al., 2022), a substantial waning of the humoral response was observed 6 months after the administration of the BNT162b2 bivalent booster. This decrease was especially important considering the XBB.1.5 subvariant. Moreover, the drop of NAbs over time coincides with the decrease of bivalent booster effectiveness against infection in the recent report of Lin *et al.* (Lin, Xu, Gu, Zeng, Sunny, et al., 2023).

Compared to the ancestral strain, the Omicron variant is characterized by the presence of around 32 mutations in the S protein (Planas et al., 2022). The S protein of BA.5 is identical to BA.2 except for 69–70 deletion, L452R, F486V and the WT amino acid at Q493 (Markov et al., 2023; Tegally et al., 2022). For the recombinant XBB subvariants, the largest proportion of S mutations is derived from the BA.2 with 10 new evolved mutations. The XBB.1.5 is characterized with a F486P substitution rather than the F486S substitution found in XBB (Ao et al., 2023). The emergence of new Omicron subvariants with substantial mutations is characterized by a

considerable immune escape and a sharp increase in infectivity, especially considering the XBB.1.5. subvariant (Ao et al., 2023).

A significant decay in the cellular response was also observed over time. Nevertheless, the proportion of samples still able to generate IFNγ in response to an *in vitro* SARS-CoV-2 stimulation remained high. This observation is consistent with the maintained and superior effectiveness against severe disease in the report of Lin *et al.* (Lin, Xu, Gu, Zeng, Sunny, et al., 2023). The fact that the release of IFNγ could represent a good surrogate of the risk of severe infection remains to be evaluated. Importantly, these data can also help competent national authorities in their recommendation regarding the administration of an additional booster.

IV.III.2 Neutralizing Antibody Response to XBB.1.5, BA.2.86, FL.1.5.1 and JN.1 Six Months After the BNT162b2 Bivalent Booster

International Journal of Infectious Diseases, 2024 ahead of print

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* Contributed equally

SUMMARY

Introduction: An increased evasion of the SARS-CoV-2 virus towards vaccination strategies and natural immunity has been rapidly described notably due to mutations in the spike receptor binding domain and the N-terminal domain.

Material and methods: Participants of the CRO-VAX HCP study who received the bivalent booster were followed at 6 months. A pseudovirus-neutralization test was used to assess the neutralization potency of antibodies against D614G, Delta, BA.1, BA.5, XBB.1.5, BA.2.86, FL.1.5.1, and JN-1.

Results: The neutralizing capacity of antibodies against Omicron variant or its subvariants was significantly reduced compared to D614G and Delta ($p<0.0001$). The lowest neutralizing response that was observed with JN-1 (GMT=22.1) was also significantly lower than XBB.1.5 (GMT=29.5, $p<0.0001$), BA.2.86 (GMT=29.6, $p<0.0001$), and FL.1.5.1 (GMT=25.2, $p<0.0001$). Participants who contracted a breakthrough infection due to XBB.1.5 had significantly higher neutralizing antibodies against all variants than uninfected participants, especially against Omicron variant and subvariants.

Conclusion: Our results confirm that JN.1 is one of the most immune evading variants to date and that the BA.2.86 subvariant did not show an increased immunity escape compared to XBB.1.5. The stronger response in BKI with Omicron variant and subvariants supports the need to use vaccine antigens that target circulating variants.

Introduction

At the end of January 2020, the D614G mutation emerged in UK and rapidly became dominant in the world. In late 2020, the Delta variant was identified, bearing 9 mutations in S (Tian et al., 2021) followed in November 2021 by the Omicron variant that presented 32 S mutations compared to the D614G strain (Planas et al., 2022). More recently (August 2023), the BA.2.86 subvariant was identified and

characterized by 60 amino acid changes, predominantly in RBD and NTD, compared to the WT strain (Lambrou et al., 2023; Lassauniere et al., 2023). The BA.2.86 subvariant has over 30 mutations in S in comparison with BA.2 and XBB.1.5 (Lasrado et al., 2023). The FL.1.5.1, also known as XBB.1.9.1.1.5.1, presents 3 additional mutations in S compared to XBB.1.5 (*i.e.,* F456L, T478R, and A701V) (Lasrado et al., 2023). In late 2023, the BA.2.86 has evolved in JN.1 and rapidly became dominant. It is characterized with one additional RBD mutation (L455S) and 3 other non-S mutations (Kaku et al., 2024; Yang et al., 2024).

The presence of these mutations raised the possibility of an increase in NAb evasion (Lasrado et al., 2023).

The aim of the study was to evaluate the impact of recent circulating SARS-CoV-2 subvariants on the NAb response of individuals who had been followed for 6 months after having received the bivalent booster.

Material and methods

Study design

The CRO-VAX HCP study is a Belgian multicenter, perspective, and interventional study that was designed to assess the humoral response in a population of HCW from 18 to 65 years having received two doses of the BNT162b2 mRNA COVID-19 vaccine followed by a homologous booster (third dose) and after by a bivalent booster (BA.1 or BA.4/5; fourth dose) (ethical approval number: 2020-006149-21) (**Appendix online**). In the present study, we compared the NAb response against D614G, Delta, BA.1, BA.5, XBB.1.5, BA.2.86, FL.1.5.1, and JN.1 6 months after the bivalent booster administration in a population of 30 participants.

