Immunisation of Children and Adolescents Against COVID-19: Immunogenicity and Reactogenicity of Heterologous and Fractional Dose Schedules

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Declaration of Contributions

I contributed to the study protocol, study design, and study documents for the Com-COV3 trial described in this thesis. I also conducted training of study site staff and carried out enrolment and study visits which included the collection of blood and mucosal samples which were subsequently used for laboratory assays. SARS-CoV-2 spike and nucleocapsid ELISA testing was carried out at UK Health Security Agency (UK HSA). ELISpots (T-cell) were conducted by Oxford Immunotec. Where sample processing took place at external or public health laboratories, this has been indicated in the thesis. I repeated the SARS-CoV-2 spike ELISA assay when conducting the avidity assay as part of my DPhil. I conducted all avidity ELISA and MSD assays with supervision from Dr. Emma Sheehan and Jennifer Alderson. I undertook all memory B cell ELISpots with oversight provided by Dr. Elizabeth Clutterbuck. Supervision of the statistical analysis performed as part of this thesis was provided by Professor Xinxue Liu. Results from Cohort A of the Com-COV3 trial were published in the Journal of Infection in 2023. Results from the publication have been included in this thesis. The graphs in the publication and in Chapter 3 were produced by the trial statisticians Melanie Greenland and Professor Xinxue Liu.

Table of Contents

Abstract

COVID-19 vaccination has been shown to protect children and adolescents against SARS-CoV-2 infection and disease, with efficacy increasing with additional doses. However, the increased risk of myocarditis associated with mRNA vaccination in adolescents, particularly following a second dose, suggests a potential role for fractional or heterologous second doses. Data regarding heterologous and fractional dose COVID-19 vaccine schedules in adolescents, however, are lacking. Additionally, the decline in vaccine-induced immunity over time, combined with the emergence of immune-evasive variants of concern underscores the need for booster doses to maintain protection against SARS-CoV-2 infection.

This DPhil aimed to investigate the humoral immune response to homologous and heterologous COVID-19 vaccination in adolescents using samples collected as part of the Com-COV3 trial. Com-COV3 was a phase II, single-blind, multi-centre, randomised-controlled trial to determine the reactogenicity and immunogenicity of COVID-19 vaccines in healthy 12-to-16-year-olds. Cohort A participants were randomised to receive either 30 µg BNT162b2 (BNT-30), 10 µg BNT162b2 (BNT-10), or NVX-CoV2373 (NVX), 8 weeks after a first 30µg dose of BNT162b2. Cohort B participants were randomised to receive either BNT162b2 30µg, BNT162b2 10µg (adult vaccine formulation), BNT162b2 10µg (paediatric formulation), NVXCoV2373, or Meningococcal B vaccine (control) as a third (booster) dose following two-30µg dose BNT162b2 primary regimen received at least 90 days prior to enrolment. The primary outcome was reactogenicity. The secondary outcome was immunogenicity. Exploratory immunological studies to investigate the humoral immune response to both homologous and heterologous second and third dose schedules were also conducted.

Reactions were mostly mild-to-moderate across both cohorts. Compared to BNT-30, a comparable antibody response was observed at day 28 following a heterologous second dose (NVX) but significantly lower following a fractional dose (BNT-10). In Cohort B, anti-spike IgG at day 28 post-third dose was similar in the 10μg BNT162b2 (adult) group and significantly lower in the 10μg BNT162b2 (paediatric) and NVXCoV2373 groups compared with 30μg BNT162b2.

SARS-CoV-2 spike-specific BMEM responses were substantially increased 28 days after both a 30μg BNT162b2 and NVX-CoV2373 third (booster) dose. Peak BMEM responses were also significantly higher following a booster dose compared to the primary series. Additionally, both homologous and heterologous primary and booster immunisation resulted in significantly increased antibody avidity. Significantly enhanced mucosal spike-specific IgG and IgA responses were also observed following both homologous and heterologous second dose schedules. Following a third (booster) dose, mucosal IgG responses were significantly elevated after mRNA vaccination, while a significant increase in mucosal spike-specific IgA was only observed in participants with prior SARS-CoV-2 infection.

This was the first study to investigate the immune response to heterologous COVID-19 vaccine schedules in adolescents. The findings demonstrate that both homologous and heterologous second and third (booster) dose schedules are highly immunogenic and demonstrate favourable reactogenicity.

List of Figures

List of Tables

Chapter 1: Introduction[1]

1.1 The SARS-CoV-2 pandemic

Three global outbreaks attributable to the family *Coronaviridae* have occurred this century: severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) in 2003, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, and SARS-CoV-2 which was first detected in December 2019 following cases of pneumonia linked to a seafood market in Wuhan, China.[1] These three viruses are among the seven coronaviruses identified to date capable of causing human disease. The other four viruses OC43, HKU1, NL63 and 229E are a frequent cause of common cold symptoms.[2] SARS-CoV-2 is responsible for COVID-19 disease (i.e., coronavirus disease 2019). Following its identification in 2019, it was renamed SARS-CoV-2 due to the significant shared sequence homology between SARS-CoV and SARS-CoV-2.[3] Following its identification, it spread rapidly across the globe and by 30th January 2020 the SARS-CoV-2 outbreak was declared a public health emergency of international concern by the World Health Organisation (WHO). By November 2021, there were greater than 257 million cases of COVID-19 reported and more than 5 million deaths had occurred due to the COVID-19 outbreak.[4]

[1] Passages of this chapter have previously been published in Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.

1.1.1 SARS-CoV-2 virus

Coronaviruses inherit their name because their spike proteins bear a resemblance to a halo or corona ("crown") when viewed using electron microscopy.[5] SARS-CoV-2 is a betacoronavirus. Other members of the betacoronavirus genus include OC43, HKU1, SARS-CoV-1 and MERS-CoV.[6] SARS-CoV-2 is a large spherical positive-sense enveloped single-stranded RNA virus. It contains a nucleocapsid protein shaped like a helix at its centre which contains the virus's genetic code. Spike proteins arrayed on the exterior of the virus are vital to the virus's ability to infect host cells, **Figure 1.1.** Two-thirds of the virus's genome encodes instructions to express 16 non-structural proteins (NSP) while one-third of its genome encodes for structural proteins S (spike), E (envelope), M (membrane), and N (nucleocapsid). E, M, and N proteins are involved in virion assembly while S protein facilitates virus entry into the host cell during infection by binding to the host cell receptor angiotensin-converting enzyme 2 (ACE2).[7]

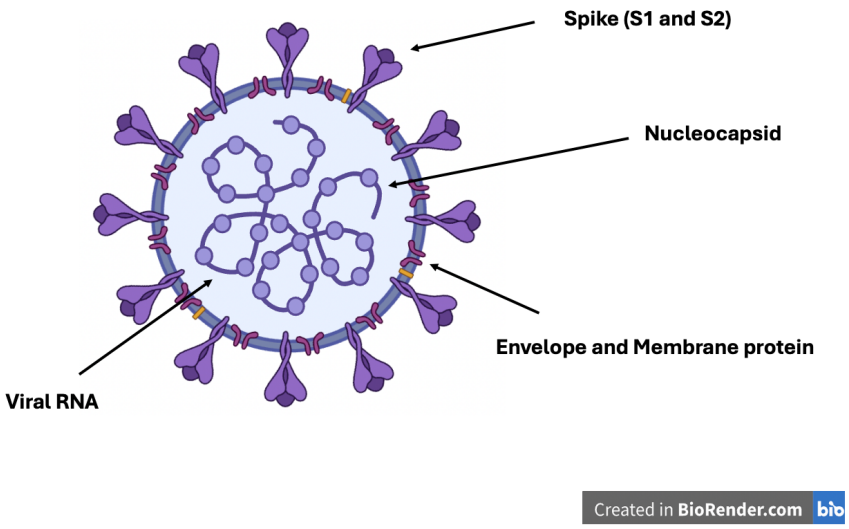


Figure 1.1 Illustration of SARS-CoV-2 spike structure

1.1.2 COVID-19 vaccination campaign

The global response to the COVID-19 pandemic led to the successful deployment of several vaccines shown to provide effective protection against severe disease and death.[8] The COVID-19 mRNA vaccine BNT162b2 (Pfizer-BioNTech) was granted emergency authorisation by the Medicines Health Regulatory Agency (MHRA) on 2nd December 2020 and was subsequently granted conditional authorisation by the European Medicines Agency (EMA) on 21st December 2020. It was authorised for use in adolescents by the MHRA on 4th June 2021 and by the EMA on 28th May 2021. The adjuvanted nanoparticle COVID-19 vaccine NVXCoV2373 (Novavax) was approved for use in adults by the MHRA on 3rd February 2022 and was later approved for use in adolescents on 26th August 2022. The Oxford/AstraZeneca ChAdOx1 nCOV-19 vaccine was granted emergency authorisation on 29th December 2020 by the MHRA. The Moderna COVID-19 (mRNA-1273) vaccine was approved by the MHRA on 8th January 2021 and later approved for use in adolescents by the MHRA on 17th August 2021. These vaccines were developed for use as homologous two-dose regimens as part of the primary immunisation series.

High rates of SARS-CoV2 infection have been shown to occur in children and adolescents.[9] Children and adolescents experienced mild symptoms early in the pandemic (fever, cough, nasal symptoms were frequently reported),[10] and immunisation of high-risk groups was prioritised initially in vaccination campaigns. However, the advent of SARS-CoV2 variants of concern (VOC) precipitated a sharp increase in paediatric hospitalisations and illness worldwide.[9,11] SARS-CoV-2 infection was also associated with the development of the potentially fatal multisystem inflammatory syndrome PIMS-TS[12] and has been associated with the long-term sequelae of a multisystemic post-COVID-19 condition ("long COVID").[13] The significant impact of COVID-19 on the education and psychological well-being of children and adolescents, the high rates of infection in this age group, and their potentially important role in transmission have been

acknowledged.[14,15] Indeed, during the pandemic, recommendations for immunisation were extended to adolescents in many countries including the USA due to the high rates of infection recorded in this age group.[16] Most COVID-19 vaccines recommended and administered to adolescents have been mRNA vaccines, predominantly BNT162b2. In the UK, following a review by the Joint Committee on Vaccination and Immunisation (JCVI), a first dose of BNT162b2 was recommended for all 12- to 17-year-olds on 13th September 2021.[17] This guidance was subsequently amended to recommend offering a second dose of BNT162b2 to all 12- to 17-year-olds on 29th November 2021.[18,19]

Early in the COVID-19 immunisation campaign however, it became apparent that receipt of an mRNA COVID-19 vaccine was associated with an increased incidence of myocarditis. An analysis of myocarditis cases reported to the Vaccine Adverse Event Reporting System (VAERS) in the USA following mRNA-based COVID-19 vaccination revealed the highest rates of myocarditis occurred after a second vaccine dose in male adolescents aged 12 to 15 years (70.7 per million doses of BNT162b2 vaccine) and 16 to 17 years (105.9 per million doses of BNT162b2 vaccine).[20] The underlying aetiology for this association remains unclear, though most reported cases are benign and resolve without intervention.[20,21] The risk of myocarditis associated with vaccination appears to be as high following a third dose of BNT162b2.[22] It has been suggested that this risk may be reduced by using another vaccine or fractional dose for the second or third vaccination, or variation to dose intervals, which might also facilitate more effective deployment of COVID-19 vaccine stocks and reduce reactogenicity.[23]

1.1.3 COVID-19 booster vaccine doses

Although COVID-19 immunisation has been shown to be highly effective at reducing the risk of severe disease and hospitalisation from SARS-CoV-2, waning of the immune response following the two-dose primary vaccine series has been shown to occur.[24,25] While adolescents are at lower risk of severe disease and death from SARS-CoV-2 infection than older populations, the emergence of more recent variants in 2022 rendered the paediatric population more vulnerable to infection and severe illness with highest rates of paediatric hospitalisation and infection at the time induced by SARS-CoV-2 VOCs Delta and Omicron.[26] Indeed, studies in both adults and children have demonstrated reduced vaccine effectiveness and a rapid decline in protection against SARS-CoV-2 variants, particularly Omicron, after the primary vaccine series (targeting wild-type strain).[27-31] Following a two-dose BNT162b2 primary vaccine series during an Omicron predominant period, vaccine effectiveness declined to 16% among adolescents within 6 months after vaccination.[30] Booster vaccination (using vaccines targeting wild-type SARS-CoV-2), however, has been shown to restore protection against hospitalisation and Omicron-related infection to levels observed against Delta.[27,30,31] These findings prompted many high-income countries to recommend a third COVID-19 booster dose to combat waning vaccine effectiveness.[28] However, policy regarding adolescent immunisation varies globally.

1.1.4 Heterologous COVID-19 vaccine schedules

In adults, heterologous vaccination schedules have been shown to be safe and immunogenic and have been implemented in Canada and northern Europe.[32] They have been approved by the WHO to enhance vaccination coverage, particularly where vaccine supplies are limited.[33] In the Com-

COV2 trial, a heterologous COVID-19 vaccine schedule utilising the Matrix-M adjuvanted recombinant nanoparticle spike protein vaccine NVX-CoV2373 as the second dose following BNT162b2, was found to be less immunogenic in 50-to-70-year-olds than two doses of BNT162b2. The BNT162b2 prime/NXV-CoV2373 boost schedule was, however, still more immunogenic than two doses of the adenoviral-vectored ChAdOx1 n-CoV-19 (Oxford/AstraZeneca) vaccine, which is highly effective against hospitalisation and death.[32,34] Heterologous COVID-19 booster vaccine schedules in adults have also been shown to elicit enhanced protection against Delta and Omicron SARS-CoV-2 variants and greater immunogenicity compared with primary and homologous boost schedules.[35,36] The COV-BOOST trial, which evaluated seven different third dose COVID-19 vaccine schedules, demonstrated robust immune responses and favourable reactogenicity following heterologous booster immunisation in adults.[37] The trial also investigated fractional dose COVID-19 booster options and showed that fractional dose BNT162b2 given as a third dose induced an immune response comparable to full dose BNT162b2.[37,38]

1.1.5 Fractional dose COVID-19 vaccine schedules

A 10µg dose of BNT162b2 has been recommended for children aged between 5 and 11 years, administered as 0.2ml using a specially designed paediatric formulation of the vaccine. A 10µg two-dose paediatric BNT162b2 regimen as the primary vaccine series has already been shown to be highly immunogenic when administered to children aged 5 to 11 years, and comparably immunogenic to a 30µg two-dose BNT16b2 adult formulation schedule administered to 18–25-year-olds.[39] A reduced dose as the second or third booster dose might reduce the risk of myocarditis associated with mRNA vaccination while providing protection against SARS-CoV-2. Prior to the Com-COV3 study (outlined in **Chapter Chapter 3:**), it was not known whether the immune response to 10µg administered using the adult formulation of BNT162b2 was non-inferior to the

same dose administered using the paediatric formulation of BNT162b2. The possibility of using a 10µg (a one-third dose) of the adult formulation of BNT162b2 as a booster might offer additional benefits in terms of cost-effectiveness, greater vaccine availability, and future pandemic preparedness, in addition to an improved reactogenicity profile. Furthermore, considering increasing global SARS-CoV-2 seroprevalence, fractional dosing may suffice for subsequent 'booster' vaccine doses in adolescents to bolster the immune response against infection, while allowing for more efficient use of vaccine supplies and schedule flexibility.

1.1.6 Impact of hybrid immunity on the immune response

SARS-CoV-2 infection prior to vaccination (i.e., hybrid immunity) has been shown to significantly influence the immune response to COVID-19 vaccination and to lead to greater protection against re-infection and disease severity.[40,41] In individuals with a previous history of SARS-CoV-2 infection, vaccination has been shown to induce an enhanced memory B cell (BMEM) and neutralising antibody response compared with infection naïve individuals.[42,43] In some cases, significant differences in the immune response between individuals with and without hybrid immunity following the primary series have been eliminated following receipt of a third (booster) vaccine dose, highlighting the importance of frequency of antigen exposure in shaping the immune response.[42] Correspondingly, in SARS-CoV2 infection naïve individuals, two doses of a COVID-19 vaccine were required to achieve an anti-spike antibody and BMEM response comparable to the response observed following one dose in individuals with a prior history of infection.[44] It is unknown whether the immune memory pool is more significantly influenced by the quality of antigen exposure (i.e., through natural infection or vaccination) or the frequency of that exposure. Previously infected children (aged-5-to-11-years) have been shown to generate increased spike-specific BMEM with activity against VOCs after mRNA-based COVID-19 vaccination compared

to infection naïve children.[45] COVID-19 vaccine-induced immune persistence and BMEM responses following heterologous vaccination in an adolescent population after second and third COVID-19 vaccine doses, and the effects of previous SARS-CoV-2 infection as well as breakthrough infection on the immune response have not yet been explored.

1.1.7 Immune responses in adolescents to SARS-CoV-2 infection and vaccination

Little data exist which specifically examine the immune response in adolescents to SARS-CoV-2 infection and/or vaccination. In children aged between 0 and 18 years, SARS-CoV-2 antibodies were detected up to one year after infection with highest levels detected in those with severe disease at presentation.[46] The study did not, however, differentiate between children and adolescents, a frequent occurrence in published studies. Similarly, Nantel et al. comparatively evaluated the adaptive immune response to SARS-CoV-2 infection between children (aged 3-17 years) and adults (19-62).[47] While binding and neutralising antibodies to SARS-CoV-2 were equivalent between both age groups, a lower frequency of spike-specific T cells was detected in children compared with adults. Interestingly, the magnitude of the cell-mediated immune response in children was proportional to that measured against seasonal beta coronaviruses. In contrast in adults, a significantly higher T cell response to SARS-CoV-2 was detected relative to that observed to the beta coronaviruses, HCoV-HKU1 and HCoV-OC43.[47] This study did not differentiate between children, adolescents, and adults in its analysis of the immune response.

Children and adolescents have been shown to possess an abundance of IgM Bmem.[48] Indeed, immunological memory is acquired through progressive antigen encounter accumulated with age and which underlies the high risk of infection and mortality associated with infancy.[48] In adults, half of circulating B cells are Bmem.[49] In children, this is estimated to be nearer to 18% and

increases with age, reaching a plateau between 12 and 18 years.[50,51] In infants, most circulating Bmem are unmutated and have few somatic hypermutations. However, additional somatic hypermutations are acquired with age and by approximately seven years of age, adult levels of IgM and class-switched Bmem have been achieved.[52]

Children and adolescents have been shown to demonstrate stronger innate immune responses and possess a higher total number of T and B lymphocytes.[53] Indeed, a progressive decline in lymphocyte numbers has been observed between infancy and adulthood.[53] In one study, CD3+ T cells were shown to increase between infancy and adolescence and to decline in elderly individuals. CD8+ T cells followed a similar pattern while CD4+ T cells declined between infancy and adolescence and rose again in adults. CD19+ B cells meanwhile declined progressively with age from childhood into adolescence and adulthood.[53]

An age-related decline in immunogenicity has also been observed following SARS- vaccination, with neutralising antibody titres almost two-fold higher in 12 to 15 year olds compared to those aged 16 to 25 year.[54] In a study comparing the humoral immune response in adolescents (aged 12 to 16 years) with adults (aged 32 to 52), significantly higher spike-specific IgG antibody responses were detected in adolescents following vaccination with BNT162b2.[55] These findings are consistent with immunosenescence (“inflammaging’), the gradual decline in the immune response with age, and which has been associated with reduced macrophage activation and neutrophil activity, as well as reduced cell-mediated immunity due to thymic atrophy.[56] A greater understanding of how these changes in the immune response with age influence vaccine-induced immunity is needed to guide vaccination strategies for the paediatric population, and may, in turn, inform future pandemic preparedness efforts.

1.2 Memory B cell response to SARS-CoV-2

Successful vaccination aims to induce durable immunological memory. Immunological memory is a key component of the adaptive immune system and refers to the rapid, potent, high affinity and specific immune response generated on secondary exposure to an antigen previously encountered. Long-lived plasma cells (LLPCs) and BMEM comprise the immunological memory compartment of the humoral immune system. BMEM have been shown to play a powerful role in the defence against infection, particularly against viruses.[57] Indeed, BMEM have been shown to confer protection against infection on repeat encounters with mutant virus strains.[58]

A key characteristic of B cells is their capacity to generate an astonishing range of highly specific immunoglobulins capable of recognising almost any antigen. This is due to the hugely diverse nature of their variable (V) regions. The B cell receptor (BCR) is composed of the same genes which encode the antibodies produced by a B cell upon activation and therefore the BCR is also referred to as surface immunoglobulin or a B-cell antigen receptor. The structure of an antibody molecule is depicted in **Figure 1.2**. The BCR is formed by a membrane-bound antibody. An antibody has two identical heavy chains and two identical light chains with a constant and variable region present on each chain. The antigen-binding site is present on the variable regions of the chains [fragment antigen binding (Fab) region]. The constant region of the heavy chains (i.e., the 'stem' of the antibody) determines the effector function of the antibody [fragment crystallisable (Fc)]. The V (variable), D (diversity) and J (joining) genes encode the variable region of the antibody and are assembled through VDJ recombination within the hypervariable regions of the variable region [also referred to as the complementarity-determining regions (CDRs)]. This combinatorial process determines the antigen binding specificity as well as the remarkable breadth of diversity achievable in BCR formation. The antibody repertoire of an individual represents the complete number of antibody specificities possible and is determined not only by the total number of B cells present but also by previous antigen encounters.[59]

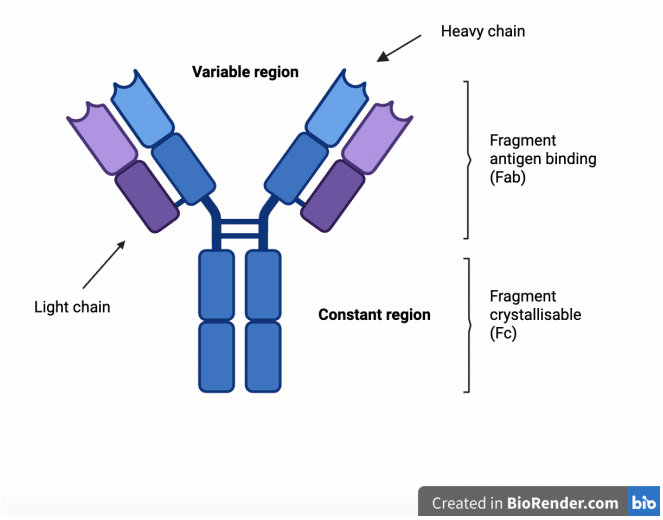


Figure 1.2 Illustration of an immunoglobulin molecule.

Antigen encounter with naïve B cells in secondary lymphoid organs precipitates B cell activation. Activated B cells then express CCR7 and EB12 that direct B cells to the border of the T-cell zone.[60] On encounter with the activating peptide presented on the B cell surface as part of an MHC class II complex, T helper cells differentiate into T follicular helper cells [T(FH) cells] which express receptors and cytokines that activate B cells and enhance B cell proliferation. Among these, CD40 ligand has been shown to play a role in the differentiation of activated B cells into BMEM.[61] Interleukin-21 (IL-21) activates the transcription factor STAT3 which stimulates B cell proliferation and is important in germinal centre (GC) development.[62] The proliferating activated B cells migrate to a primary lymphoid follicle along with T(FH) cells and form a germinal centre (i.e., a secondary lymphoid follicle).

The germinal centre is a specialised site within the follicle where B cell proliferation, somatic hypermutation, and selection for antibody affinity take place. B cells undergo somatic hypermutation within the dark zone of the GC. During this process [triggered by the enzyme activation-induced cytidine deaminase (AID)], mutations are introduced in the V genes of the V region BCR, generating antibodies with improved antigen binding.[63] Positive selection of B cells

takes place in the light zone of the GC. During this competitive selection process, BCRs with the highest affinity are directed towards clonal expansion and differentiation into plasmablasts and plasma cells.[63,64] GC B cells exit the GC as either plasmablasts (short-lived), LLPCs, or differentiate into BMEM. BMEM remain in circulation and on antigen encounter, re-enter GCs and undergo further rounds of affinity maturation.[65] There is evidence to suggest that BMEM leave the GC early and have lower affinity compared with other GC B cells.[66] This has been postulated to confer BMEM with enhanced flexibility and a broader capacity to recognise and respond to related antigens.[67] However, the mechanism by which differentiation into GC BMEM or LLPCs is determined has not yet been established.[63,68,69]

Notably, class-switch recombination takes place prior to B cell GC entry.[70] During this stage triggered by the enzyme AID, antibody function is determined by switching B cell immunoglobulin from the initial IgM to one of four other classes ('isotypes'): IgG, IgA, IgD, and IgE.[71] This takes place through a series of 'breaks' and recombination in the double-stranded DNA in "switch regions" of Ig resulting in changes to the heavy chain constant region gene.[63] It is not known what factor(s) determine which Ig heavy chains are exchanged, though BACH2 (a B cell specific transcription factor) is believed to play a role.[72]

The result of this elaborate process is the production of B cells with the ability to provide both a rapid (though transient) antibody response as well as the formation of B cells capable of differentiating into LLPCs (which take up residence in the bone marrow and can secrete neutralising antibodies for several decades) and BMEM.[73] Here, LLPCs provide durable immunity against reinfection through the production of high-affinity antibodies with evidence suggesting that LLPCs act as an initial layer of defence against re-infection.[57,74] In contrast, BMEM, also capable of producing mature, high affinity antibodies, have been shown to play a prominent role in defence during breakthrough infections, particularly on exposure to variant virus strains.[75] Indeed, on exposure to a variant strain, cross-reactive BMEM differentiate into antibody-secreting plasma cells or re-enter GCs and undergo further rounds of somatic hypermutation and affinity

maturation.[76] However, recent evidence suggests that LLPCs specific for SARS-CoV-2 were not maintained in the bone marrow up to 33 months following (mRNA) vaccination.[77] In this study by Nguyen et al., SARS-CoV-2 specific antibody secreting cells (ASCs) in LLPCs were measured following SARS-CoV-2 infection and vaccination and the results compared with vaccination-induced influenza and tetanus specific ASCs. The results showed a stark absence of SARS-CoV-2-specific ASCs in LLPC compartment following vaccination and an association between serum anti-spike IgG and IgG in non-LLPCs.[77] These findings highlight the importance of BMEM in sustaining immune protection following infection and vaccination in the absence of SARS-CoV-2-specific LLPCs in the bone marrow. Authors have also speculated that LLPCs arise from BMEM but in the context of SARS-CoV-2, fail to develop into LLPCs.[77,78]

While antibodies are essential to vaccine efficacy, physiological decline occurs after every vaccination.[79] The effectiveness of immune memory and the 'recall response' at this stage become essential. Indeed, the generation of immune memory is an essential component to the development of a successful vaccine. However, the mechanisms by which immune persistence can be optimised through COVID-19 immunisation are still under investigation. Many vaccines (and indeed infections) can generate lifelong immunity (though this is not necessarily mediated through humoral immunity). Classic examples include the measles and smallpox vaccines while inactivated Influenza vaccines require annual boosters due to significant decline in bone marrow Influenza-specific LLPCs.[80] However, the duration of immune memory following SARS-CoV-2 infection and immunisation is still unclear and a focus of active research. Most literature to date reports findings from studies carried out in adult participants. Following three dose mRNA vaccination in adults, SARS-CoV-2 specific BMEM were maintained up to 17 months after the third dose. RBD-specific BMEM were also associated with a lower incidence of breakthrough infection. Furthermore, a significant increase in SARS-CoV-2 spike-specific BMEM (and in RBD-specific BMEM) was observed at 5 months after the third dose compared with 5 months after the second dose. During this period, the RBD BMEM response increased 1.6-fold and 3.1-fold in previously infected individuals (pre-vaccination) and infection naïve participants, respectively. A further increase by

1.9-fold in infection naïve participants by 17 months after the third dose suggests a qualitative difference in the BMEM response according to infection history.[81] The finding of an expanded SARS-CoV-2-reactive $CD27^-$ $CD21^-$ atypical B-cell population during study follow up in previously infected participants further supports the hypothesis of a qualitative difference in the immune response according to infection history.

Scant data exist relating to BMEM responses in children and adolescents following COVID-19 vaccination. In healthy 5–12-year-olds following a two dose BNT162b2 primary series, spike-specific BMEM increased out to 6 months after immunisation. A third dose (given to 9 children after 1 year of follow up) resulted in no significant change in the BMEM response, suggesting age-dependent differences in BMEM responses to vaccination (though a very small number of participants received the booster dose in this study). Furthermore, no significant difference in the BMEM response was observed between participants with infection only compared with participants with infection who had received three vaccine doses by 6 months following vaccination. Furthermore, this study demonstrated SARS-CoV-2 spike-specific BMEM to be a correlate of protection against symptomatic SARS-CoV-2 infection following the primary immunisation series.[82] In children (aged up to 17 years) with chronic hepatitis B, RBD-specific BMEM were shown to remain stable over time after a two-dose primary series of inactivated SARS-CoV-2 vaccines (BBIBP-CorV/CoronaVac) and BMEM responses were significantly higher in children compared with adults.[83] However, no study has examined BMEM responses to COVID-19 vaccination in a healthy adolescent population.

Furthermore, studies have yet to examine BMEM responses following heterologous vaccination in adults or adolescents and to investigate the response according to vaccine platform and doses received (primary versus booster immunisation). Considering the significant differences in BMEM evolution over time which have been shown to take place between childhood and old age, such focussed research into COVID-19 vaccine-induced humoral immune responses in adolescents could also yield important insights in immunological memory formation in this age group.[84]

1.3 Mucosal antibody responses to SARS-CoV-2

The mucosal surface is not only the initial site of SARS-CoV-2 invasion but is also the site of entry for multiple respiratory pathogens and is thus, extremely vulnerable to infection. Fortunately, in addition to possessing a physical epithelial barrier, the mucosa has both innate and adaptive components to its immune defence system, the latter supported by mucosa associated lymphoid tissue (MALT).[85] Mucosal humoral immunity is mediated mainly through secretory IgA which exists in a dimeric form in mucosal secretions and a monomeric form in serum.[86] IgA consists of two isotypes: IgA1, present in systemic and mucosal secretions, and IgA2, which is found mostly in the mucosa.[87] IgG, which has also been detected in saliva and other mucosal secretions, has been shown to seep from the systemic circulation into the mucosa.[88] However, compared with both IgG and monomeric IgA, dimeric secretory IgA has been shown to possess more potent neutralising antibody activity against SARS-CoV-2.[89] Additionally, mucosal IgA has been shown to correlate with virus neutralisation while mucosal IgG has been shown to correlate with virus phagocytosis. Indeed, mucosal IgA responses have been associated with protection against influenza, respiratory syncytial virus (RSV) and SARS-CoV-2 infection.[90-92] If an effective and sterilising mucosal humoral immune response could be induced, it would have the potential to prohibit both initiation of viral invasion and eliminate transmission.[93] The mucosal immune response therefore has the potential to prevent viral entry and induce protective immunity against SARS-CoV-2.

On antigen exposure, the mucosal antibody response is initially comprised of IgM, IgA and IgG which correlate with serum antibody levels.[94] A more rapid decrease in mucosal secretory IgA compared with mucosal IgG levels has been illustrated following infection, though some studies have also detected mucosal IgA up to nine months after infection.[95,96] Mucosal IgA has also been

shown to dominate the neutralising antibody response to SARS-CoV-2, further emphasising its important role in defence against infection.[97] However, most studies investigating the mucosal immune response to vaccination suggest that vaccination induces minimal mucosal secretory IgA without a prior history of SARS-CoV-2 infection.[98]

Though COVID-19 vaccination has been shown to induce robust systemic immune responses, much less is known in relation to mucosal immunity. Evidence to date from studies performed in adults suggests that vaccination generates little mucosal secretory IgA in the absence of priming through infection.[98] The generation and persistence of antibody in mucosal tissue and the mechanism by which systemic vaccination could induce an effective immune response in the mucosa have not been determined.

