Using statistical modelling approaches to examine COVID-19 vaccine responses and determine temporal decay of antibodies associated with waning immunity

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December 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract – English

The COVID-19 pandemic, caused by SARS-CoV-2, sparked a global race to create vaccinations and treatments. A significant challenge in vaccine development is the rapid mutation of SARS-CoV-2. The emergence of the XBB.1.5 variant raised concerns about the effectiveness of existing vaccines. Monovalent vaccines target the ancestral strain, while bivalent vaccines target both the ancestral and specific variant strains. For example, the Pfizer-BioNTech bivalent vaccine targets the ancestral strain and the BA.4/5 Omicron subvariants. Evaluating the efficacy of bivalent compared to monovalent vaccines, and the effects of past infections in conferring additional protection against such variants is essential. This study is part of the Living Lab Seroprevalence Study, a prospective cohort analysis of the immune response to COVID-19 vaccines. A multivariate mixed-effects model was used to investigate the association between several predictors and covariates, including visit, vaccine type, recent infection, age, and sex, on ID50, a measure of neutralizing antibody induction. The model indicated that visit, specifically dose 4 ($p<0.01$), and recent infection ($p=0.014$) were the two most important predictors of ID50, or the inhibitory dilution of serum samples at which in vitro infection was reduced by 50%. ID50 levels against XBB.1.5 were 70% higher post-4^{th} dose compared to post-3^{rd} (95%CI [32%,120%]; $p<0.001$), 30% lower for monovalent 4^{th} dose compared to bivalent 4^{th} dose (not statistically significant (95%CI [-53%,5%]; $p=0.081$)), and 60% higher with recent infection (i.e., detectable anti-N-IgG) compared to undetectable anti-N-IgG 95%CI [11%,132%]; $p=0.014$). Analysis showed additional boosters elicit the best vaccine response against XBB.1.5, while recent infection enhanced this response. However, there was no significant advantage of the bivalent over the monovalent in neutralizing XBB.1.5. The nucleocapsid (N) protein, a structural protein inside SARS-CoV-2, is an important immune target. Understanding the dynamics of anti-N-IgG waning

can provide valuable insights into post-infection immunity. We investigated the kinetics of anti-N-IgG decay, addressing questions regarding waning immunity following recent infection using Living Lab subjects. Cox regression was used to estimate the half-life and time it takes for detectable anti-N-IgG to drop below the SCO cutoff, a ratio used to determine whether a sample is positive or negative. The estimated half-life of anti-N-IgG was shown to be approximately 86 days, with anti-N-IgG levels dropping below the 1.0 SCO cutoff on average at around 228 days, showing consistency with existing literature of a typical waning period of 6-8 months. The target for mRNA vaccine-induced immunity is the spike (S) protein, which triggers an immune response that makes antibodies and immune memory cells capable of recognizing and responding to the virus if encountered later on. Finally, we sought to gain insight about the overall antibody response to the vaccine. Living Lab antibody responses were assessed starting in 2021 before the administration of COVID mRNA vaccines and followed up approximately 28 days, 3 months, 6 months, and 12 months after second, third and fourth doses through serum sample collection. Graphical results show IgG-anti-spike and IgG-anti-RBD levels, as well as neutralizing antibody levels, were notably lower at 3 months and 6 months post-second dose compared to 28 days post-second dose. The most significant boost in antibody production is seen 28 days post-third dose, with antibody levels returning to similar levels observed after the second dose. Using statistical modelling, our analysis showed that additional vaccine doses significantly boost neutralizing antibody levels against XBB.1.5, with no statistically significant difference between monovalent and bivalent vaccines. We also assessed anti-N IgG waning and evaluated key patterns in the immune response to COVID-19 mRNA vaccines.

Abstrait – Français

La pandémie COVID-19, causée par SARS-CoV-2, a déclenché une course globale à la création de vaccins et traitements. Sa mutation rapide constitue un défi pour la mise au point vaccinale. L'émergence de la variante XBB.1.5 a suscité des inquiétudes quant à l'efficacité des vaccins existants. Les vaccins monovalents ciblent la souche ancestrale, tandis que les bivalents ciblent la souche ancestrale et la variante spécifique. Par exemple, le vaccin bivalent Pfizer-BioNTech cible la souche ancestrale et les sous-variantes BA.4/5 Omicron. Il est essentiel d'évaluer l'efficacité du vaccin bivalent comparée au monovalent et l'effet d'infection antérieure. Cette étude fait partie du Laboratoire Vivant, une analyse de cohorte prospective de la réponse immunitaire aux vaccins COVID-19. Un modèle multivarié à effets mixtes a été utilisé pour étudier l'association entre prédicteurs et covariables, y compris visite, type de vaccin, infection récente, âge et sexe, sur ID50, une mesure de l'induction d'anticorps-neutralisants. Le modèle indique que visite, notamment la 4e-dose ($p<0,01$), et infection récente ($p=0,014$) sont les prédicteurs les plus importants du ID50, ou la dilution inhibitrice des échantillons de sérum à laquelle l'infection in vitro est réduite de 50 %. Les niveaux d'ID50 contre XBB.1.5 étaient 70% plus élevés après la 4e-dose qu'après la 3e (IC95% [32%,120%]; $p<0,001$), 30% plus bas pour la 4e-dose monovalente que pour la 4e-dose bivalente (IC95% [-53%,5%]; $p=0,081$)), et 60% plus élevés en cas d'infection récente (IC95% [11%,132%]; $p=0,014$). Des rappels supplémentaires entraîne la meilleure réponse vaccinale contre XBB.1.5, tandis qu'infection récente renforce cette réponse. Cependant, le bivalent ne présente aucun avantage significatif concernant la neutralisation du XBB.1.5. La protéine de la nucléocapside (N) est une cible immunitaire importante. La compréhension de dynamique d'affaiblissement d'anti-N-IgG peut fournir des informations de valeur sur l'immunité post-infection. Nous avons étudié la cinétique de décroissance anti-N-IgG, en répondant aux

questions concernant l'immunité décroissante après une infection récente, en utilisant des sujets du Laboratoire Vivant. La régression de Cox a été utilisée pour estimer la demi-vie et le temps nécessaire pour que les anti-N-IgG détectables tombent en dessous du seuil SCO, un ratio utilisé pour déterminer si l'échantillon est positif ou négatif. La demi-vie estimée de l'anti-N-IgG était d'environ 86 jours, les niveaux d'anti-N-IgG tombant en dessous de 1,0 SCO en moyenne à 228 jours, une période d'affaiblissement de 6-8 mois cohérente avec la littérature existante. La cible immunitaire principale induite par le vaccin ARNm est la protéine spike (S), qui déclenche une réponse immunitaire produisant des anticorps et des cellules de mémoire immunitaire capables de reconnaître le virus et d'y répondre s'il est rencontré ultérieurement. Enfin, nous cherchons à mieux comprendre la réponse des anticorps au vaccin. Les réponses anticorps du Laboratoire Vivant ont été évaluées à partir de 2021 avant l'administration des vaccins ARNm COVID et suivies 28 jours, 3 mois, 6 mois et 12 mois après 2e, 3e et 4e-doses de vaccin par collecte d'échantillons de sérum. Les graphiques montrent que les taux d'IgG-anti-spike, d'IgG-anti-RBD et d'anticorps-neutralisants, étaient nettement plus faibles 3 et 6 mois après la 2e-dose que 28 jours après la 2e-dose. L'augmentation la plus significative d'anticorps est observée 28 jours après la 3e-dose, les taux d'anticorps revenant à des niveaux similaires à ceux observés après la 2e-dose. En utilisant la modélisation statistique, notre analyse a montré que des doses supplémentaires de vaccin augmentent de manière significative les niveaux d'anticorps neutralisants contre XBB.1.5, sans différence significative entre les vaccins monovalents et bivalents. Nous avons également évalué le déclin des IgG anti-N et les principales caractéristiques de la réponse immunitaire aux vaccins ARNm COVID-19.

Acknowledgements

I extend my heartfelt gratitude to my supervisor Dr. Bruce Mazer, whose guidance and support were the cornerstone of my journey throughout this master's program. His expertise and mentorship were pivotal in navigating this research.

My sincere thanks also go to Dr. Maria Plesa and Dr. Danbing Ke for their invaluable assistance in guiding me through the complexities of sample preparation and clinical research, as well as for helping me get started in the world of statistics.

I express deep appreciation to the Mazer lab team for the memories I will always cherish and for the opportunity to work with such a knowledgeable group of people.

Immense gratitude to my parents and sister for their unwavering love and encouragement. Their presence made this journey not just possible, but meaningful.

Lastly, thank you to all Living Lab participants whose time and willingness to participate in our study were indispensable. This work owes its completion to their contributions.

Contributions

Chapter II

The samples used come from The Living Lab Seroprevalence Study. I performed sample processing, which included PBMC extraction and serum and plasma aliquot preparation, alongside Dr. Maria Plesa. All clinical research procedures (e.g., contacting participants, setting blood draw appointments, preparation of plate shipment to the research team in Ottawa, etc.) were also done by Dr. Maria Plesa and me. I compiled the data, developed and interpreted the linear mixed effects model and its findings, and performed data analysis presented in this chapter, with advice on the modelling approach provided by Dr. Danbing Ke and Dr. Nisha Almeida. Measurements (e.g., SCO cutoff determination) and neutralizing antibody readings were determined and provided by Dr. Marc-Andre Langlois and his research team at the University of Ottawa. Dr. Mazer provided immunological feedback and editorial support.

Chapter III

The samples used for this chapter are also Living Lab study samples, encompassing similar procedures for sample processing and clinical research procedures as chapter 2, which I carried out with Dr. Maria Plesa. I compiled the data, developed the Cox regression model and performed the analysis for this chapter independently. Dr. Tanya Murphy, Dr. Xun Zhang, and Dr. Danbing Ke provided input and advice on the modelling approach, variable and sample selection. Measurements (e.g., SCO cutoff determination) and IgG readings were determined and provided by Dr. Marc-Andre Langlois and his research team at the University of Ottawa. Dr. Mazer provided immunological feedback and editorial support.

Chapter IV

Since the samples used for this chapter were Living Lab samples as well, I carried out similar sample processing and clinical research procedures with Dr. Maria Plesa as described for chapters 2 and 3. Specifically, I was involved in subject recruitment, data collection, sample preparation and preparation of the data spreadsheets. I generated personalized readings of the antibody response to COVID-19 vaccines and conceptual models to understand the various patterns in antibody response observed for all Living Lab participants. Measurements (e.g., SCO cutoff determination) and IgG readings were determined and provided by Dr. Marc-Andre Langlois and his research team at the University of Ottawa. The statistical analysis for the response to the first 3 vaccines was done by Dr. Nisha Almeida and Ian Schiller. Dr. Mazer provided immunological feedback and editorial support.

Chapter I: Literature review

COVID-19 is an infectious disease, affecting the upper respiratory tract and other body systems, caused by the SARS-CoV-2 coronavirus. It first emerged in Wuhan, China in December 2019 and quickly spread worldwide, with the World Health Organization (WHO) declaring it a global pandemic in March 2020. Symptoms associated with this illness develop 2-14 days after being infected, with the most common ones being fever, chills, sore throat, cough, and fatigue which can be accompanied by loss of taste or smell. Less common symptoms include sore eyes, abdominal pain, diarrhoea, nausea, chest pain, and appetite loss[1]. Individuals with certain underlying health conditions (diabetes, coronary artery disease, cancer or immune suppression) are at higher risk for severe COVID-19 outcomes and these comorbidities are strongly associated with increased fatality for older adults aged 65 and above[1]. SARS-CoV-2 transmission can occur through the nose, mouth or the eyes. Since it's an airborne virus, it spreads via small respiratory droplets that can affect those in close contact with an infected person who coughs or exhales[2]. The COVID-19 pandemic deeply affected international communities and global health, as well as individual daily life.

Each viral component of the SARS-CoV-2 virus plays an important role in its infectivity and interaction with host cells. Key components include the spike (S) protein and its receptor binding domain (RBD), nucleocapsid (N) protein, and envelope I protein[2]. The spike protein is composed of two subunits: S1 and S2. The S1 subunit is primarily responsible for binding to the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell surface. It contains the RBD, which is the part of the spike protein that directly interacts with the ACE2 receptor[3]. This helps the virus attach to and enter the host cell, making it a target in vaccine design. The S2 subunit, although less

variable than S1, drives the membrane fusion process. After S1 binds to ACE2, S2 undergoes structural changes that enable the fusion of the viral and host cell membranes, allowing viral entry virus to the host cell[3]. The nucleocapsid protein encapsulates the viral RNA genome, needed for viral replication and assembly. The less prominent envelope protein is involved in viral assembly and budding[2]. Viral infectivity and ability to evade the host immune response is determined mainly by the spike protein.