Seroneutralization

A pVNT was used to assess the neutralization potency of antibodies against 8 variants (D614G, Delta, BA.1, BA.5, XBB.1.5, BA.2.86, FL.1.5.1, and JN.1). The antibody titer is determined as the dilution of serum at which 50% of the infectivity is inhibited as determined by a nonlinear sigmoid regression model. Method details have been described elsewhere(Favresse, Gillot, et al., 2023).

Statistical analysis

The normality of distribution was assessed by the Anderson-Darling's test following log-transformation. Median and IQR were used to present demographic data and GMT and 95% CI to present the results of the humoral response. A multiple comparison test (two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli) was used to assess the potential difference between the type of variants. A Mann-Withney test was used to compare age between genders and to compare NAb

titers between participants who developed a BKI or not. Statistical analyses were performed using GraphPad Prism 10.2.0 (GraphPad Software, Massachusetts, USA). $p<0.05$ was considered significant.

Results

Demographics

Twenty-one were females (median age=55 years; IQR=44–59) and 9 were males (median age=54years; IQR=41–60). Ages were non-significantly different between females and males (p=0.90). Most of the participants (25 of 30; 83%) had a history of SARS-CoV-2 infection (BA.1) before the administration of the bivalent booster. Twenty-six participants received the BA.1 adapted booster while 4 received the BA.4/5 adapted booster. Six participants development a BKI due to XBB.1.5, 3–6 months after the bivalent booster administration (**Appendix online**).

Neutralizing capacity

Bivalent-booster sera obtained at 6 months neutralized D614G, Delta, BA.1, BA.5, XBB.1.5, BA.86, FL.1.5.1, and JN.1 with GMT of 319, 162, 71.6, 61.4, 29.5, 29.6, 25.2, and 22.1, respectively (**Figure IV.III.2.1**). The neutralizing capacity of antibodies against Omicron variant or subvariants was significantly reduced compared to D614G and Delta variants ($p<0.0001$). The lowest neutralizing response that was observed with JN.1 (GMT=22.1, 95% CI: 16.2–30.2) was also significantly lower compared to XBB.1.5 (GMT=29.5, 95% CI: 21.4–40.6; p=0.0002), BA.2.86 (GMT=29.6, 95% CI: 21.4–41.0; p=0.0003), and FL.1.5.1 (GMT=25.2, 95% CI: 18.1-34.9; p=0.0094) (**Figure IV.III.2.1**).

The level of NAbs against Delta, BA.1, BA.5, XBB.1.5, BA.2.86, FL.1.5.1, and JN.1 were 1.97, 4.46, 5.20, 10.81, 10.77, 12.67, and 14.43 lower compared to D614G. Compared to XBB.1.5, BA.2.86, and FL.1.5.1, the neutralizing capacity of antibodies against JN.1 was 1.33, 1.34, and 1.14-fold-decrease, respectively (**Figure IV.III.2.1**).

Participants who contracted a BKI had significantly higher NAbs against all variants (*i.e.,* cross-reactivity) compared to uninfected participants. The fold change was more pronounced with BA.1, BA.5, XBB.1.5., BA.2.86, FL.1.5.1, and JN.1 (fold change ranging 4.5–5.0) compared to D614G (fold change=2.5) and Delta (fold change=3.0) (**Figure IV.III.2.2**).