Little data pertaining to the mucosal immune response in children and adolescents to COVID-19 immunisation exist. In children aged between 5 and 11 years following a primary BNT162b2 immunisation series, a significant increase in salivary RBD-specific IgA1 was detected while infected but unvaccinated children demonstrated a significant increase in RBD-specific IgA2 displaying specificity in the pattern of the mucosal immune response elicited depending on the type (or route) of antigen exposure.[99] The study however, did not explore the longer-term maintenance of the mucosal immune response beyond 10 days after the second dose nor the spike-specific mucosal IgG response. In children younger than 5 years, a SARS-CoV-2 specific IgG response was detected within 2- and 4-weeks following mRNA-based vaccination (up to 3 doses received) in saliva samples while, similar to findings in adult studies, significant induction of spike-specific IgA occurred only in the setting of prior SARS-CoV-2 infection.[100] Similarly, stimulated tonsillar mononuclear cells (using positive SARS-CoV-2 nasopharyngeal and oropharyngeal swabs) demonstrated significantly increased anti-spike (S1) IgG and anti-RBD IgG compared with unstimulated cells. Samples were collected from 11 children aged between 3- and 14-years undergoing elective tonsillectomy. Almost all children had a previous history of SARS-CoV-2 infection, and most children were unvaccinated (8/11). Interestingly, significantly higher levels of

neutralising antibodies against both wild-type and Omicron BA.1 were detected in stimulated versus unstimulated samples in all children, including those with no history of infection. Similar findings were also detected in the stimulated tonsillar mononuclear cells of previously infected and vaccinated adults when tested.[101]

However, age specific differences in mucosal immunity have also been demonstrated between children (younger than 12 years) and adults (aged > 30 years) in nasal epithelial cells in response to SARS-CoV-2 infection.[102] In this study by Woodall et al., paediatric infected cell cultures demonstrated increased expression of interferon-stimulated genes and partial viral replication while cells which promote viral spread were found in adult infected cell cultures. Differences in mucosal immune responses between children and adults have also been detected in relation to mucosal antibody responses to SARS-CoV-2 and seasonal coronaviruses (hCoV). In children aged between 4 and 15 years with a prior history of SARS-CoV-2 infection, who had received at least one dose of a COVID-19 vaccine, SARS-CoV-2 mucosal spike IgA titres were lower in children compared with adults (who had received between 2 and 4 doses of a COVID-19 vaccine). Furthermore, mucosal hCoV IgA titres were significantly higher in adults compared with children. In contrast mucosal IgA titres specific for respiratory syncytial virus and Influenza were similar between adults and children. However, the neutralising capacity of the (spike-specific) mucosal IgA antibodies detected was similar between children and adults despite quantitative differences in the response detected. Furthermore, SARS-CoV-2 infection substantially boosted mucosal IgA responses.[103]

Authors have suggested that differences in hCoV immunity between children and adults may account for differences in age-dependent responses to SARS-CoV-2 infection and vaccination. Antibody responses to seasonal coronaviruses are short-term and re-infection occurs frequently.[104] Higher levels of cross-reactive antibodies between hCoV and SARS-CoV2 occur in children and adolescents and are likely the result of the high degree of homology which exists between SARS-CoV2 and seasonal hCoV.[105] Additionally, neutralising antibodies to SARS-CoV2 have been found

in unvaccinated children without prior SARS-CoV2 exposure.[105] However, the importance of such cross-reactivity in the immune response to infection and vaccination is unclear. It has been hypothesised that more recent exposure to HCoV may account for the differences in clinical presentation following SARS CoV2 infection in the paediatric population and may also play a role in the immune response to vaccination and other VoCs.[106,107] However, no data exist relating to hCoV serum and mucosal antibody responses following primary and booster immunisation in adolescents and whether an association exists between pre-existing hCoV immunity and COVID-19 vaccine-induced immunogenicity.

1.4 Antibody avidity response to SARS-CoV-2

Affinity is defined as the strength of binding between an antibody and a specific target epitope on the surface of an antigen and avidity as the cumulative strength of binding between a multivalent antibody (i.e., an antibody with multiple binding sites) and multiple epitopes on the antigen surface.[108,109] As discussed previously, B cell GC are specialised sites of B cell proliferation, expansion and affinity maturation. Affinity maturation results from repeated rounds of somatic hypermutation in GCs (initiated by AID, essential to this process[110]), strengthening binding between an antibody and its target epitope (antigen). Mutations introduced by AID result in point mutations in the genes of Ig variable regions resulting in sequence diversity.[111] During this Darwinian selection process, B cells are tested against the target antigen for binding (affinity) and positively selected out for further proliferation and clonal expansion based on affinity.[63] This results in the generation of antibodies with high affinity and high avidity necessary to ensure tight antibody-antigen binding interactions and optimal antibody functionality. Repeated GC antigen presentation further enhances affinity and avidity maturation.[112] Indeed, the production of high affinity antibodies underlies the anamnestic response capabilities of BMEM.[113]

Antibody avidity has been shown to play an important role in determining antibody effector functionality.[114,115] Antibodies with low avidity have been associated with failure of protection against infection and disease and high avidity antibodies have been suggested as a reliable marker of vaccine efficacy.[116-118] Several studies have demonstrated the importance of high avidity antibodies in the defence against viral infection. This includes protection against cytomegalovirus (CMV) with low avidity antibodies associated with intrauterine transmission of the virus.[119] High avidity antibodies in this study were also shown to perform an essential role in the generation of high levels of neutralising antibodies, a prominent feature of the humoral defence against infection.[116] Indeed, authors have suggested that effective neutralisation of SARS-CoV-2 necessitates the production of neutralising antibodies with high avidity.[120] High affinity serum antibodies have also been associated with highly effective vaccines.[121] Correspondingly, studies have also suggested that cases of measles vaccine failure can be identified through measurement of IgG antibody avidity.[122,123] Similarly, low varicella zoster virus antibody avidity IgG has been associated with repeated infection.[124]

Most studies to date have focussed on antibody avidity responses in adults to COVID-19 vaccination using mRNA-based vaccines. A two-dose BNT162b2 primary series has been shown to induce antibodies with high avidity in adults while a third (booster) dose resulted in antibodies with higher avidity and greater neutralising capacity against both wild-type and Omicron variant SARS-CoV-2 strains.[108,125] With respect to adenovirus-based vaccine boosters, intranasal Salnavac® booster and intramuscular (IM) Sputnik V when administered as booster doses were shown to elicit comparable avidities of serum antibodies. However, while increased avidity was observed for both wild-type and Delta variants following booster immunisation, no increase was observed for Omicron BA.4 and BA.5.[121] Hybrid immunity has also been shown to exert a substantial influence on avidity maturation with significantly higher avidity levels observed following COVID-19 vaccination in individuals with hybrid immunity compared with infection naïve individuals following the primary series (mRNA) immunisation.[126] In this and other studies,

avidity has been shown to continue to increase up to 6 months following the primary immunisation series however, while avidity is initially significantly increased following receipt of a booster dose, no change in avidity has been found between 3 and 6 months following a booster dose.[126,127]

Little data exist regarding antibody avidity responses in children and adolescents following COVID-19 vaccination. A study by Yang et al. revealed striking differences in antibody responses between children, adolescents and adults following SARS-CoV-2 infection with a negative correlation detected between anti-spike IgG, neutralising antibodies, and antibody avidity and age in a sub-cohort aged between 1 and 24 years. Although children demonstrated significantly higher antibody and neutralising antibody responses compared with adolescents and young adults, antibody avidity was not significantly different between children and adolescents.[128] In convalescent (unvaccinated) children (aged up to 18 years), frequencies and avidity of wild-type, Delta and Omicron reactive CD4 + and CD8 + T cells were shown to be similar to convalescent, vaccinated (between one and two doses received) adults, though neutralising antibodies remained significantly higher in convalescent vaccinated adults compared with (unvaccinated) children.[129] In a study which examined avidity maturation following COVID-19 vaccination in the paediatric population, adolescents who received 3 doses of CoronaVac COVID-19 vaccine (Sinovac COVID-19 vaccine, whole inactivated virus COVID-19 vaccine) administered via the IM or intradermal (ID) route, demonstrated a higher antibody avidity response after 3 doses compared with 2 doses (irrespective of vaccine administration route). Antibody avidity was significantly greater in the IM group compared with the ID group following two doses while avidity was significantly higher in the ID group following three doses.[130]

Avidity has been postulated as a method to assess vaccine success. In the setting of malaria, a model using avidity predicted vaccine efficacy against infection.[118] Similarly, in the context of pneumococcus, antibody avidity was shown to be an important determinant of vaccine efficacy and high avidity antibodies were shown to be more effective than low avidity antibodies in pathogen opsonisation and phagocytosis.[131] No study to date has compared the antibody avidity response in

adolescents between homologous and heterologous COVID-19 primary and booster immunisation schedules or examined the avidity response to mRNA-based vaccination in adolescents.

1.5 Aims

This DPhil set out to investigate COVID-19 vaccine-induced immunity in the adolescent population, to characterise the humoral immune response to homologous and heterologous COVID-19 vaccination in adolescents and to explore correlates or markers of "efficacy" using these novel vaccine schedules. This was achieved through the following aims:

1.5.1 Aim 1

To complete a phase II randomised controlled trial (RCT) in healthy adolescents to determine reactogenicity and immunogenicity of second and third dose homologous, heterologous and fractional dose COVID-19 vaccine schedules with collection of safety, reactogenicity and immunogenicity data over a ten month follow up period.

1.5.2 Aim 2

1) To characterise the BMEM response to vaccination against SARS-CoV-2 in adolescents using an ELISpot (FluoroSpot) assay and to compare the BMEM responses induced following a

primary and booster immunisation series, and according to vaccine schedule (i.e., homologous v heterologous, vaccine platform) received.

2) To examine the BMEM responses to seasonal coronaviruses (OC43, NL63) and the effect of hybrid immunity and breakthrough infection on the BMEM response to SARS-CoV-2 spike (ancestral), Delta (B.1.617.2), and seasonal coronaviruses (OC43, NL63) following immunisation.

1.5.3 Aim 3

1) To determine to what extent anti-spike mucosal IgA and IgG antibodies are induced by homologous, heterologous, and fractional dose schedules in adolescents and the effect of prior SARS-CoV-2 infection and breakthrough infections on the immune response observed.

2) To examine mucosal and serum IgA and IgG responses to hCoV OC43, HKU1, NL63, and 229E following a primary and booster COVID-19 immunisation series in adolescents.

3) To determine the effect of pre-existing (pre-vaccination) hCoV mucosal and serum antibodies on the humoral immune response to two or three doses of a COVID-19 vaccine in adolescents.

1.5.4 Aim 4

1) To assess the antibody avidity response to homologous and heterologous primary and booster COVID-19 vaccine schedules in adolescents.

2) To determine the effect of prior SARS-CoV-2 infection and breakthrough infection on antibody avidity maturation following primary and booster COVID-19 vaccination in adolescents.

3) To examine whether a correlation exists between antibody avidity and neutralising antibody responses to ancestral SARS-CoV-2 and SARS-CoV-2 variants following primary and booster COVID-19 vaccination.

1.5.5 Aim 5

To determine whether levels of BMEM, mucosal antibodies, neutralising antibodies or total binding antibodies to SARS-CoV-2 induced following primary and booster COVID-19 immunisation in adolescents represent immune biomarkers of vaccine-induced protection against SARS-CoV-2.

Chapter 2: Materials and Methods

2.1 Buffers

2.1.1 Phosphate Buffered Saline (PBS)x1 for ELISpot plate wash

Five phosphate buffered saline (PBS) tablets (P4417, Merck-Life Technologies, Sigma, Massachusetts, USA) were dissolved in 1000ml sterile, pyrogen free water (UKF7114, Baxter, USA).

2.1.2 35% Ethanol for pre-wetting ELISpot well membrane

35% ethanol was prepared by diluting 3.5ml of pure ethanol (>99.5%) in 6.5ml of deionised water (32221, UN 1170, Sigma-Aldrich, Massachusetts, USA) and stored at room temperature (Milli-Q® Ultrapure water, Sigma-Aldrich, Massachusetts, USA). The volume was adjusted depending on the number of plates prepared.

2.1.3 PBS coating buffer (ready-made) for ELISpot, ELISA, and avidity immunoassay plates

Dulbecco's phosphate buffered saline (DPBS; without calcium chloride, magnesium chloride, sterile-filtered liquid) was used as the coating buffer for the ELISpot, ELISA and avidity

immunoassay plates. It was stored at room temperature and kept sterile (D8537, Sigma-Aldrich, Massachusetts, USA).

2.1.4 Cell wash buffer (ready-made) for ELISpot

Automacs Running Buffer (PBS-EDTA and 0.5% BSA) was used as a cell wash buffer following the 72-hour stimulation period and cell harvest (Miltenyi Biotech, 130-091-221, Germany).

2.1.5 PBS + 0.05% Tween®20 (PBS-T)/plate wash for MSD, ELISA and Avidity immunoassays

Five PBS tablets (P4417, Merck-Life Technologies, Sigma, Massachusetts, USA) were dissolved in 1000ml of deionised water and 0.5ml of Tween®20 detergent (P7949, Sigma-Aldrich, Massachusetts, USA) was added.

2.1.6 MSD GOLD Read Buffer B

Meso Scale Discovery (MSD) GOLD Read Buffer B is provided ready to use as part of the MSD V-PLEX COVID-19 Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland). To each plate, 150µl/well of the MSD GOLD Read Buffer B was added immediately prior to reading the plate on the MSD instrument. No incubation period was required.

2.1.7 Extraction Buffer

Extraction buffer was used to extract mucosal lining fluid (MLF) which had adhered to a synthetic absorptive matrix (SAM) device [part of the Nasosorption FXi nasal sampling device (NSFL-FXI-13)] used to collect nasal fluid samples from participants as described in **section 2.4.4**. Extraction buffer was prepared by add 1.5g sodium chloride (NaCl, S7653, Sigma-Aldrich, Massachusetts, USA) and 0.2g of bovine serum albumin (BSA, A8022, Sigma-Aldrich, Massachusetts, USA) into a reagent bottle followed by 50ml of DPBS (D8537, Sigma-Aldrich, Massachusetts, USA). This was placed on a magnetic stirrer and allowed to mix. Once the NaCl and BSA were fully dissolved and no longer visible, 49ml of DPBS and 1ml (100x) of cOmplete™, EDTA-free Protease Inhibitor Cocktail (#C33843573, Merck KGaA, Darmstadt, Germany) were added to the solution. The solution was vortexed to mix and then filtered using a 0.22µm filter unit into a sterile reagent bottle. Extraction buffer was stored at 4^0C in the fridge for up to 5 weeks.

2.1.8 Blocking and Dilution buffer for ELISA and avidity immunoassays

Blocker Casein in PBS was used as a blocking and dilution buffer for ELISA (enzyme-linked immunosorbent assay) and avidity immunoassays (Blocker™ Casein in PBS, #37528, Thermo Fisher Scientific, UK).

2.1.9 Development buffer for the ELISA and avidity immunoassays

The development buffer for the ELISA and avidity immunoassays was made by adding one 20mg pNPP tablet (4-nitrophenyl phosphate tablet, Sigma-Aldrich, USA, N2765) to 4ml 5X diethanolamine (DEA) substrate buffer in 16ml of sterile water (#3500, Sigma-Aldrich, USA) and mixed thoroughly. The developer was made just before use and wrapped in foil once made (and protected from light until use).

2.2 Reagents

2.2.1 B cell FluoroSpot kit for the detection of IgG

The B cell FluoroSpot kit for the detection of IgG was used in the B cell FluoroSpot assay (#X-06G05R-10, Mabtech, UK). The following were supplied as part of the kit: capture antibody IgG (clone MT91/145, 0.5mg/ml), detection antibody IgG-550 (MT78/145), 240µl, FluoroSpot plates (IPFL plates), and fluorescence enhancer. These antibodies and regents have been outlined in detail in the following sections.

2.2.2 Fluorescence enhancer for the ELISpot assay

The FluoroSpot fluorescence enhancer increases the spot quality in the FluoroSpot assay. It contains 0.002% Kathon CG (a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one) and is supplied ready to use as part of the Mabtech human IgG FluoroSpot kit (#X-06G055R-10, Mabtech, UK).

2.2.3 Immunostimulatory agents: R848 and IL-2

R848 (TLR 7/8 agonist) and IL-2 are immunostimulatory agents that can stimulate activation and proliferation of B cells. They are supplied as part of the Mabtech B cell stimulation kit (#3660-1, Mabtech, UK) as 1mg/ml in 100µl (R848) and 1.0µg/ml in 1ml (IL-2). IL-2 required reconstitution by adding 1ml distilled water (15230-162, Gibco, UK) to obtain 1µg/ml. To make the simulation mix, R848 was diluted to 1:500 to give 2µg/ml (final well concentration 1µg/ml) and IL-2 was diluted to 1:50 to give 20ng/ml (final well concentration 10ng/ml).

2.2.4 Complete Medium (CM), sterile

Fifty millilitres was discarded from 500ml of RPMI-1640 with phenol red indicator and 25mM hepes modification, without L-glutamine (Merck-Life Technologies; R-5886) and to which 50ml of batch tested foetal bovine serum-heat inactivated (FBS-HI) was added (#F9665, Merck-Life Technologies, Germany). To this was added the following: 5ml penicillin-streptomycin solution (Merck-Life Technologies P-4458, Germany), 5ml L-glutamine (Merck-Life Technologies, Germany, G-7513; replenished after 14 days), 5ml MEM NEAA [MEM (minimum essential medium) non-essential amino acids (NEAA), (100x); Merck Life Technologies, Germany, 11140035), 5ml sodium pyruvate (100mM, Merck Life Technologies, Germany, 11360039), and 500µl 2-mercaptoethanol (50mM, Merck Life Technologies, Germany, 31350010). The medium was stored at 4^0C for up to one month. It was warmed to room temperature or 37^0C and checked for contamination before use.

2.2.5 Cell Count and Viability kit

The Muse Count & Viability Assay Kit (MCH600103, Cytek, California, US) was used for the quantitative analysis of cell count and viability on the Guava Muse cell analyser during the ELISpot assay. To 380µl of the Cell Count & Viability reagent supplied ready-made as part of the kit was added 20µl of the peripheral blood mononuclear cell (PBMC) cell suspension (detailed in **Section 2.4.2.3**).

2.2.6 MSD V-PLEX COVID-19 Coronavirus Panel 3 (IgG) kit

The MSD V-PLEX COVID-19 Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland) is a multiplex assay that can be used to measure IgG and IgA antibodies to nine SARS-CoV-2 related antigens: SARS-CoV-2 nucleocapsid, SARS-CoV-2 spike (wild-type), SARS-CoV-2 S1 receptor binding domain (RBD), SARS-CoV-1 spike, human coronavirus (hCoV)-HKU1 spike, hCoV-OC43 spike, hCoV-NL63 spike, hCoV-229E spike, and MERS-CoV spike. A BSA control is also supplied as part of the kit. The following reagents are provided: Serology Control 1.1, Serology Control 1.2, Serology Control 1.3, Reference Standard 1, SULFO-TAG anti-human IgA and SULFO-TAG anti-human IgG, Coronavirus Plate 3, Diluent 100, MSD GOLD Read Buffer B, MSD Blocker A, and MSD Phosphate Buffer 5X.

Three levels of Serology Controls containing assigned concentrations of human IgG and IgA against the antigens in the V-PLEX COVID-19 kit are supplied. The Serology Controls are provided at working concentration and do not require dilution prior to use. The Reference Standard 1 is used to establish a calibration curve in the assay to calculate the concentration of IgG and IgA responses to antigens contained in the kit. A 7-point calibration curve is created using a 4-fold serial dilution. The plates (Coronavirus Plate 3) are supplied pre-coated with 9 antigens and a BSA control coating a 10-spot MULTI-SPOT® 96-well plate.

2.2.7 Diluent 100

Diluent 100 is a reagent supplied as part of the MSD V-PLEX COVID-19 Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland) and used as a diluent for samples, calibrators (Reference Standard 1), and samples as part of the MSD immunoassay described in **section 2.4.3**.

2.2.8 MSD Blocker A solution

MSD Phosphate Buffer (5X) (R93SA-2) and MSD Blocker A (R93BA-2), supplied as part of the MSD V-PLEX COVID-19 Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland), were used to prepare the MSD Blocker A solution. The MSD Blocker A solution consists of a mixture of BSA (MSD Blocker A dry powder) in a PBS-based buffer (MSD Phosphate Buffer) that has been optimised for use in the MSD MULTI-SPOT assay. The solution blocks non-specific binding of proteins to the plate surface, reducing background in the assay and improving assay sensitivity. The MSD Blocker A solution was prepared by adding 200ml of deionised water to the MSD Blocker A dry powder (provided in a 250ml bottle). A magnetic stir bar was added and the solution placed on a magnetic stirrer until all the protein was resuspended. This step took between 30 minutes and 2 hours. Once all the protein had dissolved, the solution appeared pale yellow and clear. The entire contents of the MSD Phosphate Buffer (5X) bottle (i.e., 50ml) were then added to the 250ml bottle containing the MSD Blocker A and deionised water solution and stirred for an additional 10 minutes. The MSD Blocker A solution was next vacuum filtered using a 0.2µM filter into a clean storage container and stored at 4^0C. The MSD Blocker A solution was stable for 5 weeks following reconstitution.

2.2.9 70% v/v Ethanol

300ml of distilled water (15230-162, Gibco, UK) was added to 700ml of 100% ethanol (32221, Sigma-Aldrich, USA) and stored at room temperature.

2.2.10 Recombinant Coating Protein for ELISA and avidity immunoassays

SARS-CoV-2 stabilised full-length spike GP trimer was used as the coating antigen for the ELISA and avidity immunoassays (REC31966-500; Lot: 21051410P, Native Antigen Company, UK). It was stored at -80^{0}C in 33µl aliquots (0.670 mg/ml stock concentration). Each aliquot provided enough antigen to coat 4 plates.

2.2.11 2% Virkon

Four Virkon tablets were added to one litre of water (Rely+On™ Virkon™ tablets, Lanxess, US).

2.3 Antibodies

2.3.1 The ELISpot assay

2.3.1.1 Coating of the ELISpot plates (refer to section 2.4.2.5)

For total immunoglobulin (Ig) IgG coated wells, the capture antibody used in the ELISpot assay was a monoclonal mouse anti-human Ig (Mabtech, UK) IgG (clone MT91/145, 0.5mg/ml) supplied

in PBS with 0.02% sodium azide. The capture antibody was supplied as part of the Mabtech human IgG FluoroSpot kit (Mabtech, UK, #FS-050617-10).

The coating antigens used in the ELISpot assay were: SARS-CoV-2 stabilised spike glycoprotein (full-length), His-Strep-Tag (HEK293) (#REC31966-500, Native Antigen Company, UK), a recombinant antigen containing 6 mutations in the S2 domain of the spike protein which stabilises the protein as a pre-fusion trimer and supplied in DPBS; SARS-CoV-2 nucleoprotein, His-Tag (E.coli), a recombinant antigen expressed and purified from E. coli as full-length nucleoprotein presented in 20mM sodium phosphate, 25mM potassium carbonate pH10.0, 150mM NaCl (0.71mg/ml, #REC31851-500, Native Antigen Company, UK); human coronavirus (hCoV) OC43 Spike Glycoprotein (full-length), Sheep Fc-Tag (HEK293) a recombinant HCoV-OC43 nucleocapsid protein manufactured in E. coli cells and supplied in 20mM phosphate monobasic, 25mM potassium carbonate pH 10.0, and 150mM NaCl (4.17mg/ml, #REC31857-500, Native Antigen Company, UK); SARS-CoV-2 Delta (B.1.617.2; AY.1, AY.2, AY.3) stabilised spike glycoprotein (trimeric) His-Strep-Tag (HEK293) supplied in DPBS, His-Strep-Tag (HEK293), (this spike protein contains T19R, T95I, G142D, E156G, del157-158, W258L, N417K, L452R, T478K, D614G, P681R, D950N mutations) (1.41mg/ml, #REC31975-500, Native Antigen Company, UK); hCoV NL63 spike glycoprotein (full-length), sheep Fc-Tag (HKE293) a recombinant hCoV-NL63 spike protein, with sheep Fc-tag manufactured in HEK293 cells and supplied in DPBS pH7.4, (1.10mg/ml, #REC31879, Native Antigen Company, UK).

2.3.1.2 Antibodies for the detection of IgG antibody secreting cell spots

Antigen-specific IgG secreted by BMEM bound to the antigen-coated plates were detected by monoclonal antibody anti-human IgG-550 (MT78/145), Mabtech, UK. The detection antibodies used in the ELISpot assay were diluted to 1:500 in PBS containing 0.5% foetal bovine serum.

2.3.1.3 Anti-human IgG (γ-chain specific) alkaline phosphatase

Anti-human IgG alkaline phosphatase (produced in goat; A3187, Merck, Germany) was used as the detection antibody for the anti-spike IgG ELISA assay. The working concentration was 1:1000 dilution in Casein. This was generated by diluting 5µl of antibody in a final volume of 5ml of Casein (5ml required per plate, volume adjusted according to the number of plates prepared).

2.3.2 The MSD immunoassay

2.3.2.1 SULFO-TAG anti-human IgG/A antibody

The SULFO-TAG anti-human IgG (IgA antibody for the IgA assay) was provided as part of the MSD V-PLEX COVID-19 Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland). It is a recombinant monoclonal antibody that binds to human IgG (or IgA). Anti-human antibodies (IgG or IgA) conjugated with MSD SULFO-TAG™ label are used to detect antibodies bound to antigens on the spots in the wells. The plate is read using the MSD instrument which measures the light emitted from the MSD SULFO-TAG. The detection antibody is provided as a 200X stock solution. The working solution is 1X. To prepare a 1X solution of the detection antibody, 30µl of 200X SULFO-TAG anti-human Ig antibody (IgG or IgA) was added to 5970µl of

Diluent 100. For each plate, 6 ml of detection antibody was required. The quantity of antibody prepared was adjusted according to the number of plates prepared.

2.4 Laboratory methods

2.4.1 Sample collection and processing

Phlebotomy was performed using a 23G butterfly needle and syringe to ensure accurate aliquoting of samples and that the required volume of venous blood was collected from paediatric study participants. All blood samples were labelled with the participant ID number and the corresponding study timepoint. Site specific labels were supplied with a range of participant IDs for each site. Processing of serum samples took place at individual study sites. Following sample collection, serum samples were delivered to the laboratory and stored at 4°C until sample processing took place. All serum samples were processed within 24 hours of collection. Samples were centrifuged at 3000g for 15 minutes [or for 10 minutes if a gold-top vacuette tube (containing clot activator and separation gel) was used]. Serum samples were aliquoted and stored at -80°C before shipping to UK Health Security Agency (UK HSA) and Churchill Centre for Vaccinology and Tropical Medicine for further testing.

Lithium heparin blood samples were shipped directly to Oxford Immunotec without any local processing. Samples were transferred to Oxford Immunotec at room temperature. Due to the addition of T-cell *Xtend* reagent (added to extend PBMC survival), samples could be shipped on the day of collection or stored overnight at room temperature and processed (with T-cell *Xtend* reagent, as per the manufacturer's instructions) within 32 hours of venepuncture.

2.4.2 FluoroSpot assay for detection of antigen-specific IgG-memory B cells

2.4.2.1 Preparation of peripheral blood mononuclear cells

Sample processing for PBMC isolation and storage was performed at Oxford Immunotec. T-Cell Xtend reagent (Oxford Immunontec, UK) was added to blood samples (25µl/ml of blood) and incubated for 20 minutes prior to Ficoll separation. The reagent aids in the enrichment of specific T cell populations from whole blood which has been stored at room temperature prior to processing, (the maximum time between venepuncture and addition of T cell Xtend was 32 hours). The Ficoll separation (Ficoll : blood mixture = 1:3, centrifuged at 1800xg for 22 minutes at room temperature) results in a layer of enriched T cells which are removed for the IFNγ T cells ELISpot. Remaining PBMCs are rosetted out in the red blood cell layer (T cell Xtend crosslinks the non-T cells to RBCs). The red cell layer is then lysed and the resulting PBMCs washed, counted and cryogenically frozen in freezing media. PBMC samples were stored in liquid nitrogen (-196^{0}C) or ultra-low temperature freezer at < - 135^{0}C.

2.4.2.2 Defrosting of cryopreserved peripheral blood mononuclear cells from liquid nitrogen storage

Defrosting of cryopreserved PBMCs was performed in a microbiological safety cabinet (MSC) class II hood. In 15 mL falcon tubes, 10mL aliquots of complete medium (CM) and 2µL benzonase® nuclease (E1014, Merck-Life Technologies, USA) were warmed to 37^0C. Cryopreserved PBMCs were rapidly thawed at 37^0C using a water bath (beaker of warm water heated to 37^0C). When samples were almost thawed, 1mL of the warmed CM containing benzonase® nuclease (added to prevent cell clumping and improve cell recovery) was added to each cryovial and used to defrost the remaining ice in the tube. The contents were then transferred to the previously prepared 15mL falcon tube containing warmed CM and benzonase® nuclease. To optimise cell recovery and viability, defrosting of PBMCs was carried out within (max.) 10 minutes of defrosting. Samples were then centrifuged at 300g for 10 minutes at room temperature. This wash step was repeated following removal of the supernatant and the addition of 10mL of warmed CM.

2.4.2.3 Cell counts

PBMCs were counted prior to use in the FluoroSpot using the Guava Muse counter using the Cell Count and Viability kit, according to the manufacturer's instructions. Each PBMC sample was re-suspended using 2mL of CM and diluted (1:20) by adding 20µL of this cell suspension to 380µL of the Cell Count and Viability reagent. The gating template was set and a staining dilution factor of 1 in 20 chosen. The viable cells/mL, total viable cells (10^6) and % viability were recorded. To calculate the volume of CM required to dilute the cells to 2 x 10^6 cells/mL, the volume of total viable cells (10^6) was divided by 2 and the volume in which the cells were already suspended subtracted. PBMC viability was assessed and samples with >65% viability were retained.

2.4.2.4 Differentiation of Memory B cells into antibody secreting cells by polyclonal stimulation of B cells

BMEM require a differentiation step into antibody secreting cells (ASC) for detection via FluoroSpot. A stimulation mix (Mabtech B cell stimulation kit, #3660-1, Mabtech, UK) containing immunostimulatory agents R848 and IL-2 was prepared by diluting R848 in CM (1:500) yielding a concentration of 2µg/ml (final well concentration 1µg/ml), and by diluting IL-2 in CM (1:50), resulting in a concentration of 20ng/ml (final well concentration 10ng/ml). The stimulation mix was added to PBMCs in round bottom, tissue culture plates in a 1:1 ratio (i.e., 100µl each of cell suspension and stimulation mix added to each well for each sample). The cell concentration was 200,000 cells/well. Plates were incubated at 37^0C with a CO2 level of 5% and 95% humidity for 72 hours.