The spike protein is the most mutation-prone component of SARS-CoV-2. Many variants exhibiting different spike-related mutations emerged since the beginning of the pandemic and were classified into several groups: Alpha, Beta, Gamma, Delta, and Omicron. The Alpha variant, first identified in the UK in late 2020, and the Beta variant showed increased transmissibility and resistance to neutralizing antibodies[4]. The Gamma variant carries mutations N501Y and E484K within the spike RBD, a combination contributing to its ability to partially evade immune responses to earlier variants and spread more efficiently[4], while the Delta variant, first detected in India, demonstrated a greater ability to cause severe illness. The Omicron variant emerged in late 2021 and is characterized by a large number of spike mutations compared to other variant groups. Notably, over 30 mutations in the spike protein alone contribute to its substantial immune escape and high transmissibility[4]. Omicron has led to more breakthrough infections among those vaccinated than any other variant. The substantially higher number of mutations in Omicron distinguish it from its Alpha, Beta, Gamma, and Delta counterparts, highlighting its unique evolutionary trajectory. While there are many SARS-CoV-2 variants, as it is a rapidly-mutating virus, the second chapter of this thesis focuses on the XBB.1.5 Omicron variant, known for having a greater immune escape and increased transmissibility compared to earlier variants. XBB.1.5 has

demonstrated a higher capacity to evade neutralizing antibodies generated by previous infection or vaccination, making it a target for understanding vaccine and immune responses.

In an effort to curb the spread of SARS-CoV-2, many researchers around the world began working rapidly and extensively on vaccine development. Vaccines are preventative therapeutic treatments that help protect against infectious diseases by stimulating the immune system to recognize and fight specific pathogens. Using vaccination, the immune response is triggered by the viral antigens presented to the immune system in a controlled manner. As a result, numerous studies sought to understand the response to vaccines, particularly in the context of evolving SARS-CoV-2 variants and waning immunity.

The pandemic has highlighted the critical role vaccines play in managing infectious disease outbreaks. They are the most important countermeasure to protect against COVID-19. Since the pandemic, significant progress has been made in reducing severe disease outcomes and reducing the virus' spread. However, concern has emerged regarding the durability of vaccine-induced immunity and the effectiveness of different vaccines available today. Some of the more widely administered vaccines include the Pfizer-BioNTech (Comirnaty) and Moderna (Spikevax) mRNA vaccines, and the Oxford-AstraZeneca viral vector vaccine. mRNA vaccines use lipid nanoparticles to deliver and facilitate the fragile mRNA's entry into host cells. Lipid nanoparticles are small, spherical particles made of a mixture of lipids and other materials that allow it to pass through the cell membrane. The messenger RNA, a small piece of the virus' genetic material, instructs host cells to produce the spike protein found on the viral surface in a stabilized pre-fusion conformation[5]. The immune system then recognizes this protein as foreign and begins producing antibodies and immune cells against it. If the actual virus is encountered later on, the immune

system can mount a quicker response to prevent severe illness. mRNA vaccines were instrumental in controlling the spread of SARS-CoV-2. Their innovative design resulted in adequate protection against COVID-19. A multinational, placebo-controlled mRNA vaccine trial published in the New England Journal of Medicine recruited 21,720 participants who received the Pfizer-BioNTech vaccine while 21,728 participants received the placebo. Findings have shown that 8 participants were infected in the vaccinated group, whereas 162 participants were infected in the placebo group, demonstrating that the vaccine was 95% effective in preventing COVID-19 in this cohort. Among those infected, 10 were severely ill, 1 in the vaccinated group and 9 in the placebo group[6].

Many real-world studies highlight mRNA vaccines' positive effect on humoral and cellular immunity. The humoral immune response, which is mainly mediated by antibodies, is important for immune defense against COVID-19. B cells become activated after encountering the spike protein and differentiate into antibody-secreting plasma cells. The first antibody made after an infection is IgM, while IgG is the most abundantly found antibody in circulation and provides long-term protection. IgG can help prevent reinfection for a period of time, with levels often rising in response to an infection or vaccination. Since mRNA vaccines target the viral spike protein, S-specific and RBD-specific IgG rise after vaccination, while N-specific IgG rises after an infection, as well as anti-S-IgG. It is expected that those who contracted COVID-19 will mount a quicker response to vaccines compared to those who are naïve. Ebinger et al. observed that spike-specific IgG antibody levels elicited by a single Pfizer-BioNTech vaccine dose in those with previous SARS-CoV-2 infection were similar to levels observed after two doses of vaccine in individuals without previous infection[7].

The immune response involves a production of antibodies with different recognition sites and different functions. While IgG and IgM isotypes are important in the context of vaccination and infection, antibodies can be classified into non-neutralizing and neutralizing depending on their functions. Non-neutralizing antibodies contribute to the immune response by marking the virus for destruction or enhancing its clearance through mechanisms like antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis[8]. In contrast, neutralizing antibodies specifically target and bind the spike protein on the viral surface, preventing the virus from attaching and entering host cells, thereby blocking infection. Neutralizing antibodies directly prevent infection, while non-neutralizing antibodies support broader immune functions to help control and eliminate the virus. Researchers in Japan wanted to investigate whether sufficient neutralizing antibodies against Omicron were induced after 2 and 3 doses of the Pfizer-BioNTech mRNA vaccine. In a cohort of 82 participants, 28% and 6% had neutralizing antibodies against Omicron at 2 months and 7 months, respectively, after 2 mRNA vaccine doses. After receiving a booster vaccine, however, all participants acquired much higher neutralizing antibody levels irrespective of their age[9]. Although mRNA vaccines elicit an increased production of neutralizing antibodies needed to 'block' the virus, the Omicron subvariants, like BA.4/5, have been shown to possess a strong ability to evade neutralizing antibodies[10,11].

The mRNA vaccines also stimulate cellular immunity. In a study by Datwani et al., spike-specific T cell responses were compared after two and three mRNA vaccine doses. The third vaccine dose significantly boosted spike-specific $CD4^+$ and $CD8^+$ T cell frequencies compared to the second dose in both the older and younger groups, and these frequencies were not significantly different between both groups after either dose[12]. $CD4^+$ helper T cells aid in the activation and

differentiation of B cells into antibody-secreting plasma cells and present antigens to $CD8^+$ cytotoxic T cells, the latter being primarily responsible for directly killing infected cells.

Additional mutations have led to Omicron subvariants, including the BA, BQ and XBB sub-lineages. Specifically, BQ.1, BQ.1.1 and XBB.1.5 were responsible for over 50% of infections in the US as of February 2023[13]. Omicron exhibits increased transmissibility and the ability to evade neutralizing antibodies, which led to recommendations for those at risk to receive regular booster doses. The emergence of these new subvariants prompted recommendations for a booster, or third, dose, shown to confer greater protection against Omicron compared to two mRNA doses. One particular US study estimated vaccine effectiveness of two and three mRNA vaccines using regression models during the Omicron-dominant period. Their regression modelling concluded vaccine effectiveness (calculated as (1 − adjusted odds ratio) × 100%) to be 21% for two doses and 62% for three doses of an mRNA vaccine[14]. Three doses provided substantial protection when the Omicron variant became the main circulating variant in the US. A study by Tauzin et al. demonstrated that a third dose of mRNA vaccine did elicit spike recognition and neutralization against some Omicron subvariants, but not all subvariants tested even when accounting for previous infection[15]. Neutralization activity of plasma was measured using an ID50 parameter at 4 weeks and 4 months post vaccination. At 4 weeks after the third dose, previously infected (PI) individuals neutralized all tested spike variants more efficiently than the naïve, or no previous infection, group, while the breakthrough infection (BTI), or infection after vaccination, group exhibited an intermediate level of neutralizing antibodies. At 4 months after the third dose, no significant differences were observed between the BTI and PI groups. However, the naive group neutralized D614G spike, which is associated with the Delta variant, and Omicron subvariant

spikes less efficiently. Although neutralizing activity was higher in the BTI and PI groups compared to naive, the BA.4/5, BA.2.75, BA.4.6, and BQ.1.1 spikes were significantly less neutralized than the D614G and BA.2 spikes in all boosted groups[15].

Bivalent vaccines, which target both the ancestral stain and Omicron variants BA.4 and BA.5 or BA.1, include the Pfizer-BioNTech BA.4/5 and the Moderna Spikevax bivalent vaccines. When they were first created, bivalent vaccines were widely encouraged and the overall response towards the strains they target had been positive. However, bivalent boosters were not found to elicit superior immune responses compared to the monovalent ones. This will be discussed further in the second and fifth chapters. In a recent study, researchers used a parameter called $FFRNT_{50}$ to measure neutralization activity against different Omicron variants in their cohort. They define $FFRNT_{50}$ as the minimal serum dilution that suppressed 50% of fluorescent foci. The higher this parameter, the greater the neutralization provided by the BA.5 bivalent booster. At 14-32 days after the booster for those without prior infection, the BA.5 bivalent booster resulted in high $FFRNT_{50}$ against the BA.4/5 variant. However, the booster did not produce robust neutralization against emerging variants BA.2.75.2, BQ.1.1 nor XBB.1. For those with previous infection, the neutralizing antibody response elicited by the bivalent booster was enhanced against all variants tested. However, while emerging variants were less neutralized compared to BA.4/5, a prior infection did result in greater $FFRNT_{50}$ values against these variants compared to those without prior infection[16]. These studies highlight two important points: receiving additional doses may be necessary to better neutralize emerging variants and immunity acquired from previous infection provides protection. It has been documented that XBB.1.5, which comes from the same sub-lineage as XBB.1, exhibits greater immune escape and additional mutations compared to previous

Omicron variants. There were not many studies in the literature looking at the neutralizing antibody response conferred by current bivalent vaccines to XBB.1.5. Therefore, in chapter 2, we aimed to measure the vaccine response to XBB.1.5 and examine which booster vaccine, monovalent or bivalent, affects the neutralizing antibody response optimally using samples from our prospective cohort.

The 'Living Lab Seroprevalence Study' is a prospective cohort launched in July 2020 to evaluate prevalence of COVID-19 infection in a group of volunteers at the RI-MUHC. We recruited more than 300 physicians, nurses, and other clinical and research staff with the objective of assessing immune responses to vaccination. Blood sample collection began in December 2020 and continued at defined intervals. We evaluated antibody responses at the following time points: pre-vaccine dose; approximately 28 days, 3 months, 6 months, and 12 months post-vaccine dose[17]. The majority of the participants provided samples after the first vaccine dose (n = 272) and a smaller number provided samples before the first dose (n = 50). Results and graphical analyses generated from the Living Lab cohort will be discussed in detail in chapter 4.

Viral vector vaccines employ a different approach than mRNA vaccines; for example, the Astra-Zeneca vaccine used a weakened chimpanzee adenovirus as a vector. It was modified to carry DNA encoding the COVID-19 spike protein and when administered, the vector entered host cells and delivered the genetic material. Once inside, the DNA was transcribed into mRNA, which is then translated into the spike protein, triggering an immune response[5]. The most widely administered viral vector vaccine during the pandemic was the Oxford-AstraZeneca vaccine. Following its rollout, healthy vaccine recipients began experiencing thrombotic events coupled

with thrombocytopenia, in a term later coined vaccine-induced immune thrombotic thrombocytopenia (VITT). This condition was found to be associated with high levels of IgG directed against platelet factor 4 (PF4), a platelet chemokine[18]. PF4 can bind to heparin and inhibit its anticoagulant effects, thereby promoting blood clotting. A study published in the New England Journal of Medicine used a standard ELISA assay to detect PF4–heparin antibodies in patients who experienced severe thrombotic events 5-16 days after receiving the ChAdOx1 nCov-19 (Oxford-AstraZeneca) vaccine, as well as a PF4-enhanced platelet-activation test to detect platelet-activating antibodies. None of the patients were exposed to heparin prior to symptom onset, and all who tested positive for antibodies against PF4–heparin tested positive on the platelet-activation assay[19]. Another group of researchers reported on five patients who presented with venous thrombosis and thrombocytopenia 7-10 days after receiving their first ChAdOx1 nCoV-19 dose. All patients had high levels of antibodies to PF4–heparin complexes, although they were not previously exposed to heparin either[20]. The DNA from the adenovirus used in the viral vector vaccine could be a potential trigger of these antibodies. After a multitude of VITT studies came out, viral vector vaccines against COVID-19 were deemed risky, with many choosing not to receive them.