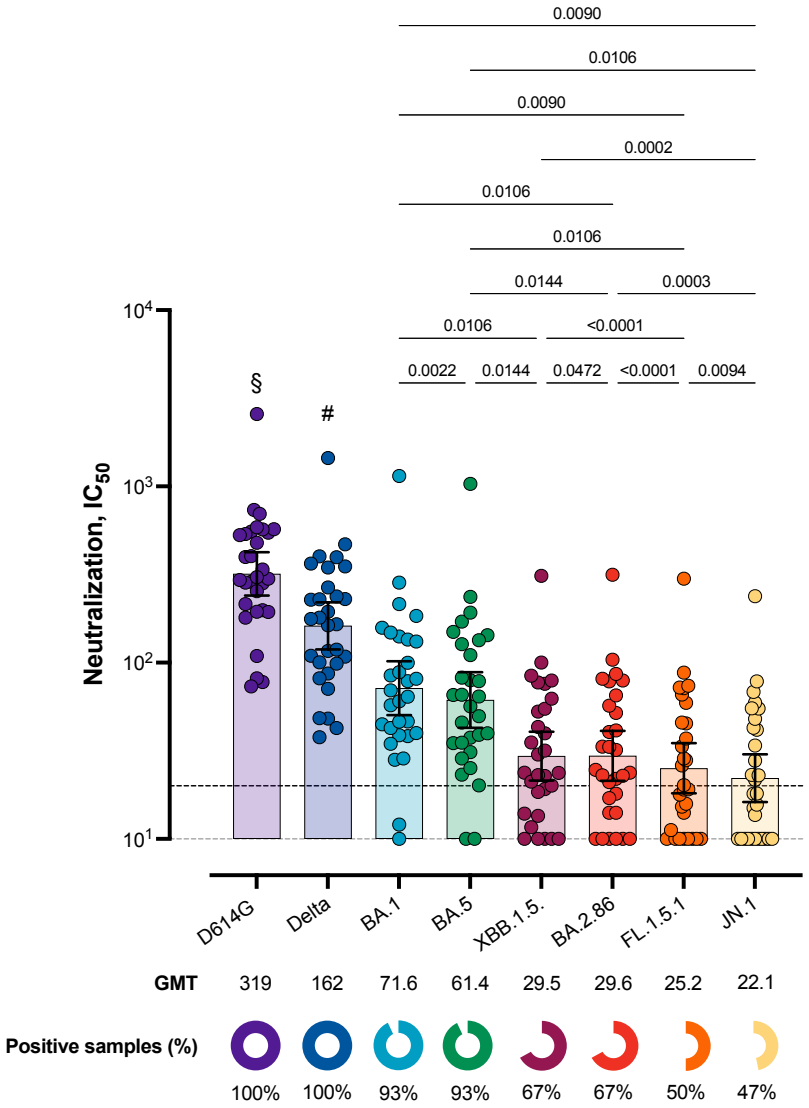

Figure IV.III.2.1: *Comparison of the neutralizing capacity against the D614G strain, the Delta variant, the BA.1 Omicron variant, the BA.5 Omicron variant, the XBB.1.5 Omicron subvariant, the BA.2.86 Omicron subvariant, the FL.1.5.1 Omicron subvariant, and the JN.1 Omicron subvariant in a population of 30 healthy volunteers 6 months after having received the bivalent booster. The proportion of detectable NAbs was 100%, 100%, 93%, 93%, 67%, 67%, 50%, and 47%, respectively. GMT ± 95% CI and percentage of positive samples are represented. The black dotted line represents the positivity cut-offs for NAbs (IC_{50} of 1:20). The grey dotted line represents the LOD of the assay (IC_{50} of 10). § = significantly higher compared to all other variants (p<0.0001). # = significantly higher compared to all other variants (p<0.0001) except for the D614G strain (p<0.0001).*

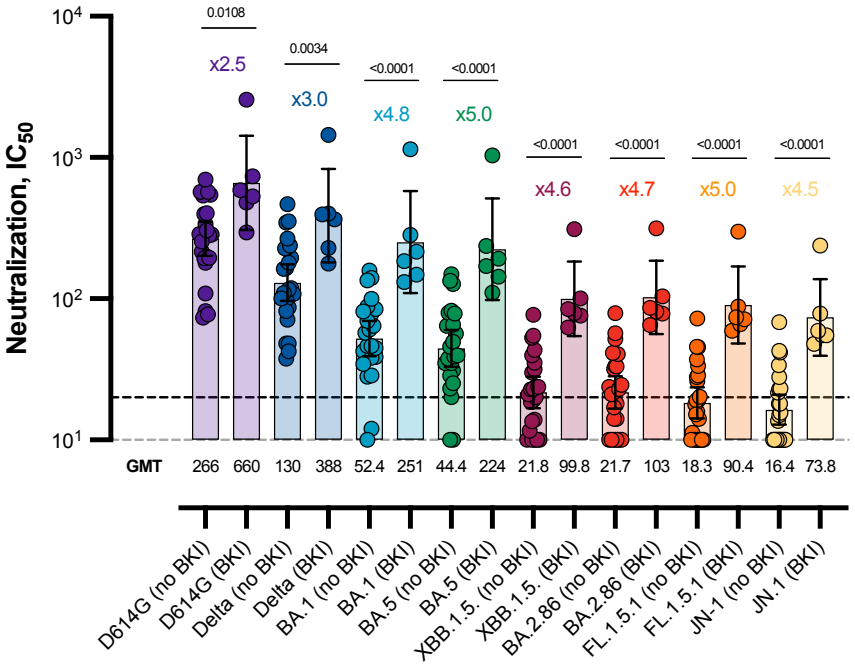

Figure IV.III.2.2: *Comparison of the neutralizing capacity against the D614G strain, the Delta variant, the BA.1 Omicron variant, the BA.5 Omicron variant, the XBB.1.5 Omicron subvariant, the BA.2.86 Omicron subvariant, the FL.1.5.1 Omicron subvariant, and the JN.1 Omicron subvariant in individuals who developed a BKI following administration of the bivalent booster or not. Blood was collected 6 months after having received the bivalent booster. GMT ± 95% CI are represented as well as the fold-change between groups. The black dotted line represents the positivity cut-offs for NAbs (IC_{50} of 1:20). The grey dotted line represents the LOD of the assay (IC_{50} of 10).*

Discussion

The administration of COVID-19 vaccines allowed the reduction of SARS-CoV-2 infections, complications and deaths. A gradual decline in vaccine efficacy (VE) against infection was however rapidly observed. This waned efficacy was consistent with the decrease of neutralizing antibodies, supporting their role as a strong correlation of COVID-19 protection from infection. The decrease in VE was further heightened by the emergence of variants, especially the Omicron variant and its subvariants (Favresse, Gillot, et al., 2023; Lasrado et al., 2023; Planas et al., 2022; Willett et al., 2023). For instance, a 23.1-fold decrease of NAbs against BA.5 was observed between one and 6 months after the bivalent booster ($T_{1/2}$=16.1 days) (Favresse, Gillot, Closset, et al., 2024).

Recently, some concerns were formulated following the identification of the BA.2.86 subvariant that could more easily escape immunity because of its high number of additional mutations compared to XBB.1.5 (Lambrou et al., 2023; Lasrado et al.,

2023). This has not happened (Q. Wang et al., 2024) and was confirmed in our study and by others by the identification of similar neutralizing antibody titers between BA.2.86 and XBB.1.5 (Lasrado et al., 2023; Willett et al., 2023). In accordance with our evaluation, Hu *et al.* identified that neutralizing antibody response of FL.1.5.1 was also lower compared to BA.2.86 and XBB.1.5. in a population of 48 individuals 14–32 days after the bivalent booster (Hu et al., 2023).