2.4.2.5 Preparation of FluoroSpot plates as part of the B cell FluoroSpot assay

FluoroSpot plates (96-well PVDF membrane, IPFL plates, Millipore, Mabtech, United Kingdom, MSIPS4510) were pre-wetted with 35% ethanol, 15µl/well for max. 1 minute and washed five times with sterile water (200 µl/well). The plates were coated with a volume of 100µl/well of 5.0µg/mL of the following proteins (antigens): SARS-CoV-2 stabilised spike glycoprotein (full-length) (REC31966-500), SARS-CoV-2 nucleocapsid protein (REC31851-500), human coronavirus OC43 spike glycoprotein (full-length)(REC31877-500), SARS-CoV-2 Delta B.1.617.2, stabilised spike glycoprotein (REC31975-500), human coronavirus NL63 spike glycoprotein (full-length)(REC31879-500) and 100µL of 15µg/mL of the following capture monoclonal antibodies for the total IgG coated wells: IgG (clone MT91/145, 0.5mg/ml), (to

measure total IgG secreting BMEM), **Table 2.1**. Coating antigens and capture antibodies were diluted to the required concentration using sterile DPBS (D8537, Sigma-Aldrich, Massachusetts, USA). DPBS was added to the antigen 'blank' wells (i.e., the negative control). Coated plates were incubated at 4-8 ^{0}C overnight.

Table 2.1 Layout of antigen-coated plate, along with subsequent control cell suspension dilutions.

	1	2	3	4	5	6	7	8	9	10	11	12
A	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu
B	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63
C	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP
D	OC43	OC43	OC43	OC43	OC43	OC43	OC43	240	OC43	OC43	OC43	OC43
E	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta
F	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS
G	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10
H	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100

C2wu = SARS-CoV-2 stabilised spike glycoprotein; NL63 = human coronavirus NL63 spike glycoprotein; CoV2-NP = SARS-CoV-2 nucleocapsid protein; OC43 = human coronavirus OC43 spike glycoprotein; IgG = total IgG control; 1:10, 1:100 cell dilution from 2x106 cell/ml; DPBS = Dulbecco's phosphate buffered saline.

2.4.2.6 Cell harvesting and B cell FluoroSpot assay

To prevent nonspecific binding, following overnight incubation, plates were washed five times with PBS (200µL/well), blocked with CM (200µL/well) and incubated for at least 30 minutes at 37°C. PBMCs were harvested after 72 hours stimulation, into 15mL cell wash buffer and centrifuged at 1000xg for 10 minutes. The cell pellet was resuspended in 1 mL of cell wash buffer, the tube topped up to 10mL with cell wash buffer, and the sample centrifuged again at 300g for 10 minutes. This wash step was then repeated. The cell pellet was resuspended in 1mL CM and cells counted using the Guava Counter and the Cell Count and Viability reagent. Cells were counted and re-suspended in CM to a final concentration of $2x10^6$ PBMC/ml. The CM used to block the plate was retained in the wells and then 100µl per well of cell suspension ($2x10^5$ cells) was added to the FluoroSpot plate. Where possible (depending on the volume of cell suspension available) each sample was tested in triplicate for IgG-ASC for each antigen/capture antibody. For each sample, dilutions of 1:10 and 1:100 were prepared and the cells added to the plates (100µL/well) to measure total IgG-ASC responses (i.e., total Ig positive control wells). Plates were incubated overnight at 37°C in 5% CO2 and 95% humidity.

2.4.2.7 Detection of antigen-specific memory B cells derived IgG-ASC by FluoroSpot assay

Following overnight incubation, plates were washed five times with 200µl/well of PBS. ASCs were detected using fluorescent monoclonal antibodies anti-IgG-550 (MT78/145). The antibody solution was filtered using a 0.2µm low protein binding filter and 100µl/well of detection antibody solution added to the FluoroSpot plate. Plates were incubated with detection antibody for 2 hours at room temperature and covered to limit light exposure.

Plates were washed five times with 200µL/well of PBS following addition of detection antibody solution. To increase the intensity of the fluorescent spots, a FluoroSpot enhancer was added to the plates (50µL/well) and the plates incubated for 15 minutes at room temperature. Excess FluoroSpot enhancer was removed by flicking the plate over a waste container and firmly tapping the plate against clean paper towels. The underdrain was removed and the plate left in the dark to dry. The plate was kept in the dark at room temperature until scanning.

2.4.2.8 FluoroSpot counting

Spots were counted using the FluoroSpot reader AID iSpot Spectrum v8.0. Count settings were optimised to capture maximum number of spots per well. Settings used for each antigen are shown in **Table 2.2**. Identical pre-defined count settings were used for all plates. Well images were edited to remove well edge artefacts and reflections by adjusting the area of interest (AOI, yellow circle) and any remaining debris removed using the 'eraser' tool.

The cell quantity specific for each antigen and isotype and the mean of duplicate and triplicate (ASCs/well) were calculated for each sample. The background value (i.e., from the antigen 'blank' wells) was subtracted from each antigen-specific result for each participant sample. The final mean spot counts are expressed per 10^6 PBMCs and calculated from the raw counts as illustrated in **Table 2.3**. If the final total IgG spots/10^6 PBMCs was <1000/10^6 PBMCs, the sample was excluded from the final analysis. The FluoroSpot assay has a sensitivity of one antigen-specific B cell per 250,000 PBMCs. Samples with ASC counts that were too numerous to count (TNTC) were assigned a value equivalent to one standard deviation above the upper limit of quantification (ULOQ) for that antigen. Antigen-specific ASC counts below the lower limit of quantification (LLOQ) were assigned a value corresponding to half the LLOQ. The coefficient of variation (CV; standard deviation/average x 100) of the means for each sample was calculated and values furthest

away from the mean excluded if the CV was >20%. Final ASC results are reported as ASCs/10^6 PBMCs, **Table 2.3**.

Table 2.2 FluoroSpot reader AID count settings used in the memory B cell FluoroSpot assay

Antigen	Isotype	Intensity (min.)	Size (min.)	Gradient (min.)	Emphasis (target spot size)
SARS-CoV-2 spike	IgG	50	45	10	Big
OC43	IgG	50	45	10	Big
NP	IgG	50	45	10	Big
NL63	IgG	50	45	10	Big
PBS	IgG	50	45	10	Big
Ig	IgG	50	45	10	Big
Delta	IgG	20	45	1	Small

NP = nucleocapsid protein; Ig = total immunoglobulin; PBS = phosphate buffered saline.

Table 2.3 Calculation of final spot counts per 10^6 PBMCs

	Raw counts (no. PBMCs added per well)	**Factor to give spots/10^6 PBMCs**
Specific Ag	Spots/2x10^5 PBMCs	Multiply x 5
Ig 1:10	Spots/2x10^4 PBMCs	(Multiply x 5) x 10
Ig 1:100	Spots/2x10^3 PBMCs	(Multiply x 5) x 100

Ag = antigen, PBMC = peripheral blood mononuclear cells, Ig = total Ig.

2.4.3 MSD multiplex immunoassay

The MSD immunoassay was carried out using the MSD V-PLEX COVID-19 serology kit Coronavirus Panel 3 kit (Meso Scale Diagnostics LLC, Rockville, Maryland) described in **section 2.2.6**. Plates and Diluent 100 were brought to room temperature before use. Samples, the reference standard and controls were thawed on ice. Plates were blocked using 150µL/well of reconstituted MSD Blocker A solution. Plates were sealed using an adhesive plate seal and incubated at room temperature with shaking at ~700 rpm for a minimum of 30 minutes. During this time the standards, controls and samples were prepared. Samples were diluted using the dilution factors outlined in **Table 2.4** and **Table 2.5**. The supplied Reference Standard 1 was used to establish a calibration curve for the assay. For IgG, a 10-fold dilution of Reference Standard 1 was used to create the highest point on the standard curve (CAL-01). For IgA, undiluted Reference Standard 1 was used as the highest point on the standard curve.

Table 2.4 Cohort A serum and mucosal sample dilution factors according to study timepoint, sample matrix and isotype

Matrix	Timepoint	Isotype	Dilution factor
Mucosal	D0, 56, 70	IgA, IgG	1:50
Serum	D0, 56, 70	IgA	1:1667
Serum	D0	IgG	1:1667
Serum	D56, 70	IgG	1:10,000

Samples were diluted using Diluent 100. D0 = pre-first dose, D56 = pre-second dose; D70 = 14 days post-second dose.

Table 2.5 Cohort B serum and mucosal sample dilution factors according to study timepoint, sample matrix and isotype

Matrix	Timepoint	Isotype	Dilution factor
Mucosal	D0, 28, 182, D210*	IgA	1:50
Mucosal	D0, 182	IgG	1:50
Mucosal	D28, 210*	IgG	1:75
Serum	D0, 28, 182, 210*	IgA	1:1700
Serum	D0	IgG	1:30,000
Serum	D28, 182, 210*	IgG	1:80,000 – 1:100,000

Samples were diluted using Diluent 100. D0 = pre-third dose; D28 and D182 following enrolment/vaccination D0 visit; ***D210 = 28 days following receipt of the Original/Omicron BA.1 bivalent vaccine in the control arm only****.*

To prepare CAL-01, the highest point on the calibration curve for IgG, Reference Standard 1 was diluted 10-fold as follows: 20µl of Reference Standard 1 was added to 180µl Diluent 100 and then vortexed briefly to mix. 150µl of Diluent 100 was added from CAL-02 to CAL-08 labelled Eppendorf tubes. To prepare CAL-02 to CAL-08, 50µl of CAL-01 was added to 150µl of Diluent

100 and this was vortexed briefly to mix. The 4-fold serial dilutions (i.e., 50µl of previous calibrator into 150µl Diluent 100) were repeated to create CAL-03 through to CAL-07. 150µl of Diluent 100 served as the 'blank' in CAL-08. Preparation of the calibrators necessary to create the calibration curve for IgG is illustrated in **Figure 2.1**.

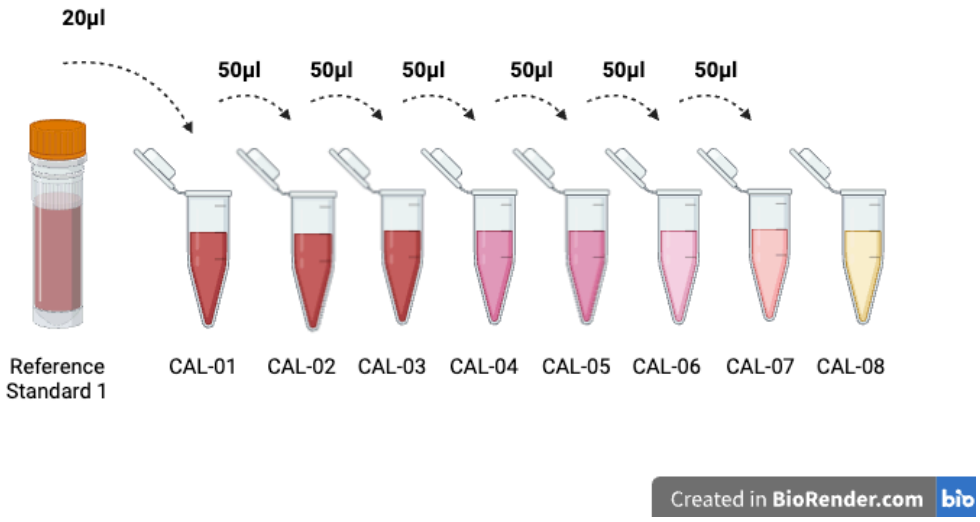


Figure 2.1 Preparation of calibrator solutions to create the calibration curve for the IgG assay using a 10-fold dilution of Reference Standard 1 to produce CAL-01.
Adapted from MSD® MULTI-SPOT Assay System COVID-19 Serology Kits package insert Dilution Schema for preparation of calibrator solutions, Meso Scale Diagnostics LLC, Rockville, Maryland. Created in BioRender.com.

With respect to the IgA assay, to create CAL-01, Reference Standard 1 was used without dilution. Reference Standard 1 was vortexed and 200µl added to an Eppendorf tube labelled CAL-01. 150µl of Diluent 100 was added to CAL-02 to CAL-08 labelled Eppendorf tubes. CAL-02 was prepared by adding 50µl of CAL-01 to 150µl of Diluent 100. To create CAL-03 to CAL-08, a series of 4-fold dilutions were performed as per the IgG assay, i.e., 50µl of the previous calibrator was added to 150µl of Diluent 100 to create CAL-03 to CAL-07. 150µl of Diluent 100 was used as the 'blank' (CAL-08), **Figure 2.2**

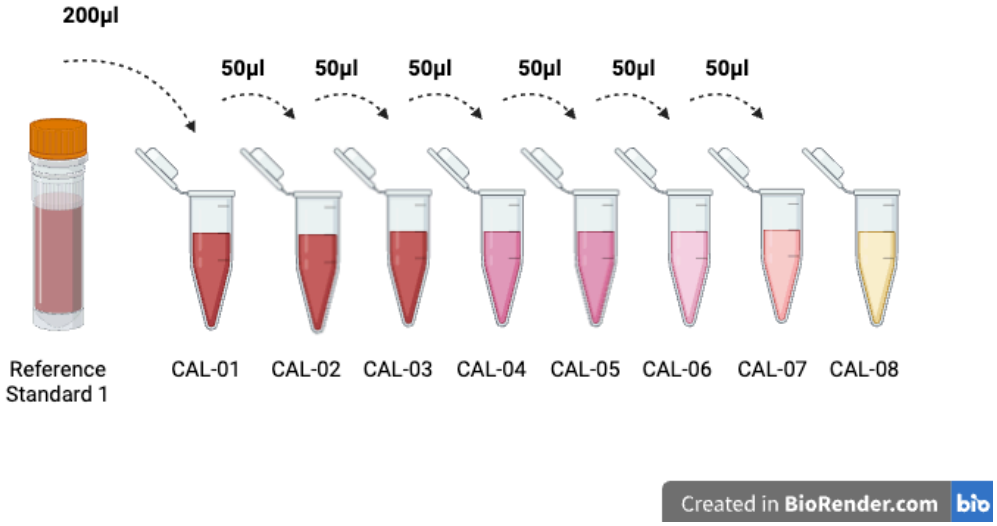


Figure 2.2 Preparation of calibrator solutions to create the calibration curve for the IgA assay using undiluted Reference Standard 1 to produce CAL-01.
Adapted from MSD® MULTI-SPOT Assay System COVID-19 Serology Kits package insert Dilution Schema for preparation of calibrator solutions, Meso Scale Diagnostics LLC, Rockville, Maryland.

Following the incubation period, plates were handwashed three times with 150µL/well PBS + 0.05% Tween®20 (PBS-T). To each plate was added (in duplicate) 50µL/well of diluted samples, controls and calibrators according to the plate layout shown in **Figure 2.3**. The plate was then sealed using an adhesive plate seal and incubated at room temperature with shaking at ~700rpm for 2 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL-01		Sample 1		Sample 9		Sample 17		Sample 25		Control 1	
B	CAL-02		Sample 2		Sample 10		Sample 18		Sample 26		Control 2	
C	CAL-03		Sample 3		Sample 11		Sample 19		Sample 27		Control 3	
D	CAL-04		Sample 4		Sample 12		Sample 20		Sample 28		Sample 33	
E	CAL-05		Sample 5		Sample 13		Sample 21		Sample 29		Sample 34	
F	CAL-06		Sample 6		Sample 14		Sample 22		Sample 30		Sample 35	
G	CAL-07		Sample 7		Sample 15		Sample 23		Sample 31		Sample 36	
H	CAL-08 (blank)		Sample 8		Sample 16		Sample 24		Sample 32		Sample 37	

Figure 2.3 MSD plate layout.
Samples were plated in duplicate. CAL = calibrator for calibration curve. CAL-01 represents the highest point on the calibration curve.

The detection antibody solution was prepared during this time by adding 30µl of SULFO-TAG anti-human IgG/IgA antibody (provided as 200X stock solution) to 5970µl of Diluent 100, generating a working solution of 1X. Following the sample incubation step, plates were handwashed three times with 150µL/well PBS-T, and 50µL/well of SULFO-TAG conjugated anti-human Ig antibody solution was added to the plates. Plates were then sealed and incubated at room temperature with shaking (~700rpm) for 1 hour. Following the detection antibody incubation step, plates were handwashed three times with 150µL/well PBS-T, and 150µL/well MSD GOLD Read Buffer B [undiluted; required for the generation of electrochemiluminescence signals (ECL)] was added to each plate. Plates were then immediately transferred to the MSD instrument. The assay uses electrochemiluminescence labels (i.e., SULFO-TAG™) which are conjugated to the detection antibodies. Electricity applied to the plate electrodes by the MSD instrument, stimulates light emission by the SULFO-TAG™ labels. The light intensity emitted is then measured to quantify the antibody concentration present in each sample. With respect to the mucosal samples, all washes and pipetting of samples took place in an MSC Class II.

The calibration curve used to calculate antibody concentrations was established by fitting signals from the calibrators to a 4-parameter logistic (or sigmoidal dose-response) model with a 1/Y2 weighting using the DISCOVERY WORKBENCH 4.0 Analysis Software (Meso Scale Diagnostics LLC, Rockville, Maryland). Controls and sample antibody unit concentrations were determined from their ECL signals backfitted to the calibration curve. With respect to samples, by correcting for the dilution factor, the final antibody concentrations in undiluted samples were provided in arbitrary units (AU/ml).

2.4.4 Elution of nasal mucosal lining fluid adhered to synthetic absorbent matrix strips

Mucosal samples were collected using a 4.5mm Nasosorption FXi nasal sampling device (NSFL-FXI-13) which uses SAM swabs to absorb MLF. SAM strips are short strips of flexible fabric attached to a plastic holder which acts as a cap to its own cryotube. Samples were collected by placing the absorbent swab into one nostril and gently pressing the nostril closed for up to 30 seconds. The swab was then removed and returned to the container. Samples were chilled immediately after collection (2-8^{0}C) and stored at -80^{0}C.

All handling and processing of SAM strips took place in an MSC Class II. The tubes containing the SAM strips were removed from the -80^{0}C freezer and thawed on wet ice. Each tube was opened and the SAM strip cut with blunt scissors just below the plastic handle. The scissors were decontaminated by plunging them in 2% Virkon and 70% ethanol and wiping them after each sample. To each tube containing the SAM strip was added 500µl of extraction buffer. The lid was replaced and the tube vortexed for 30 seconds and placed back on ice for 30 minutes incubation. During the 30 minutes incubation period, 2ml Eppendorf tubes with spin-x filter chamber were prepared and the microcentrifuge cooled to 4^{0}C. The tube contents were removed with a sterile 1000µl tip and placed into the top chamber of the spin-x tube along with the SAM strip. Each SAM strip was then carefully transferred using a flat-end forceps which was decontaminated using 70% ethanol after each sample. The samples were centrifuged for 10 minutes at 13,000 rpm in the chilled microcentrifuge (+4^{0}C). Equal volumes (i.e., 250µl) of the eluent were aliquoted into two pre-labelled sterile Eppendorf tubes and placed in the -80^{0}C freezer for storage until analysis.

2.5 Anti-SARS-CoV-2 Spike Glycoprotein IgG standardised ELISA

The serum antibody concentrations of samples intended for avidity analysis were determined using an in-house optimised ELISA assay. Although samples had already been processed at the UK Health Security Agency (UKHSA) to generate anti-spike IgG antibody data as part of the Com-COV3 trial, I repeated this assay to determine the dilution factor required to standardise serum antibody concentrations.

SARS-CoV-2 Spike GP trimer (0.670mg/ml stock solution stored at -80^{0}C) was thawed and prepared by adding 7.5µl of Spike GP trimer to 4.9925ml of DPBS to give 1µg/ml of coating antigen in DPBS sufficient to coat one plate (the volume was adjusted according to the number of plates, e.g., 15µl in 9.98ml DPBS to coat 2 plates). 50µl/well of prepared coating antigen was added to each plate and the plates covered with clingfilm and stored overnight at 4^{0}C for at least 16 hours.

Following the incubation period, the residual coating solution was gently tapped into the sink, and the plates washed 6 times with DPBS-T using a handheld plate washer. Plates were inverted and tapped dry between washes and after the final wash. The plates were blocked with 100µl/well of Casein (Blocker™ Casein in PBS, #37528, Thermo Fisher Scientific, UK). The blocked plates were covered with cling film and incubated for one hour at room temperature.

The reference standard dilutions and serum sample dilutions were prepared during the incubation period. The reference plasma used as the reference standard for the assay was sourced from a pool of COVID-19 convalescent/vaccinee plasma samples and was stored at - 80^{0}C and thawed prior to use. The standard curve was created through a two-fold dilution series with an initial dilution of 1:300 from the reference plasma pool. The standards were prepared by diluting the reference

plasma pool to 1:300 in a final volume of 1200µl in 'Tube 1' (i.e., 4µl reference plasma plus 1196µl Casein). 600µl of Casein was added to the remaining tubes (Tube 2 to Tube 10). 600µl was transferred from Tube 1 to Tube 2 and mixed by vortexing 10 times. 600µl was transferred from Tube 2 to Tube 3 and this procedure was repeated across the rest of the tubes. 600µl was discarded from the last tube (Tube 10). The positive control was a 1:1600 dilution prepared from the 1:300 standard dilution and corresponded to standard 5. It was prepared by adding 50µl of the 1:300 standard into an Eppendorf tube containing 750µl Casein and mixed by vortexing. The sample dilutions used are shown in **Table 2.6** and were based on the results from previous testing performed at UKHSA. Cohort A serum antibody sample processing was originally performed using Roche Elecsys® anti-SARS-CoV-2 spike ECLIA at UKHSA and Cohort B serum antibody concentrations were originally analysed using an ELISA (validated at Nexelis, Laval, QC, Canada) at UKHSA.

Table 2.6 Cohort A and Cohort B sample dilution factors used in the anti-spike IgG ELISA immunoassay.
The dilution factor was calculated based on the anti-spike IgG concentrations attained through previous testing at UKHSA.

Anti-spike IgG, units	Dilution factor
Cohort A	
< 5000 BAU/ml	1:1000
5000 to 50,000 BAU/ml	1:50,000
>50,000 BAU/ml	1:100,000
Cohort B	
< 10,000 ELU/ml	1:10,000
10,000 to 80,000 ELU/ml	1:50,000
> 80,000 ELU/mL	1:100,000

ELU = ELISA units; BAU = binding antibody units.

After the blocking period, the blocker casein was flicked off and the plates tapped dry. 50µl/well of vortexed diluted samples were added to the plate in triplicate along with the blank control (Casein only), standards and positive control according to the plate layout shown in **Table 2.7**. The plates were stacked with an empty blank plate on top and covered with cling film and left for 2 hours at room temperature.

Table 2.7 Anti-spike IgG ELISA plate layout.
Diluted samples were plated in triplicate. Blue boxes show standard curve dilutions from D1/E1 to D10/E10.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
D	St 1:100	St 1:200	St 1:400	St 1:800	St 1600	St 1:3200	St 1:6400	St 1:12800	St 1:25600	St 1:51200	Blank	Blank
E	St 1:100	St 1:200	St 1:400	St 1:800	St 1600	St 1:3200	St 1:6400	St 1:12800	St 1:25600	St 1:51200	Blank	Blank
F	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Positive control
G	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Positive control
H	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Positive control

St = (reference) standard; blank = Casein.

The secondary antibody was prepared during the sample incubation period (see **section 2.3.1.3**). Following the 2-hour sample incubation period, the plates were washed 6 times with DPBS-T as before. After the final wash, the plates were inverted and tapped firmly to remove residual liquid from the wells and 50µl/well of secondary antibody added. The plates were stacked and covered with cling film and incubated for 1 hour at room temperature. During this time, the development buffer was prepared as described in **section 2.1.9**. Following the secondary antibody incubation period, the plates were washed 6 times with DPBS-T as previously described and 100µl/well of development buffer added. Samples were read at absorbance OD405 using the Bio-tek ELx800 microplate reader (using Gen5 ELISA software).

The plates took approximately 20-30 minutes to develop. When the positive control reached an OD405 reading of 0.7, the plate was read continuously until the positive control and curve parameters fulfilled the following criteria:

- Positive control OD 0.9-1.3

- Standard curve interpolation X value 0.9 – 1.3
- St 1:100 OD >3.5
- ODs of blank wells <0.2
- Standard curve reaches 4 parameter ranges:

Parameters	A	B	C	D	R^2
Ideal range	0.001-0.2	0.6-1.54	1-15	2-8	>0.996

Any plate that did not fulfil all the above criteria was repeated. A sample with any of the following was also repeated:

- An OD > 2.5 (sample was repeated at a higher dilution)
- CV of OD405 values for replicates of a sample >20%
- An OD <0.25. If the dilution factor was higher than 1:100, the sample was repeated at a lower dilution factor.

2.6 SARS-CoV-2 Spike total IgG avidity ELISA

ELISA plates were coated, washed and blocked as described in **section 2.5**. Serum dilutions were calculated by determining the concentration of serum required to give an ELISA OD reading of 1.0. Samples were diluted using Blocker™ Casein. To standardise the antibody concentrations of samples and to minimise the influence of varying antibody concentrations on antibody avidity, serum samples were diluted to contain an antibody concentration of 1 EU (ELISA unit). The positive control was prepared by making a 1:4800 dilution of the standard pool used in the ELISA immunoassay (see **section 2.5**) by adding 31µl of 1:100 diluted standard pool serum to 1469µl DPBS. The diluted samples and positive controls were plated according to the plate layout shown in

Table 2.8, each well containing 50µl sample. Casein was used as the 'blank'.

Table 2.8 Anti-spike IgG avidity ELISA plate layout.

<>	1	2	3	4	5	6	7	8	9	10	11	12	
A	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6	0
B	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6	1.6
C	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6	2.4
D	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6	3.2
E	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 10	Sample 10	Pos Ctrl	Pos Ctrl	B	B	0
F	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 10	Sample 10	Pos Ctrl	Pos Ctrl	B	B	1.6
G	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 10	Sample 10	Pos Ctrl	Pos Ctrl	B	B	2.4
H	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 10	Sample 10	Pos Ctrl	Pos Ctrl	B	B	3.2

Pos Ctrl = positive control; 'B' = blank. Numbers '0' to '3.2' represent sodium thiocyanate concentrations used in each plate row.

The sodium thiocyanate (NaSCN) dilutions were prepared during the 2-hour sample incubation period. The NaSCN concentrations were prepared from 8M stock in DPBS according to the dilutions shown in **Table 2.9.**

Table 2.9 NaSCN dilutions for avidity immunoassay.

Conc (M)	DPBS	8M NaSCN
1.6	3.2	0.8
2.4	2.8	1.2
3.2	2.4	1.6

NaSCN = sodium thiocyanate; DPBS = Dulbecco's phosphate buffered saline; 'conc' = concentration of sodium thiocyanate (M = molarity).

Following the sample incubation period, the plates were washed five times in PBS-T and tapped dry. 50µl/well of sodium thiocyanate dilution was added according to the plate layout shown in **Table 2.8**. The plates were sealed, protected from light using aluminium foil, and left for 15 minutes. Following the NaSCN incubation period, the plates were washed six times in PBS-T and 50µl/well of secondary antibody added as previously described in **section 2.5**. The plates were again sealed and left at room temperature for 1 hour. Following the secondary antibody incubation period, the plates were washed six times and 100µl/well development buffer added as described in **section 2.5**. Samples were read at absorbance OD405 using the Bio-tek ELx800 microplate reader (using Gen5 ELISA software) until an OD405 of 1.0 was reached in the average of each of the

positive control and test sample 0M wells. Plates were considered passed if the OD405 of the controls was between 0.8-1.2 and the value of the blank wells was <0.2. For each sample, the CV of the two replicates for each NaSCN concentration was calculated and the sample repeated if the CV was >20%. The sample was deemed to have passed if the OD405 of the 0M sample was between 0.8-1.2.

The avidity index was calculated as the percentage of IgG detected following treatment with NaSCN (i.e., the average OD of the sample treated with NaSCN) divided by average OD of the untreated sample (i.e., '0M test sample') and multiplied by 100. The IC50 was defined as the NaSCN concentration required to reduce the OD405 of the 'OM' sample (i.e., the untreated sample) by 50%. As demonstrated in **Figure 2.4** the IC50 strongly correlated with all concentrations of NaSCN used in the assay. Results generated (i.e., the avidity index or % binding following NaSCN treatment) following treatment with 2.4M NaSCN were chosen for further analysis as the results attained using this concentration of NaSCN demonstrated an almost perfect correlation with the IC50, **Figure 2.4** (see **Chapter 6:** An Exploration of Biomarkers of Protection against SARS-CoV-2 Infection, and the Kinetics of Antibody Avidity and Neutralising Antibody Responses.).

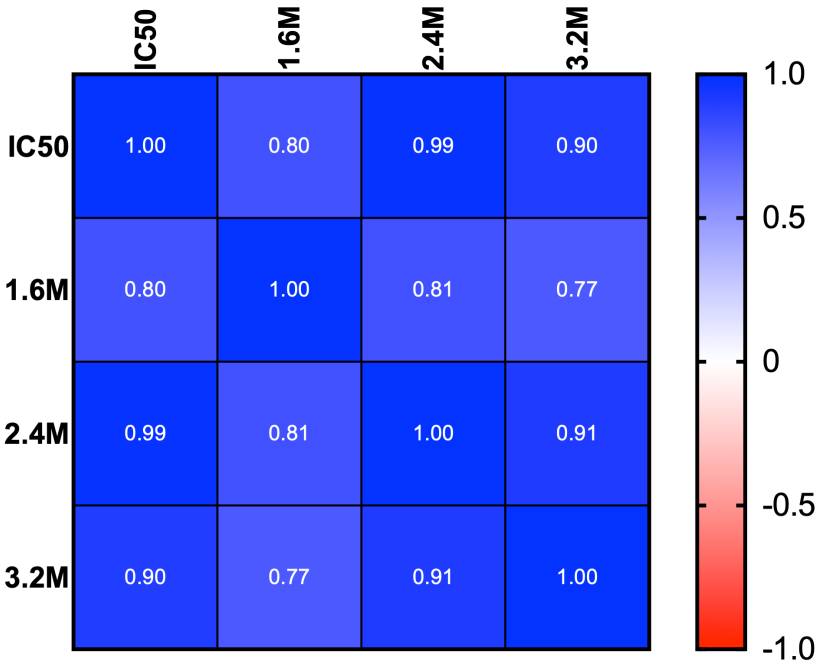


Figure 2.4 Correlation between results of avidity immunoassay generated using three different concentrations of NaSCN with the IC50.

2.7 Statistical Methods

2.7.1 Analysis of immunological data

Descriptive statistics were used to characterise the results of the immunoassays performed. Categorical/binary variables were summarised using frequencies and percentages. Data were tested for normality using a Shapiro-Wilk test. The data were normally distributed if the p-value was >0.05 Skewed data were log-transformed to render a normal distribution, and parametric testing was used when testing for statistical significance. Data below the lower limit of detection were imputed by a value half the lower limit of detection, prior to log transformation. Skewed continuous variables were summarised using medians/geometric mean concentrations and associated 95% confidence intervals (where appropriate), inter-quartile ranges and range values. A

t-test was used to compare the means between two groups and to determine whether the means of the two groups were statistically significantly different. For paired data, for example, before and after vaccination, a paired t-test was used to compare the means of two paired groups. Since the nature of this project is hypothesis-generating and exploratory (except for **Chapter 3**:A Clinical Study Comparing COVID-19 Vaccine Schedule Combinations in Adolescents, Com-COV3: A Randomised Controlled Trial, reporting results from the RCT), the significance level was set to be 0.05 to claim statistical significance with no adjustment for multiple testing. Correlations were performed using Spearman's rank correlation for skewed data and the correlation coefficient ('r') expressed as a non-parametric measure of correlation between two continuous variables. Pearson's correlation coefficient was used to determine the strength of the correlation between two variables when the data were normally distributed. Scatter plots were used to visualise the relationship between the two variables.

With respect to samples in the BMEM ELISpot assay with results that were TNTC, the range of BMEM frequencies specific for the antigen was determined and the standard deviation calculated and added to the highest result attained and this value substituted for sample values TNTC. Regarding MSD samples with results above the upper limit of quantification (ULOQ), the ULOQ for the antigen tested was multiplied by the highest dilution factor used and this value substituted for the sample result.