Antibodies naturally undergo degradation over time, leading to reduced levels in the bloodstream, as they get broken down and cleared from circulation. The gradual decline in IgG can impact the duration of the immune response, a key factor for waning immunity. This phenomenon occurs when protective effects conferred by previous vaccinations or infections diminish over time. The immune system reacts differently to vaccines across individuals, and one must consider age, comorbidities, and lifestyle when trying to assess waning as these play an important role in

determining vaccine response. As a result, establishing an overall mechanism for IgG decay becomes harder. For instance, researchers in England conducted a study on staff (median age 48 years) and older residents (median age 84 years) in an assisted living facility. They observed a significant boost in spike-specific antibody production among staff and residents after a third vaccination. Despite this observation, antibody titers in older residents without a prior infection were 42% lower than staff and the difference was statistically significant[21]. One cohort involving healthcare workers in Spain determined how long anti-S IgG persist for after a third dose of mRNA vaccine. They established two groups: with and without prior infection. They found that all healthcare workers were anti-S IgG positive 8 months after receiving the booster dose of the vaccine[22]. A US multistate, longitudinal cohort of almost 13,000 adults assessed the duration of anti-spike antibodies after COVID-19 and found that anti-spike antibodies persisted for up to 284 days, almost 9.5 months, with only 2.4% having undetectable levels[23]. Despite the significant advancements made in understanding the temporal dynamics of waning immunity, there remain gaps in the literature regarding the precise mechanisms of waning and timelines for different vaccines and specific antibodies involved. Further research is required to elucidate how quickly immunity wanes in various populations and in different cases (i.e. comorbidities, immunosuppression, etc.), while considering the variation between antibodies that target different SARS-CoV-2 viral components. Since the N protein encapsulates the viral RNA genome, which is released into the host cell cytoplasm upon infection, antibodies made against the N protein serve as markers for past infection. Many questions remain about the persistence of these N-specific IgG and the duration of immunity following an infection, which has been shown to be very variable. Waning immunity of anti-N IgG will be addressed in chapters 3 and 5. In chapter 3, we used

statistical modelling to model the temporal decay of anti-N IgG and determine the average time to waning for the Living Lab cohort.

In the context of evolving variants, monitoring antibodies that target more stable viral components may be necessary to maintain effective protection and ensure sustained immunity. The SARS-CoV-2 N protein is predominantly found in the viral cytoplasm, making it less exposed to the external environment compared to the S protein found on the viral surface. This allows for the N protein to be generally more conserved across different viral variants[24]. The structure of the S protein is more complex and undergoes frequent mutations, especially in functional areas like the RBD, affecting its stability but may allow the virus to escape detection and infect more efficiently. Being exposed to the external environment renders it susceptible to degradation under conditions like temperature and pH changes. The N protein is thus, generally less affected by such environmental factors.

Researchers are increasingly exploring the N protein as a potential target for vaccine development[24]. By developing N protein-targeted vaccines less susceptible to the antigenic drift observed with S protein-targeted vaccines, the durability and efficacy of vaccines against diverse SARS-CoV-2 variants may improve. One approach focuses on recombinant nucleocapsid protein vaccines, which use genetically engineered versions of the N protein expressed in *E. coli.* A group of researchers recombinantly expressed the N protein in E. coli and found that the BALB/c mice they immunized elicited high levels of N-specific IgG and IgM, as well as IFN-γ[25]. The Convacell vaccine is the only N-based vaccine currently licensed for human use in Russia, after researchers observed an N-specific $CD4^+$ and $CD8^+$ T cell response in marmoset monkeys and increased anti-

N IgG associated with lower viral proliferation in Syrian hamsters following immunization[26]. Taking note of these emerging studies, an interesting question arises regarding how long these vaccine-induced N-specific IgG antibodies can last after immunization, which has yet to be answered.

The COVID-19 pandemic, caused by the spread of the SARS-CoV-2 virus, emphasized the importance of vaccine therapies to fight a rapidly mutating virus that overwhelmed healthcare systems worldwide. COVID-19 mRNA vaccines were successful in providing robust protection against COVID-19, reducing the risk of hospitalization and severe illness. However, their effectiveness was challenged by the rapid emergence of new variants, with the Omicron variant exhibiting a substantially higher number of mutations compared to other variants. Omicron subvariants, namely XBB.1.5, were found to evade vaccine-induced responses. Although bivalent vaccines were developed to counter the threats posed by emerging variants, they were ultimately shown to be less effective than anticipated. While hybrid immunity, which is acquired from both a previous infection and vaccination, offers a broader immune response to COVID-19 compared to either type of immunity alone, immunity still wanes over time. Anti-N IgG, antibodies made against the N protein, serve as a marker for previous infection. These antibodies can persist for varying periods of time post-infection, with levels gradually declining over time. Researchers continue to investigate how long anti-N IgG remains detectable in sera and their potential role in assessing waning immunity. To address the challenges surrounding variant-induced immune escape, some researchers are currently exploring the potential of N protein-targeted vaccines.

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Introduction to Chapter II

The rapid evolution of SARS-CoV-2, marked by the emergence of variants like XBB.1.5, presents ongoing challenges for vaccine development and public health responses. In the following chapter, we evaluate the effectiveness of the BA.4/BA.5 bivalent vaccine against XBB.1.5 and examine the different predictor variables that influence the induction of neutralizing antibodies in forty-four Living Lab participants with varying vaccine types and infection statuses. We used a parameter called ID_{50}, which represents the dilution of serum at which neutralization is reduced by half. The higher the ID_{50}, the higher the overall neutralization. It was found that nearly all participants produced neutralizing antibodies against XBB.1.5, with the fourth dose yielding significantly higher antibody levels than the third dose. Additionally, no significant advantage was observed between bivalent and monovalent vaccines in inducing these antibodies, though a recent infection was associated with higher neutralizing antibody levels. The analysis and findings from chapter 2 have been published in the *Journal of Allergy and Clinical Immunology*.[5]

Chapter II: Booster Doses Of COVID-19 Vaccine Enhance Neutralization Efficiency Against XBB.1.5

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Acknowledgements: This study was performed through funding by the McGill University Health Centre Foundation.

Conflicts of Interest: The authors report no conflicts of interest for this study.

The swift emergence of the XBB.1.5 variant highlights the rapid mutation dynamics of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Renewed urgency led to development of bivalent vaccines to counter the potential threat of XBB.1.5. Considering that most individuals received earlier versions of coronavirus disease 2019 (COVID-19) vaccine that contained only the original Wuhan SARS-CoV-2 strain spike antigen, the effectiveness of those versions in protecting against XBB.1.5 remains uncertain. We assessed whether available booster vaccines induce neutralizing antibodies against XBB.1.5 and the Wuhan wild-type strain and whether bivalent vaccines (BVs) which also contain the BA.4/5 strain spike are superior to earlier monovalent vaccines (MVs) in inducing XBB.1.5-neutralizing antibodies.

Our prospective cohort study included 44 participants from the Living Lab Seroprevalence Study (Almeida et al., 2023, *Frontiers in Immunology*). Matched serum samples were obtained 28 days after the third dose and 28 days after the fourth dose. We used surrogate neutralization ELISAs[1] that had been optimized for compatibility with a Hamilton Robotic liquid handler. Plates were coated with XBB.1.5 or Wuhan spike proteins. We calculated the median inhibitory dilution [ID_{50}], utilizing a 5-point sample titration and a 3-parameter log-logarithmic regression to determine the reciprocal dilution factor needed for inhibiting 50% of angiotensin-converting enzyme 2 (ACE2) antigen binding.

A multivariable mixed-effects model interrogating age, sex, vaccine type (MV vs BV), blood sample procurement after the third and fourth doses ("visits"), and recent infection (defined as positive if the anti–N-IgG signal-to-cutoff ratio was greater than 1.0) was used to examine associations between different predictor variables and the log-transformed outcome variable

ID_{50} for XBB.1.5 and Wuhan. Sensitivity analyses were conducted using linear regression for model validation. The statistical modeling packages lme4 and lm within R, version 4.2.3, were used for model fitting.

Almost all of the subjects produced neutralizing antibodies against XBB.1.5 (Fig 1). Multivariable mixed-effects modeling indicated that the ID_{50} levels against XBB.1.5 were 70% higher after the fourth dose than after the third dose (95% CI = 32%-120% [$P < .001$]). Participants who received a fourth dose of MV had ID_{50} levels against XBB.1.5 that were 30% lower than those who received a fourth dose of BV, which was not a statistically significant difference (95% CI = –53% to 5% [$P < .081$]). The ID_{50} for sera just before the fourth dose were significantly below the levels achieved 28 days after dose 3 ($P < .003$). Moreover, participants with recent infection (i.e., detectable anti–N-IgG) had ID_{50} levels 60% higher (95% CI = 11%-132% [$P = .014$]) than those of participants without detectable anti–N-IgG. Similar results were obtained for the Wuhan ancestral strain. Figures depicting the neutralizing antibody response to the Wuhan ancestral strain are presented in the Appendix, along with the descriptive characteristics of this study.

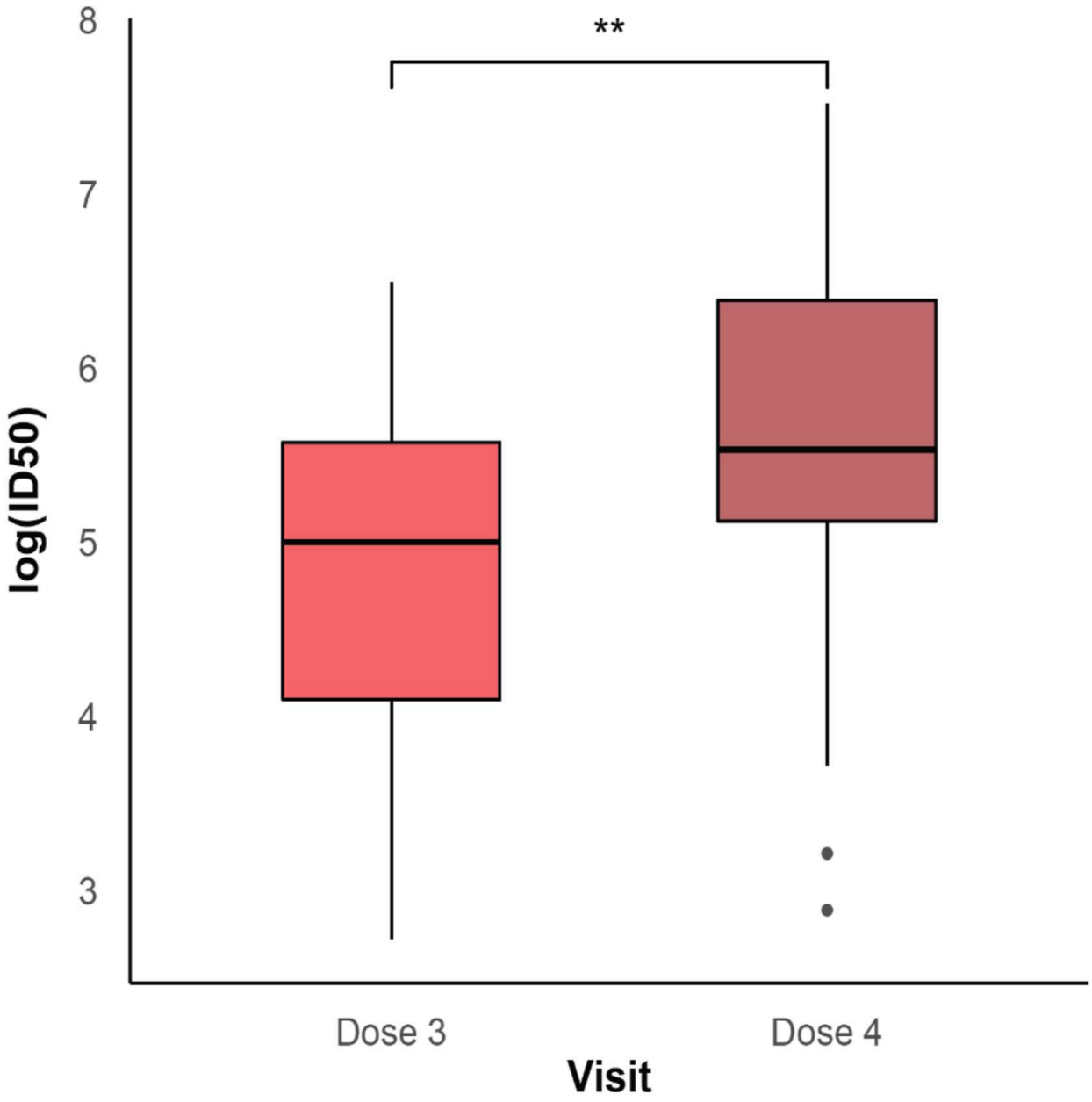

Fig 1 COVID-19 vaccine neutralization efficiency against XBB.1.5 measured after the third and fourth vaccine doses. A fourth vaccine dose of either the MV or BV vaccine is associated with higher ID_{50} values, thus inducing a greater number of neutralizing antibodies against XBB.1.5 than a third dose. **$P < .01$.