Compared to BA.2.86, the JN.1 has become the predominant subvariant (Jeworowski et al., 2024; Kaku et al., 2024). Yang *et al.* found an enhanced immune escape of JN.1 compared to BA.2.86 (1.1 to 2.1-fold decrease in the 50% neutralization titer (NT_{50}) (n=54)(Yang et al., 2024). Kaku *et al.* pointed a 4.5-fold decrease in NT_{50} of JN.1 compared to BA.2.86 3-4 weeks after XBB.1.5 vaccination (n=19) (Kaku et al., 2024). Planas *et al.* confirmed a 2.0-fold neutralization decrease between BA.2.86 and JN.1 after 3 doses of BNTA62b2 (n=13) and 6 months after the BA.5 bivalent booster (n=8) (Planas et al., 2024). A less pronounced decreased of 1.15-fold was also observed by Jeworowski *et al.* compared to BA.2.86 (Jeworowski et al., 2024) and is in line with the present study.

Interestingly, individuals who developed a XBB.1.5 BKI in our study presented a boost in neutralizing antibodies against all variants and subvariants compared to uninfected individuals, but mostly on Omicron variants and subvariants, supporting the need to consider the use of vaccines that are adapted to circulating variants, as recommended by the WHO and the FDA (Hu et al., 2023; Willett et al., 2023). Similar results have been found in two studies in individuals who developed an XBB.1.5 BKI or who received the XBB .1.5 monovalent vaccine (Planas et al., 2024; Q. Wang et al., 2024).

The sample size of our study was low, especially considering the subcohort of participants who developed a BKI. This represents a limitation.

Conclusion

Our results confirm that JN.1 is one of the most immune evading variants to date and that the BA.2.86 subvariant did not show an increased immunity escape compared to XBB.1.5. The fact that only the reduced neutralizing capacity might be the only mechanism that could explain why JN.1 rapidly became predominant need further evaluations. The boost in neutralizing antibody titers observed in subjects who developed a BKI after the administration of the bivalent booster was mostly superior considering Omicron variants and subvariant. This supports the need to use vaccine antigens that target circulating variants.

CHAPTER V: CONCLUSION AND PERSPECTIVES

Humoral response as a correlate of protection

Adaptive humoral and cellular responses to infection are commonly specific to a pathogen and protect against reinfection (Krammer, 2021). Vaccination allows humans to acquire these protective responses without suffering from an infection. A CoP is defined as "an immune marker statistically correlated with VE that may or may not be a mechanistic causal agent of protection" (Krammer, 2021; Plotkin & Gilbert, 2012). Most CoPs are based on antibody measurement (*i.e.,* humoral immune response) for example in the case of hepatitis A or B, measles, polio, rotavirus, influenza, yellow fever, etc. (Plotkin, 2010). CoPs are divided into mCoPs (mechanistic CoPs) and nCoPs (nonmechanistic CoPs), depending on the presence or absence of a mechanistic cause or not. The nCoP typically predicts VE through its correlation with another immune response for which a mechanistic protection has been found (Plotkin & Gilbert, 2012). The cellular component could also represent a CoP (zoster, tuberculosis), but antibodies are much easier to measure and therefore preferred for clinical purposes (Krammer, 2021). The search for a CoP, as well as for an absolute CoP threshold, was urgently needed in the context of SARS-CoV-2 (Krammer, 2021; Plotkin, 2010) to enable more efficient vaccination management, by recommending additional boosters in case of too low antibody titers for example. This can also serve to select convalescent serum to administer in patients with severe COVID-19. A CoP can also help to determine the percentage of the population that is protected and may lead to interventions such as vaccination campaigns if the percentage of immune individuals is considered too low (Kent et al., 2022; Krammer, 2021). Extending a vaccine to other populations who were not included in randomized Phase 3 trials is possible when a CoP is available. The approval of new vaccine strains and booster doses should also be based on studies showing non-inferiority or superiority using a CoP endpoint (mostly NAbs) (Gilbert et al., 2022).

Khoury *et al.* and *Earle et al.* were the first to identify a CoP for SARS-CoV-2 (Earle et al., 2021; Khoury et al., 2021). Significant correlations between VE and NAbs were reported. Binding antibody titers (anti-S or RBD IgG) were also highly correlated with VE. Importantly, the CoP may also differ depending on the endpoint evaluated (symptomatic infection, severe disease, or mortality) and most studies identified a correlation between NAbs and symptomatic disease. The CoP may also depend on the pathogen and its potential circulating variants, on timing (because antibodies tend to weaken over time) and on the vaccine product (Perry et al., 2022; Regev-Yochay et al., 2023). Additionally, it might also be influenced by the laboratory method used for antibody measurement since there is frequently no international standardization (Perry et al., 2022).

The CRO-VAX HCP study results found high positive correlations between NAbs directed against Omicron (r=0.89) and binding antibodies (r=0.83) and symptomatic infection (Favresse, Gillot, et al., 2023). A meta-analysis carried out by Cromer *et al.* that included 15 clinical study trials, four vaccines, and pre-Delta, Delta and Omicron variants reported a high correlation between NAbs and the symptomatic efficacy (ρ=0.95). They also identified a significant correlation between severe efficacy (ρ=0.72) and the lower NAb titer needed to predict protection (Cromer et al., 2023). The fact that NAbs are correlated with severe outcomes is not surprising because antibody administration to symptomatic patients helped reduce hospitalization or death rates by up to 85% (Gupta et al., 2021). Interestingly, changes in NAbs over time and against new circulating variants was found to be predictive of efficacy changes.