2.7.2 Analysis software

MSD data were exported using Methodical Mind software and analyses performed using Stata statistical software version: Release 18.0. StataCorp LLC. Graphs were produced using Stata statistical software version: Release 18.0. StataCorp LLC and GraphPad Prism version 10.0.

Chapter 3: A Clinical Study Comparing COVID-19 Vaccine Schedule Combinations in Adolescents, Com-COV3: A Randomised Controlled Trial

3.1 Introduction

The global response to the COVID-19 pandemic has led to the successful deployment of several vaccines shown to provide effective protection against severe disease and death.[132] Although immunisation of high-risk groups was initially prioritised in vaccination campaigns, the significant impact of COVID-19 on the education and psychological well-being of children and adolescents, the high rates of infection in this age group, and their potentially important role in transmission have now been widely acknowledged.[14,15]

As outlined in **Chapter 1**:**Introduction**, the use of heterologous COVID-19 vaccine schedules has already been studied in several trials in adults and shown to induce robust immune responses.[32,34,37,133] The Com-COV studies demonstrated that priming with ChAdOx1 nCOV-19 with the Pfizer-BioNTech vaccine (BNT162b2) as the second dose was highly immunogenic, and this regimen has been used in Canada and many northern European countries.[32] Similarly, Torre et al. in their study showed that ChAdOx1 nCOV-19 followed by BNT162b2 induced higher anti-spike IgG titres compared with the homologous ChAdOx1 nCOV-19 primary vaccine series.[134]

Vaccine efficacy following a two-dose primary COVID-19 vaccine series has been shown to decline over time.[24,25] Combined with the advent of SARS-CoV-2 variants, this finding prompted

many high-income countries to recommend a third dose COVID-19 booster to maintain protection. Booster doses have been shown to provide the strongest protection against SARS-CoV-2 infection and hospitalisation and to restore protection against severe disease and hospitalisation to levels comparable to those attained following the primary COVID-19 immunisation series.[31,135]

Com-COV3 was the first RCT to investigate the reactogenicity and immunogenicity of heterologous and fractional dose primary and booster COVID-19 vaccine schedules in adolescents. The study consisted of two cohorts: Cohort A and Cohort B. Cohort A examined reactogenicity, immunogenicity, and SARS-CoV-2 breakthrough infections in adolescents receiving 30µg BNT162b2 as a first dose and a second dose of either 30µg BNT162b2 (schedule hereafter referred to in this chapter as BNT-30), 10µg BNT162b2 (schedule henceforth in this chapter referred to as BNT-10), or NVX-CoV2373 (schedule hereafter in this chapter referred to as NVX) given at least 8 weeks later. Cohort B of the trial investigated whether a 10µg (a one-third dose) of the adult formulation of BNT162b2 (hereafter referred to as 'adult BNT-10') was non-inferior to 10µg administered using the paediatric formulation of BNT162b2 (hereafter referred to as 'paediatric BNT-10') when given as a third (booster) dose in adolescents, and examined the reactogenicity and immunogenicity of homologous, heterologous, and fractional third dose vaccine schedules in adolescents when administered at least three months after a two-30µg dose BNT162b2 primary vaccine series.

I was the lead clinical research fellow on all aspects of the Com-COV3 trial preparation and delivery and performed my own analysis using trial data. The graphs used in this chapter were produced by the trial statistician and have been taken from the published manuscript.[136] Sections of this chapter have previously been published in Kelly, E., et al. (2023).[136] This chapter presents the immunogenicity results of Cohort A and Cohort B of the Com-COV3 trial. Results relating to reactogenicity and safety from the trial can be found in the **Appendix (page 305)**. Results of further exploratory immunological analysis carried out using samples from the trial are described in detail in subsequent chapters of this thesis.

3.2 Methods

3.2.1 Primary and Secondary Outcome Measures

The primary outcome of the trial (both Cohort A and Cohort B) was reactogenicity measured through solicited systemic reactions for 7 days after vaccination. With respect to Cohort B, the co-primary outcome was to determine whether the immune response to adult BNT-10 (0.1mL) was non-inferior to paediatric BNT-10 (0.2mL). Secondary outcomes included immunogenicity (assessed through anti-spike antibodies and cellular immune responses via ELISpot) and safety [assessed through serious adverse events (SAEs), adverse events of special interest (AESIs]. The incidence of SARS-CoV-2 infection and live virus neutralising antibody (VNA) titres were exploratory outcomes.

3.2.2 Study Design (including amendment to study design)

The Com-COV3 study was a single-blind, randomised, multi-site phase II trial. Ethical approval for the study was granted by South-Central Berkshire Research Ethics Committee (21/SC/0310) and the Medicines and Healthcare products Regulatory Agency (MHRA). The trial was conducted in accordance with the principles of the Declaration of Helsinki and the International Council for Harmonisation Good Clinical Practice guidelines.

Cohort A of the study examined reactogenicity and immunogenicity of homologous and heterologous COVID-19 vaccine schedules in 12-to-16-year-olds using either BNT162b2 (full or one-third dose), or NVXCoV2373 as the second vaccine dose administered 8 weeks after the first 30µg BNT162b2 vaccination. Recruitment to Cohort A commenced on 27th September 2021 and took place across seven UK National Health Service (NHS) and academic institutions. Study recruitment was completed in November 2021, and the final study visit took place in September 2022.

The planned total sample size of Cohort A was 270 with 90 participants allocated to each of the three study arms, **Table 3.1**. However, it was intended that the primary analysis of the study would be descriptive and therefore no formal sample size calculation was carried out. The number chosen was based on practical constraints. Following a change to the UK national immunisation policy on 29th November 2021 recommending that all 12-to-15-year-olds should be offered a second dose of 30µg BNT162b2, the study design was reviewed with the Trial Steering Committee (TSC). The TSC recommended ceasing recruitment to the NVXCoV2373 arm, and the study was amended to focus on the immune response to BNT162b2 (thus the pre-planned sample size was not met). Prior to the amendment, 117 participants had been recruited. Following the study amendment, 15 enrolled participants who had not yet received their second vaccine were randomised to receive either BNT-30 or BNT-10. Participants were no longer randomised to the NVXCoV2373 study arm to prioritise those groups more likely to inform UK immunisation policy, **Table 3.2**.

Table 3.1 Cohort A study design prior to 29th November 2021

Total participants	Arm	Prime (Day 0)	Boost (day 56)
n = 270	1 (n = 90)	BNT-30	BNT-30
	2 (n = 90)	BNT-30	BNT-10
	3 (n = 90)	BNT-30	NVXCoV2373

BNT-30 = 30µg BNT162b2; BNT-10 = 10µg BNT162b2; n = target sample size.

Table 3.2 Cohort A study design following 29th November 2021

Total participants	Arm	Prime (Day 0)	Boost (day 56)
	1	BNT-30	BNT-30
	2	BNT-30	BNT-10

BNT-30 = 30µg BNT162b2; BNT-10 = 10µg BNT162b2.

Participants in Cohort B were randomised to receive either 30µg BNT162b2, 10µg BNT162b2 (given as 0.1mL of the adult formulation of the vaccine), 10µg BNT162b2 (given as 0.2mL of paediatric formulation of the vaccine), NVXCoV2373 (full dose) or to two doses of 4CMenB (Meningococcal Group B vaccine, given 3 months apart, i.e., the control group) as their third dose following a two-dose 30µg BNT162b2 primary COVID-19 vaccine regimen received at least three months prior, **Table 3.3**. The control group were offered the Pfizer-BioNTech bivalent vaccine [Comirnaty Original/Omicron BA.1] six months after study enrolment. Recruitment to Cohort B took place across eleven UK NHS and academic institutions between 01 June 2022 and 30 June 2023. The final Cohort B study visit occurred in February 2024.

The Cohort B sample size was based on the primary outcome to determine non-inferiority between the two BNT162b2 10µg formulation arms (adult vs paediatric) with paediatric as the reference group. Based on Cohort A Day 28 (post-second dose) anti-spike antibody data (Roche S assay), the expected standard deviation was 0.30 in all participants. A non-inferiority margin of 0.67 with a power of 90%, a type I error of two-sided 0.05, and 15% of loss of follow up was initially planned, which would require 76 participants to be recruited per arm, for an effective sample size of 64 per arm. However, due to recruitment challenges during the study, the type 1 error was changed from a two-sided to a one-sided significance level of 0.05 which would require 62 participants per arm for an effective sample size of 52 per arm to achieve the co-primary outcome of non-inferiority between the two 10µg BNT162b2 formulation (adult vs paediatric) groups. The same sample size of up to 62 per arm was used for the remaining three groups to simplify randomisation and trial delivery. The total target sample size was, therefore, up to 310 participants.

Table 3.3 Com-COV3 Cohort B study design
Adapted from the Com-COV3 Protocol v10.0 31 Aug 2023

Study arm	Dose 1&2 (received in the community)	Dose 3 given at study D0 (3 months post dose 2)	3 months (Day 84)	6 months (Day 182)
1 (up to 62)	BNT162b2 30µg x 2	**BNT162b2 30 µg**	-	-
2 (up to 62)	BNT162b2 30µg x 2	**BNT162b2 10 µg** (1/3 dose, adult formulation)	-	-
3 (up to 62)	BNT162b2 30µg x 2	**BNT162b2 10 µg** (paediatric formulation)	-	-
4 (up to 62)	BNT162b2 30µg x 2	**NVXCoV2373**	-	-
5 (up to 62)	BNT162b2 30µg x 2	**4CMenB Control**	-	**Comirnaty Original/Omicron BA.1 15/15 µg**

3.2.3 Study participants

Adolescents aged 12-to-16 years inclusive, who were COVID-19 vaccine naïve or who had received a single dose of 30µg BNT162b2, were eligible to enrol in Cohort A of the Com-COV3 trial. 'High-risk' individuals advised to receive additional doses of BNT162b2 as part of the UK COVID-19 vaccination programme (e.g., individuals with a confirmed, or suspected immunosuppressive condition or serious chronic illness, or receiving immunosuppressant medication) were excluded. Previous SARS-CoV-2 infection was not an exclusion criterion. A complete list of the exclusion criteria can be found in the published paper.[136] Participants with well-controlled or mild co-morbidities were permitted to enrol in the trial if they did not belong to the 'high risk' cohort described above.

With respect to Cohort B, healthy adolescents aged between 12 and 15.5 years who had received two 30µg doses of BNT162b2 at least 90 days prior to enrolment were eligible to enrol in the trial. Participants with well-controlled mild-to-moderate comorbidities were permitted to take part. In addition to the exclusion criteria relevant to Cohort A, those who had received more than two doses of a COVID-19 vaccine (or a COVID-19 vaccine other than 30µg BNT162b2) or had previously received the 4CMenB vaccine were not eligible to enrol in the trial.

3.2.4 Blinding and randomisation

Computer-generated randomisation lists were prepared by the study statistician prior to study commencement. Cohort A participants were randomised 1:1:1 at the time of their second vaccination to the three study groups. After 29th November 2021, when UK national immunisation policy changed to offer all 12-to-15-year-olds a second dose of BNT162b2, a protocol amendment

was implemented and participants who had already received their first dose of BNT162b2 within the study were randomised 1:1 to receive either 30µg BNT162b2 or 10µg BNT162b2 as a second dose. Randomisation was performed using block randomisation. Block sizes of three and six were used before 29th November 2021, and block sizes of two and four were used thereafter. Randomisation was stratified by the study site and baseline anti-nucleocapsid IgG serostatus.

Both Cohort A and B participants were blinded to their study group allocation (Cohort A until one month after the second dose; Cohort B until 56 days after their study vaccine). To maintain the blind, vaccines were prepared out of sight of the participant, and masking tape applied to the vaccine syringe. Although laboratory staff were blinded to the study arm, staff involved in the study delivery were not. Statisticians were also unblinded throughout the trial.

Cohort B participants were initially randomised (1:1:1:1:1) to receive 30µg BNT162b2, adult BNT-10 (administered giving one-third the volume of the adult vaccine formulation), paediatric BNT-10 (administered using the paediatric formulation), full dose NVXCoV2373 (heterologous schedule), or two doses of the 4CMenB vaccine given 3 months apart (i.e., the control group). The control group received the Comirnaty Original/Omicron BA.1 (15/15µg)/dose vaccine as their third COVID-19 vaccine 6 months after enrolment. Due to ongoing recruitment challenges and following discussion with the TSC, study randomisation was changed to 1:3:3:1:1 to prioritise recruitment to the fractional dose BNT162b2 groups to achieve the participant numbers required to meet the study's co-primary endpoint (i.e., non-inferiority between the two 10µg BNT162b2 formulation groups). The change to randomisation was proposed on 5th December 2022 and approved as part of a substantial amendment to the trial on 9th June 2023. Due to recruitment difficulties, the TSC recommended ceasing recruitment on 30th June 2023. There were 5 participants recruited using the new randomisation. Despite these recruitment challenges, the sample size required to fulfil the co-primary objective of the trial was achieved.

Cohort B participants were randomised using block randomisation. Initially, block sizes of 5 and 10 were used. Randomisation was performed at the time of the first study visit and stratified by study site and history of a positive COVID-19 test. After the randomisation ratio was changed to 1:3:3:1:1, a random block size of 9 was used.

3.2.5 Study procedures and vaccines

3.2.5.1 Cohort A

With respect to Cohort A, informed consent was obtained from participants aged 16 years or from parents or guardians if the participant was younger than 16 years. Written assent was obtained from participants aged 12-to-15 years. COVID-19 vaccine naïve participants attended a screening/enrolment visit and those eligible received a 30µg BNT162b2 first dose. Participants who had received 30µg BNT162b2 in the community attended a screening visit 8 weeks afterwards. All participants were randomised and vaccinated at least 8 weeks after their first dose. Two vaccines were used in the study and administered by IM injection in the upper arm. 30µg BNT162b2 was given as 0.3 ml, 10 µg BNT162b2 as 0.1 ml, and NVXCoV2373 as 0.5 ml injection.

3.2.5.2 Cohort B

Six vaccines were used in Cohort B and all vaccines were administered by IM injection in the upper arm. BNT-30 was given as a 0.3mL IM injection, paediatric BNT-10 as 0.2mL, adult BNT-10 as 0.1mL, NVXCoV2373 as 0.5mL, 4CMenB as 0.5ml and Comirnaty Original/Omicron BA.1

(15/15µg)/dose as 0.3mL injection. All study vaccines were administered using a 23-gauge (25mm) needle.

3.2.5.3 Applicable to Cohort A and Cohort B

Eligible participants were invited to attend a screening and enrolment visit (day 0). A detailed medical history was taken from participants at their enrolment visit and a physical examination performed if required. All participants and parents had the opportunity to read the participant information sheet (PIS) (customised versions were available for parents, participants aged 16 years, and 12-to-15 years to enhance comprehensibility) and to discuss trial participation with a member of the study team before signing the consent form. A video presentation of the PIS was also made available to participants and their parents at the first study visit. Consent was taken by clinical staff (registered doctor or nurse) with appropriate experience and training. The final eligibility assessment was performed by a study doctor. Study group randomisation and vaccination took place at the day 0 visit. Blood samples for COVID-19 immunogenicity assays (anti-spike IgG, anti-nucleocapsid IgG, cellular responses) were taken at the day 0 visit, prior to vaccination. Participants also took a lateral flow test (LFT) at this visit. If this was positive, the remainder of the visit was deferred for at least 4 weeks.

Participants were observed for at least 15 minutes after vaccination. Participants were supplied with an oral thermometer, tape measure, and a link to an electronic diary (ediary) at the day 0 visit. They were requested to record solicited adverse events (AEs) for 7 days, unsolicited adverse events for 28 days, and medically attended adverse events throughout the trial. During the Cohort A study, community testing for SARS-CoV-2 was free, widely available and conducted either in response to symptoms or as screening. Cohort A participants were requested to record, using the ediary, all SARS-CoV-2 infections (classified as AESIs) detected by community-based self-testing with either PCR or rapid antigen test. Cohort B participants were provided with LFT kits and asked to perform

an LFT every week for 4 weeks between the first and second visits. Diaries were reviewed daily by a study doctor for AEs, AESIs, and SAEs.

Participants randomised to the control arm of the study received their COVID-19 vaccine [Comirnaty Original/Omicron BA.1 (15/15µg)/dose] at the day 182 visit. Control group participants were also required to record solicited, unsolicited, and medically attended adverse events in an e-diary for 28 days after this visit. They were provided with LFT kits and asked to perform an LFT every week between the day 182 visit and their final study visit (day 210), 28 days after vaccination.

3.2.5.4 Sample collection

An overview of the Cohort A study design, sample collection and study timepoints are shown in **Figure 3.1**. Blood samples were collected at all study visits while mucosal fluid samples were collected at day 0, day 56, and day 70 visit timepoints. Details relating to the samples collected are outlined in **section 3.2.6**.

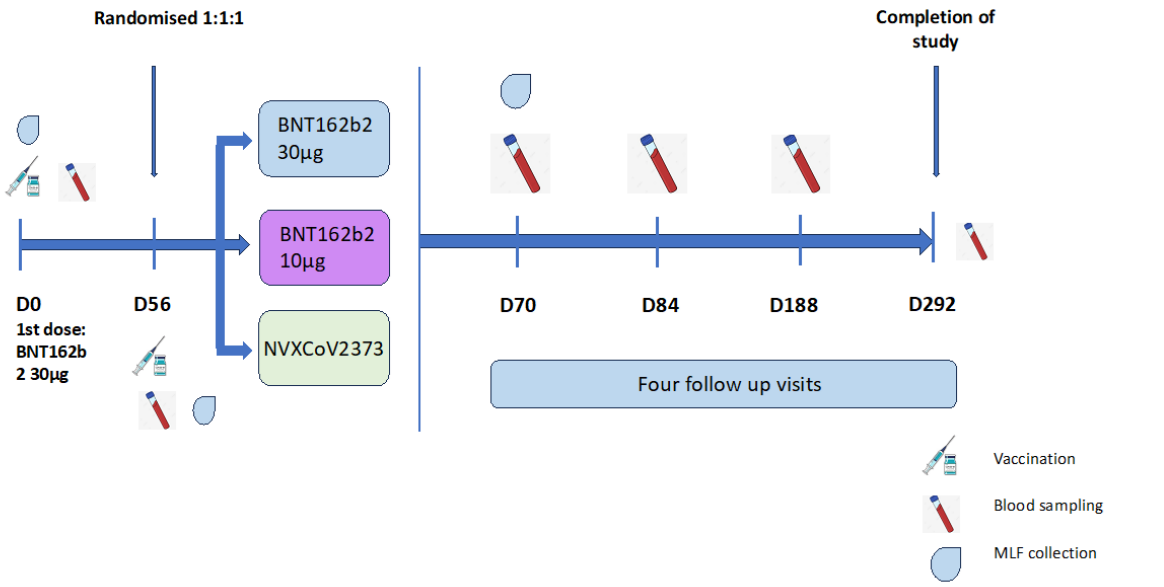


Figure 3.1 Com-COV3 Cohort A study design schematic.
Participants received their first dose (30µg BNT162b2) at day 0 (D0) and their second vaccine dose at day 56 (D56). MLF = mucosal lining fluid.

All Cohort B participants received a study vaccine following enrolment at day 0. As shown in **Figure 3.2**, blood and mucosal fluid samples were collected at all study timepoints. Participants randomised to receive a COVID-19 vaccine at their enrolment (day 0) visit had three follow up study visits while participants in the control arm had four study visits following their enrolment visit. Only participants in the control arm attended a study visit at day 210, i.e., 28 days after they had received their COVID-19 vaccine. Full details relating to the samples collected are outlined in **section 3.2.6**.

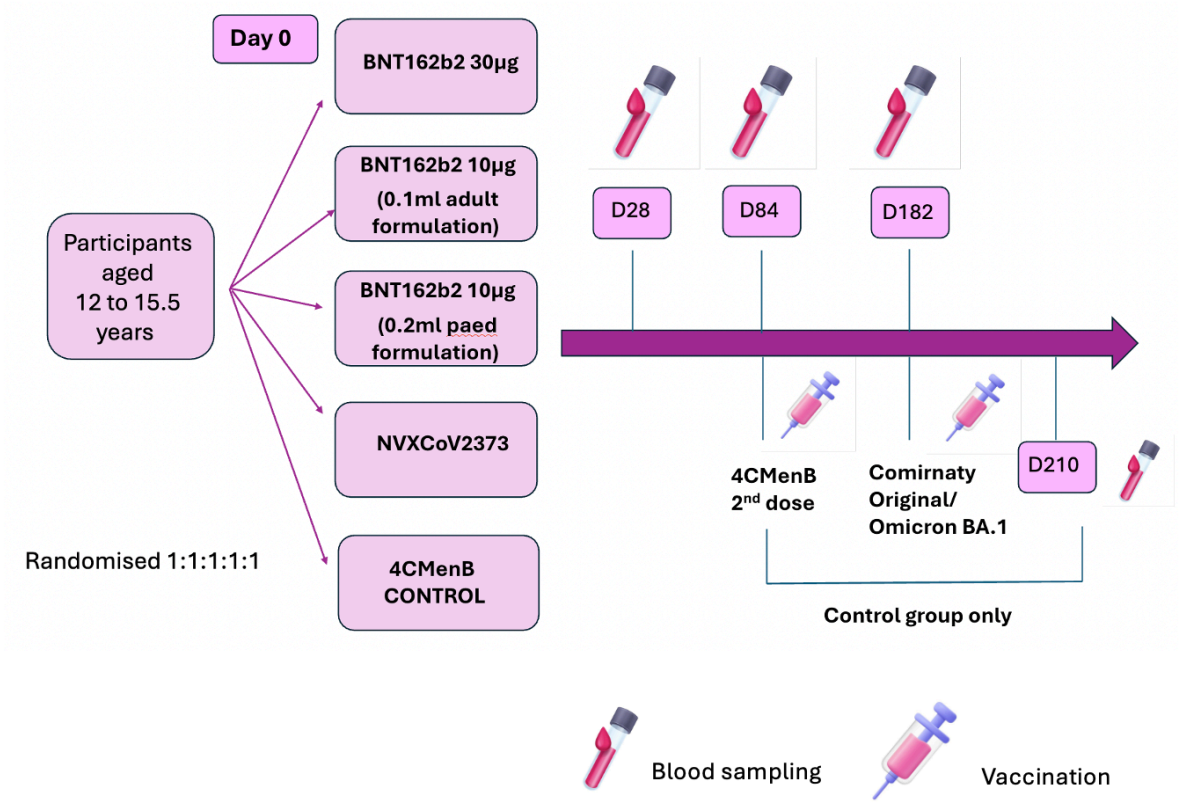


Figure 3.2 Com-COV3 Cohort B study design schematic.
Mucosal samples (not indicated here) were also collected at all study timepoints. Participants received their third (booster) dose at day 0. Control group participants received their first dose of 4CMenB at day 0 and their second dose at day 84. Control group participants received the bivalent vaccine Comirnaty Original/Omicron BA.1 at day 182 following enrolment.

3.2.5.5 Mucosal fluid sample collection – applicable to Cohort A and Cohort B

Collection of MLF samples took place at two study sites (Oxford and Bristol during Cohort A, Oxford and Southampton during Cohort B) and this was offered to participants as an optional study procedure. Informed assent was obtained from study participants (and written consent from parents) prior to sampling. Nasal fluid samples were collected using a 4.5mm Nasosorption™ FXi nasal sampling device (NSFL-FXI-13) which uses SAM swabs to absorb the mucosal fluid

collected, **Figure 3.3**. Samples collected were stored at 2-8^{0}C until freezing was possible at –80^{0}C. For details regarding the mucosal sampling procedure, please refer to **section 2.4.4**.

Figure 3.3 Nasosorption™ FXi nasal sampling device (NSFL-FXI-13)
The SAM strip (shown) is a short strip of soft flexible fabric attached to a plastic holder which acts as a cap to its own cryotube.

3.2.6 Laboratory methods

3.2.6.1 Cohort A

Sera were analysed using electrochemiluminescence immunoassays (ECLIA) at the UKHSA, Porton Down. Anti-nucleocapsid antibodies were determined using Roche Elecsys® Anti-SARS-CoV-2 ECLIA with a cut-off index (COI) value of 1.0 or greater considered positive. Antibodies (total Ig) against the SARS-CoV-2 spike (S) receptor binding domain (RBD) were measured using the Elecsys® anti-SARS-CoV-2 spike assay, specifically targeting the RBD of the SARS-CoV-2 spike protein. Samples ≥0.8 U/ml were considered positive, with results 1:1 to Binding Antibody Units/ml (BAU/ml).[137] Serum neutralising ability against SARS-CoV-2 Victoria (a Wuhan-related strain isolated early in the pandemic), Omicron BA.1 or BA.2 strains was measured using Focus Reduction Neutralisation Test (FRNT50) as previously described.[138] Blood sample collection took place at baseline (day 0), immediately prior to the second dose (day 56), and at days 14, 28, 132, and 236 following the second dose, **Table 3.4**.

3.2.6.2 Cohort B

Humoral immune responses were measured by testing serum samples at all study visit timepoints for anti-SARS-CoV-2 spike IgG antibodies using a validated ELISA at UKHSA, Porton Down (reported as ELISA laboratory units [ELU]/mL), **Table 3.5**. This assay was validated at Nexelis (Laval, QC, Canada) and the technology transferred to UKHSA. Sera were also analysed at days 0, 84, and 182 to assess anti-nucleocapsid IgG serostatus at Porton Down, UKHSA by ECLIA (Cobas platform, Roche Diagnostics). An assay COI below 1.0 was reported as seronegative. Samples collected at days 28, 84, 182, and 210 (control group only) were tested using a microneutralization assay to assess 50% focus reduction neutralisation titres (FRNT50) for live SARS-CoV-2 virus lineage Victoria and Omicron sublineages BA.5 and XBB.15 at the University of Oxford, Oxford, UK, as previously described.[139] Testing was performed in 4 study groups: 30µg BNT162b2, adult BNT-10, paediatric BNT-10, and NVXCoV2373.

3.2.6.3 Applicable to Cohort A and Cohort B

IFN-γ secreting T-cells specific to whole spike protein epitopes, designed based on the Wuhan-Hu-1 sequence (YP_009724390.1), were detected using T-SPOT-Discovery, ELISpot Test 14 (full spike, Wuhan) at Oxford Immunotec (Abingdon, UK). PBMCs to which T-cell Xtend reagent had been added to extend PBMC survival, were tested within 32 hours of venepuncture. T-cell frequencies were reported as spot-forming cells (SFC) per 250,000 PBMCs per well with a lower limit of detection (LLOD) of one in 250,000 PBMCs.

Table 3.4 Cohort A blood and mucosal fluid sampling and visit schedule

Study timeline	D0	D56	D70 (D14 post 2nd dose)	D84 (D28 post 2nd dose)	D188 (D132 post 2nd dose)	D292 (D236 post 2nd dose)
Secondary endpoints*	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG
	Anti-nucleocapsid IgG	Anti-nucleocapsid IgG	Anti-nucleocapsid IgG		Anti-nucleocapsid IgG	Anti-nucleocapsid IgG
	T cell ELISpot	T cell ELISpot	T cell ELISpot		T cell ELISpot	T cell ELISpot
	MLF	MLF	MLF			
				PBMC for storage		

**Immunological endpoints; MLF = mucosal lining fluid; PBMC = peripheral blood mononuclear cells collected at one timepoint.*

Table 3.5 Cohort B blood and mucosal fluid sampling schedule in relation to study visits and study vaccination
Adapted from Com-COV3 Protocol V10.0 31 Aug 2023

Study timeline	D0	D28	D84	D182	D210*
COVID-19 vaccination	X			X*	
4CMenB vaccination*	X*		X*		
Immunological endpoints	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG	Anti-spike IgG
	MLF	MLF	MLF	MLF	MLF
	Anti-nucleocapsid IgG		Anti-nucleocapsid IgG	Anti-nucleocapsid IgG	
	T cell ELISpot	T cell ELISpot	T cell ELISpot	T cell ELISpot	T cell ELISpot
	PBMC for storage	PBMC for storage		PBMC for storage	PBMC for storage

**Control group only; MLF = mucosal lining fluid; PBMC = peripheral blood mononuclear cells.*

3.2.7 Statistical analysis

3.2.7.1 Cohort A

Cohort A immunogenicity analyses were performed using the modified intention-to-treat (mITT) populations at 28, 132 and 236 days following the second vaccination overall and stratified by pre-second dose serostatus (defined by anti-nucleocapsid value pre-second dose or pre-first dose if missing). The Cohort A mITT populations excluded participants who withdrew, had no blood sample, received a third dose in the community before their visit or self-reported a SARS-CoV-2 infection within 14 days after the second vaccination. Immunogenicity outcomes were summarised using geometric mean concentrations (GMCs) and 95% confidence intervals (CIs). Adjusted geometric mean ratios (aGMRs) and 95% CIs were calculated comparing groups to BNT-30 as the reference, adjusting for study site in the linear regression models.

A 'breakthrough infection' between the second dose and day 236 visit was defined as either: a self-reported SARS-CoV-2 infection>14 days after second dose, a two-fold rise in anti-nucleocapsid IgG, a two-fold rise in anti-spike antibodies, or a seroconversion of anti-nucleocapsid IgG serostatus. Distributions according to the different definitions of SARS-CoV-2 infections during follow-up were presented across the study arms.

3.2.7.2 Cohort B

Cohort B was designed to detect non-inferiority between adult BNT-10 and paediatric BNT-10 at 28 days post-third dose. The sample size calculation assumed a standard deviation of 0.3 of anti-spike IgG on a log10 scale based on cohort A data (as previously described), a non-inferiority

margin of 0.67, and the true mean difference between the two study groups to be zero (or a GMR of one). Originally, the study aimed to recruit 76 participants in the co-primary outcome study groups to achieve 90% power at a two-sided 5% type I error level assuming a 15% attrition rate for an effective sample size of 64 participants in each group. Following recruitment challenges, the type I error level was relaxed from two-sided to one-sided following discussion with the TSC, which reduced target recruitment to 62 participants per arm in the two BNT-10 groups, with an effective sample size of 52 participants in each group.

The immunogenicity co-primary outcome analysis was non-inferiority of anti-spike IgG for adult BNT-10 compared with paediatric BNT-10. The primary analysis was conducted on the per-protocol (PP) population at 28 days post-third dose. All participants who received a third vaccine as randomised, with endpoint data available, no self-reported COVID-19 infection within 28 days post-vaccination, and no protocol deviations of timing of blood sample were included. The aGMR for the co-primary outcome was presented with a one-sided 95% CI. Non-inferiority was concluded if the lower limit of the CI lay above the 0.67 non-inferiority margin.

Cohort B secondary immunogenicity outcome analyses were performed using the mITT populations days 0, 28, 84, 182, and 210 post-third vaccination overall and stratified by pre-third dose serostatus. Cohort B immunogenicity outcomes were summarised as for Cohort A. Data below the LLOD were imputed with a value equal to half the threshold before transformation. Data above the upper limit of detection were included in analyses as they were deemed meaningful. Adjusted GMRs and CIs were calculated as the antilogarithm of the difference between the mean of the log10 transformed titre in study groups compared to the reference study group, the control arm. The 30µg BNT162b2 study arm was also used as a reference group. The linear regression models were used to estimate the difference in means after log10 transformation, adjusting for study site, previous positive COVID-19 test, age, interval between second and third dose, and baseline immunogenicity value as fixed effects.

Anti-nucleocapsid IgG in the mITT populations were analysed to determine the frequency of seropositivity at enrolment and seroconversion throughout the study. A 'breakthrough infection' in Cohort B was defined as either: a self-reported SARS-CoV-2 infection after 28 days following a third dose, a two-fold rise in anti-nucleocapsid IgG between day 28 and day 84 and between day 84 and day 182, a two-fold rise in anti-spike antibodies between day 28 and day 84 and between day 84 and day 182, or a seroconversion of anti-nucleocapsid IgG serostatus. Distributions according to the different definitions of SARS-CoV-2 infections during follow-up were presented across study arms.