Our results confirm that COVID-19 boosters, even those not targeting XBB.1.5, induce neutralizing antibodies against this variant. This may be a positive consequence of epitope spreading, namely, the development of an immune response to epitopes distinct from and non–cross-reactive with previously encountered pathogens,[2] whereby the immune system recognizes similar epitopes on different viral variants. Immune responses generated against ancestral viral strains may cross-react with new variants, conferring partial protection. Additionally, we observed no statistical advantage of the BV over the MV. This may be due to immune imprinting, when memory B-cell responses expand following exposure to related viral variants.[3] Hojjat Jodaylami et al showed that vaccination and confirmed infection enhance antibody levels and improve cross-reactive inhibition of variants.[4] Despite more than 30% of subjects having a history of COVID-19 at blood procurement after the fourth dose, only those with detectable anti–N-IgG had augmented responses to XBB1.5, suggesting that more recent infection may contribute to production of neutralizing antibodies. Considering that 20% to 54% of Americans have received 3 doses of COVID-19 vaccines, our findings show that 4 doses elicit greater immune responses. Upcoming boosters target XBB1.5, and as BA2.63 is emerging, our data suggest that receiving a booster, regardless of type, can provide protection against severe COVID-19 outcomes.

Disclosure statement

Supported by the McGill University Health Centre Foundation.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

We thank the Living Laboratory Serology Study research team (Ian Schiller, Dr Danbing Ke, Dr Maria Plesa, Maria Bazan, Dr Corey Arnold, and Yannick Galipeau) for their invaluable contribution.

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Introduction to Chapter III

There are several important findings that should be highlighted from chapter 2 concerning different COVID-19 vaccines against XBB.1.5. First, although additional booster doses are essential for enhancing the immune response, there was no statistically significant difference between the BA4/BA5 bivalent and monovalent vaccines in their ability to produce neutralizing antibodies against the XBB1.5 variant constructs in our assays. This underscores the complexity of vaccine immune responses and predictions of protection against severe disease in the face of rapidly evolving viral variants, which should lead to the continuous assessment of vaccine strategies.

Second, the effectiveness of vaccines, as well as protection against viral variants, are not solely dependent on the vaccine type administered, but also on the underlying dynamics of the immune response over time; thus, we need to equally consider the broader context of individual infectious exposures and their immune response. Only when anti-N IgG was detectable did it influence the response to the booster vaccine, as discussed in chapter 2. Consequently, an essential aspect of COVID-19 studies is brought forth: the waning of immunity following an infection. While the preceding chapter addressed the immediate vaccine response to variants such as XBB.1.5, the virus and its variants are constantly mutating and dynamic, which requires a comprehensive understanding of vaccine efficacy and evaluation of how the immune response evolves over time. The N protein is the most abundantly expressed protein[1] in SARS-CoV-2-infected cells, and anti-N IgG antibodies are produced in response to an infection. They are then detectable in the sera of

infected individuals, serving as a reliable indicator of a recent or ongoing infection. Looking at anti-N IgG waning can reveal additional subtleties related to the maintenance of protection.

In the following chapter, we study a Living Lab subset of 34 participants from their first positive sample, where anti-N IgG > 1.0 SCO, until their first negative samples post infection or last sample provided for the study. A Cox regression model will be employed to investigate the temporal decay of anti-N-IgG levels and provide valuable information on how immunity evolves post-infection.

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Chapter III: Temporal decay modelling of anti-N IgG antibodies

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Acknowledgements: This study was performed through funding by the McGill University Health Centre Foundation.

Conflicts of Interest: The authors report no conflicts of interest for this study.

Background

Anti-N IgG antibodies are produced against SARS-CoV-2's nucleocapsid (N) proteins in response to infections. When the virus infects host cells, the N protein gets released into the host cell cytoplasm. When infected cells die, the N protein gets released into circulation, allowing for IgG antibodies to be made against this viral component[1]. Therefore, the production of anti-N IgG initially peaks at high levels subsequent to an individual becoming infected. Through the natural process of antibody decay, anti-N antibodies get degraded and eliminated from the body[2]. The amount of time needed for half of the antibody levels to degrade is known as the half-life, and for anti-N IgG, determining the half-life will allow for a better understanding of how long an immune response against the internal components of the virus persists post-infection.

Several studies have suggested that anti-RBD and anti-S IgG decay at a slower rate than anti-N IgG[2,3], as anti-S and anti-RBD IgG can be protective for a prolonged period of time. This could be partially attributed to anti-S IgG having a higher peak level compared to anti-N IgG, which may contribute to their longer persistence[2]. In contrast, anti-N IgG antibodies primarily serve as markers of previous infection, as they are unlikely to provide direct protection against reinfection. Although the N protein is abundantly expressed during infection, antibodies produced against it are not capable of neutralizing SARS-CoV-2[4]. The temporal characteristics of anti-N IgG decay, such as how quickly these antibodies fall below a detection limit cutoff (such as SCO in our studies[5]) and how this correlates with long-term immunity, remain less characterized in the literature. We attempt to answer these questions for participants of our Living Lab Seroprevalence Study[6] cohort using Cox regression modelling. SCO, or signal cutoff, is a ratio used for determining whether a sample has a positive or negative result. The SCO for this cohort was

established on > 500 pre-pandemic samples, enabling all cutoffs to be set at 1.00 for all conditions and antigens tested. Samples with signals above 1.0 SCO are considered positive for the presence of nucleocapsid-specific IgG, while samples with signals below 1.0 SCO are considered negative.

Methods

We studied a subset of 34 participants who had at least one positive anti-N IgG sample (i.e. an infection) throughout the study period to examine the waning of anti-N IgG. Living Lab participants provided serum samples at defined timepoints: 28 days, 3 months, 6 months, and 12 months after their first dose of vaccine against SARS-CoV2. Anti-N IgG responses were assessed by chemiluminescent ELISA[4]. Fluorescent detection above 1.0 SCO was considered positive for anti-N IgG, while values below this cutoff were considered negative. For our analysis, we used the continuous SCO values, allowing us to track the gradual decline of anti-N and measure average time to waning. We evaluated subjects with positive anti-N IgG from the time of their first positive sample until their first negative samples, or last sample provided for the study, and assessed when anti-N antibodies were no longer detectable (i.e., dropped below the SCO cutoff). If the last sample provided by the subject was positive for anti-N IgG, they were excluded from the analysis, as we would not be able to measure waning of anti-N IgG.

A Cox regression model is generally used to determine the relationship between the time it takes for an event to occur and the specific predictor variables that are being studied. We, therefore, employed Cox regression modelling to determine the half-life and average time to waning of anti-N IgG antibodies following an infection. The outcome of interest was when a subject's samples went from detectable Anti-N IgG (>1.0 SCO) to undetectable (<1.0 SCO) for each participant. We

were interested in assessing the impact of the predictor 'days post infection' and covariates, such as age and sex, simultaneously using the regression model. The predictor variable 'days post infection' was defined relative to the participant's first positive sample, which we designated as day 0. Since the exact timing of infection is unknown, the first positive sample serves as a reference point to measure changes in anti-N IgG over time. Subsequent samples were collected at intervals following day 0, and each sample was classified based on the number of days post-day 0. If a participant's first provided sample was positive, the analysis still proceeded as described, using their first positive sample as the baseline for measuring subsequent anti-N IgG changes. The model also accounted for repeated measures within participants.

A second objective was to quantify the daily decrease in anti-N IgG levels over time for our cohort. Because our data were non-linear and not normally distributed, log-transformation was utilized. Using the slope derived from log-transformed anti-N IgG, we determined the number of units by which anti-N IgG decreased per day. We used the statistical modelling packages Survival, Survminer and lm within R, version 4.2.3, for model fitting. A Shapiro-Wilk normality test was done to confirm that our data were not normally distributed before proceeding with a log-transformation. According to the p-value of 3.158e-09, the test indicates the data were not normally distributed. By log-transforming our initial non-linear and non-normal data, a linear model could be used to determine the relationship between log anti-N IgG and predictor variable 'days post infection'. The linear model also generates a value for the slope, which quantifies the daily drop in anti-N IgG for participants of the Living Lab.

Results

Results from the Cox regression model reveal an estimated half-life (median survival time) for anti-N IgG of approximately 86 days, and an average time to waning, when anti-N IgG levels drop below the 1.0 SCO cutoff on average, of 227.64 (~228) days, which is roughly 7.5 months. Our analysis demonstrates a statistically significant negative relationship between 'days post infection' and anti-N IgG levels ($P < .001$), indicating a gradual decrease in anti-N IgG over time. The slope derived from log-transformed anti-N IgG was found to be -0.0033, suggesting anti-N IgG levels decrease approximately 0.0033 SCO units per day. Figure 2 depicts a time-series plot, which aids in visualizing how anti-N IgG changes over time. The negative trend between anti-N IgG and 'days post infection' is clearly represented in the longitudinal plot. Moreover, by plotting the individual trajectories of each participant, the overall antibody level decline over time is clearly shown. Re-exposure can also be observed in a limited number of participant trajectories, but an eventual decline in anti-N IgG was consistent for all subjects. The implications of the model's findings are discussed in chapter 5.

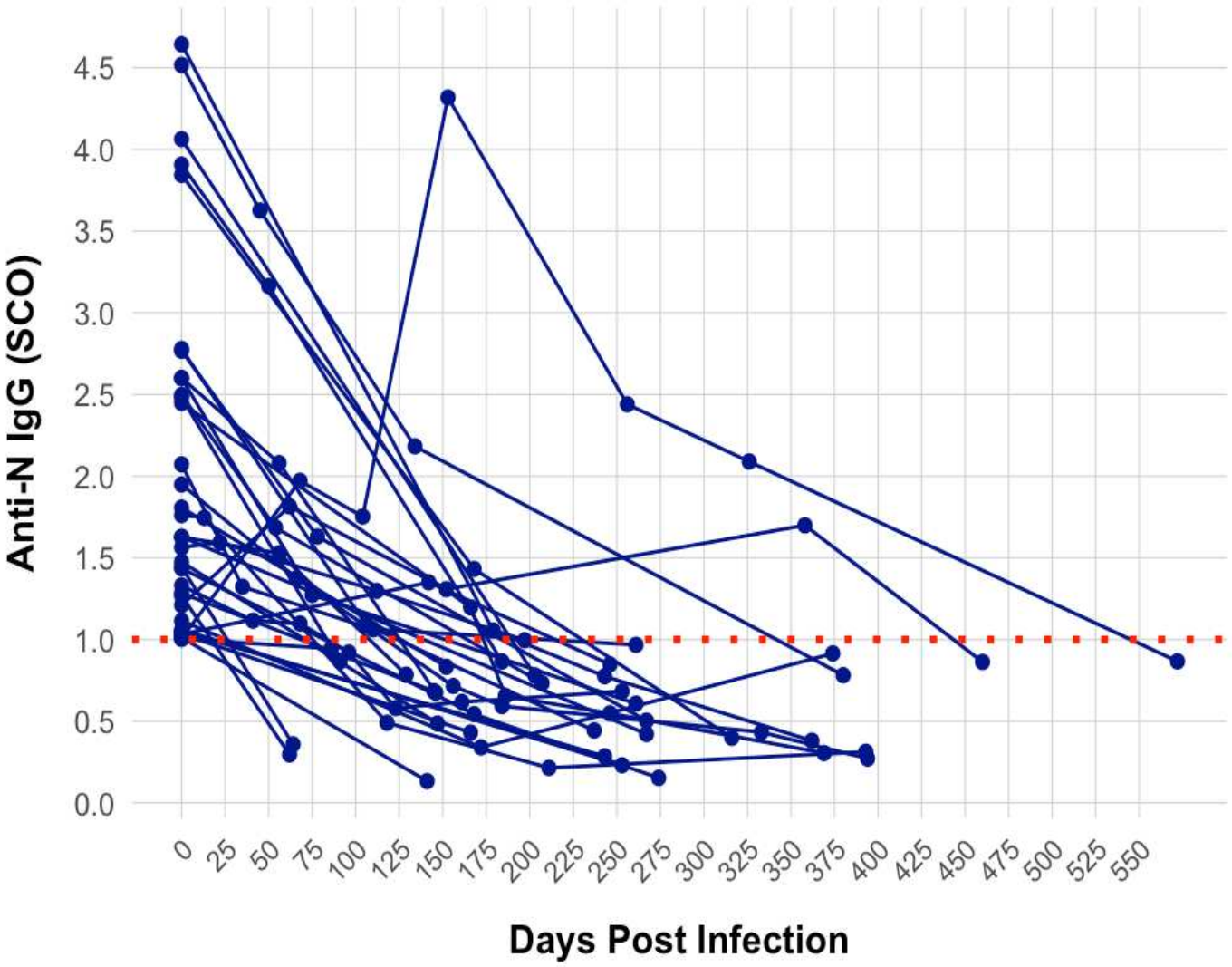

Fig 2 Longitudinal time-series plot depicting the negative relationship between anti-N IgG levels and days post infection. As days post infection increase, anti-N IgG (SCO) levels decrease, highlighting waning immunity (i.e. anti-N IgG does not persist at high levels indefinitely).