There are however multiple reports describing high levels of pre-infection anti-S or NAbs in individuals who had developed a BKI despite vaccination or recent infection. This suggests that antibodies could represent only one component of protection, as the presence of antibodies reduces the risk but does not eliminate it (Perry et al., 2022). The role of T-cells and of non-neutralizing antibodies should therefore be more studied (Zhang et al., 2023).

Whether NAbs must be considered as a mCoP against initial infection acquisition remains to be determined. Lingas *et al.* found that the progressive increase of NAbs was associated with the shortening of the $T_{1/2}$ of infected cells and viral particles (Lingas et al., 2024). Some authors proposed that mucosal anti-S IgA could be the mCoP while serum markers could be the nCoP (Gilbert et al., 2022).

Binding antibodies instead of NAbs?

Even if most studies searched for a CoP based on NAb measurement, some studies found significant correlations between VE and binding antibodies as well (Bergwerk et al., 2021; Earle et al., 2021; Favresse, Gillot, et al., 2023; Gilbert et al., 2022; Regev-Yochay et al., 2023). Compared to binding antibody measurement, NAbs are much more difficult to quantify. These tests are generally reserved to specialized laboratories, require high workload, intense operators training, present a low throughput and are quite expensive (Favresse, Gillot, Di Chiaro, et al., 2021). Most laboratories measure binding antibodies in clinical practice since they can be quantified on rapid high throughput automates, do not require very specific training or installations, and are cheaper to quantify. The possibility of measuring binding antibodies in more convenient matrices like dried blood spots with high performance compared to standard blood samples also exists (Castelletti et al., 2024).

In three distinct papers of this thesis, we provided data in favor of NAbs as a better CoP compared to binding antibodies. On the one hand, we found a slightly better correlation between NAbs against Omicron and VE (r=0.89) as compared to binding antibodies (r=0.83). On the other hand, the ratios of BKI cases and controls were lower considering NAb measurement compared to binding antibodies within 3 months after either the BNT162b2 or mRNA-1277 homologous booster (0.12 versus 0.64; p=0.02 and 0.55 versus 0.82; p=0.0033, respectively) (Favresse, Gillot, et al., 2023; Gillot et al., 2023; Gillot et al., 2024). This last observation was also made in the report by Bergwerk *et al.* (Bergwerk et al., 2021).

Preliminary studies that have been published before the arrival of variants sought to compare binding antibodies and NAbs. Although not perfect, significant correlations were identified in multiple studies on infected patients (Favresse, Gillot, Di Chiaro, et al., 2021), mostly by using binding IgG against the S or RBD antigen (Bayart, Douxfils, et al., 2021; Douxfils et al., 2021; Legros et al., 2021; Padoan, Zuin, et al., 2020).

To predict seroneutralization, thresholds of commercial binding assays should be updated since insert kits mostly refer to a seroprevalence threshold to determine a post-infectious status (Douxfils et al., 2021; Favresse, Gillot, et al., 2023). Using the Elecsys kit from Roche for anti-S1 RBD binding antibodies, Rus *et al.* identified a cut-off of 133 BAU/mL associated with a sensitivity and specificity of 84% and 74.5%, respectively (Resman Rus et al., 2021). This cut-off is much higher compared to the insert kit cut-off of 0.8 BAU/mL.

The appearance of variants was characterized by a high mutation rate, especially since the emergence of the Omicron variant in November 2021, which presented more than 30 S mutations compared to the D614G strain and the more recent subvariants including XBB.1.5 and JN.1 (Favresse, Gillot, Cabo, et al., 2024; Planas et al., 2022). As previously mentioned, these mutations have been related to immunity escape and reduced VE. Moreover, the presence of these mutations may also jeopardize the ability of current commercial binding assays to detect S or RBD antibodies (Lippi, Adeli, et al., 2021). Indeed, S and RBD antigens used in current commercial assays are still based on the original SARS-CoV-2 lineage detected in Wuhan in 2019 (Hempel et al., 2024; Lippi, Adeli, et al., 2021). Following the administration of the homologous BNT162b2 booster, CRO-VAX HCP study participants were followed up at regular intervals and NAbs against Omicron (BA.1) as well as binding antibodies using the kit from Roche were measured. A cut-off of 8,434 BAU/mL was found to be the best predictor of NAb presence with a sensitivity and a specificity of 83.1% and 63.4%, respectively (Favresse, Gillot, et al., 2023). The possibility of adapting the antigens present in the kit should be explored in the future to assess whether this will better predict NAb presence.

An attractive alternative to the laborious measurement of NAbs through cell-based culture models is to use a sVNT (Tan et al., 2020). This method is based on the antibody-mediated blockage of the interaction between ACE2 receptor protein and the RBD. sVNTs can be automated, can present a high throughput, does not need for a BSL2 or BSL3 laboratory, and can be performed by technicians without an intensive training program. Correlations with conventional techniques were found to be excellent in some studies (Favresse, Gillot, Di Chiaro, et al., 2021; Tan et al., 2020). However, other reports presented lower performance, with sensitivities and specificities of 80.4-87.67% and of 72.6-84.5%, respectively (Graninger et al., 2023; Simon et al., 2023). In one of our studies, we even showed that the correlation between sVNT (targeting Omicron) and binding IgG against RBD (targeting WT) (r=0.90) was superior to that of sVNT versus pVNT, both targeting Omicron (r=0.52) (Simon et al., 2023).