3.3 Cohort A Results

3.3.1 Recruitment

Between 27th September and 29th November 2021, 179 volunteers were screened across seven UK sites, and 148 participants were enrolled. Of these participants, 81 received their first vaccination in the trial. Sixteen participants withdrew prior to randomisation before second vaccination. Prior to 29th November, 117 participants were randomised to three study arms, **Figure 3.4**. Following an amendment to the trial to remove the NVX arm, a further 15 participants were randomised to the two study arms (BNT-30 and BNT-10). In total, 132 participants were randomised and received a second dose in the trial. Recruitment occurred prior to a period of increased SARS-CoV-2 infections, initially due to the Delta Variant, and subsequently Omicron BA.1 and BA.2.

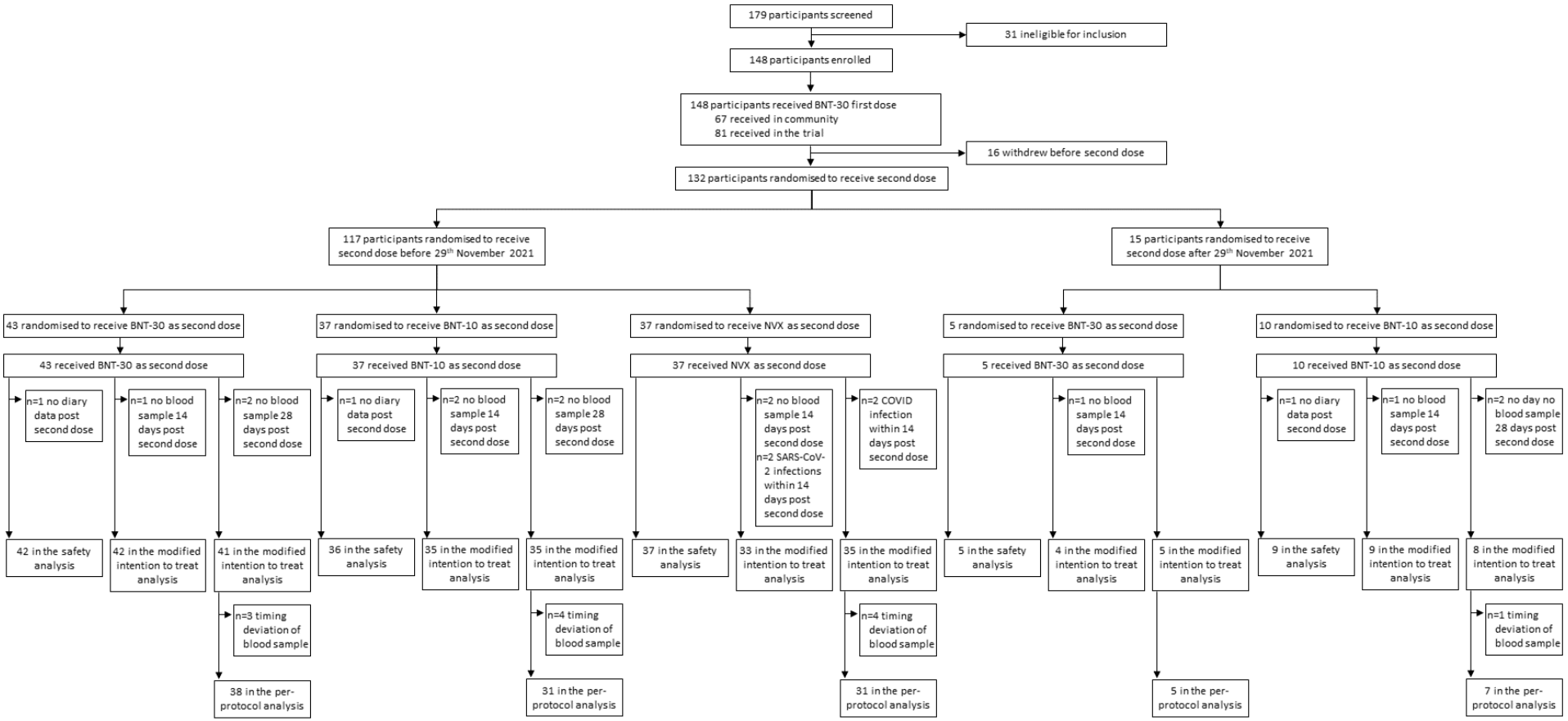


Figure 3.4 Cohort A Consolidated Standards of Reporting Trials (CONSORT) Flow Diagram and Study Design.[136]
BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373.

Of all 148 participants enrolled, the median age was 14 years (range 12–17), 62% were female, and six participants (4%) were from an ethnic minority group (**Table 3.6**). Forty-eight participants were randomised to BNT-30, 47 to BNT-10 and 37 to NVX. The median interval between two doses was 59 days (range 56–109) and was similar across the study arms. Of 132 participants randomised, 65 received their first dose in the trial, of whom 19% were positive for SARS-CoV-2 anti-nucleocapsid IgG prior to their first dose. Seropositivity increased to 30% prior to the second dose.

Table 3.6 Study participant characteristics according to study arm.

Characteristic	BNT-30 (N=48)	BNT-10 (N=47)	NVX (N=37)	Total randomised to second	Not randomised to receive	All enrolled† (N=148)
Age (years)						
Mean (SD)	14.6 (1.3)	14.4 (1.4)	14.6 (1.4)	14.5 (1.4)	14.0 (1.3)	14.5 (1.4)
Median (range)	15 (12, 17)	14 (12, 17)	14 (12, 17)	14 (12, 17)	14 (12, 16)	14 (12, 17)
Gender						
Female	28 (58.3%)	28 (59.6%)	24 (64.9%)	80 (60.6%)	11 (68.8%)	91 (61.5%)
Male	20 (41.7%)	19 (40.4%)	13 (35.1%)	52 (39.4%)	5 (31.3%)	57 (38.5%)
Ethnicity						
White	44 (91.7%)	47 (100%)	36 (97.3%)	127 (96.2%)	15 (93.8%)	142 (95.9%)
Asian	1 (2.1%)	0 (0%)	0 (0%)	1 (0.8%)	1 (6.3%)	2 (1.4%)
Mixed	3 (6.3%)	0 (0%)	1 (2.7%)	4 (3.0%)	0 (0%)	4 (2.7%)

Characteristic	BNT-30 (N=48)	BNT-10 (N=47)	NVX (N=37)	Total randomised to second	Not randomised to receive	All enrolled† (N=148)
Anti-nucleocapsid IgG serostatus pre-first dose*†						
Seropositive	5 (19.2%)	5 (19.2%)	2 (15.4%)	12 (18.5%)	2 (12.5%)	14 (17.3%)
Seronegative	20 (76.9%)	21 (80.8%)	11 (84.6%)	52 (80%)	13 (81.3%)	65 (80.3%)
Unknown	1 (3.8%)	0 (0%)	0 (0%)	1 (1.5%)	1 (6.3%)	2 (2.5%)
Anti-nucleocapsid IgG serostatus pre-second dose						
Seropositive	15 (31.3%)	14 (29.8%)	10 (27.0%)	39 (29.5%)	-	39 (26.4%)
Seronegative	32 (66.7%)	32 (68.1%)	27 (73.0%)	91 (68.9%)	-	91 (61.5%)
Unknown	1 (2.1%)	1 (2.1%)	0 (0%)	2 (1.5%)	16 (100%)	18 (12.2%)
Days between two doses, median	58 (56, 95)	61 (56, 105)	59 (56, 109)	59 (56, 109)	-	59 (56, 109)

BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373; SD: standard deviation.
** Anti-nucleocapsid IgG serostatus pre-first dose includes participants who received their first and second doses in the study (denominator of 65).*
† Anti-nucleocapsid IgG serostatus pre-first dose includes participants who received their first dose in the study (denominator of 81).

3.3.2 Immunogenicity

3.3.2.1 Peak humoral and cellular immune responses

The highest anti-spike antibody concentrations occurred in the BNT-30 and NVX groups at 28 days after the second dose, with GMC of 19,005 BAU/ml (95% CI: 15,916, 22,694) and 20,172 (95% CI: 16,128, 25,230) in each study group, respectively (**Figure 3.5**) (aGMR for NXV versus BNT-30: 1.09 [95% CI: 0.84, 1.42]). By contrast, anti-spike antibody concentrations in the BNT-10 group were 14,408 BAU/ml (95% CI: 12,438, 16,689; aGMR versus BNT-30: 0.78 [95% CI: 0.61, 0.99]). Among participants who were anti-nucleocapsid antibody negative prior to their second dose (hereafter referred to as seronegative participants), anti-spike antibody concentrations 28 days after the second dose were significantly lower in BNT-10 compared to BNT-30 recipients (aGMR 0.70 [95% CI: 0.53, 0.93]). Conversely, seronegative NVX recipients tended to have higher anti-spike antibody concentrations than BNT-30 recipients (aGMR 1.33 [95% CI: 0.98, 1.79]), although not reaching statistical significance. Among seropositive pre-second dose participants (hereafter referred to as seropositive participants), anti-spike antibody concentrations were similar between BNT-10 and BNT-30 recipients (aGMR 0.98 [95% CI: 0.65, 1.46]), however seropositive NVX participants had lower concentrations than BNT-30 recipients (aGMR 0.60 [95% CI: 0.37, 0.95]) and had the lowest anti-spike antibody response among any serostatus and study arm subgroup (GMC 11,723 BAU/ml [95% CI: 7573, 18,146]).

The aGMRs between the VNA titres (FRNT50) against the Victoria (ancestral) strain, relative to BNT-30, followed a similar pattern to those of binding antibodies at 28 days after second dose, **Figure 3.5**. However, NVX participants demonstrated higher titres compared with BNT-30 against both Omicron strains with aGMRs of 1.7 (95% CI: 1.07, 2.69) (BA.1) and 1.43 (95% CI: 0.96, 2.12) (BA.2), **Figure 3.6**. This was most pronounced in seronegative NVX recipients with aGMRs of 1.95 (95% CI: 1.18,

3.23) (BA.1) and 1.74 (95% CI: 1.07, 2.84) (BA.2). For BNT-10 recipients, titres were similar to BNT-30 recipients irrespective of serostatus prior to the second dose.

Cellular immune responses against wild-type virus at 14 days after the second vaccination were greatest in the NVX group (GMC 121 SFC/106 PBMCs [95% CI: 73, 200]), followed by BNT-30 (GMC 75 SFC/10^6 PBMCs [95% CI: 50, 114]) (**Figure 3.5**). Similar patterns were seen in both seropositive and seronegative subgroups; however, responses were consistently higher in seropositive participants across all groups.

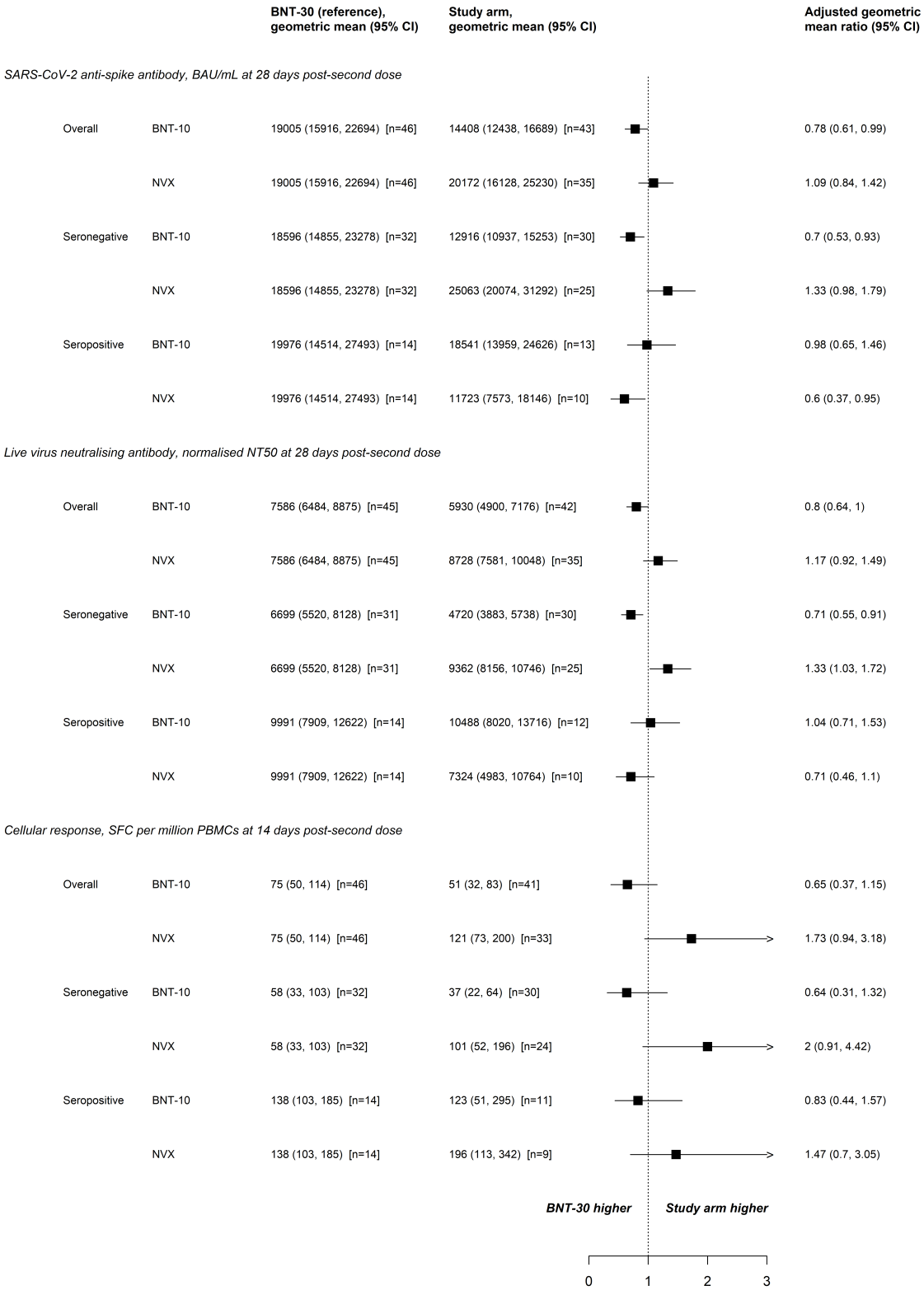


Figure 3.5 Anti-spike antibody and live virus neutralising antibody (Victoria strain) response at day 28 and cellular immune response at day 14 after the second vaccine dose by study arm and pre-second serostatus in the day 28 mITT population.

BNT-10: BNT162b2 10 µg; NVX: NVX-CoV2373; CI: confidence interval. Data presented are the geometric means, adjusted geometric mean ratios and the corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios between BNT-30 and either BNT-10 or NVX are adjusted for the study site as a fixed effect. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference BNT-30 study arm. Figure taken from Kelly E, et al. (2023).[136]

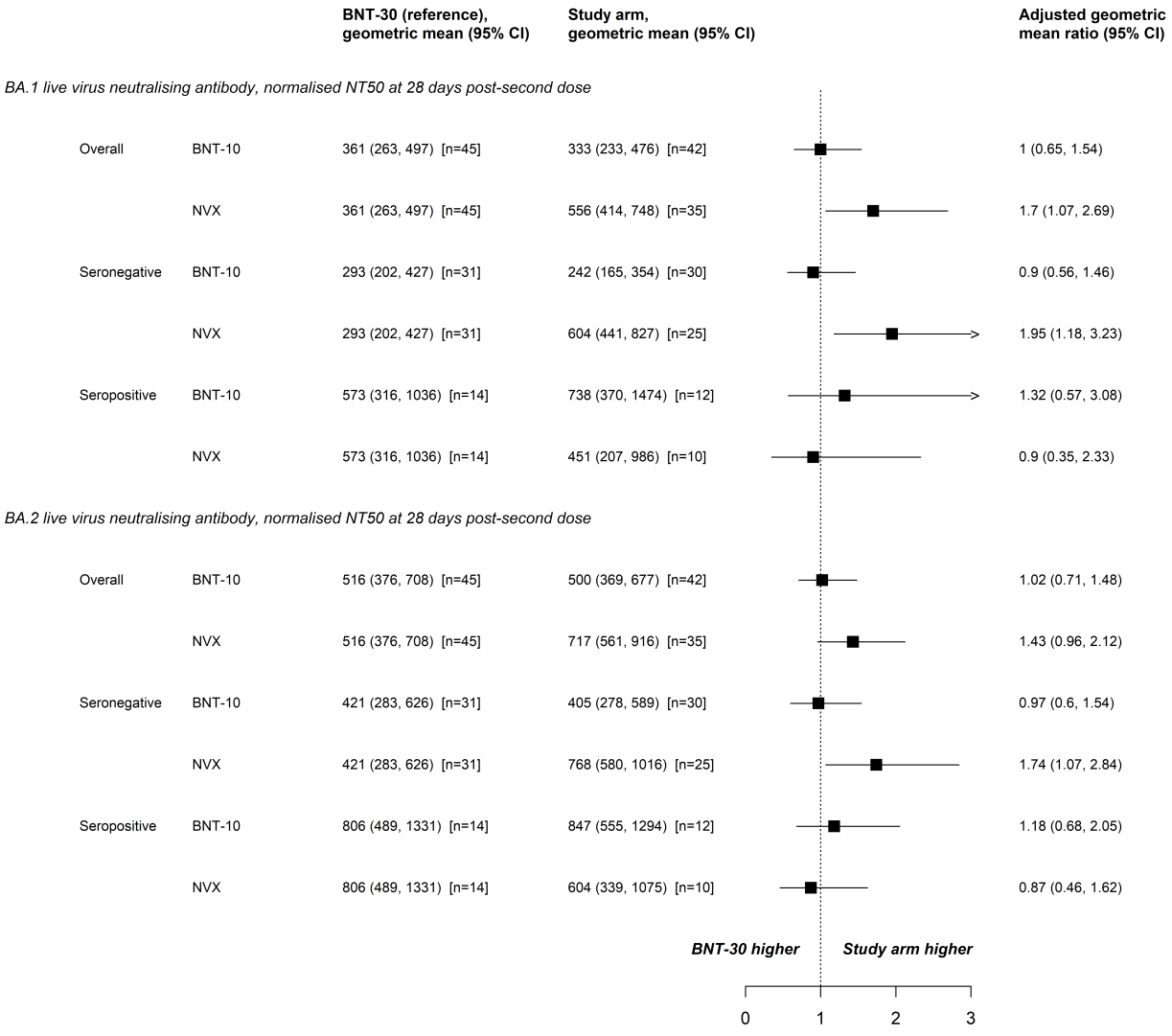


Figure 3.6 Virus neutralising antibody activity against Omicron BA.1 and BA.2 variants by study arm and pre-second dose serostatus at 28 days after the second vaccine dose in the day 28 modified intention-to-treat population.

BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373; CI: confidence interval. Data presented are the geometric means, adjusted geometric mean ratios and their corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios between BNT-30 and either BNT-10 or NVX are adjusted for study site as a fixed effect. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the

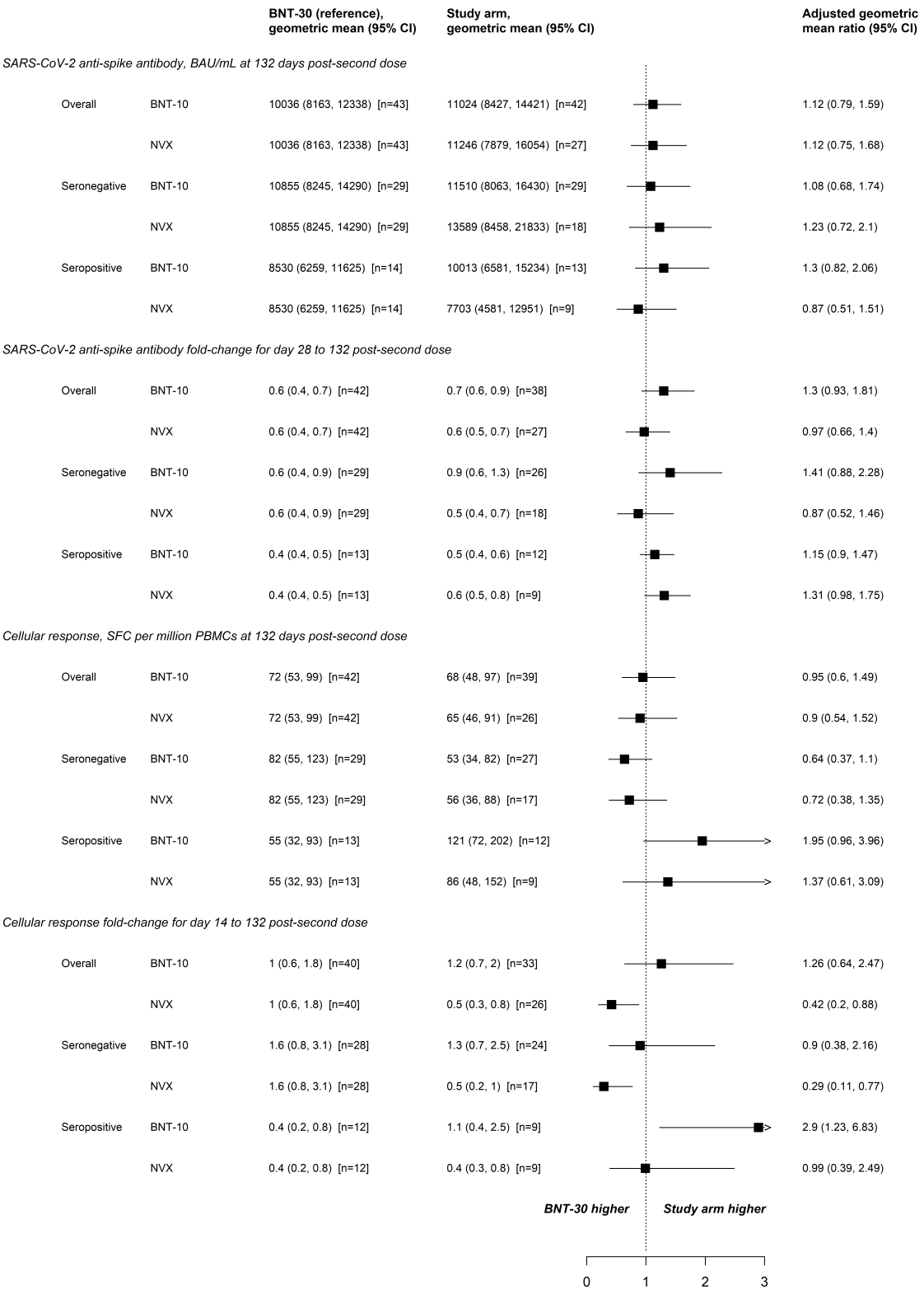
geometric mean concentrations between the study arm and the reference BNT-30 study arm. Figure taken from Kelly E, et al. (2023) [136]

3.3.2.2 Persistence of the humoral and cellular immune response

The persistence of humoral and cellular immune responses following immunisation was assessed at 132 and 236 days after the second dose. In the mITT population, anti-spike antibody values were similar for BNT-10 and NVX compared with BNT-30 participants at each timepoint, irrespective of serostatus (**Figure 3.7**). However, anti-spike antibody values were highest for seronegative NVX participants at both timepoints, **Figure 3.7**. There were no differences in fold-changes after the second dose across the groups.

The cellular immune response against wild-type virus at 132 days after the second dose was also similar across the groups, **Figure 3.7 (A)**; however, responses tended to be highest in seropositive BNT-10 and NVX participants compared with BNT-30. Conversely, seronegative BNT-30 participants tended to have higher responses compared with BNT-10 and NVX participants. Cellular responses across the study arms were similar at day 236. Responses for NVX participants were almost halved from day 14 (GMC 121 SFC/106 [95% CI: 73, 200]) to 236 after the second dose (GMC 75 [95% CI: 49, 114], fold-change 0.4 [95% CI: 0.3, 0.6]) (**Figure 3.7 (B)**).

A) Day 132 post-second dose



(B) Day 236 post-second dose

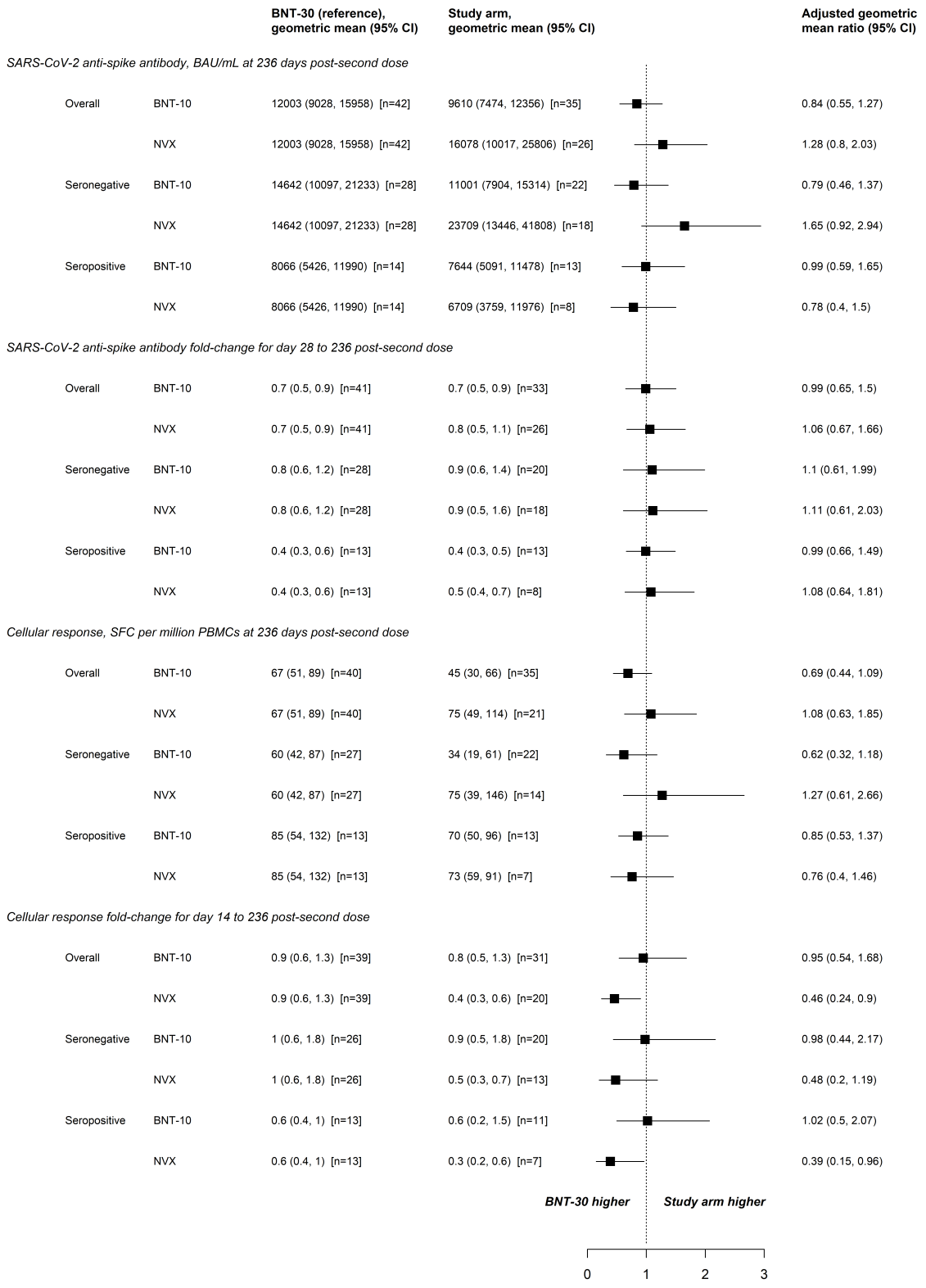


Figure 3.7 Anti-spike antibody and cellular immune responses by study arm and pre-second dose serostatus in the modified intention-to-treat populations, (A) day 132 post-second dose, (B) day 236 post-second dose.

BNT-10: BNT162b2 10 µg; NVX: NVX-CoV2373; CI: confidence interval. Data presented are adjusted geometric mean ratios and the corresponding 95% confidence intervals. The circles indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios between BNT-30 and either BNT-10 or NVX are adjusted for the study site as a fixed effect. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference BNT-30 study arm. Figure taken from Kelly E, et al. (2023).[136]

3.3.3 Breakthrough infection

In participants randomised before 29th November in the mITT population, 36 self-reported 'breakthrough infections' occurred during follow-up (n = 32/36 had serological evidence of infection) and there was a difference in proportions across groups, with rates highest in BNT-10 and lowest in NVX recipients, **Table 3.7**. Nearly all self-reported 'breakthrough infections' occurred in seronegative participants (n = 33/36). An additional 25 participants had serological evidence of infection without self-reporting, such that the proportion of probable 'breakthrough infections' by any definition up to day 236 was 66% in BNT-30, 69% in BNT-10% and 62% in the NVX group (**Table 3.7**). When limited to seronegative participants, this was 81%, 89% and 72% respectively.

When participants with breakthrough infection were removed from the analysis, seronegative BNT-10 participants in the day 132 mITT population with no breakthrough infections (and therefore, no boosting by infection) were shown to have lower anti-spike antibody concentrations compared with BNT-30 participants (aGMR 0.54 [95% CI: 0.31, 0.94]), **appendix**, **Supplementary Figure 5**.

Table 3.7 SARS-CoV-2 infections following second dose for participants randomised to three study arms before 29th November 2021 in the day 236 modified intention-to-treat population.

	BNT-30	BNT-10	NVX	Total
All participants in the day 236 modified intention-to-treat population	**N=38**	**N=29**	**N=26**	**N=93**
Self-reported infection up to day 132	9 (24%)	13 (45%)	3 (12%)	25 (27%)
Self-reported infection up to day 236	13 (34%)	15 (52%)	8 (31%)	36 (39%)
Seroconversion of anti-n status from second dose to day 132 post second dose	9 (24%)	13 (45%)	5 (19%)	27 (29%)
Seroconversion of anti-n status from day 132 to day 236 post second dose	8 (21%)	2 (6.9%)	4 (15%)	14 (15%)
2-fold rise in anti-n value from second dose to day 132 post second dose	12 (32%)	16 (55%)	7 (27%)	35 (38%)
2-fold rise in anti-n value from day 132 to day 236 post second dose	16 (42%)	6 (21%)	6 (23%)	28 (30%)
2-fold rise in anti-s value from day 28 to day 132 post second dose	3 (7.9%)	4 (14%)	0 (0%)	7 (7.5%)
2-fold rise in anti-s value from day 132 to day 236 post second dose	10 (26%)	3 (10%)	7 (27%)	20 (22%)
Infection by any definition up to day 132	13 (34%)	16 (55%)	7 (27%)	36 (39%)
Infection by any definition up to day 236	25 (66%)	20 (69%)	16 (62%)	61 (66%)
Seronegative participants in the day 236 modified intention-to-treat population	**N=26**	**N=18**	**N=18**	**N=62**
Self-reported infection up to day 132	9 (35%)	13 (72%)	1 (5.6%)	23 (37%)
Self-reported infection up to day 236	13 (50%)	14 (78%)	6 (33%)	33 (53%)
Seroconversion of anti-n status from second dose to day 132 post second dose	9 (35%)	13 (72%)	5 (28%)	27 (44%)
Seroconversion of anti-n status from day 132 to day 236 post second dose	8 (31%)	2 (11%)	4 (22%)	14 (23%)
2-fold rise in anti-n value from second dose to day 132 post second dose	10 (38%)	14 (78%)	5 (28%)	29 (47%)
2-fold rise in anti-n value from day 132 to day 236 post second dose	14 (54%)	5 (28%)	5 (28%)	24 (39%)
2-fold rise in anti-s value from day 28 to day 132 post second dose	3 (12%)	4 (22%)	0 (0%)	7 (11%)
2-fold rise in anti-s value from day 132 to day 236 post second dose	9 (35%)	3 (17%)	7 (39%)	19 (31%)

	BNT-30	BNT-10	NVX	Total
Infection by any definition up to day 132	11 (42%)	14 (78%)	5 (28%)	30 (48%)
Infection by any definition up to day 236	21 (81%)	16 (89%)	13 (72%)	50 (81%)

Events are >14 days after second dose. BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373.