For a Cox survival regression, data are considered "censored" when a subject has not experienced the outcome event during the study period. Usually, Kaplan-Meier curves help visualize distributions of different groups in the survival analysis (those who experienced the outcome event versus those who did not). However, we do not have censored data because all participants in the cohort experienced the outcome event, which is when anti-N IgG drops below 1.0 SCO. The Kaplan-Meier curve generated from our data would, therefore, be a null curve. Instead, the survival analysis focused on the timing of the outcome event rather than censoring.

A limitation of our analysis is the inability to determine the exact timing of infection for each participant. The precise timing of infection relative to the first positive sample for anti-N IgG remains uncertain. While this introduces some variability in the estimation of day 0, the assay used to measure anti-N IgG in sera is reliable in providing accurate continuous values for anti-N IgG (SCO) at the time of sample procurement. Therefore, estimates for day 0, defined as the first positive sample, provided a dependable reference for tracking changes in anti-N IgG levels.

An interesting observation is highlighted in figure 2: anti-N IgG levels appear to wane at the same rate, regardless of the height of the initial level. Future directions will involve incorporating continuous anti-N IgG levels as a predictor in the Cox regression model. Specifically, we will test whether the rate of antibody waning (i.e., time to fall below the SCO cutoff) differs based on initial anti-N IgG levels by including an interaction term between 'anti-N IgG' and 'days post infection'. This will determine if the slope of the decay varies for participants with higher versus lower initial antibody levels. If the interaction term is significant, it means the rate of decline differs for the two groups, suggesting that initial antibody levels influence the dynamics of antibody decay. However,

if this interaction is not significant, the rate of decline would be similar for both groups, indicating that initial antibody levels do not significantly affect the temporal dynamics of waning immunity. Additionally, we will explore non-linear effects of anti-N IgG using natural splines to model potential non-linear relationships between baseline antibody levels and rate of waning.

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Introduction to Chapter IV

As the half-life is revealed to be 86 days with an average time to waning of around 228 days, temporal decay modelling highlights the relatively short-lived nature of anti-N IgG compared to anti-S IgG, which can persist up to 10 months after infection[1]. The duration of IgG antibodies varies depending on the virus. For instance, measles-specific IgG antibodies peak approximately two weeks after rash onset and can persist for life in an unvaccinated individual following a measles infection[2]. In contrast, antibodies targeting the influenza virus show a pattern more akin to anti-N IgG. The influenza nucleoprotein (NP) is essential for viral replication and the anti-NP antibody response can last for up to one year, for so influenza vaccines need to be updated annually[3].

The gradual decline in these antibodies is reflected in the data, with a focus on the variability in how long anti-N IgG remains detectable following an infection. The decline of these antibodies necessitates a focus on longer-lasting protective measures. Unlike anti-N IgG, anti-RBD and anti-S IgG persist for an extended period of time, thus offering a more sustained immune defense. One particular study has shown that both IgG and neutralizing antibodies can be correlates of protection against SARS-CoV-2 infection following Pfizer–BioNTech vaccination[4]. Their cohort consisted of 1,461 subjects who received one or two doses of the Pfizer–BioNTech vaccine and had valid baseline IgG and neutralizing antibody results. They found that higher IgG antibody concentrations and neutralizing antibody titres were significantly associated with a reduced probability of disease severity. Specifically, IgG concentrations higher than 500 BAU/mL correlated with a reduced probability of infection and moderate disease severity. If neutralizing antibody titres were greater

than 1024, this correlated with an 8% probability of infection and a 2% probability of moderate disease severity[4].

Vaccines are meant to elicit a lasting and robust antibody response to these key viral components. The Living Lab cohort tracked antibody responses after vaccination and provided an opportunity to evaluate how longer-lasting antibodies, like anti-S and anti-RBD IgG, compare with the short-lived anti-N IgG. Detailed graphical analyses for each individual participant in the cohort were generated to observe the varying patterns of vaccine-induced anti-S and anti-RBD IgG, as will be discussed in chapter 4.

The graphical representations generated reveal diverse trends and patterns among participants and in the overall cohort. A comprehensive view of the immune landscape can be gained by integrating our graphical analyses with our understanding of anti-N IgG decay. It is important to focus on vaccines that promote sustained antibody responses, as chapters 2 and 3 emphasize the limitations of relying solely on anti-N IgG for assessing long-term and durable protection. These crucial observations and insights can guide future vaccination strategies aimed at maintaining adequate protection against SARS-CoV-2.

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Chapter IV: Assessing variability in immune responses to COVID-19 vaccines following serial booster vaccinations

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Acknowledgements: This study was performed through funding by the McGill University Health Centre Foundation.

Conflicts of Interest: The authors report no conflicts of interest for this study.

Background

After the first reported case of SARS-CoV-2 infection in December 2019, COVID-19 rapidly spread globally, with the World Health Organization (WHO) declaring a global pandemic in March 2020. As a result, the international community began to prioritize vaccine development, which has been successful and effective in managing COVID-19. Millions worldwide safely received the vaccine, while severe complications associated with COVID-19, such as hospitalizations and death, decreased significantly relative to the number of people infected[1]. For our study, we focused on mRNA vaccines, which use synthetic SARS-CoV-2 mRNA to instruct cells to make antibodies that target the spike protein, enabling the immune system to respond efficiently to the virus if encountered again.

Methods

Participants of the 'Living Lab Seroprevalence Study[2] provided serum samples via either venipuncture or self-collection with cards for dried blood spots (DBS), which could be performed at home and mailed to the laboratory. For those opting for venipuncture, we collected 10 ml of venous blood in clot activator red-capped blood collection tubes. We centrifuged blood samples twice at 1200 rpm for 12 minutes, collected serum from these samples and stored them in aliquots at -80°C, until processing. Those opting for DBS were provided with home kits that contained ethanol swabs, lancets and filter paper. We included detailed procedure instructions and participants could mail or deliver the sample to our study center. We stored cards in desiccation boxes at room temperature until processing[2].

We evaluated antibody responses at the following time points: pre-vaccine dose; approximately 28 days, 3 months, 6 months, and 12 months post-vaccine dose. We then assessed S-specific and RBD-specific IgG, N-specific IgG, as well as neutralizing antibodies, based upon and optimized from assays described by Colwill and colleagues[3]. Automated chemiluminescent enzyme-linked immunosorbent assays (ELISA) were performed at the University of Ottawa, Faculty of Medicine, using Hamilton MicroLab Star Robotic Liquid Handlers and a 405 TS/LS LHC2 plate washer (Biotek Instruments)[3].

The immune response to vaccines can differ significantly among individuals. The main objective of the Living Lab study was to determine whether shorter or longer intervals between vaccine doses resulted in higher antibody levels (published in Almeida et al., 2024)[2]. Precisely, the main objective was to investigate the impact of delaying the second dose on antibody responses to COVID-19 mRNA vaccines. In collecting this data, we generated individual anti-S and anti-RBD curves for each participant to observe and analyze the variability in their vaccine responses.

Results

Using multivariable statistical modelling, we show that anti-S IgG was 31% higher (95% CI = 12%-53%) and anti-RBD IgG was 37% higher (95% CI = 14%-65%) in the long (> 89 days) versus short (≤ 89 days) interval participants, across all time points, indicating that extending the dose interval beyond 89 days (approximately 3 months) provides stronger antibody responses than intervals less than 89 days. As expected for most participants, IgG anti-spike and anti-RBD antibody levels peaked at 28 days post second dose and decreased significantly over the following 6 months. Analysis shows IgG anti-spike and anti-RBD were notably lower at 3 months and 6

months post second dose in comparison to 28 days post second dose. The most significant boost in antibody production is seen after the third dose, which is necessary to maintain protective anti-S and anti-RBD IgG levels. The third dose led to greater anti-S and anti-RBD IgG production compared to the second dose. Similar patterns were observed for the fourth, fifth, and sixth doses. Graphs were generated to represent these results, namely for IgG anti-spike and anti-RBD, for each Living Lab cohort participant. Some representative graphs are shown, as follows.

We observed that sex, age and previous infection were significant factors influencing antibody responses. Female participants had higher antibody levels compared to males, as mean anti-S IgG was 25% higher, anti-RBD IgG was 37% higher, and neutralizing antibody levels were 52% higher for females[2]. Age had a negative correlation with antibody levels, as every 10-year increase in age was associated with a 10% decrease in anti-S IgG and a 16% decrease in anti-RBD IgG[2]. Finally, those who were previously infected exhibited 53% higher anti-S IgG, 66% higher anti-RBD, and 87% higher neutralizing antibody levels than those without a previous infection[2]. Graphs depicting the participant results, as well as the descriptive characteristics for the cohort, can be found in the published paper[2]. As a contributor in this project, I was involved in participant recruitment, data collection and organization, and sample preparation, but was not the primary person responsible for analysis of the overall project. However, my role included collation of all data and generation of individual vaccine response graphs for all participants.

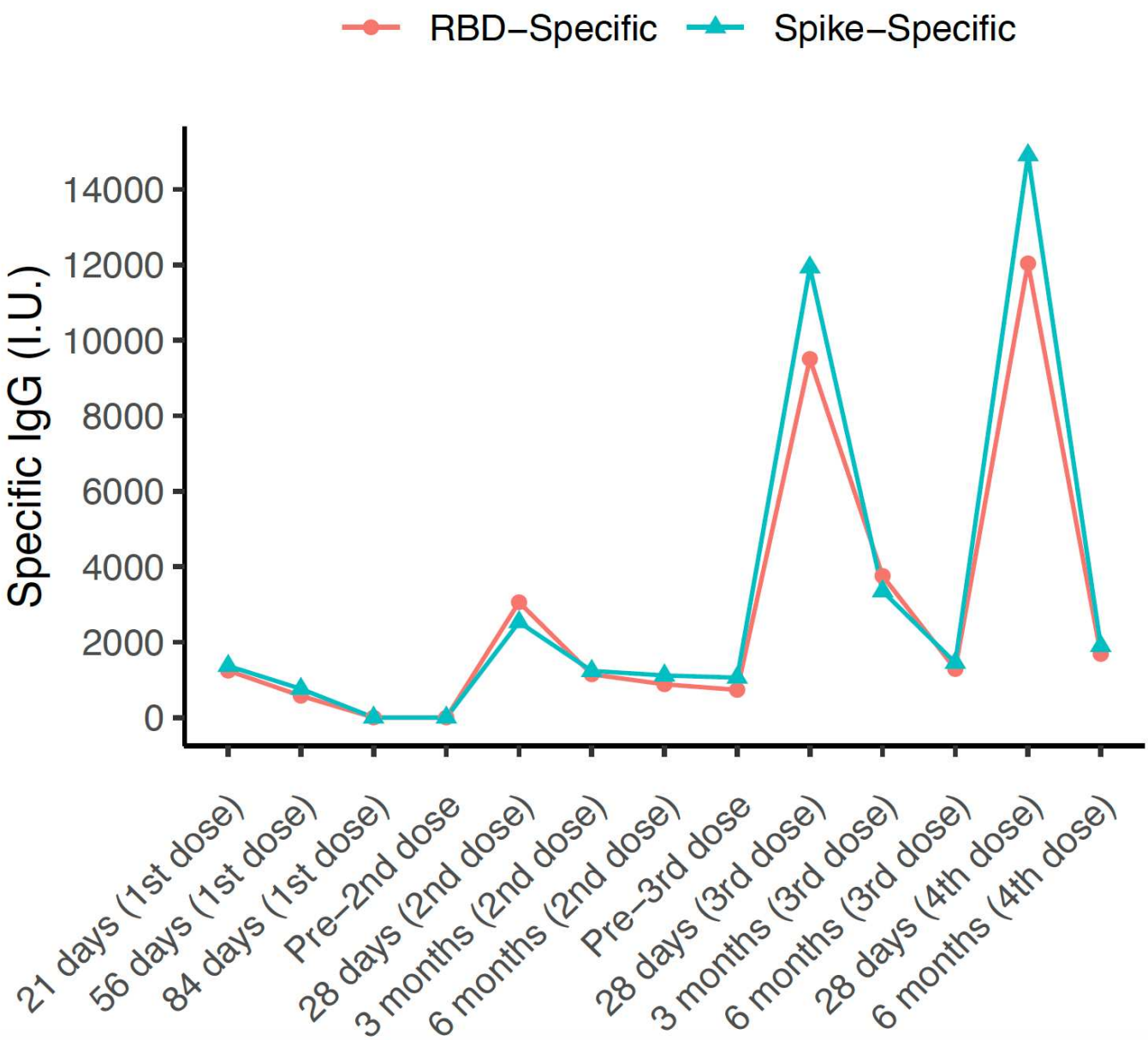

Fig 3 Antibody response graph for participant demonstrating a vaccine response without infection. High S-specific and RBD-specific IgG peaks are observed 28 days after the third and fourth doses, with levels declining over the next 6 months. A large difference is observed between the second and third dose, as repeated antigen exposure leads to greater IgG production.