Even if the literature tends to favor NAbs as the best predictor of infection and disease severity, there is no definitive evidence yet regarding the fact that the best CoP is NAbs compared to binding antibodies, and this also depends on the method used for NAbs as well. However, the FDA and EMA already made up their decision and accepted NAb titer as a CoP for emergency use authorization of SARS-CoV-2 variant vaccine booster. This implies that there is no need for additional new randomized trials to confirm clinical benefit (Follmann et al., 2023; Kent et al., 2022).

The quest for harmonization of antibody results

Comparing the immunogenicity of infection or vaccination is challenging given that there are different methods for the measurement of SARS-CoV-2 antibodies (Favresse, Eucher, et al., 2021; Hempel et al., 2024; Kristiansen et al., 2021).

In December 2020, major efforts have been made for the creation of an international standard (IS) (*i.e.,* WHO IS for anti-SARS-CoV-2 Ig; IS 20/136). The IS is based on pooled human plasma from convalescent patients. The product is lyophilized in ampoules and one ampoule is equivalent to 250 IU for neutralizing activity and to 1000 BAU/mL for binding antibody measurement (Kristiansen et al., 2021). These ampoules are therefore useful for assay calibration at the international level. Neutralizing and binding assays should use the IU/mL and BAU/mL units, respectively (Kristiansen et al., 2021). Using the WHO IS for anti-SARS-CoV-2 Ig reduced inter-laboratory variability by more than 50 times for neutralization (live and pseudotype based neutralization assays) and 2,000 times for binding antibodies (ELISA and rapid tests) (Kristiansen et al., 2021).

This step was major, especially in the quest for a protective threshold. However, even though the WHO IS reduced the variability, most laboratories and researchers are not

using it. Moreover, even if some of the variability has been reduced, some is still present, making it still difficult to find a threshold in 2024 (Follmann et al., 2023; Fong et al., 2023; Hempel et al., 2024).

The final goal would also be to use only one unit for antibody measurement, by referring to the assay method (neutralizing or binding assays, including Ig type) and to the target used (*i.e.,* N, S or RBD) (Hempel et al., 2024).

In the context of antigen mutations, a new reference panel for variants (22/270) has been developed. It contains antibodies targeting and capable of neutralizing Alpha, Delta, Gamma, and Omicron variants (Hempel et al., 2024; NIBSC, 2023). The suitability of this new reference panel for more recent variants, including BA.2.86 and JN.1, needs to be studied.

Cellular response as a correlate of protection

Despite a substantial decline in humoral immunity, vaccination still shows robust protection against severe COVID-19 disease (*i.e.,* hospitalization and death), even against highly mutated variants (Arbel et al., 2022; Bar-On, Goldberg, & Milo, 2022; Ferdinands et al., 2022; Goel et al., 2021; Keeton et al., 2022; Lin et al., 2022; Giuseppe Lippi et al., 2022; J. Liu et al., 2022; Maringer et al., 2022; Moss, 2022; Underwood et al., 2023; Wherry & Barouch, 2022; Zhang et al., 2022).

Compared to NAbs, T cells cannot prevent host cells from becoming infected, but they can respond rapidly once infection has occurred to limit virus replication and propagation (Wherry & Barouch, 2022). T cells require antigen presentation for activation and subsequent antiviral activity (Kent et al., 2022).

Accumulating evidence suggests that T cell response (helper CD4+ and cytotoxic CD8+ T cells) could play a key role in reducing the severity and controlling the infection (Ledford, 2022; Giuseppe Lippi et al., 2022; Wherry & Barouch, 2022). Memory B cells are also highly durable and may contribute to protection along with memory T cells (Wherry & Barouch, 2022; Zhang et al., 2022). In mouse models lacking antibodies but with functional B cells and lymphoid organs, Fumagalli *et al.* found that prior infection or mRNA vaccination could protect against SARS-CoV-2 infection (Fumagalli et al., 2024). Authors reported that CD8+ T cells were important against severe infection, while CD4+ T cells were more useful to manage milder infectious cases. IFNγ also had an essential role in the defense process, without requiring antibodies. Situations where antibodies can be absent include patients with agammaglobulinemia, patients treated with B-cell-depleting agents (*i.e.,* cancer, multiple sclerosis), and in cases where variants escape NAbs (Fumagalli et al., 2024). In these populations, T cells responses are generally conserved (Sette et al., 2023).

In comparison to NAbs, T cells recognize many more epitopes throughout the sequence of the S protein of SARS-CoV-2, which are therefore not limited to the S RBD and NTD, in which most mutations occur (Wherry & Barouch, 2022). T cells are more resilient against highly mutated variants, with more than 80% of epitopes conserved (Keeton et al., 2022; Ledford, 2022; Moss, 2022; Tarke et al., 2022; Wherry & Barouch, 2022).

The cellular response can be measured by different methods. It can be assessed using antigen-specific T cell responses by using assays measuring cytokine production after antigen stimulation, such as the enzyme-linked immunosorbent spot (ELISpot) assay and intracellular cytokine staining, or through the activation-induced marker assays. Compared to these more sophisticated and complex methodologies, the use of a specific IGRA is easier to implement in clinical routine. Significant and positive correlations have been reported between IGRA and more sophisticated methods (Gatti et al., 2023; Lochmanova et al., 2023; Wakui et al., 2022).