3.4 Cohort B Results

3.4.1 Recruitment

Of the 298 participants screened between 01 June 2022 and 30th June 2023, 283 participants were eligible to enrol in the trial. At the day 0 visit, one participant was excluded, and 282 participants underwent randomisation, **Figure 3.8**. Baseline characteristics were balanced across the study arms (**Table 3.8**). The mean age of participants was 14.1 (range 12.4-15.5) years. Most participants were Caucasian (89%) and 57% were female. The mean interval between second and third COVID-19 vaccination was 244.8 (range 91-501) days. More than half of participants (64%) reported at least one previous COVID-19 infection, and the proportion was balanced between the study arms.

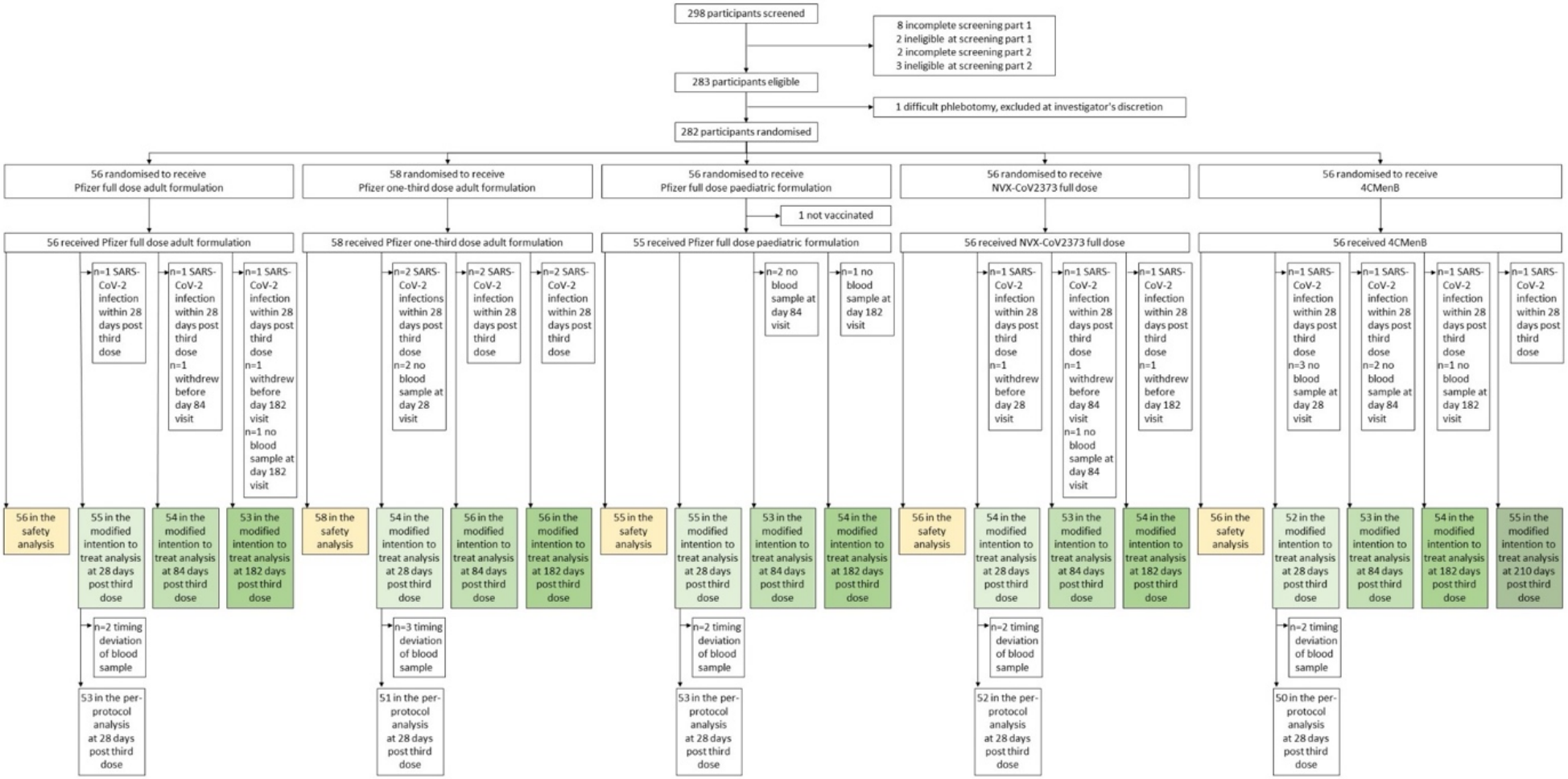


Figure 3.8 Cohort B Consolidated Standards of Reporting Trials (CONSORT) Flow Diagram and Study design

Characteristic	Pfizer full dose adult formulation N = 56	Pfizer 1/3 dose adult formulation N = 58	Pfizer full dose paediatric formulation N = 55	NVX-CoV2373 full dose N = 56	4CMenB N = 56	Total N = 281
Age (years)						
Mean (SD)	14.2 (0.8)	14.2 (0.9)	14.1 (0.8)	14.1 (0.9)	14.0 (0.9)	14.1 (0.9)
Range	12.4, 15.4	12.6, 15.5	12.5, 15.5	12.6, 15.5	12.5, 15.3	12.4, 15.5
Sex						
Male	24 (43%)	24 (41%)	22 (40%)	28 (50%)	24 (43%)	122 (43%)
Female	32 (57%)	34 (59%)	33 (60%)	28 (50%)	32 (57%)	159 (57%)
Ethnicity						
White	46 (82%)	55 (95%)	48 (87%)	49 (88%)	53 (95%)	251 (89%)
Mixed	4 (7.1%)	1 (1.7%)	1 (1.8%)	4 (7.1%)	2 (3.6%)	12 (4.3%)
Asian	4 (7.1%)	2 (3.4%)	3 (5.5%)	2 (3.6%)	0 (0%)	11 (3.9%)
Other	2 (3.6%)	0 (0%)	3 (5.5%)	1 (1.8%)	1 (1.8%)	7 (2.5%)
Prefers not to give	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Days since previous COVID-19 vaccination						
Mean (SD)	235.7 (96.4)	240.1 (78.7)	248.5 (96.0)	255.1 (90.2)	244.6 (97.4)	244.8 (91.5)
Range	98.0, 487.0	93.0, 459.0	91.0, 496.0	108.0, 493.0	94.0, 501.0	91.0, 501.0
Previous positive COVID-19 test						
No	16 (29%)	18 (31%)	18 (33%)	16 (29%)	17 (30%)	85 (30%)
Yes	40 (71%)	40 (69%)	37 (67%)	40 (71%)	39 (70%)	196 (70%)
Baseline serostatus						
Seropositive	48 (86%)	51 (88%)	48 (87%)	51 (91%)	45 (80%)	243 (86%)
Seronegative	8 (14%)	7 (12%)	7 (13%)	5 (8.9%)	11 (20%)	38 (14%)

Table 3.8 Cohort B Demographics and baseline characteristics by study arm.

Data presented are frequency (percentage) for categorical variables and mean (standard deviation) and range for continuous variables, SD: standard deviation.

3.4.2 Immunogenicity

3.4.2.1 Third dose adult BNT-10 is non-inferior and superior to paediatric BNT-10

In the PP population, anti-spike IgG GMC at 28 days post-boost in paediatric BNT-10 was 41906 (95%CI 34636-50702) and in adult BNT-10 was 62635 (95%CI 51907-75579) ELU/ml. Compared with paediatric BNT-10, adult BNT-10 was not only non-inferior with aGMR of 1.50 (one-sided 95%CI: 1.25, ∞), but also superior (aGMR: 1.50, two-sided 95%CI: 1.21, 1.85). The sensitivity analysis in the mITT population showed similar results, **Figure 3.9**.

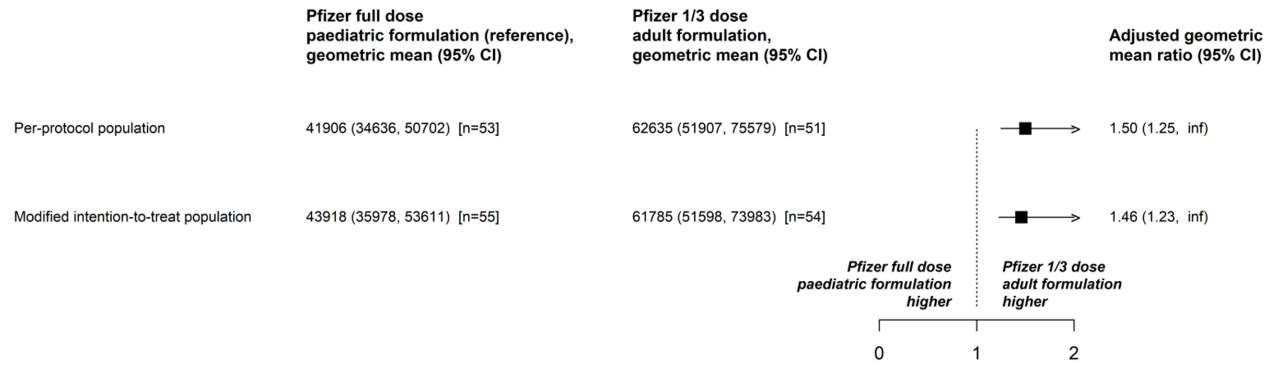


Figure 3.9 Co-primary immunogenicity outcome – SARS-CoV-2 anti-spike antibody, ELU/mL at 28 days post-third vaccination.
Data presented are the geometric means and their corresponding 95% confidence intervals, and the adjusted geometric mean ratios and their corresponding one-sided 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios are adjusted for study site, age, interval between second and third COVID-19 vaccinations, any previous positive COVID-19 test, and baseline immunogenicity value as fixed effects. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the geometric mean concentrations between adult 10µg BNT162b2 study arm and the reference paediatric 10µg BNT162b2 study arm. CI: Confidence interval.

3.4.2.2 Humoral immune response

Immunogenicity analysis in Cohort B was not performed according to participant pre-vaccination anti-nucleocapsid IgG serostatus as more than 80% of Cohort B participants were seropositive prior to vaccination. Compared with the control arm, humoral and cellular responses were significantly boosted at 28 days post-third dose in the four COVID-19 vaccine arms (**Figure 3.10**). In the four COVID-19 vaccine arms, the aGMRs of anti-spike IgG in all participants ranged from 2.55 (95%CI: 2.06-3.16) in paediatric BNT-10 to 4.04 (95%CI: 3.26-5.00) in 30µg BNT162b2. By day 182, the anti-spike IgG response persisted and was significantly higher in the COVID-19 vaccine arms compared with the control arm, the aGMR ranging from 1.42 (95%CI: 1.12-1.81) in paediatric BNT-10 and 1.88 (95%CI: 1.48-2.39) in 30µg BNT162b2. A similar pattern was also observed for cellular responses with significantly higher T-cell responses seen at 28 days in the COVID-19 vaccine arms compared with the control arm. This difference was no longer statistically significant by 182 days post-vaccination.

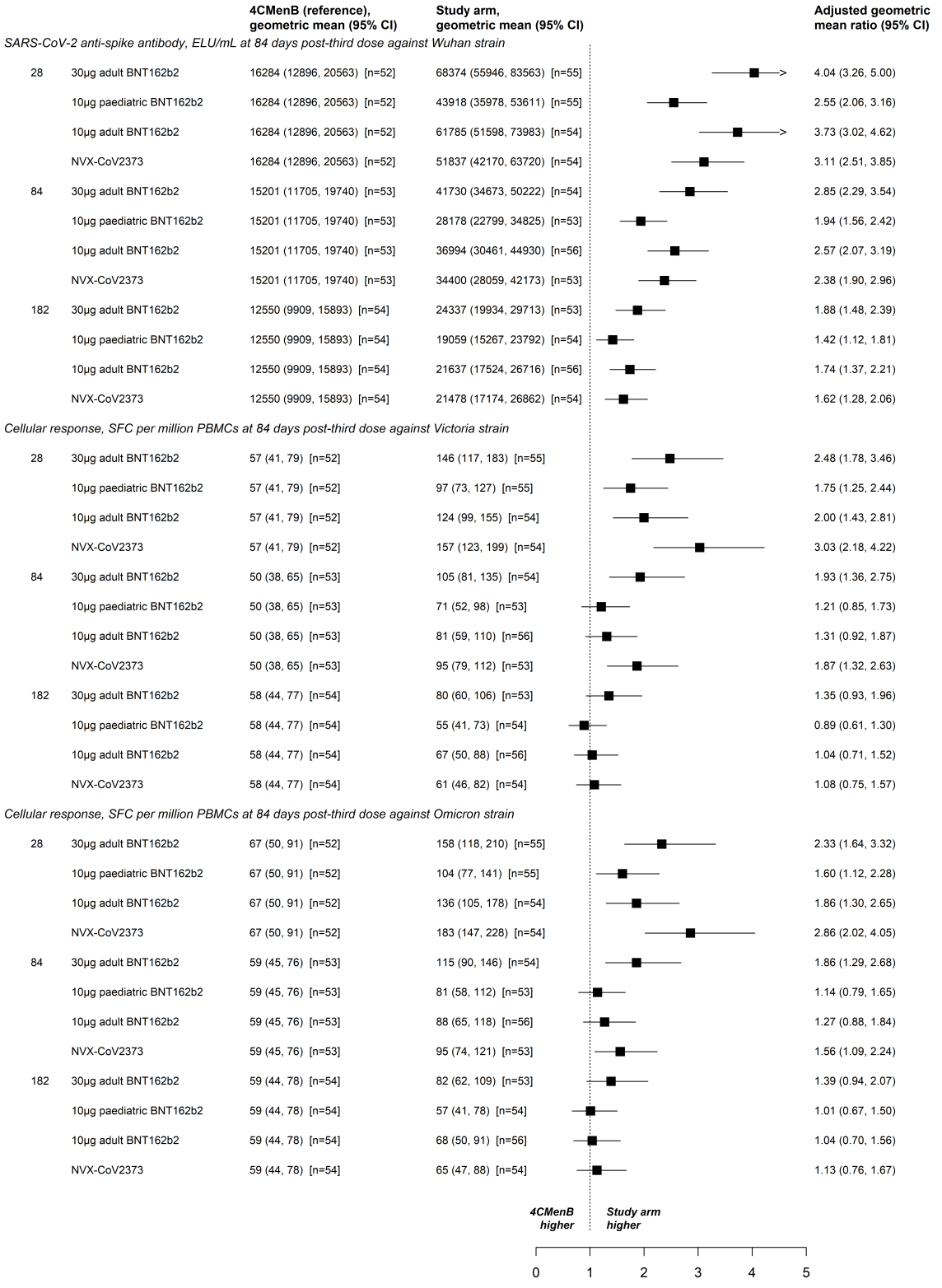


Figure 3.10 - Immune responses following third dose vaccination by study arm in the modified intention-to-treat population.

The control arm is the reference group. Data presented are the geometric means, adjusted geometric mean ratios and their corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios are adjusted for study site, age, interval between second and third COVID-19 vaccination, any

previous positive COVID-19 test, and baseline immunogenicity value as fixed effects. SARS-CoV2 anti-spike antibodies against Wuhan variant were used as a proxy for baseline immunogenicity values in the case of live virus neutralising antibody models where corresponding baseline immunogenicity values were not available. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference study arm. CI: Confidence interval.

Across the four COVID-19 vaccine arms, anti-spike IgG concentrations post-vaccination were similar between 30µg BNT162b2 and adult BNT-10 during the follow-up: GMC at 28 days post-boost was 68374 ELU/mL (95%CI 55946-83563) and 61785 (51598-73983) in BNT-30 and adult BNT-10, respectively, with an aGMR of 0.93 (95%CI 0.75-1.14), with 30µg BNT162b2 as the reference group. On the other hand, the GMC in paediatric BNT-10 was significantly lower than the BNT-30 arm during the whole study period with aGMRs of 0.63 (0.52-0.78), 0.68 (0.55,0.85), and 0.76 (0.61,0.96) at 28-, 84-, and 182-days, respectively, post-boost. For NVXCoV2373, the anti-spike IgG GMC was significantly lower at 28 days (aGMR of 0.77, 95%CI: 0.63-0.95), but this difference was no longer significant at 182 days post-boost (aGMR of 0.86, 95%CI: 0.68-1.09), **Figure 3.11**.

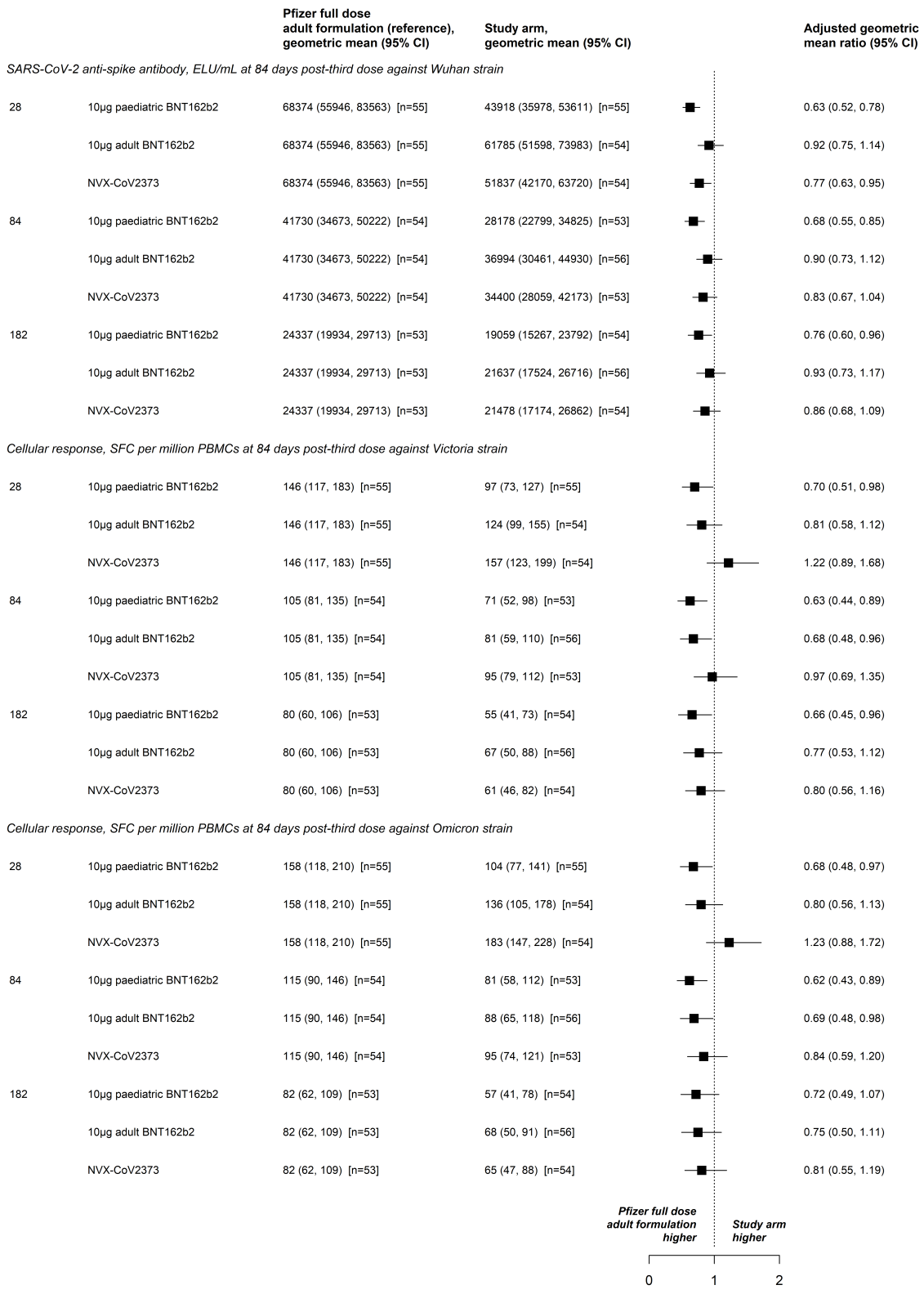


Figure 3.11 Anti-spike IgG and cellular responses to the Victoria and Omicron strains) following third dose vaccination by study arm in the modified intention-to-treat populations.
<u>The 30µg BNT162b2 study arm is the reference group</u>*. Data presented are the geometric means, adjusted geometric mean ratios, and their corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios are adjusted for study site, age, the interval between second and third COVID-19*

vaccination, any previous positive COVID-19 test, and baseline immunogenicity value as fixed effects. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and does not intersect the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference study arm. Baseline serostatus was defined using anti-nucleocapsid data at day 0 pre-vaccination. CI: Confidence interval.

Live VNA titres against Victoria were significantly lower in BNT-10 and NVXCoV2373 groups compared with 30µg BNT162b2 at 28 days post-boost (**Figure 3.12**). However, across the four COVID-19 vaccine arms, VNA titres against Victoria were not significantly different by the end of the study. There was no statistically significant difference between BNT-10, NVXCoV2373 and 30µg BNT162b2 groups across all time points for VNA titres against both Omicron BA.5 and XBB.15 except for the paediatric BNT-10 arm, which had significantly lower VNA titres at day 84 against Omicron BA.5 and XBB.15.

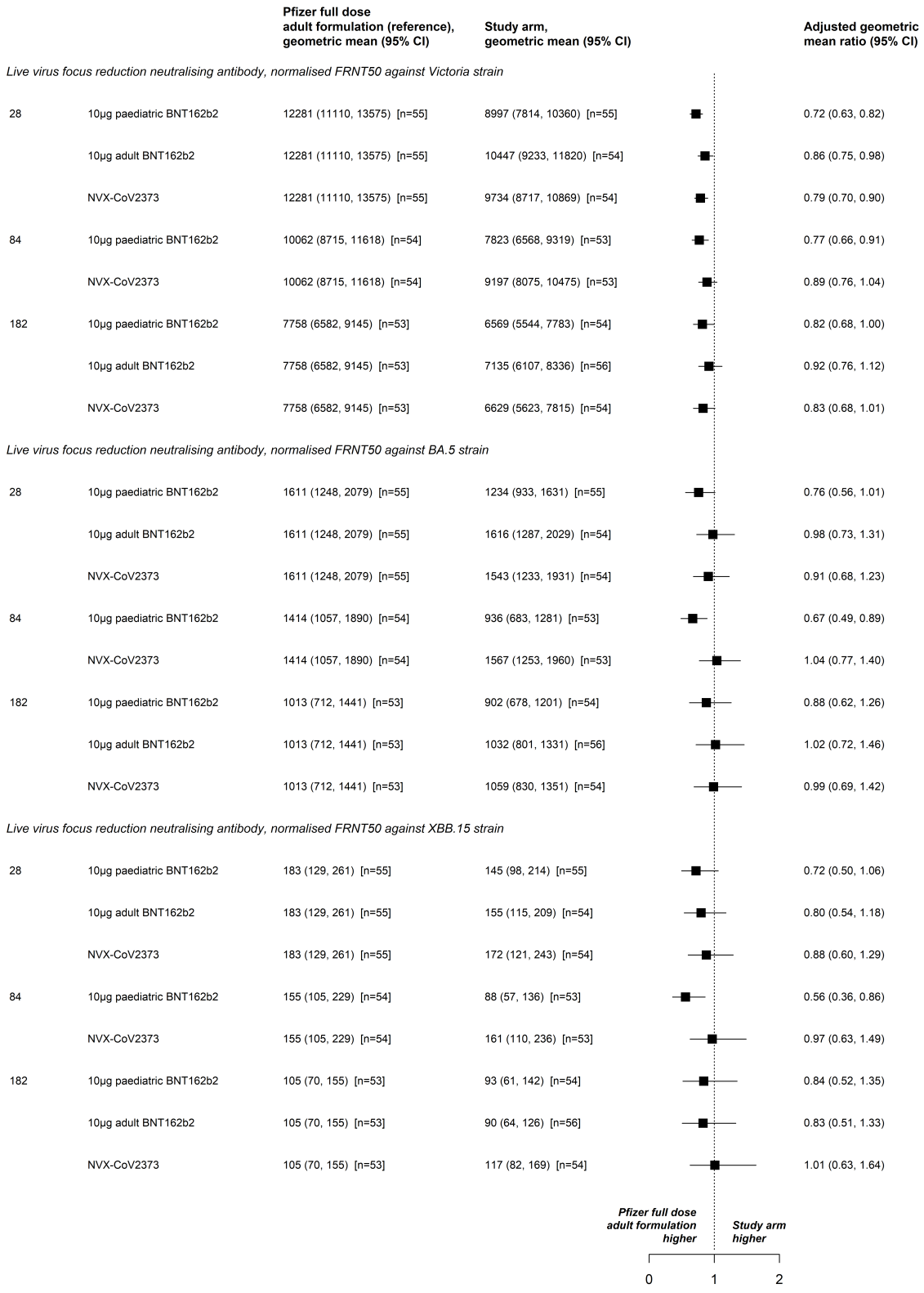


Figure 3.12 Live virus neutralising antibody responses post-third dose by study arms in the modified intention-to-treat populations.

The 30µg BNT162b2 study arm is the reference group. *Data presented are the geometric means, adjusted geometric mean ratios, and their corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios are adjusted for study site, age, the interval between second and third COVID-19*

vaccination, any previous positive COVID-19 test, and baseline immunogenicity values as fixed effects. SARS-CoV2 anti-spike antibodies against the ancestral strain were used as a proxy for baseline immunogenicity values in the case of live virus neutralising antibody models where corresponding baseline immunogenicity values were not available. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and does not intersect the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference study arm. CI: Confidence interval.

3.4.2.3 Cellular immune response

Overall, cellular responses to the Victoria strain were lower in adult and paediatric BNT-10 across all time points compared with 30µg BNT162b2, but only responses in paediatric BNT-10 reached statistical significance (**Figure 3.11**). There was no statistically significant difference in cellular responses between NVXCoV2373 and 30µg BNT162b2 arms. A similar cellular response pattern to Omicron was observed between vaccine groups (**Figure 3.11**).

3.4.2.4 Immune response to Comirnaty Original/Omicron BA.1 vaccination

Anti-spike IgG, VNA against Victoria strain, and cellular responses to Victoria and Omicron strains at day 210 (28 days following Comirnaty Original/Omicron BA.1 vaccination) in the control arm were similar to the immune responses at day 28 in the 30µg BNT162b2 arm. However, significantly higher VNA titres against Omicron BA.5 and XBB.15 were observed following Comirnaty Original/Omicron BA.1 vaccination [aGMR of 2.00 (95%CI 1.47-2.71) and aGMR 3.30 (95%CI 2.20-4.95), respectively],

Figure 3.13.

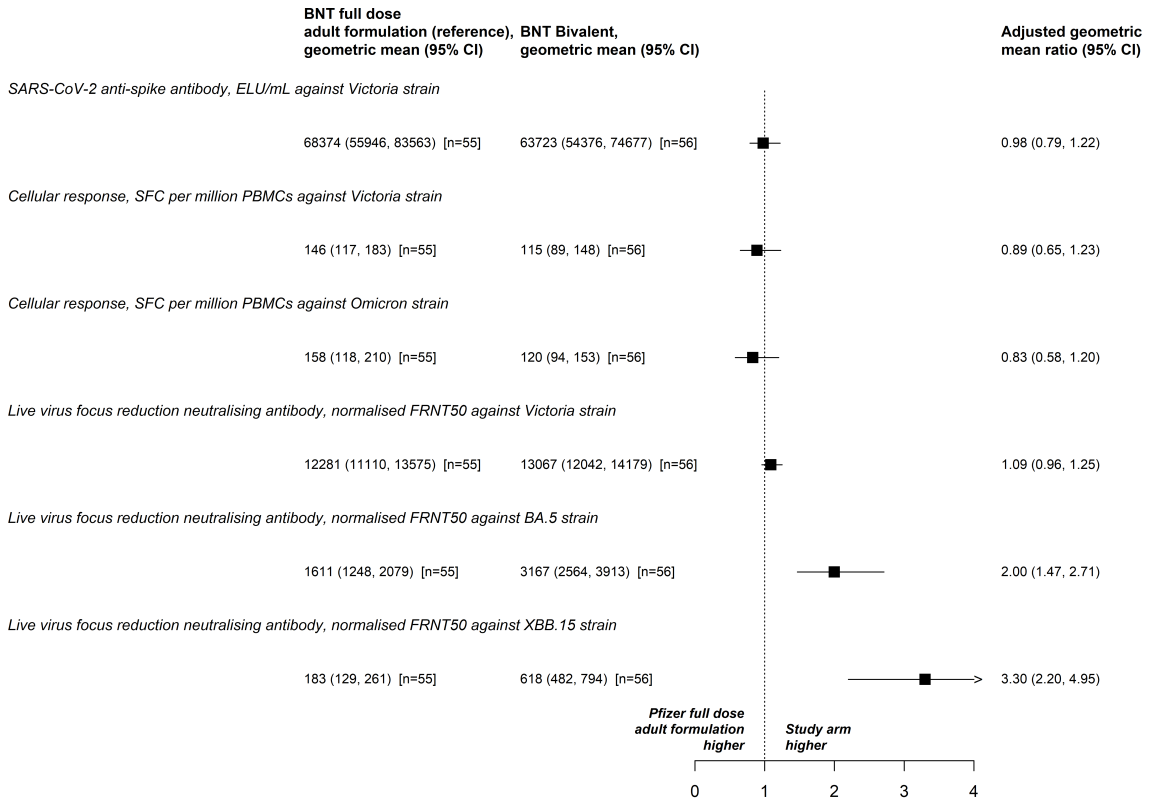


Figure 3.13 Humoral and cellular immune responses at day 28 following bivalent COVID-19 vaccination compared with BNT-30 (reference group) in the modified intention-to-treat population. *Data presented are the geometric means, adjusted geometric mean ratios, and their corresponding 95% confidence intervals. The boxes indicate the adjusted geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios are adjusted for study site, age, any previous positive COVID-19 test, and baseline immunogenicity value as fixed effects. SARS-CoV2 anti-spike antibodies against the ancestral strain were used as a proxy for baseline immunogenicity values in the case of live virus neutralising antibody models where corresponding baseline immunogenicity values were not available. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and does not intersect the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference study arm. CI: Confidence interval.*

3.4.3 Breakthrough infection

Overall, 33% of participants experienced breakthrough infection by any definition after vaccination, and all had a two-fold increase in anti-nucleocapsid IgG (**Table 3.9**). The proportion of breakthrough infections by any definition was similar across all study groups, including the control arm [range 14

(26%) in 30µg BNT162b2 to 20 (36%) in adult BNT-10]. Overall, 6.3% (17/271) of participants self-reported infection detected by LFT with the lowest proportion observed in the 30µg BNT162b2 (1/53, 1.9%) and the highest in the control arm (7/54, 13%). A similar pattern was also observed for breakthrough infections defined by a two-fold rise in anti-spike IgG.