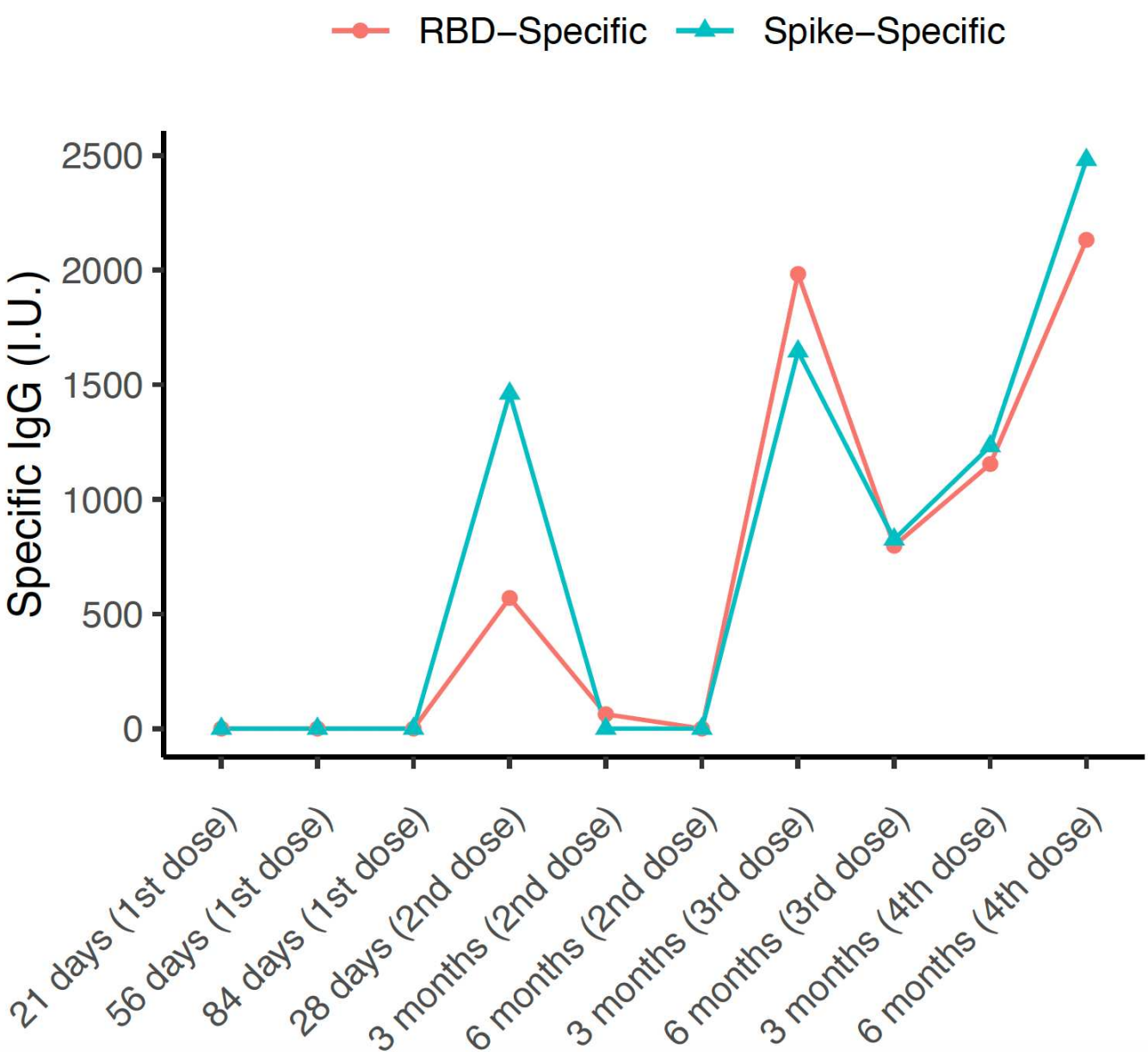

Fig 4 Antibody response graph for participant showing an overall lower S-specific and RBD-specific IgG production. The specific IgG levels reflect the participant's immunocompromised status based on intake questionnaires, indicating a slower immune response to the vaccine and their overall antibody production after vaccination is lower compared to healthy individuals[4].

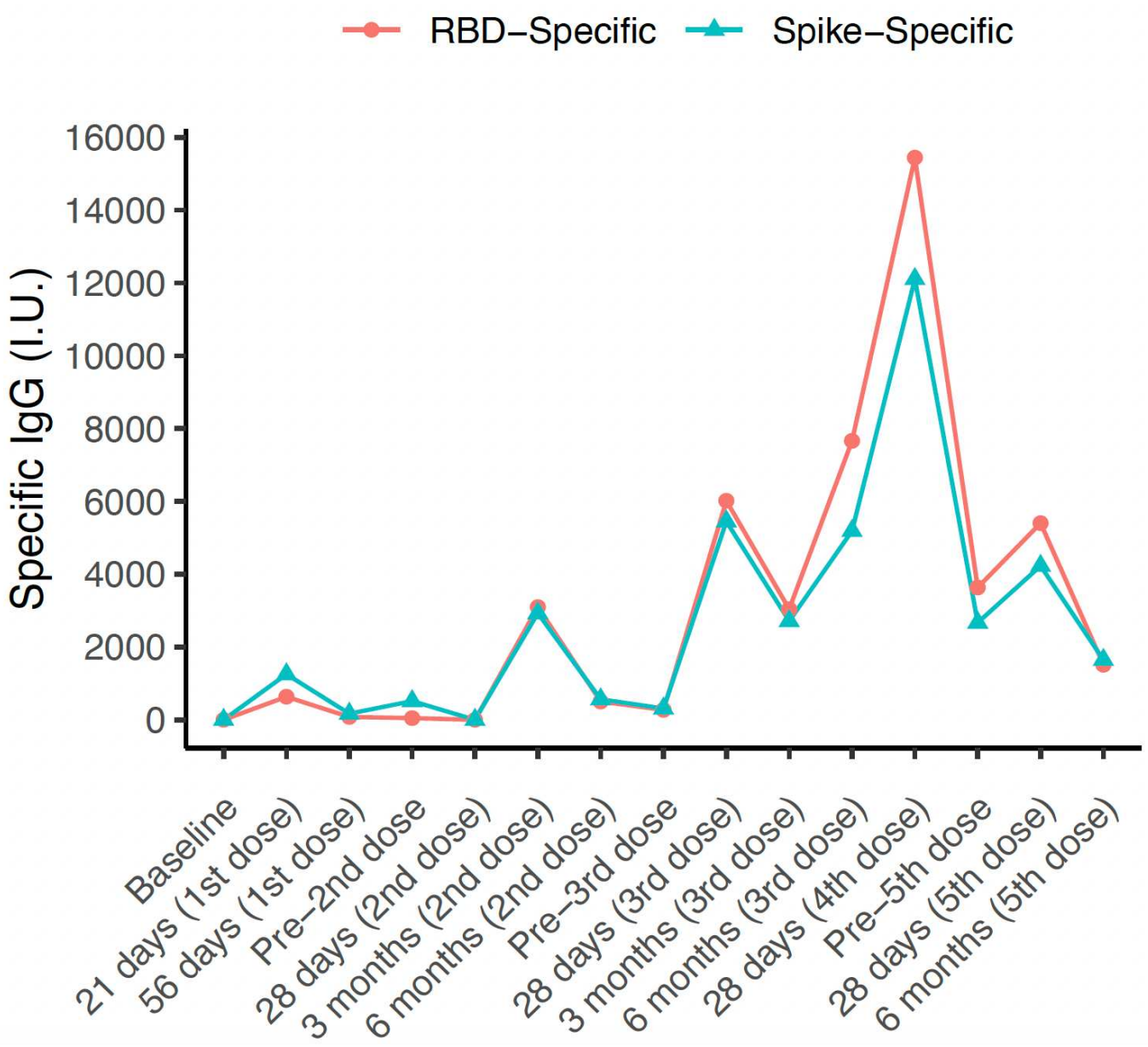

Fig 5 Antibody response graph for participant with an infection detected at the 6 months (3rd dose) and 28 days (4th dose) timepoints. A large increase in IgG production at 6 months (3rd dose) and 28 days (4th dose) is observed, which is a result of the infection. Usually, these timepoints don't exhibit such high IgG levels from the vaccine alone.

Figure 3 depicts the antibody response from a participant who had not been infected throughout their participation in the study, as suggested by the absence of anti-N-IgG. We observed a pronounced antibody response 28 days post second vaccine dose, as the first dose was meant to prime the immune system as the initial exposure to the antigen. Antibody levels then decreased gradually over the next 6 months before the third dose. A significant increase in antibody production is seen 28 days post third dose, as the immune system has now been exposed to the spike antigen several times from vaccination. As expected, IgG levels declined afterwards due to waning. The greatest peak for this participant, however, was seen 28 days after the fourth dose, as each additional vaccine dose stimulated the production of more antibodies. After reaching a peak, the S-specific and RBD-specific IgG typically declined, but consistently remain above baseline levels recorded after the first and second doses.

Our second example is an immunocompromised participant, whose antibody responses are shown in figure 4. Theoretically, depending on the reason for the immune compromise, individuals may experience less robust vaccine responses compared to healthy individuals, with their S-specific and RBD-specific IgG levels being lower, potentially reducing the effect of the vaccine. Their IgG response following the 3rd dose is not the typical response we would observe in healthy participants; it is much lower. For instance, the participant from figure 3 had a specific IgG response around 4000 I.U. for the 3 months post 3rd dose sample, whereas figure 4 indicates a specific IgG response of approximately 1500-2000 I.U. at the same timepoint. Overall, this participant exhibits a less pronounced vaccine effect on their IgG levels, as their immune system might not react as strongly or as quickly to spike antigen exposure.

We have also observed antibody responses for participants who had been infected during their participation in the cohort, as portrayed in figure 5. The following participant received boosters consistently and apparently had been infected with SARS-CoV-2 based on newly detected anti-N IgG with their blood samples just prior to their 4th dose and 28 days following their 4th dose samples. The participant exhibits a peak instead of the expected decline in Anti-S and RBD-IgG levels after a vaccine; usually, no significant boost in antibody production in seen at 6 months post 3rd dose. Additionally, IgG levels at 6 months after their 3rd dose are higher in comparison to 3 months following their 3rd dose. For the 28 days post 4th dose sample, IgG levels for the uninfected participant illustrated in figure 3 were approximately 12000 I.U., but for this infected and vaccinated participant, IgG levels were around 16000 I.U. Other infected and vaccinated participants have even exhibited IgG levels upward of 25000 I.U. These observations are consistent across all infected participants in our cohort and the implications of these observations are discussed in chapter 5.