The cellular response received less attention in the literature than to the humoral response (Kent et al., 2022; Maringer et al., 2022; Moss, 2022). This could be attributed to the fact that methodologies used for cellular response assessment are not readily accessible, require a rigorous pre-analytical procedure (*i.e.,* fresh whole blood samples), entail a prolonged sample processing, and are expensive. Furthermore, the development of such assays requires greater efforts than antibody measurement (Fonseca Brito et al., 2023; Kent et al., 2022; Moss, 2022; Salvagno, Pighi, et al., 2023). Peripheral T cells analysis (as compared to specific anatomical locations) is considered a valid approach as a surrogate for the global T cell response (Sette et al., 2023). Moreover, as for antibody measurement, there is no standardization for T cell response measurement (Kent et al., 2022).

In mild disease, CD4+ and CD8+ T cell responses were detected in most infected individuals. The strong dominant CD4+ and CD8+ T cell response to S protein that was also observed had positive implications given that vaccine development was only based on the S protein. The induction of humoral response as well as CD4+ and CD8+ T cell responses was therefore expected. Additional antigens are also recognized by T cells like N, M, E, and non-structural protein antigens (Sette et al., 2023). The number of epitopes recognized by human T cells exceeds 2,000. The large breath of CD4+ and CD8+ T cells responses against SARS-CoV-2 makes viral escape unlikely in the context of epitope mutation.

In the majority of patients, the SARS-CoV-2 infection leads to adaptive antigen-specific responses, viral clearance, and creation of immune memory (Sette et al., 2023). However, in some situation, and especially in elderly people, the infection is characterized by strong deregulatory effects with delayed innate IFNγ responses, delayed adaptive immunity and extreme inflammatory responses. It is estimated that

memory CD4+ and CD8+ T cells are maintained for at least 6 months after infection, regardless of disease severity (Sette et al., 2023).

Early reports have shown that IFNγ generation occurs rapidly after the first vaccination with BNT162b2 with a peak response around 1 month (Lim et al., 2022; Sahin et al., 2021), which was consistent with the percentage increase in S specific CD8+ and CD4+ T cells (Zhang et al., 2022). A robust but decreasing cellular response was then observed after several months (Herzberg et al., 2022; Lim et al., 2022; Maringer et al., 2022; Naaber et al., 2022; Sahin et al., 2021; Vogel et al., 2022; Zhang et al., 2022). After 6 months, 84% (CD4+) and 85% (CD8+) of memory T cell responses were preserved against Omicron (Tarke et al., 2022). Among the different type of vaccine types available, mRNA vaccines induced stronger CD4+ and CD8+ T cell responses.

After the homologous BNT162b2 booster, a significant increase in the cellular response (IFNγ and S-specific CD8+ and CD4+ T cells) was observed in two studies (Herzberg et al., 2022; Naaber et al., 2022). In contrast, Maringer *et al.* identified that the S-specific T cell responses (*i.e.,* IFNγ by ELISpot) after different COVID-19 vaccination regimens were not further enhanced by booster vaccination, suggesting that booster vaccination was mostly useful to increase antibody response (Maringer et al., 2022).

Administration of the monovalent or the bivalent booster also increased the CD4+ and CD8+ T cell responses (Collier et al., 2023; Favresse, Gillot, Closset, et al., 2024; Salvagno, Pighi, et al., 2023; N. H. Tan et al., 2023). Participants who had never developed a SARS-CoV-2 infection (Favresse, Cabo, et al., 2023) or who presented a lower residual cellular response at baseline, were those who most benefited from the second booster (Favresse, Gillot, Closset, et al., 2024; Salvagno, Pighi, et al., 2023). Six months after the bivalent booster, and even if a significant decrease in IFNγ was observed, the proportion of individuals with detectable IFNγ levels was still high (*i.e.,* 95.0%) (Favresse, Gillot, Closset, et al., 2024). This kinetics was completely different from that of NAbs (Favresse, Gillot, Closset, et al., 2024; Sette et al., 2023).

Cellular response measurement (including IFNγ) as a suitable surrogate for the risk of severe COVID-19 still deserves further investigations. Screening individuals with a decreased cellular response to prioritize vaccination could, for example, be a potential application that needs to be explored. Nevertheless, the cost-effectiveness of such strategy also needs to be challenged. Along with the assessment of NAbs, cellular response measurement should also be encouraged in vaccine development programs. Incorporating additional T cell antigens in future vaccines might be interesting for the prevention of severe disease along with antibodies (Sette et al., 2023).

The role of T cells might also be indirect since the help of CD4+ T cells is required (differentiation and selection of B cells) for the generation of high titers of NAbs (Cromer et al., 2023; Kent et al., 2022; Sette et al., 2023). Since NAbs and cellular responses are typically correlated, it is also possible that the correlation is present but below the LOD of most assays (*i.e.,* serum dilution of 1:10 or 1:20) (Cromer et al., 2023; Kent et al., 2022). This can also be explained by the rapid protection onset after the first vaccine dose, when NAbs are supposed to be absent (or below LOD) or at very low levels (Perry et al., 2022).

The potential role of non-neutralizing antibodies (Fc-dependent effector functions) as a CoP against either symptomatic disease and/or severity should also be further investigated (Zhang et al., 2023).