Table 3.9 SARS-CoV-2 infections between day 0 to day 182 post-third dose by study arm and baseline serostatus in the day 182 modified intention-to-treat population

	Pfizer full dose adult formulation	Pfizer 1/3 dose adult formulation	Pfizer full dose paediatric formulation	NVX-CoV2373 full dose	4CMenB	Overall
All participants in the day 182 mITT population	N=53	N=56	N=54	N=54	N=54	N=271
Two-fold rise in anti-n value between day 0 to day 84 visit, or day 84 to day	14 (26%)	20 (36%)	19 (35%)	19 (35%)	18 (33%)	90 (33%)
Sero-conversion of anti-n status from day 0 to day 84, or day 84 to day	3 (5.7%)	4 (7.1%)	4 (7.4%)	2 (3.7%)	7 (13%)	20 (7.4%)
Two-fold rise in anti-s value between day 28 to day 84 visit, or day 84 to day	0 (0%)	1 (1.8%)	1 (1.9%)	2 (3.7%)	6 (11%)	10 (3.7%)
Self-reported infection from day 28 to day 182 visit	1 (1.9%)	2 (3.6%)	4 (7.4%)	3 (5.6%)	7 (13%)	17 (6.3%)
Infection by any definition up to day 182 visit	14 (26%)	20 (36%)	19 (35%)	19 (35%)	18 (33%)	90 (33%)

3.5 Discussion

This was the first RCT to investigate the immune response of adolescents to heterologous and fractional dose primary and booster COVID-19 vaccine schedules and demonstrated that heterologous COVID-19 vaccine schedules in adolescents are highly immunogenic and elicit a comparable immune response to the licensed homologous schedule for both prime and boost vaccination. The Cohort A study demonstrated that NVX following a first dose of 30 µg BNT162b2 elicited robust humoral and cellular immune responses, with higher neutralising titres against Omicron BA.1 and BA.2 variants compared with BNT-30. Seropositive BNT-10 participants demonstrated similar antibody responses to BNT-30 and, irrespective of serostatus, elicited similar VNA titres to BNT-30 against Omicron BA.1 and BA.2 variants.

Cohort B was the first study to demonstrate that a 10µg dose administered using the adult BNT162b2 vaccine formulation was non-inferior and superior to a 10µg dose administered using the paediatric BNT16b2 formulation when given as a third (booster) dose (assessed through superiority of the anti-spike IgG response at day 28 post-boost). Though all Cohort B COVID-19 vaccine study arms elicited a robust immune response, peak immune responses in the paediatric BNT-10 were notably less immunogenic.

3.5.1 Fractional dose BNT162b2 vaccine as a second or third booster dose is immunogenic in adolescents

Although BNT-10 as a second dose was immunogenic in adolescents, anti-spike antibody concentrations were significantly lower in seronegative BNT-10 participants compared with BNT-30. Higher rates of breakthrough infection were also found in seronegative BNT-10 recipients. Correspondingly, Dorabawila et al. reported a more significant decline in protection against infection caused by VOCs following two doses of 10µg BNT162b2 in 5-to-11-year-olds than following 30µg BNT162b2 in 12-to-17-year-olds.[140] However, the SARS-CoV-2 infection naïve status of the Cohort A Com-COV3 study population is now historical and largely unique, considering the current global situation of almost universal SARS-CoV-2 seropositivity (indicative of previous SARS-CoV-2 infection).[141] Indeed, in Cohort A seropositive participants, anti-spike antibodies were similar between the BNT-10 and BNT-30 study groups with few breakthrough infections in either group.

The performance of fractional dose BNT162b2 in seropositive Cohort A participants is also consistent with the previously demonstrated immune enhancing effects of "hybrid immunity", shown to provide greater protection against symptomatic re-infection.[142] As highlighted in this study and in Cohort B, the frequency of antigen encounter, either through infection or vaccination, has a significant effect on the humoral immune response, and the performance of fractional dose BNT162b2, either as a second dose in seropositive participants or as a third booster dose, may support the use of lower (fractional) vaccine doses when boosting immunity against infection. In adults, fractional doses of BNT162b2 as part of booster vaccination campaigns have already been shown to elicit comparable immunogenicity to full dose vaccine schedules.[37,143] This approach of reduced dosing as part of booster immunisation schedules has already been adopted by the mRNA vaccine manufacturer Moderna for its monovalent booster vaccines. However, Moderna still utilises a 50µg dose in its (monovalent) booster vaccines

[greater than that of the full dose (30µg) BNT162b2 studied here] and for its bivalent Omicron variant containing vaccines, which contain 25µg each of the two target strains.[144]

This is the first clinical trial to show that adult BNT-10 elicited a superior immune response to paediatric BNT-10 and a similar immune response to BNT-30 when given as a third dose. The significant difference in immunogenicity between the formulations may be accounted for by differences in vaccine constitution or preparation. However, the mechanism of action, active ingredients and list of excipients are identical for both vaccines.[145,146] Vaccine preparation was followed in accordance with the trial vaccine preparation and administration standard operating procedure. Both vaccines share similar preparation steps though differ in the concentration of solution used for dilution: 1.8mL (adult BNT162b2) versus 1.3mL (paediatric BNT162b2) of sodium chloride (0.9%). Following dilution, one adult BNT162b2 vial contains 2.25mL from which 6 doses of 0.3mL can be extracted while one paediatric BNT162b2 vial contains 2.6mL from which 10 doses of 0.2mL can be extracted. It is possible that the differences in diluent may have contributed to the significant difference in immunogenicity observed between the formulations with a more concentrated formulation inducing more potent responses.

This was the first study to date to directly compare the immune response between full and fractional vaccine dose primary and booster COVID-19 vaccine schedules in adolescents. In this study, the adult BNT-10 and 30µg BNT162b2 third dose groups demonstrated similar immunogenicity. This is consistent with the comparable immune response previously observed between a two-10µg BNT162b2 dose prime schedule in 5- to 11-year-olds and a two-30µg BNT162b2 schedule in 18- to 25-year-olds.[39] Although BNT-10 in Cohort A was notably less immunogenic compared with BNT-30, most participants in Cohort B (unlike Cohort A) were seropositive prior to vaccination. More participants in the Cohort A BNT-10 group also had a breakthrough infection than in the BNT-30 group. In Cohort B, no significant difference in the proportion of breakthrough infections was observed between the fractional dose BNT162b2 study groups and the 30µg BNT162b2 group. These results suggest that hybrid immunity and frequency of antigenic exposure influence vaccine-induced

immunogenicity and provide support for the use of fractional doses as part of booster immunisation campaigns.

The use of fractional vaccine doses in booster immunisation schedules has already been studied in adults and the results compared with full doses. Puga et al. showed that a fractional dose of BNT162b2 (15µg or 10µg) as a booster dose was non-inferior to full dose BNT162b2 in adults aged 18-60 years, though this was dependent on the priming schedule received.[143] A recent systematic review and meta-analysis using SARS-CoV-2 VNA levels to predict vaccine efficacy in studies involving adults participants, examined the relationship between immunogenicity and protection following fractional dose COVID-19 vaccination.[147] The results of this review suggested that fractional vaccine doses could provide at least partial protection against SARS-CoV-2 and variants. Vaccine efficacy was also shown to rise with increasing dose fractions used. [147] In Cohort B, both the 10µg adult [61785 (95%CI 51598-73983) and 10µg paediatric BNT162b2 [43918 (95%CI 35978-53611)] adolescent groups demonstrated higher peak anti-spike IgG concentrations compared with those observed following third dose 30µg BNT162b2 in seropositive adults (aged over 30 years) [37916 (95%CI 26907-53429)] in the COV-BOOST trial, measured using the same immunoassay.[37] Greater immunogenicity in children compared with adults has already been demonstrated following vaccination.[39,148] Combined with increasing global SARS-CoV-2 seroprevalence, fractional dosing may suffice for subsequent 'booster' doses in adolescents to bolster the immune response against infection, while allowing for more cost-effective and efficient use of vaccine supplies as well as greater vaccine availability and schedule flexibility.

3.5.2 NVXCoV2373 as a second or third booster dose is highly immunogenic

In Cohort A, the highest humoral and cellular immune responses were observed in the NVX group, and this pattern persisted out to day 132. Correspondingly, the lowest rate of self-reported and serologically confirmed infections was also recorded in this group. Furthermore, when compared to the number of serologically confirmed infections, a lower number of self-reported infections were reported in the NVX group, suggesting these participants also experienced milder symptoms on infection. NVX-CoV2373 has already been shown to be highly immunogenic in adult populations and to provide protection against VOCs.[149] These findings concur with the results from the adolescent PREVENT-19 Phase 3 trial in which NVX-CoV2373 demonstrated protective efficacy of 79.5% against SARS-CoV-2% and 82% vaccine efficacy against the Delta variant.[150] In addition, results from the auxiliary observational study to the phase 3 PREVENT-19 trial ("SNIFF") showed that a two-dose NVXCoV2373 primary series demonstrated efficacy of 72.8% against SARS-CoV-2 during a Delta predominant period of the pandemic.[151] It has been postulated that the success of the vaccine's performance may be attributable to the presence of the novel Matrix M adjuvant, previously shown to enhance immunogenicity.[152]

The results of Cohort A also revealed significant differences in the immune response in adolescents to heterologous second dose NVXCoV2373 compared with 50-to-70-year-olds. The Com-COV study in adults, which also examined the immunogenicity of heterologous COVID-19 vaccine schedules, showed that the anti-spike IgG response following second dose NVX in seronegative adults was inferior compared with BNT-30 (aGMR 0.53).[32] In contrast, in adolescents, anti-spike antibodies following second dose NVXCoV2373 were higher compared with BNT-30 (aGMR 1.33). The T-cell response in adolescents was also significantly greater (121 SFC/106, aGMR 1.73 compared to BNT-30) compared with adults [29 SFC/106, aGMR 0.6 compared to BNT-30)].[32]

Scant data exist regarding the immune response to NVXCoV2373 when administered as a third dose in the paediatric population. One preprint was identified which compared medically attended events (MAEs) related to COVID-19 following homologous third dose BNT162b2 or heterologous third dose NVXCoV2373 in 12-18-year-olds in South Korea. The study showed that for third dose NVXCoV2373 compared with BNT162b2, the adjusted hazard ratio for MAEs related to COVID-19 was 0.68 (0.54-0.84), suggesting greater protection against SARS-CoV-2 following NVXCoV2373 as a third (booster) dose.[153] In Cohort B, although third dose NVXCoV2373 was immunogenic, anti-spike IgG concentrations at day 28 post-boost remained significantly lower compared with BNT-30. There was no significant difference however, in the cellular immune response between BNT-30 and NVX during study follow up nor any significant difference in the number of breakthrough infections between the groups. This is discussed in more detail in **section 3.5.3**.

3.5.3 Heterologous COVID-19 vaccine schedules induce robust immune responses in adolescents

This was the first trial in adolescents to investigate the immunogenicity of heterologous and fractional second dose COVID-19 vaccine schedules. The Com-COV2 (adult) study demonstrated that a heterologous COVID-19 vaccine schedule using NVX-CoV2373 as the second dose following BNT162b2 had a favourable reactogenicity profile and elicited higher antibody concentrations than two doses of the adenoviral-vectored ChAdOx1 n-CoV-19 vaccine.[32] Results from Com-COV3 Cohort A showed that a heterologous vaccine schedule in adolescents using NVXCoV2373 as the second dose was comparably immunogenic to the licensed homologous BNT162b2 schedule and provides further supportive evidence for its use as part of COVID-19 immunisation campaigns.

Despite the impressive performance of second dose NVXCoV2373 in Cohort A, day 28 anti-spike IgG concentrations were notably lower following a third dose of NVXCoV2373 compared with 30µg

BNT162b2, though by day 182, anti-spike IgG concentrations were not significantly different between the groups. Additionally, there was no significant difference in VNA titres and cellular responses between NVXCoV2373 and 30μg BNT162b2 third dose groups. When compared with results from the (adult) COV-BOOST trial,[37] higher anti-spike IgG GMC were observed following third dose NVXCoV2373 in adolescents compared with adults at all timepoints,

Table 3.10.[2] The results of this study are consistent with previous studies in adults showing that heterologous third dose vaccination in adolescents elicits a robust immune response and similar immunogenicity when compared with a homologous booster vaccine schedule.[37,154]

[2] Although Table 3.10 reports anti-spike IgG results from baseline seropositive COV-BOOST adult participants, more than 80% of Com-COV3 Cohort B participants were also seropositive at baseline.

Table 3.10 Anti-spike IgG (ELU/ml) at day 28, day 84 and day 182 following third dose 30µg BNT162b2 and third dose NVXCoV2373 in adolescents and at day 28, day 84 and day 242 following third dose 30µg BNT162b2 and third dose NVXCoV2373 in (baseline) seropositive adults (COV-BOOST).
All participants were primed with two-dose homologous 30µg BNT162b2.

	Com-COV3 Cohort B	**COV-BOOST**	**Com-COV3 Cohort B**	**COV-BOOST**
Age range	12-15years	>30 years	12-15 years	>30 years
SARS-CoV-2 anti-spike IgG ELU/ml, GM (95%CI)				
Schedule	**BNT/BNT/BNT**		**BNT/BNT/NVX**	
Day 28	68374 (55946-83563) [n=55]	37916 (26907-53429) [n=13]	51837 (42170-63720) [n=54]	21700 (15089-31208) [n=12]
Day 84	41730 (34673-50222) [n=54]	22167 (14981-32800) [n=12]	34400 (28059-42173) [n=53]	14483 (9705-21613) [n=12]
Day 182†/Day 242‡	24337 (19934-29713) [n=53]	7207 (3440-15102) [n=8]	21478 (17174-26862) [n=54]	8560 (4233-17309) [n=7]

†Day 182 applies to Com-COV3 Cohort B only; ‡day 242 applies to COV-BOOST trial. BNT/BNT/BNT = third dose 30µg BNT162b2 following homologous BNT162b2 prime vaccination; BNT/BNT/NVX = third dose NVXCoV2373 following homologous BNT162b2 prime vaccination; n = participant number; GM = geometric mean; 95%CI = 95% confidence interval; ELU = ELISA units.

Only one other study which examined heterologous third-dose IM vaccination in adolescents has been published. The study investigated the immunogenicity of 30µg, 15µg, and 10µg BNT162b2 when given as a third (booster) dose following CoronaVac/BNT162b2 prime vaccination.[155] In this study, third dose BNT162b2 vaccination induced robust VNA activity against the Omicron variant while no significant difference in VNA titres was detected between the fractional BNT162b2 groups [GMRs of 0.82 (0.44–1.53) in 15µg BNT162b2 arm and 0.74 (0.39–1.39) in 10µg BNT162b2 arm, BNT-30 as the reference group]. The paediatric formulation of BNT162b2 was used to administer the 10µg dose in this study.[155] The findings of this study are consistent with the results of Cohort B which showed

that 30µg BNT162b2 and 10µg BNT162b2 elicited similar VNA titres against Omicron. The Cohort B study also demonstrated that heterologous third dose COVID-19 vaccination elicited robust humoral and cellular immune responses, and that persistence of the immune response was well maintained out to six months.

3.5.4 Bivalent vaccine is comparably immunogenic to monovalent BNT162b2 booster

Similar peak humoral and cellular immune responses to the Victoria strain were observed between the bivalent Original/Omicron BA.1 and 30µg BNT162b2 vaccines. However, significantly higher VNA titres against Omicron BA.5 and XBB.15 were observed in the bivalent Original/Omicron BA.1 group compared with 30µg BNT162b2. In line with previous studies, these results suggest that boosting with bivalent vaccines targeting variant strains generates enhanced VNA activity against related subvariants.[156] However, the longer interval prior to vaccination and the occurrence of SARS-CoV-2 infections during this time may have influenced these findings.

3.5.5 Limitations and Conclusion

This study had multiple limitations. A pragmatic approach to the study design of Cohort A was adopted with no formal sample size calculation performed. The change to the UK national immunisation policy during the study also necessitated a change to the study design and resulted in fewer participants recruited than originally planned. The smaller sample size also limits the study power. The differences in self-reported infection should be interpreted with caution as both Cohort A and Cohort B were not powered to assess efficacy, and Cohort A BNT-10 participants may have been

more likely than other groups to self-test once unblinded at day 28 following vaccination. However, the pattern of infection across groups remained consistent for serologically defined infections which were not influenced by testing behaviour. Considering the varied vaccine volumes used in the study, there is also a risk that participants may have been inadvertently unblinded at the time of vaccination. However, to maintain the blind, vaccines were prepared out of sight and the syringes covered with masking tape, thereby minimising this risk.

With respect to Cohort B, although the study achieved the sample size required to fulfil the primary objective of the trial, recruitment was challenging and was stopped after one year following discussion with the TSC. Most study participants were Caucasian, and therefore not representative of the general population, limiting the wider applicability of the results. The age range of participants included in the study was also narrow, limiting the generalisability of the results to younger children. Although the effect of vaccine interval on immunogenicity was examined in the control group, the follow up period for this group was too short to assess immune persistence. Similarly, a follow-up period of just six months following the third dose in the vaccine arms did not allow for a longer-term assessment of immune persistence.

In conclusion, this study demonstrated that heterologous and fractional second and third dose COVID-19 vaccine schedules in adolescents were highly immunogenic and well-tolerated. Heterologous second dose NVX demonstrated the highest peak humoral and cellular immune response and the highest neutralising activity against Omicron BA.1 and BA.2. Furthermore, adult BNT-10 as a third booster dose was shown to be superior to paediatric BNT-10 and comparably immunogenic to BNT-30. This study provides support for the use of heterologous second and third dose COVID-19 vaccine schedules in adolescents and for fractional adult BNT162b2 as an alternative to the paediatric formulation in adolescent booster campaigns.

Chapter 4: Memory B Cell Responses to Homologous and Heterologous COVID-19 Vaccination and the Influence of Hybrid Immunity and Breakthrough Infection

4.1 Introduction

The COVID-19 pandemic, triggered by the outbreak of SARS-CoV-2 in December 2019, precipitated a rapid expansion in vaccine development and the subsequent successful global implementation of COVID-19 vaccination campaigns which resulted in a 59% reduction in deaths, saving approximately 1·6 million lives.[157] Despite the enormous success of these COVID-19 vaccination programmes, SARS-CoV-2 re-infection is common following vaccination and vaccine effectiveness has been shown to wane with time.[27] Immunological memory in the form of LLPCs and BMEM is proposed to persist and provide long-lasting protection against infection.[158] Indeed, LLPCs and BMEM are capable of secreting neutralising antibodies (known to confer protection against SARS-CoV-2) for many years following vaccination (and/or infection) and thus induction of LLPCs and BMEM represent an important mechanism for maintenance of elevated antibody levels.[73-75,159] Furthermore, recent studies have highlighted the critical role of BMEM in protecting against re-infection, particularly against variant strains.[76,160]

Most effective vaccines induce potent germinal centre B cell responses (essential for the generation of high affinity BMEM) and long-term immunity by generating LLPC and BMEM.[161] However, the duration of immune memory following COVID-19 vaccination and the mechanisms by which its efficacy and persistence can be optimised through immunisation are uncertain. Published data suggest

that BMEM are expected to persist and confer long-term protection against SARS-CoV-2, ameliorate the severity of subsequent re-infections and enhance the immune response to variant strains.[162] Turner et al. reported high frequencies of SARS-CoV-2 spike-specific germinal centre B cells and plasmablasts in draining axillary lymph node aspirates for up to 12 weeks following second dose mRNA vaccination.[163] Similar findings were reported by Mudd et al. for T-follicular helper cell responses, which strongly correlated with the size of lymph node germinal centre B-cell populations, and which persisted for at least 6 months following second dose mRNA vaccination.[164] Correspondingly, enhanced germinal centre B-cell reactions and differentiation of B cells into LLPC and BMEM have been observed in mice following mRNA vaccination, but have not been found following vaccination with a recombinant protein-adjuvanted vaccine, suggesting that vaccine-induced B cell response and durability may be platform-dependent.[161]

Third 'booster' dose mRNA SARS-CoV-2 vaccination has also been shown to elicit robust B-cell germinal centre reactions and antigen-specific BMEM responses.[165] The long-term duration of BMEM responses following booster vaccination remains uncertain, but studies have reported detecting SARS-CoV-2 specific BMEM responses up to 10 months following third dose vaccination.[166] Scant data exist regarding BMEM responses to the Matrix-M adjuvanted recombinant nanoparticle spike protein vaccine NVXCoV2373 but data from animal studies suggest enhanced BMEM responses are detectable at 7 months following third dose NVXCoV2373 vaccination.[167] Longer-term BMEM response duration elicited through vaccination remains unknown.

Conversely, it is well established that hybrid immunity, defined as immunity induced through a combination of SARS-CoV-2 infection and vaccination, is associated with greater vaccine-induced immunogenicity and protection against SARS-CoV-2 infection than immunity elicited through either vaccination or infection alone.[168] In individuals with a history of SARS-CoV-2 infection, a single dose of an mRNA vaccine resulted in an immune response comparable to that elicited in response to two-dose mRNA vaccination in infection naïve participants.[169] Goldberg et al. demonstrated that the risk of SARS-CoV-2 infection was four to five times lower in individuals with hybrid immunity compared

with individuals who had received two or three doses of an mRNA vaccine.[170] Correspondingly, robust BMEM responses and increased frequencies of IgA-switched, RBD-specific BMEM have been found in convalescent participants following vaccination.[171] With respect to differences in functional immune memory, vaccination in the setting of hybrid immunity has been shown to result not only in a greater frequency of SARS-CoV-2 spike-specific BMEM but also in more broadly neutralising antibodies against variant strains and distinct populations of CD4+ memory T cells.[42]

Immune imprinting can have a similarly beneficial effect on vaccine-induced immunity. Immune imprinting refers to the influence that first exposure to a viral antigen (via infection or vaccination) has on the subsequent immune response generated on re-exposure to related strains or variants of the original antigen.[172] In the context of breakthrough SARS-CoV-2 infection, this can result in reactivation of BMEM that recognise shared epitopes between ancestral and variant lineages, resulting in a more rapid response to antigenic challenge.[173,174] In some instances however, this anamnestic cross-reactive immune response may be detrimental if it prevents de novo B cell activation in response to a new variant, resulting in immune evasion. Preferential de novo B cell activation (in place of cross-reactive immunity) is largely dependent on antigenic distance between strains. Johnston et al. demonstrated that exposure to variants BA.5 and XBB.1.5 following wild-type SARS-CoV-2 mRNA vaccination elicited B cell responses targeting conserved epitopes shared between the ancestral strain and BA.5. [175] A similar pattern was observed with XBB.1.5 exposure however, some individuals exhibited a low frequency of XBB.1.5-specific B cells. Notably, individuals with higher levels of pre-existing cross-reactive B cells mounted weaker XBB.1.5-specific responses, highlighting the significant influence of prior SARS-CoV-2 immunity on B cell activation in response to emerging variant strains.[175]

In contrast, a paucity of data exists examining BMEM responses to SARS-CoV-2 infection and vaccination in the paediatric population. Only one study was found which investigated BMEM responses in adolescents following SARS-CoV-2 vaccination and which demonstrated no significant increase in the BMEM response following either a two-dose homologous BNT162b2 regimen or a

CoronaVac prime, BNT162b2 second dose schedule.[176] Conversely, higher spike-specific BMEM frequencies were detected in 5- to 12-year-olds compared with adults at 6 months following two-dose BNT162b2 vaccination. However, a subsequent third booster dose was found to have no effect on spike-specific BMEM frequencies in infection naïve children (i.e., when participants with SARS-CoV-2 breakthrough infection after vaccination were excluded from the analysis).[82] No studies were found which examined BMEM responses induced by heterologous third dose vaccination in either children or adolescents.

BMEM are not actively secreting antibody and require a differentiation step into ASC. Therefore, to enable detection of circulating, antigen-specific BMEM in peripheral blood, the isolated PBMCs require in vitro, polyclonal stimulation with a combination of TLR ligand and IL-2, for 72 hours. In this case the TLR ligand is R848 which binds to TLR7/8 which is upregulated on BMEM in comparison to naïve B cells. IL-2 binds to CD25, which is also upregulated in BMEM, and acts as a growth factor. As no B cell Receptor ligand is included in the pre-stimulation culture, it preferentially drives the growth and differentiation of BMEM into ASC (secreting any antibody isotype), **Figure 4.1**. Since the stimuli are polyclonal, the system drives the differentiation of all BMEM, which allows detection of responses to multiple antigens, by multiple antibody isotypes, from one sample, in the subsequent FluoroSpot Assay. In this particular assay, the readout is the frequency of BMEM-derived ASC (secreting IgG) specific to each antigen tested. Since a single BMEM may proliferate and differentiate into multiple ASCs, this process enhances the detection of rare, antigen-specific cells at selected time points following vaccination in comparison to pre-vaccine/baseline samples.

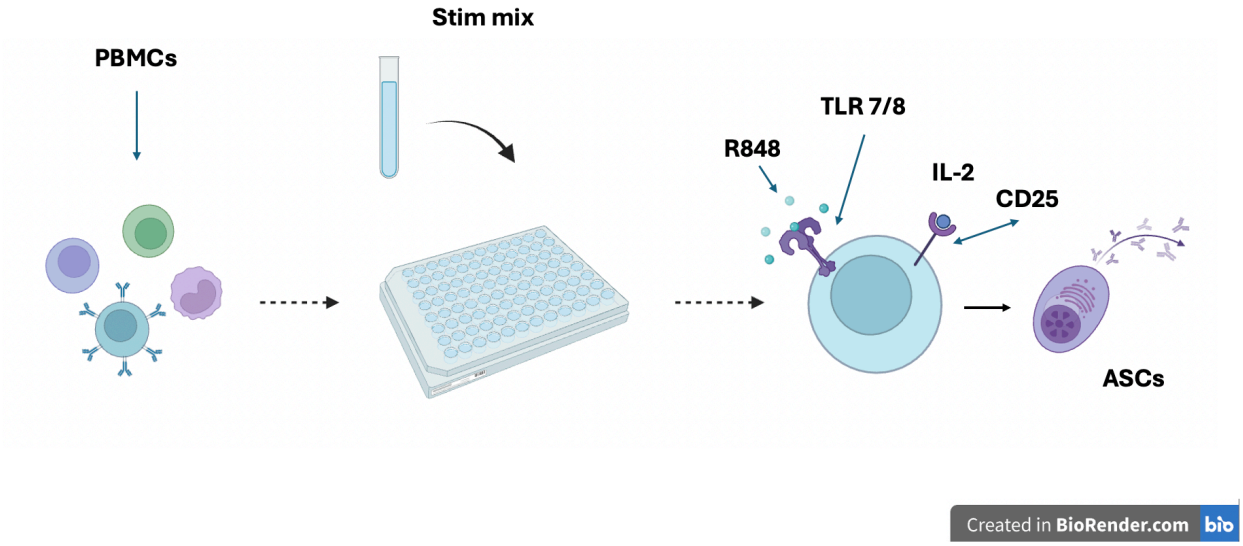


Figure 4.1 PBMC stimulation and differentiation of memory B cells into antibody secreting cells.
PBMC = peripheral blood mononuclear cells; ASC = antibody secreting cells; 'stim mix' = mixture of R848 and IL-2 added to PBMCs in tissue culture plate.

This chapter describes results which relate to the following objectives:

1. To investigate SARS-CoV-2 spike-specific IgG BMEM responses and kinetics to homologous and heterologous primary and booster COVID-19 vaccine schedules in adolescents.
2. To investigate the impact of prior SARS-CoV-2 infection on BMEM response following COVID-19 vaccination.
3. To assess the impact of breakthrough SARS-CoV-2 infection on BMEM response following COVID-19 vaccination.
4. To evaluate whether COVID-19 vaccination induces cross-reactive BMEM responses to hCoVs and the Delta variant in adolescents.

4.2 Methods

4.2.1 Sample selection

PBMCs isolated from heparinised samples taken from participants enrolled in the Com-COV3 trial were used for the B cell FluoroSpot assay described in detail in **Chapter 2**: **section 2.4.2**, and, in brief, in this chapter. PBMC samples were collected from Cohort A participants at day 28 following the second vaccine dose. Cohort B participant samples were collected at days 0, 28, 182 and 210. Study-related procedures, sample types collected, secondary endpoints and sample collection timepoints have previously been discussed in detail in Chapters 2 and 3 (please refer to **sections 2.4.1**, **3.2.1**, **3.2.5**, **3.2.5.5**). The study designs of Cohort A and Cohort B are illustrated in **Figure 4.2** and **Figure 4.3**, respectively.

To compare BMEM responses following homologous and heterologous COVID-19 vaccine schedules, Cohort A samples were selected from the second dose 30µg BNT162b2 and NVXCoV2373 study groups. Similarly, Cohort B samples were chosen from participants who had received either 30µg BNT162b2 or NVXCoV2373 as their third (booster) dose at least three months following completion of a two-dose 30µg BNT162b2 primary vaccine series. Cohort B samples from the 'control arm' were included as a reference group. The control group received two doses of the 4CMenB, the first dose at enrolment (day 0) and the second dose at day 84. Control group participants received their COVID-19 vaccine (i.e., Comirnaty bivalent Original/Omicron BA.1 vaccine) at day 182. Samples from the study timepoints outlined above were analysed to assess peak BMEM responses and longer-term kinetics.

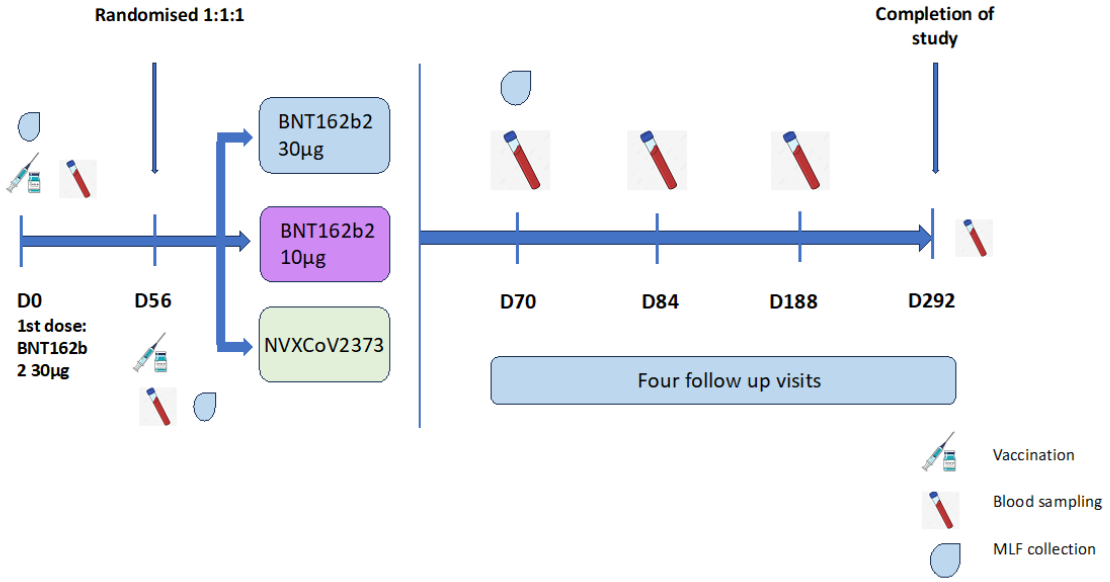


Figure 4.2 Com-COV3 Cohort A study design schematic.
Participants received their first dose (30µg BNT162b2) at day 0 (D0) and their second dose at day 56 (D56). MLF: mucosal lining fluid.