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Chapter V: Discussion

With the rise of the highly transmissible variant XBB.1.5, this main project for this thesis aimed to determine the effectiveness of the bivalent vaccines being administered at the time in providing protection against this evolving strain. Multivariable mixed-effects modeling allowed us to assess the relationship between an outcome variable, ID_{50}, and different predictors and covariates. By employing statistical modelling, we sought to evaluate the effectiveness of BA.4/BA.5 bivalent vaccines and determine which predictors significantly affect the induction of neutralizing antibodies against XBB.1.5. Importantly, despite histories of infection in many subjects, only when anti-N IgG was still detectable did past infection appear to influence the response to the booster vaccine. This led to my second analysis using data from our cohort: we aimed to model the decay of anti-N IgG and determine the average time to waning for the Living Lab study using a Cox regression. This statistical method is particularly useful for analyzing and interpreting time-to-event data, which for our study was used to look at time-to-waning for anti-N IgG. In chapter 4, we generated graphical representations to analyze diverse trends and patterns of the COVID-19 mRNA vaccine response among participants, which allowed for a comprehensive view of the immune landscape.

In chapter 2, we underscored the utility of receiving additional booster doses of the COVID-19 mRNA vaccine. We demonstrated that third and fourth vaccine doses not only elicited neutralizing antibodies against the wild-type virus, but against the XBB.1.5 variant irrespective of the use of bivalent or monovalent vaccines for the fourth dose, and irrespective of infection status. Our

multivariable model did not show any statistically significant advantage of the bivalent compared to the monovalent vaccine in the induction of neutralizing antibodies.

Cross-reactivity occurs when the immune response initially generated against a specific antigen expands to target other related antigens. This occurs when the immune system recognizes similar epitopes on different viral strains. The immune response generated against the ancestral viral strain can thus cross-react with new variants, conferring partial protection against new variants, despite the immune response being initially targeted at the ancestral strain. Although XBB.1.5 exhibits new mutations compared to the ancestral strain, there are viral epitopes that remain conserved or only slightly modified. The immune response generated against the ancestral strain, through infection or immunization, may recognize these shared epitopes on the XBB.1.5 variant. This phenomenon is a key reason why receiving additional booster vaccines can elicit a strong neutralizing antibody response against emerging variants, even if not identical to the ancestral strain, which helps protect against infection by blocking viral entry as discussed in chapter 1.

The bivalent and monovalent vaccines' comparable performance and the improvement in vaccine response seen with booster doses regardless of type can be explained by a phenomenon known as imprinting, or original antigenic sin, where the immune system of previously vaccinated or infected individuals is primed to mount an immune response to the SARS-CoV-2 ancestral strain. Despite extensive efforts, data surrounding bivalent vaccines developed in response to certain Omicron variants of SARS-CoV-2 have not shown major differences from the monovalent vaccine in the immune response. Studies assessing the effect of bivalent mRNA boosters, which target the BA.4-BA.5 spike and wild-type spike, showed that boosting with bivalent mRNA vaccines did not

result in a significantly greater neutralizing antibody response compared to the original monovalent vaccines[1,2]. During imprinting, memory cells can bind to shared epitopes between the ancestral strain and BA.4-BA.5, leading to antibody production that specifically targets these shared epitopes. Therefore, these individuals' immune response was likely targeting the shared epitopes, rather than the BA.4-BA.5 epitopes, which provides another explanation for the lack of advantage for bivalent over monovalent vaccines in our cohort. Administering additional booster vaccines results in the most effective vaccine response against XBB.1.5 and leads to greater induction of neutralization antibodies, according to our multivariable model. Over time, antibody levels triggered by vaccination can decrease, leading to reduced protection against SARS-CoV-2. Booster doses can help reinforce the immune response and provide prolonged protection.

Our multivariable mixed-effects model detailed in chapter 2 reveals 'recent infection', as defined by detectable anti-N antibodies, was a significant predictor variable that immunologically enhanced the vaccine response. A study looking at the immune response generated by mRNA vaccines against Omicron variant BQ.1.1 has suggested that hybrid immunity acquired from recent infection and vaccination gave stronger humoral responses against BQ.1.1 spike compared to vaccination alone. They have shown that all participants with a recent breakthrough infection, who also received a fourth dose, developed neutralizing antibodies against BQ.1.1 spike[3]. Therefore, hybrid immunity, particularly when acquired from a recent infection, plays an important role in inducing greater neutralizing antibodies, whereas prior infection may contribute less effectively due to waning of the antibody response over time.

Unlike the S and RBD proteins, the N protein is intracellular, encapsulating the viral RNA genome and is not targeted by RNA-based vaccines. Therefore, the presence of anti-N IgG antibodies in sera serves as a marker for past infection. At some timepoint after the infection, these antibodies get degraded and eliminated from the body, reducing anti-N IgG in the bloodstream to near undetectable levels over time. Anti-N antibody levels can persist for several months post-infection. In chapter 3, we find that average time to waning for anti-N IgG in the Living Lab study was 7.5 months. Other research corroborates these findings, although the rate and extent of this decay may slightly vary in the literature. Similar to our work, a study by Van Elslande et al. showed that while anti-N IgG levels waned over time, they were still detectable in individuals 8 months after SARS-CoV-2 infection. The researchers found that anti-N IgG levels peaked around 1 month after symptom onset, followed by a steady decline, with 70.6 % of patients testing positive up to 8 months after infection (i.e. anti-N IgG levels > 1.4 SCO cutoff)[4]. Similarly, another Van Elslande et al. study found that while anti-N IgG provides insight into recent infections, anti-S IgG antibodies persist longer and their prolonged presence in sera can be protective against re-infection, making them good indicators of long-term immunity[5]. The median time to seronegativity was found to be greater than 2 years for anti-S compared to less than a year for anti-N IgG[5]. These results, which highlight the importance of incorporating broader serological assessments in vaccination strategies, support our model's findings. Several other studies have tried to assess the gradual decline of anti-N IgG in infected individuals. They have shown high levels of anti-N antibodies following infection that exhibited stable neutralizing potential for at least a 3-month period, even in elderly people, with a pronounced decline in anti-N IgG antibodies 18 months after infection compared to 2 months[6,7].

In Chapter 2, we observed that only when anti-N IgG was detectable did it influence the response to the booster vaccine, underscoring the importance of tracking nucleocapsid (N) protein waning to understand the duration of vaccine-induced immunity. Unlike the spike (S) protein, which is highly mutable, the N protein is less susceptible to mutations. Therefore, tracking N protein waning can help determine how long the protective effects of the vaccine persist and determine the necessity for periodic booster doses. Our findings from chapter 3 indicate that anti-N IgG wanes below detectable levels around the 8-month period. As a result, regular annual boosters could help maintain robust immunity and ensure continued protection against evolving variant strains.

The Living Lab cohort has allowed us to visualize and examine the immune response following several doses of COVID-19 vaccines. By improving cellular immunity and generating high neutralizing antibody levels, mRNA vaccines have most likely contributed to a remarkable decline in infection rates and hospitalizations. Researchers have demonstrated that the immune response is robust after receiving second and third doses of mRNA COVID-19 vaccines and may produce significant amounts of neutralizing antibodies and memory B cells, both of which are crucial for long-lasting protection[8]. Other researchers decided to study the antibody response in a group of 46 elderly living facility residents (mean age 85 years) who received four COVID-19 mRNA vaccines. Their analysis shows serum anti-RBD IgG levels were significantly higher after the fourth dose compared to the third dose ($P = 0.03 < 0.05$) and neutralizing levels against the ancestral strain and the BA.1 Omicron variant increased significantly after the fourth dose compared to the third (ancestral: $P = 0.002 < 0.05$, BA.1: $P = 0.018 < 0.05$)[9]. These findings indicate that booster doses of the original mRNA vaccine were beneficial in maintaining protection against the ancestral viral strain and emerging variants, as demonstrated previously in chapter 2.

When it comes to vaccines, it is useful if we can determine a 'correlate of protection' i.e., a certain IgG concentration generally considered protective. While exact thresholds can differ depending on the study design and lab measurements, it is commonly recognized that higher anti-S IgG levels correlate with increased protection. One American study with a similar experimental design and objective as the Living Lab cohort determined a 'correlate of protection (CoP)' for their vaccinated study participants: anti-S IgG levels were considered protective if > 3800 BAU/mL (or I.U.) and if > 5750 BAU/ml for anti-RBD IgG[10]. In reviewing the data in preparation for chapter 4, the majority of our participants exhibited S-specific and RBD-specific IgG levels greater than 15000 I.U., indicating that the mRNA COVID-19 vaccine appeared to be successful at initiating protective levels of antibodies for a period following vaccination.

For COVID-19, neutralizing antibody titers against the spike protein have been suggested as a reliable CoP and validated in a few studies [11,12]. There are two approaches used to understand the relationship between neutralizing antibody levels and protection. The vaccine-comparison approach compares mean neutralization titers from early vaccine trials with observed vaccine efficacy to derive a protection curve, while the breakthrough-infection approach examines neutralization titers in people who have experienced breakthrough infections compared to those who have not[11]. It is important to note, however, that there is no universally standardized assay for measuring neutralization titers and discrepancies can arise due to differences in assay methodologies and other study-specific factors. There is no set threshold above which someone is considered fully protected, as protection is probabilistic and varies with antibody levels. Moreover, defining a CoP for COVID-19 is complex because of variability in viral exposure and waning of antibody levels. An attempt to normalize and compare data from different studies involves

adjusting for variations and aligning the data to a common scale to facilitate comparison. There is ongoing research to define CoPs for new Omicron variants, assess long-term protection, and evaluate other immune responses like mucosal IgA[12].

Over the last 4 years, we have accumulated hundreds of samples from our participants. In the future, these samples can be used to conduct interesting experiments, such as potentially carrying out a spike-specific ELISPOT to detect antigen-specific B cells following vaccination. The assay would help quantify the number of B cells that recognize and respond to the spike antigen and assess how well vaccine-induced B cells respond to different viral variants. A question to be answered would be whether there are more antigen-specific B cells being produced after a particular vaccine dose or whether it is the same number of cells making more antibodies.

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Conclusion

The COVID-19 pandemic, instigated by SARS-CoV-2, quickly became one of the most significant global health crises of our generation, overwhelming healthcare systems and infecting millions worldwide. Over recent years, the pandemic has driven unprecedented scientific collaboration and innovation, particularly in vaccine development and public health strategies. Throughout this thesis, we address the efficacy of current COVID-19 vaccines in inducing greater neutralizing antibodies against the XBB.1.5 variant and analyze the duration of infection-induced immunity using statistical modelling. Published findings demonstrated that four doses of a COVID-19 vaccine significantly increased the neutralizing antibody response against XBB.1.5 compared to three doses, irrespective of booster type as there was no statistically significant difference between monovalent and bivalent vaccines. A previous infection was also shown to enhance this response. Additionally, the Cox regression model determined the temporal decay of anti-N IgG antibodies for the 'Living Lab Seroprevalence Study', revealing a half-life of 86 days and average time to waning of approximately 228 days. Reflecting on the research process, the combination of statistical modelling and graphical representations of the antibody response following vaccination provided a holistic understanding of vaccine-induced and infection-induced immunity. Important patterns were identified by looking at the anti-S and anti-RBD response to several doses of the vaccine in cohort participants. The confirmation that additional boosters are effective against emerging variants, regardless of type, supports continued booster administration to sustain immunity, especially in high-risk populations. Moreover, understanding the temporal dynamics of anti-N IgG antibodies emphasizes the need for timely booster doses to counteract infection-induced waning immunity.

Appendix

Supplementary material for Chapter II

This appendix contains supplemental tables and figures that provide additional details for the methods and results presented in chapter 2. It presents descriptive characteristics of the XBB.1.5 cohort from the Living Lab Seroprevalence Study. Additionally, it includes several figures comparing the neutralizing antibody response between the XBB.1.5 variant and the ancestral Wuhan strain at vaccine dose 3 and dose 4, as well as comparisons between the bivalent and monovalent vaccine at each dose.