Continuous surveillance of variants and vaccine adaptation

Since the emergence of the SARS-CoV-2 in Wuhan in 2019, the virus has considerably evolved and different VOC have been identified (Alpha, Beta, Gamma, Delta, and Omicron) (Quarleri et al., 2024; Tian et al., 2021). Variant emergence has been one of the most significant events of the pandemic. The Omicron variant was first identified in November 2021 and was responsible for a worldwide surge of infections. The first vaccines (including BNT162b2), elaborated on the sequence of the WT SARS-CoV-2, proved to be less effective against Omicron because of its considerable immune escape. The VE also tended to decrease over time after the first vaccination doses. Taken together, many countries decided to deploy a third vaccine dose to boost immunity (*i.e.,* homologous booster) (Favresse, Gillot, et al., 2023). Nevertheless, booster efficacy after one month was at most around 65% against symptomatic disease and decreased afterwards (Favresse, Gillot, et al., 2023). Subsequent variants (or subvariants) were then identified and were also responsible for infection waves (BA.2, BA.4/5, XBB.1.5, BA.286, JN.1). In fact, it is estimated that over 90% of individuals have likely been infected by at least one Omicron subvariant (Quarleri et al., 2024). At that time, the idea was to adapt the vaccine formulation to boost the protection against circulating variants. These adapted boosters (Pfizer-BioNTech and Moderna) containing two mRNA components of the virus (*i.e.,* one original strain and one in common between the BA.4 and BA.5 lineages) obtained an emergency use authorization in August 2022. In Europe, a bivalent booster containing a combination of WT and BA.1 was also used before approval of the WT-BA.4/5 by the EMA. We found that the administration of either the BA.1 or BA.4/5 adapted booster led to the same NAb levels (Favresse, Gillot, Cabo, et al., 2024).

On September 2023, the CDC recommended the update of vaccines to contain a component from the Omicron XBB.1.5 lineage. Compared to previous adapted

boosters containing a combination of WT and Omicron (BA.1 or BA.4/5), the last version of the vaccine does not contain the ancestral strain and is therefore monovalent (Link-Gelles et al., 2024). This decision was based on the increased immune escape of XBB.1.5 compared to BA.4/5 (*i.e.,* significant reduction of NAb levels) as reported by several reports, including ours (Favresse, Gillot, Cabo, et al., 2024; Favresse, Gillot, Closset, et al., 2024; Gayed et al., 2024).

The continuous monitoring of circulating variants is therefore an important task that should be continued in the future. Identification of variants that most escape the immunity system must be studied in detail to determine whether vaccines need to be adapted or not. An interesting case was that of BA.2.86, which was first detected in August 2023. It was anticipated that the BA.2.86 might further escape immunity due to the presence of more than 30 mutations in S in comparison with BA.2 and XBB.1.5 (Lasrado et al., 2023). However, this did not happen in real life (Q. Wang et al., 2024), as confirmed by studies having evaluated the neutralizing capacity of antibodies against this variant compared to XBB.1.5. Indeed, no significant reduction in NAb activity was observed (Favresse, Gillot, Cabo, et al., 2024; Willett et al., 2023). In late 2023, the BA.2.86 evolved in JN.1 and rapidly became dominant in several countries. JN.1 features an additional RBD mutation (L455S) and three other non-S mutations (Kaku et al., 2024; Yang et al., 2024). This variant was characterized by an increased immune escape due to significantly reduced NAb levels compared to XBB.1.5 and BA.2.86 (Favresse, Gillot, Cabo, et al., 2024; Kaku et al., 2024; Yang et al., 2024) and VE against COVID-19 was significantly reduced after the JN.1 became dominant in the USA (Shrestha et al., 2024).

Considering the above-mentioned elements, should we therefore adapt the vaccination strategies and move from monovalent XBB.1.5 to JN.1? This is not necessary. Indeed, several studies found that there is a cross-neutralizing activity of serum antibodies in individuals who developed XBB.1.5 BKI or in individuals who received the XBB.1.5 monovalent booster (Favresse, Gillot, Cabo, et al., 2024; Planas et al., 2024; Quarleri et al., 2024; Q. Wang et al., 2024). Accordingly, VE against JN.1 was not significantly different compared to XBB.1.5 (Link-Gelles et al., 2024).

The FDA anticipates that the composition of COVID-19 vaccines may need to be updated every year (FDA, 2023). Seasonal vaccination (between October and December) with the latest adapted vaccine (*i.e.,* monovalent XBB.1.5 from Pfizer-BioNTech) was associated with a VE against hospitalization of 70.7% and a VE against ICU admission of 73.3% (van Werkhoven et al., 2024). Continuous and active surveillance to guide vaccine strain selection, based on *in vitro* neutralizing studies and real-life studies evaluating VE, remains crucial. The possibility of using a vaccine that could act against both COVID-19 and influenza has been explored and could represent a valuable option to boost the seasonal immunity (Y. Wang et al., 2024).

Results of the two surveys of the European Federation of Clinical Chemistry and Laboratory Medicine Task Force Preparation of Labs for Emergencies published in 2023 showed that the number one threat that is more likely to disrupt again laboratory activities is thought to be related to epidemics or pandemics (Lippi, Cadamuro, et al., 2023a, 2023b). The continuous surveillance of new threats is mandatory (reference laboratories and continuous expert meetings) to limit the spread of new respiratory viruses in the future. The lessons learned from COVID-19, from its rapid first description in early 2020 in *The Lancet* (Chan et al., 2020), to the shortage of reagents and/or supplies, the release of *in vitro* assays without formal approval, as well as the release of vaccines at an unprecedented speed and the urgent creation of vaccination centers should serve in the future to be better prepared.

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