Table A1: Descriptive characteristics of participants in the XBB.1.5 cohort from the Living Lab Seroprevalence Study

	28 days post-3rd dose (N=44)	**28 days post-4th dose (N=44)**
Age, mean (SD), yrs	54.1 (11.2)	
Female, %	65.9	
Vaccine type monovalent at time of visit, %	100	61.4
Recent infection positive at time of visit, %	22.7	29.5
Interval between 3rd and 4th vaccine dose, mean (SD), days	236.9 (73.9)	

Figure A1

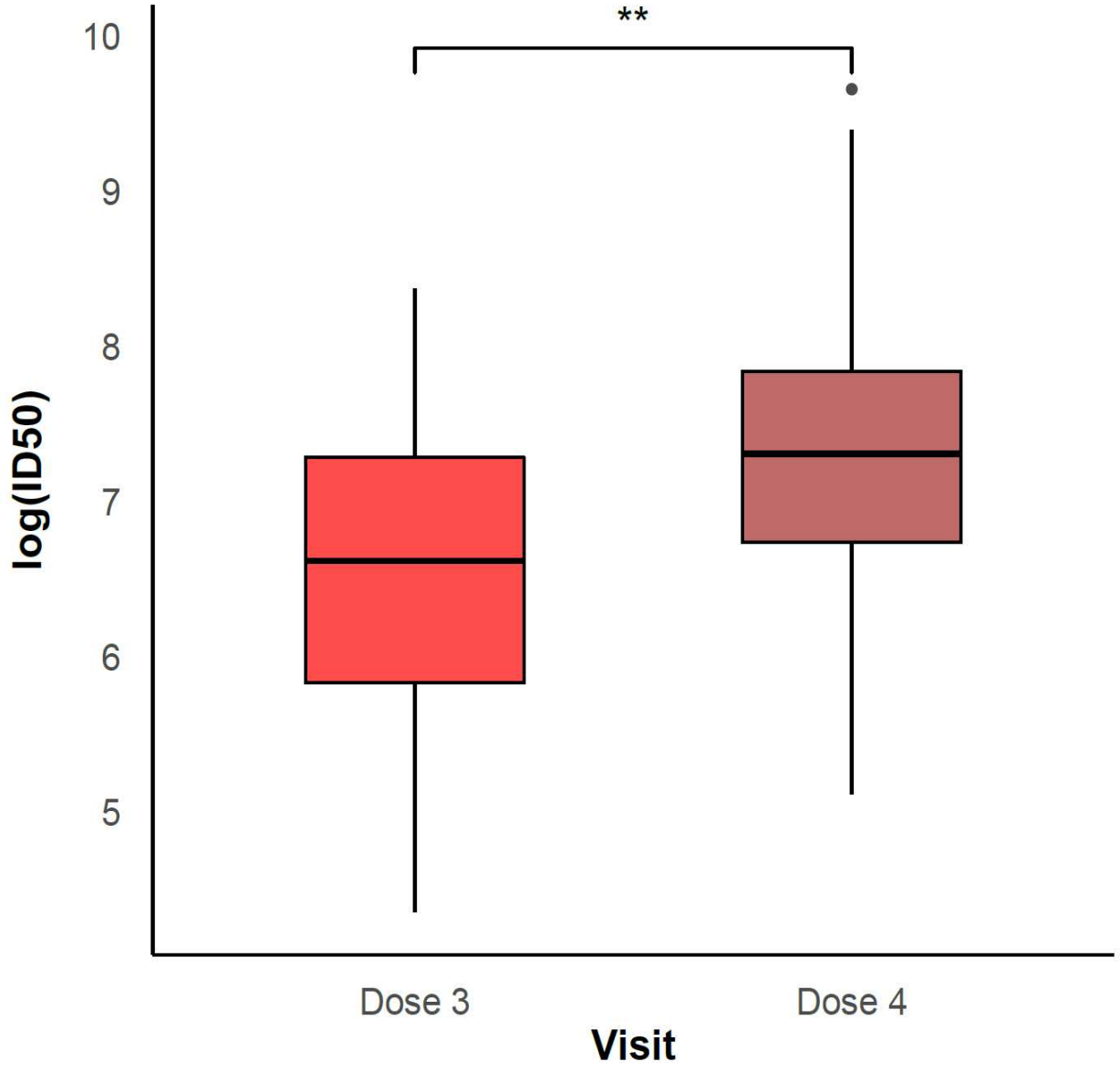

Fig A1 COVID-19 vaccine neutralization efficiency against the Wuhan ancestral strain measured after the third and fourth doses. A fourth vaccine dose is associated with greater ID_{50} values, therefore inducing a greater number of neutralizing antibodies against the Wuhan ancestral strain than a third dose. ***P < .01.*

Figure A2

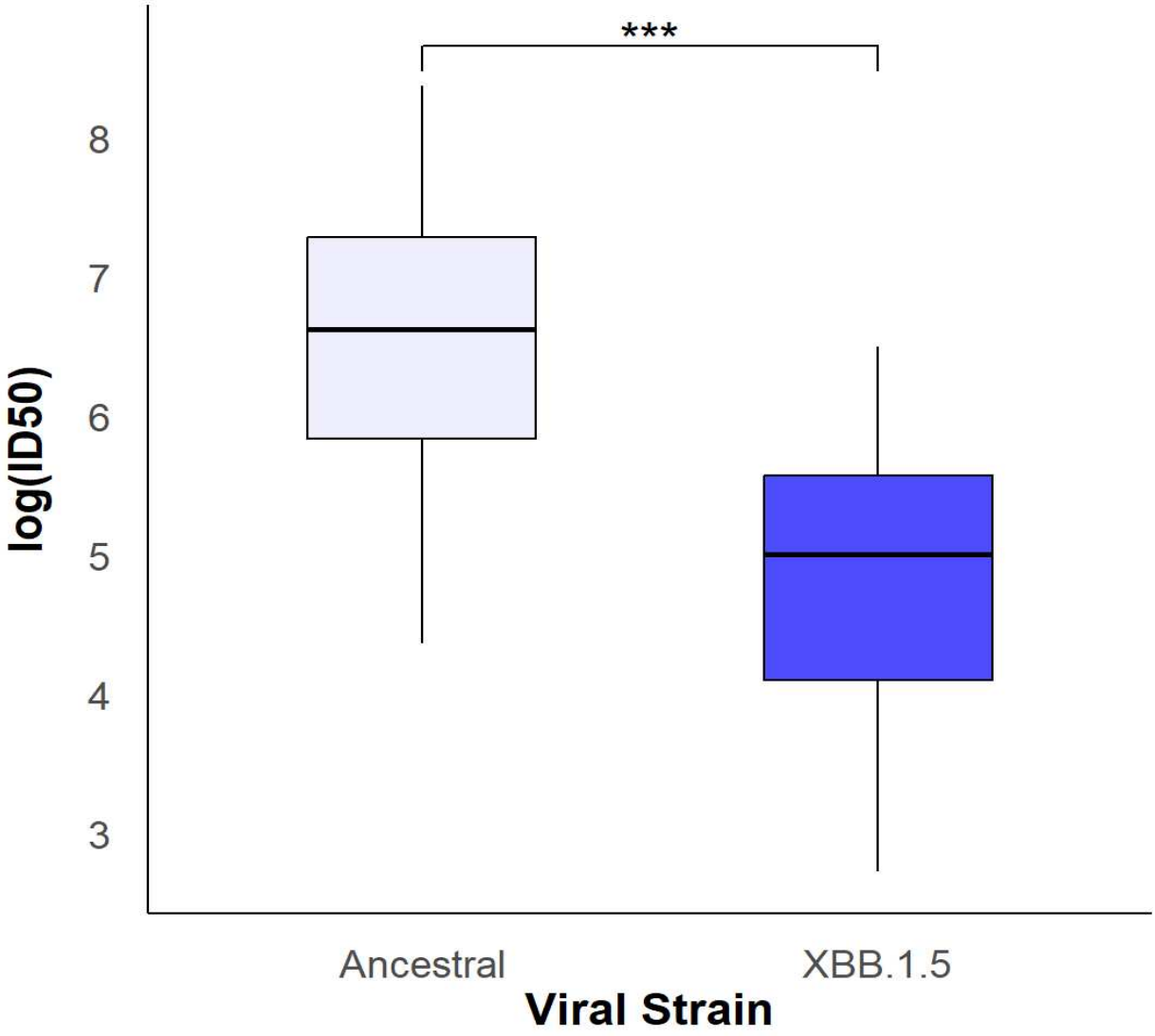

Fig A2 Lower neutralizing antibody response after dose 3 for XBB.1.5 compared to the Wuhan ancestral strain. ID_{50} values are higher after the third dose for the Wuhan ancestral strain compared to XBB.1.5, eliciting a greater neutralizing response for the ancestral strain. All participants received the monovalent for the third dose, which was accounted for in our multivariable mixed-effects model. ****P < .001.*

Figure A3

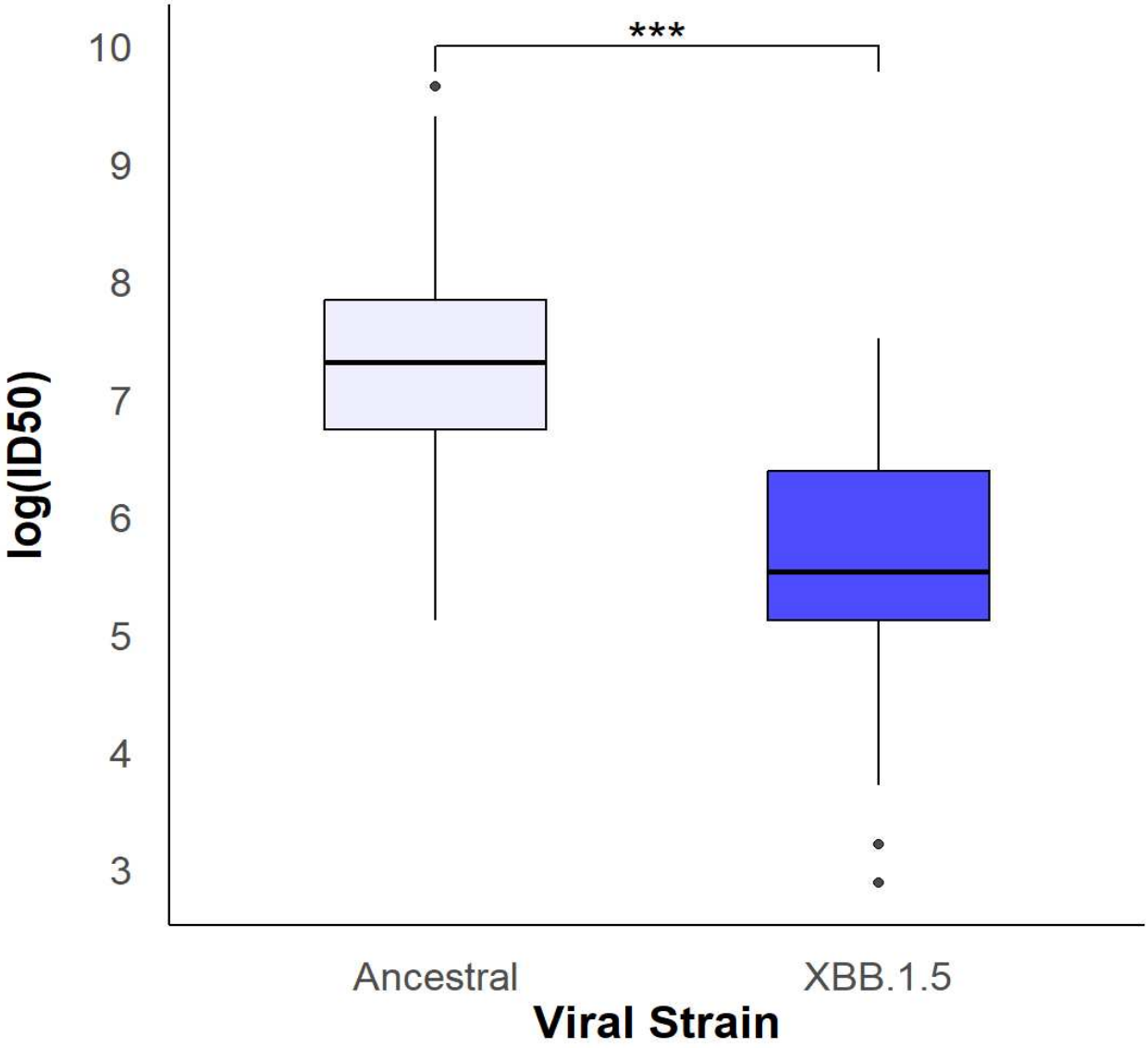

Fig A3 Lower neutralizing antibody response after dose 4 for XBB.1.5 compared to the Wuhan ancestral strain. ID_{50} values are higher after the fourth dose for the Wuhan ancestral strain compared to XBB.1.5, eliciting a greater neutralizing response for the ancestral strain. ******P < .001.***