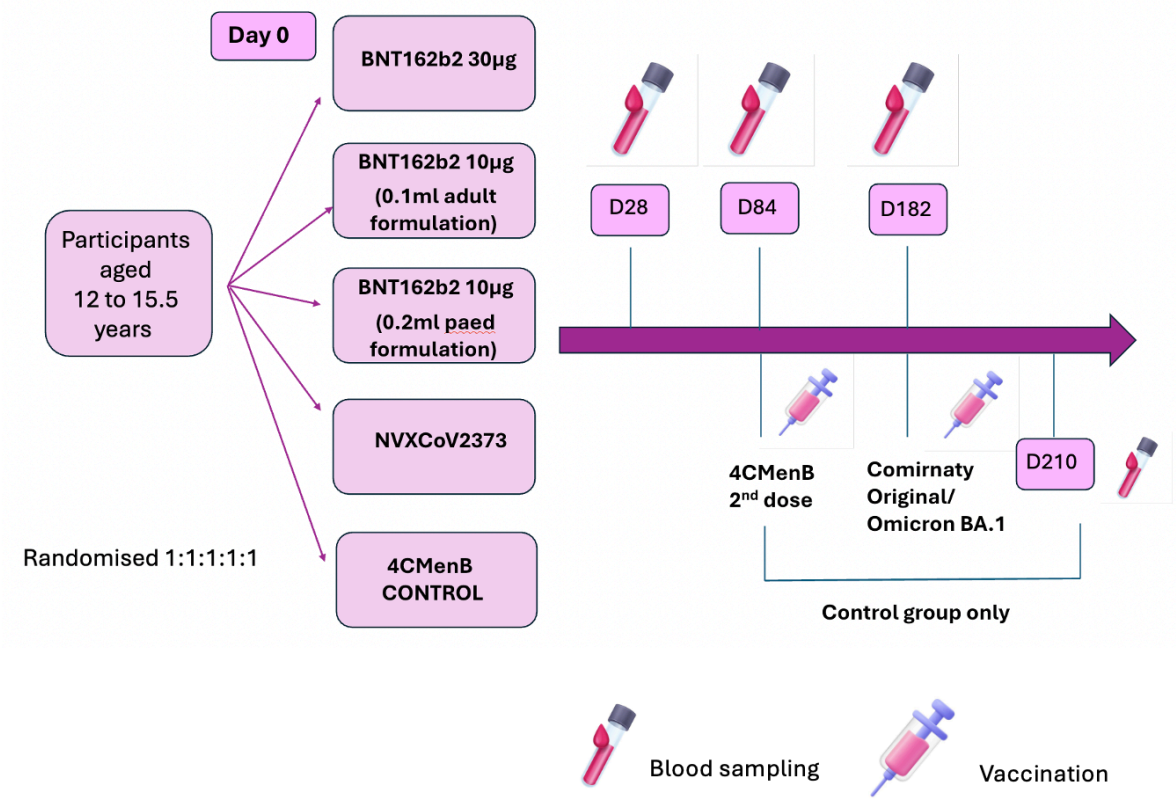


Figure 4.3 Com-COV3 Cohort B study design schematic.

PBMC samples were collected at days 0, 28, 182 and 210. Participants received their third (booster) dose at day 0. Participants in the control group received the meningococcal group B (4CMenB) vaccine at day 0 and day 84 and their COVID-19 booster dose (Comirnaty Original/Omicron BA.1 vaccine) at day 182.

To evaluate the impact of prior SARS-CoV-2 infection (i.e., hybrid immunity) and breakthrough infection on the BMEM response to vaccination, participant samples were also selected based on participant 'SARS-CoV-2 infection status'. Samples were selected and analysed according to the following categories:

- SARS-CoV-2 "infection naïve" (i.e., seronegative throughout): anti-nucleocapsid IgG seronegative at enrolment and/or tested negative on a SARS-CoV-2 lateral flow test at their enrolment visit (prior to vaccination) and no breakthrough infection throughout the study
- 'Seropositive' throughout: anti-nucleocapsid IgG seropositive at study enrolment and no breakthrough infection throughout the study
- Seronegative with breakthrough infection: anti-nucleocapsid IgG seronegative at enrolment and evidence of breakthrough infection during the study
- Seropositive with breakthrough infection: anti-nucleocapsid IgG seropositive at enrolment and evidence of breakthrough infection during the study

A 'breakthrough infection' was defined as either: a self-reported SARS-CoV-2 infection >14 days after a second dose or >28 days after a third dose, a two-fold rise in anti-nucleocapsid IgG, a two-fold rise in anti-spike IgG antibodies, or seroconversion of anti-nucleocapsid IgG serostatus. Participants with evidence of SARS-CoV-2 infection within 14 days after a second dose or 28 days after a third vaccine dose were excluded from the analysis.

An outline of the terminology used in this chapter is shown in **Figure 4.4**. The sample numbers analysed according to Cohort, study arm and 'SARS-CoV-2 infection status' are illustrated in

Figure 4.5 and

Figure 4.6. This study was exploratory in nature and sample selection was based on PBMC sample availability and practical limitations. Balanced sample representation across study groups was prioritized within the constraints of sample availability.

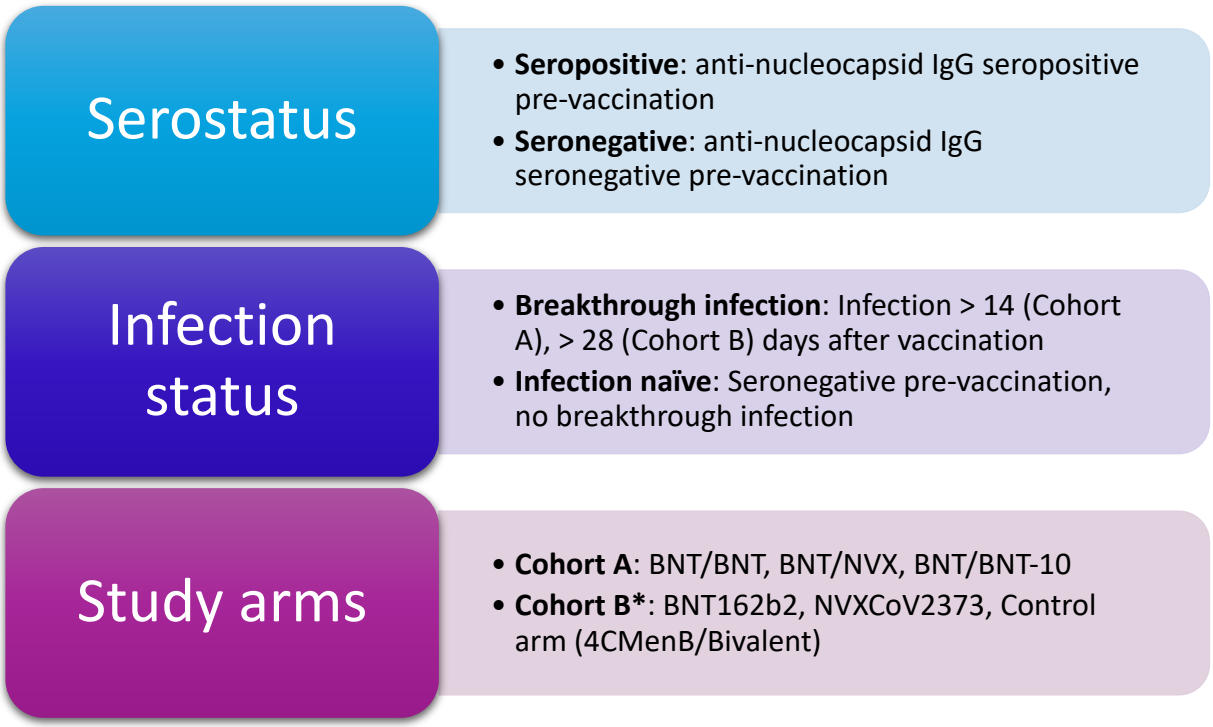


Figure 4.4 A summary of terminology used in this chapter.
*All Cohort A participants were primed with a two-dose 30μg BNT162b2 primary vaccine series. *Cohort B study groups referred to as BNT/BNT/BNT; BNT/BNT/NVX, and BNT/BNT/Bivalent in figures for clarity. BNT = 30μg BN162b2; NVX = NVXCoV2373; BNT-10 = 10μg BNT162b2; 4CMenB = meningococcal group B vaccine; bivalent vaccine = Original/Omicron BA.1 vaccine.*

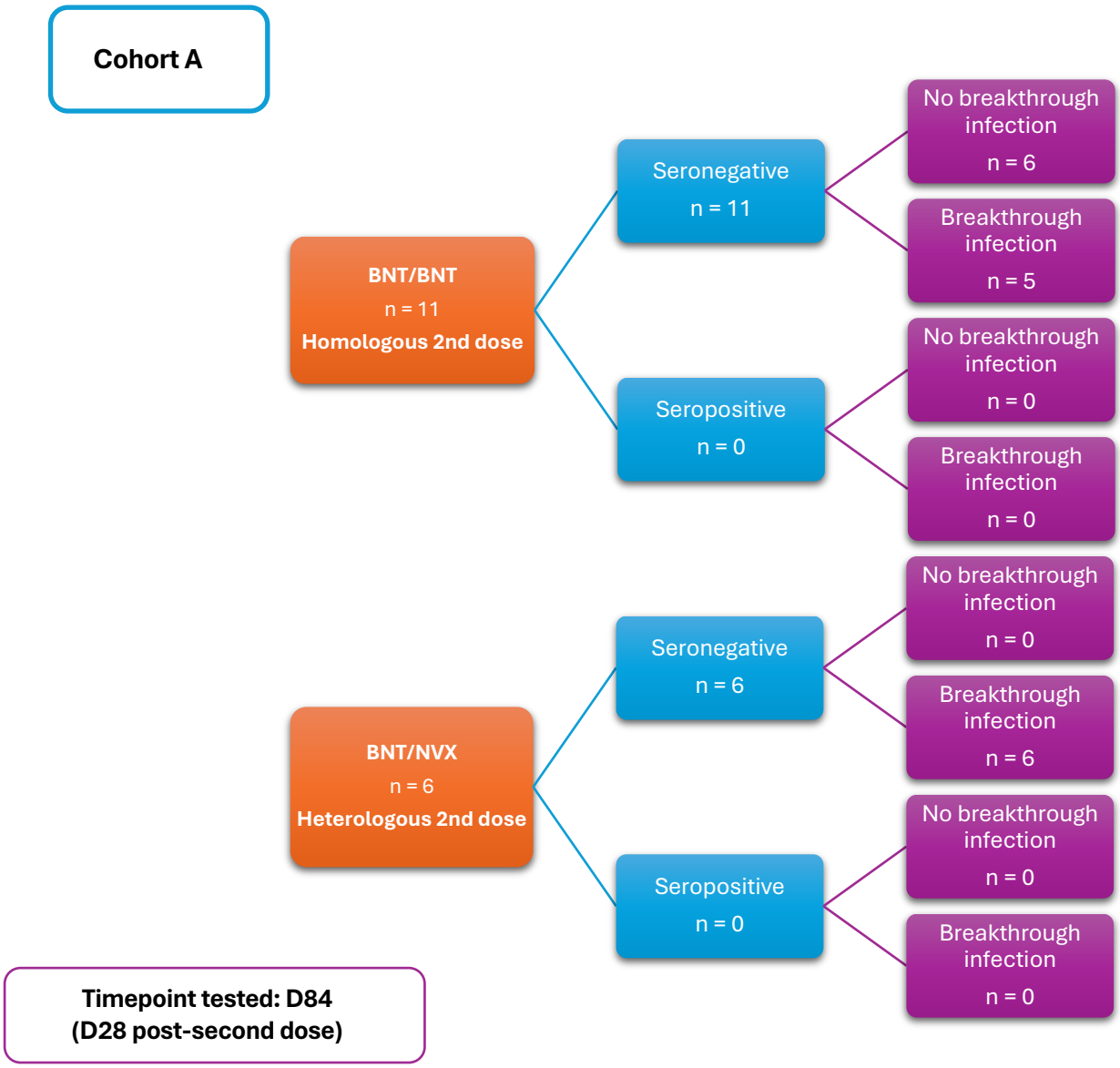


Figure 4.5 Cohort A sample analysis by study arm, serostatus and breakthrough infection status; n = number of participant samples analysed.
Seronegative refers to pre-second dose anti-nucleocapsid IgG seronegative; seropositive refers to pre-second dose anti-nucleocapsid IgG seropositive. Samples were tested at day 84 (day 28 post-second dose). Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56. BNT = 30µg BNT162b2; NVX = NVXCoV2373.

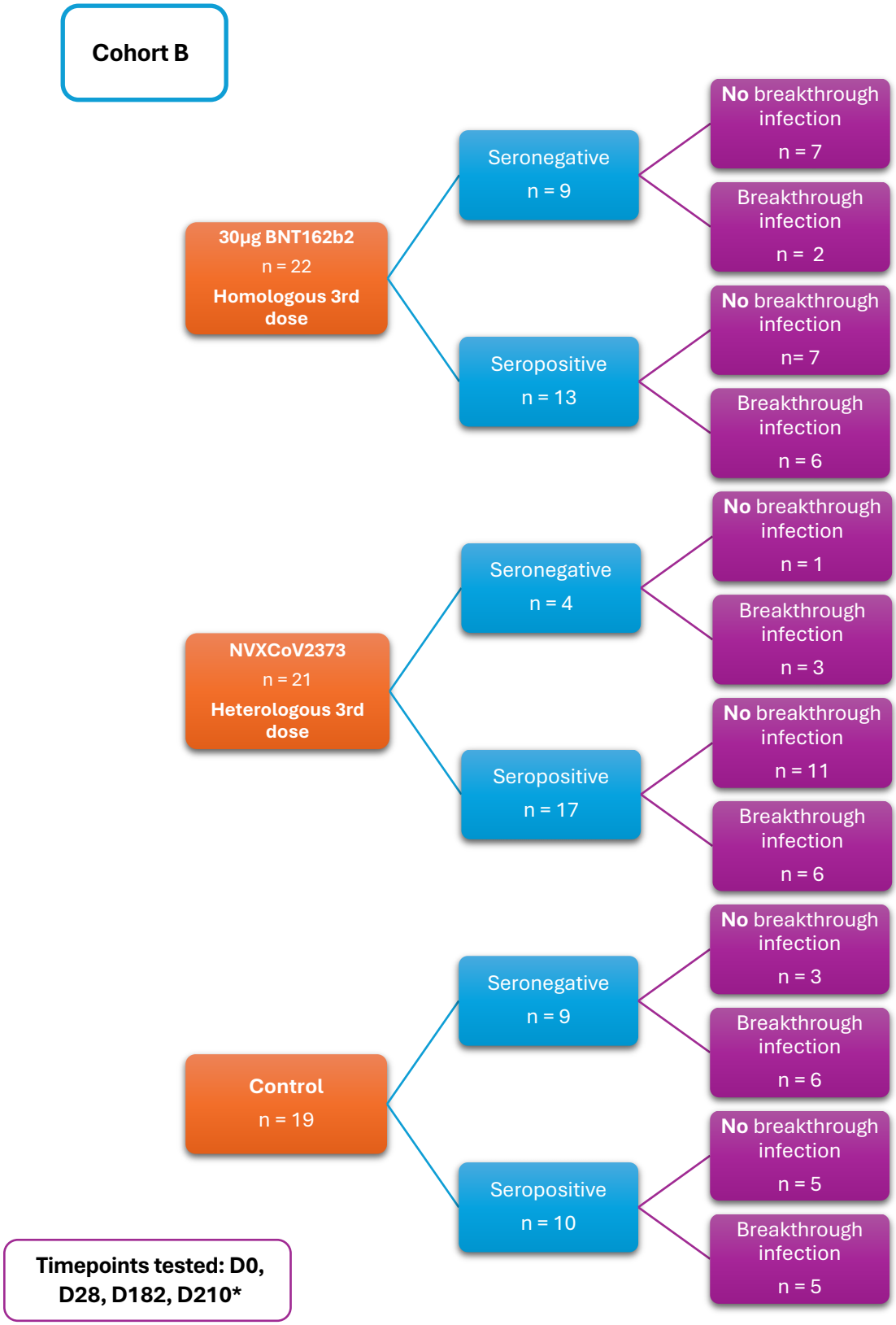


Figure 4.6 Cohort B sample analysis by study arm, serostatus and breakthrough infection status
*n = number of participant samples analysed. Seronegative/seropositive refers to pre-third dose anti-nucleocapsid IgG serostatus. Participants received their third (booster) dose at day 0. Samples were tested at day 0, day 28, and day 182. *Only control group samples were tested at day 210 (28 days following receipt of the bivalent vaccine in this group).*

4.2.2 Sample collection and storage

Samples were collected at study visits as outlined in **Figure 4.5** and **Figure 4.6**. PBMCs were isolated from heparinised blood samples at Oxford Immunotec as described in detail in **Chapter 2: section 2.4.2.2**. Samples were stored in liquid nitrogen at -196^{0}C before use. For full details relating to sample collection and storage, please refer to the following sections in **Chapter 2**: **sections 2.4.1** and **2.4.2.1**.

4.2.3 Investigation of vaccine-induced antigen-specific memory B cell responses

IgG antigen-specific BMEM responses were assessed using a FluoroSpot assay on PBMC samples collected 28 days after the second vaccine dose (Cohort A), and at days 0, 28, 182 and 210 following the third dose (Cohort B). Vaccine-induced BMEM responses to the following proteins (antigens) were tested: SARS-CoV-2 spike (wild-type), SARS-CoV-2 Delta B.1.617.2 spike, SARS-CoV-2 nucleocapsid protein, hCoV NL63 spike, and hCoV OC43 spike. Total IgG-secreting BMEM were also measured. SARS-CoV-2 BMEM derived IgG-ASC will be referred to as IgG-ASC henceforth in this chapter and throughout the thesis.

For full details regarding the BMEM FluoroSpot assay performed, please refer to Chapter 2, **section 2.4.2**.

4.2.4 Optimisation of a FluoroSpot B-cell assay for the detection of IgG SARS-CoV-2-specific BMEM

The FluoroSpot assay for detecting antigen-specific IgG using fluorescent-labelled anti-IgG-Cy3 -antibodies, using dual wavelength fluorescence for detection, was provided as a kit. The FluoroSpot assay protocol, using the MabTech B cell FluoroSpot kit and stimulation kit, was optimised for detection of ASCs specific for SARS-CoV-2-related antigens. However, additional optimisation steps were required for several reasons:

1) Viable cell yields were low after defrosting cryopreserved PBMCs.

2) This resulted in lower yields in viable cells following stimulation and cell harvest.

3) During plate analysis, spot formation appeared blurred, with evidence of pooling of cells at well edges, and merging of cells on the plates.

Therefore, several steps were taken to resolve these issues and to optimise the assay to generate accurate results using these samples.

Optimisation of spot appearance and counting: Donor PBMCs from two adult volunteers (obtained under ethically approved Protocol OVC002, Ethics Ref 18/LO/0415) were used in this experiment. The plate layout and plate antigen coating concentrations used in the experiment are depicted below (**Table 4.1**). Convergence and blurring of spots occurred mainly in the SARS-CoV-2 spike protein coated wells, therefore two SARS-CoV-2 protein coating concentrations were tested: 5.0µg/mL (1:120 dilution factor) and 10µg/mL (1:60 dilution factor), to enhance the capture of antibody secreted by the ASC. Since the confluence of spots observed may have been the result of a high proportion of cells secreting in the wells, the cell concentration per well was also reduced (see plate layout below, **Table 4.2**) to a 1:10 (2×10^4) and 1:100 (2×10^3) dilution of the original cell suspension (2×10^6 cells/mL). A 'neat' (i.e., 2×10^5 cells/well) concentration of sample was also tested. A negative control (PBS

only ‘blank’ wells) and positive control (anti-IgG capture antibodies coated to detect total IgG secreting BMEM) were included in the experiment. The results of this experiment and the subsequent corrective actions taken have been outlined in **Results, section 4.3**.

Table 4.1 Layout of antigen-coated plate

A	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	**5ug/mL; 1:120**
B	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	
C	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	**10ug/mL; 1:60**
D	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	
E	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	
F	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	
G	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	
H	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	

C2wu = SARS-CoV-2 stabilised spike glycoprotein, PBS = phosphate buffered saline, Ig = total IgG control, 1:10, 1:100 cell dilution from 2x10^6 cell/ml.

Table 4.2. Layout of cells on the plate.

sample 1	A	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat
sample 1	B	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10
sample 1	C	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100
sample 2	D	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat
sample 2	E	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10
PBS	F	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat	neat
IgG	G	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10	1in10
IgG	H	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100	1in100
			sample 1 PBS+IgG dilutions						sample 2 PBS+IgG dilutions				

Sample 1 = tested in SARS-CoV-2 spike protein coated wells rows A to C; Sample 2 = tested in SARS-CoV-2 spike protein coated wells rows D to E; Sample 1 = tested in PBS and IgG capture antibody coated wells rows F to H, columns 1 through 6; Sample 2 = tested in PBS and IgG capture antibody coated wells rows F to H, columns 7 through 12; ‘neat’ = concentration of sample tested 2 x 10^5 cells/well, ‘1in10’ = concentration of sample tested 2 x 10^4 cells/well, ‘1in100’ = concentration of sample tested 2 x 10^3 cells/well; PBS = phosphate buffered saline, IgG = total IgG secreting memory B cells.

A further technical issue encountered when using the FluoroSpot assay was the presence of artefact in the wells. Although easy to remove with the masking tool when reviewing plates, the use of a different type of paper towel when drying plates reduced the amount of dust exposure to the plates

and strands from the paper towels which had been detected in the wells. An uneven distribution of spots in wells resulting in spots gathering at well edges in a crescent moon shape was also observed. This higher concentration of cells near the edges of the well also negatively impacted spot counting. This effect was counteracted by carefully pipetting directly into the centre of the well when adding cells to pre-coated FluoroSpot plates and by ensuring that plates were placed flat in the incubator to prevent cells from pooling to one side.

The IPFL FluoroSpot plates were coated with 100µL/well of the proteins (antigens) of interest as described in Chapter 2, **section 2.3.1.1**. The FluoroSpot plates contain a PVDF membrane at the bottom of each well capable of binding capture antibodies and proteins following pre-treatment with ethanol (causing the membrane to become hydrophilic). According to the manufacturer's kit instructions, plates should be pre-wetted with freshly prepared 35% ethanol to activate the membrane. To remedy the absence of spot formation in a well resulting from 'bubble' formation in the centre of the well, where the membrane had not been properly activated with ethanol prior to coating, plates were carefully tapped following the addition of ethanol to ensure even distribution over the membrane surface.

4.2.5 A FluoroSpot B-cell assay for the detection of IgG SARS-CoV-2-specific vaccine-induced memory B cells: Results of assay optimisation

Results of the experiment outlined previously to optimise the performance of the FluoroSpot assay (**Methods section 4.2.4, Table 4.1**, **Table 4.2**) demonstrated that using a higher SARS-CoV-2 protein coating concentration 10µg/mL (1:60 dilution factor) yielded well-defined spots and helped to prevent merging of spots in the wells (**Figure 4.7 A, B, E, F**). Furthermore, using a reduced cell number in wells with a high proportion of responsive cells resulted in more accurate cell counts and prevented

the formation of a confluence of spots in the well. As illustrated in **Figure 4.7 G**, an uneven of coating of ethanol in the wells led to a 'bubble' formation in the wells, a result of capture antibody or antigen being unable to bind to the membrane. This was resolved by ensuring an even distribution of ethanol over the membrane surface prior to plate coating.

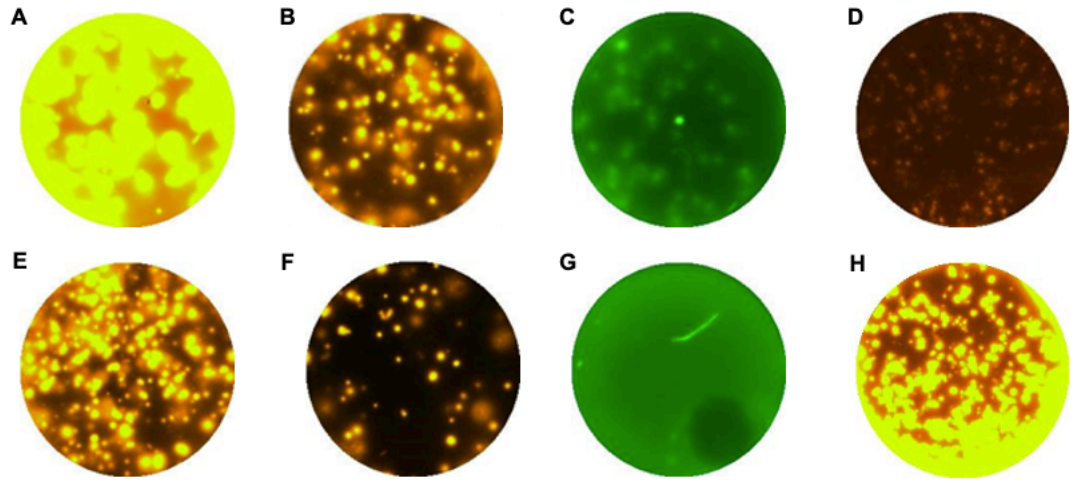


Figure 4.7 Spot appearance and results of assay optimisation experiment using a memory B cell FluoroSpot.
(A) Merging of spots in the well due to a high proportion of reactive B cells. (B) Following assay optimisation, use of a lower concentration of cells and a higher coating concentration of antigen resulted in discrete, well-defined spots. (C) The appearance of 'blurry spots' in wells due to uneven ethanol treatment, preventing the membrane from becoming activated (D) The appearance of 'blurry spots' as outlined in image (C) in an IgG isotype specific well. (E & F) Two concentrations of samples were placed in the SARS-CoV-2 ancestral spike coated wells following the optimisation experiment: a 'neat' concentration of sample (i.e., 2 x 10^5 cells/well) (E) and a 1:10 (2 x 10^4) dilution. (G) 'Bubble' formation due to uneven distribution of ethanol in the well. The presence of artefact is also evident. (H) Pooling of cells at the edge of the wells. This was resolved by pipetting directly into the centre of the well when adding cells to pre-coated FluoroSpot plates and by ensuring that plates were placed flat in the incubator to prevent cells from pooling to one side.

Based on the results of this experiment, the plate layout was amended as shown in **Table 4.3**. Two rows of the plate were coated with SARS-CoV-2 ancestral spike: a 'neat' (i.e., 2 x 10^5 cells/well) concentration of sample was placed in the first row while a 1:10 (2 x 10^4) dilution of the original cell suspension of sample was placed in the second row. To facilitate this change to the plate layout, the hCoV NL63 spike protein coating was omitted from the plate following this experiment. To maximise binding of IgG, secreted by ASC, to the plate coating antigen, the antigen protein coating

concentration was increased to 10µg/ml which improved the appearance of spots in the wells. The results of the experiment and the changes made to the assay following the experiment are shown in **Figure 4.7**, **Table 4.3** and **Table 4.4**.

Table 4.3 Original memory B cell FluoroSpot plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu	c2wu
B	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63	NL63
C	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP
D	OC43	OC43	OC43	OC43	OC43	OC43	OC43	240	OC43	OC43	OC43	OC43
E	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta
F	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS	DPBS
G	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10
H	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100

C2wu = SARS-CoV-2 stabilised spike glycoprotein; NL63 = human coronavirus NL63 spike glycoprotein; CoV2-NP = SARS-CoV-2 nucleocapsid protein; OC43 = human coronavirus OC43 spike glycoprotein; IgG = total IgG control; 1:10, 1:100 cell dilution from 2x10^6 cell/ml; DPBS = Dulbecco's phosphate buffered saline.

Table 4.4 Amended memory B cell FluoroSpot plate layout following assay optimisation experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'	c2wu 'neat'
B	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10	c2wu 1:10
C	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP	CoV2-NP
D	OC43	OC43	OC43	OC43	OC43	OC43	OC43	240	OC43	OC43	OC43	OC43
E	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta	Delta
F	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
G	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10	IgG 1:10
H	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100	IgG 1:100

C2wu = SARS-CoV-2 stabilised spike glycoprotein; 'neat' = 2 x 105 cells/well; CoV2-NP = SARS-CoV-2 nucleocapsid protein; OC43 = human coronavirus OC43 spike glycoprotein; IgG/A = total IgG and IgA control; 1:10, 1:100 cell dilution from 2x106 cell/ml; DPBS = Dulbecco's phosphate buffered saline.

4.2.6 Statistical analysis

The frequency of BMEM-derived ASC was expressed as median with interquartile ranges (IQR). Data were log-transformed prior to statistical testing and parametric tests were used to assess statistical significance. Differences in the frequency of BMEM-derived ASC between two study groups at different timepoints, as well as comparisons between cohort A and cohort B, were determined using a t-test. A p-value of < 0.05 was considered statistically significant. Correlation analyses were performed using Spearman's rank correlation coefficient and the correlation coefficient ('r') was reported.

4.3 Results

4.3.1 SARS-CoV-2 spike-specific IgG memory B cell responses are enhanced following homologous and heterologous booster vaccination

SARS-CoV-2 spike-specific IgG-ASC levels at day 28 following BNT/NVX [median 151.3 ASC/10^6 PBMCs, (IQR 78.8-295.6), n=6] were higher compared with BNT/BNT [median 125.0 ASC/10^6 PBMCs, (IQR 56.7-237.5), n=11] however, this difference did not reach statistical significance, likely due to the limited sample size, **Table 4.5** and **Figure 4.8**.

Table 4.5 SARS-CoV-2 spike-specific and Delta-specific IgG-ASC levels at day 28 following homologous and heterologous two-dose primary vaccine series and booster (third) dose vaccination.
Spike- and Delta-specific IgG-ASC responses are shown as median values with interquartile ranges (IQR).

Day 28 post-boost	BNT162b2 Homologous 2nd/3rd	NVXCoV2373 Heterologous 2nd/3rd	Control arm (Cohort B only)
Wild-type SARS-CoV-2			
Cohort A	125.0 (56.7-237.5)	151.3 (78.8-295.6)	N/A
Cohort B	355 (184.2-486.7)	383.3 (172.5-653.3)	252.5 (94.6-419.8)
Baseline seronegative (Cohort B)	355.0 (170.8-487.1)	N/A*	207.5 (50.8-615.2)
Baseline seropositive (Cohort B)	352.5 (202.5-509.5)	364.2 (163.1-689.5)	260.0 (108.3-381.7)
Delta-variant specific BMEM			
Cohort A	101.7 (75.0-272.5)	160.0 (128.7-418.8)	N/A
Cohort B	316.7 (147.1-713.8)	455.0 (127.5-983.3)	207.5 (123.7-552.5)
Baseline seronegative (Cohort B)	355.0 (170.8-487.1)	N/A*	207.5 (50.8-615.2)
Baseline seropositive (Cohort B)	352.5 (202.5-509.5)	364.2 (163.1-689.5)	260.0 (108.3-381.7)

**Only one participant in this group. Day 28 refers to day 28 following a second or third vaccine dose. BMEM = memory B cells.*

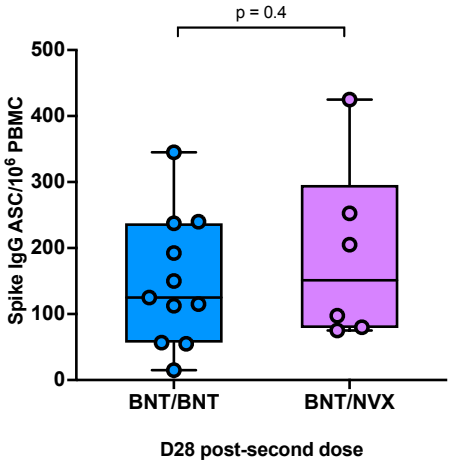


Figure 4.8 SARS-CoV-2 spike-specific IgG-ASC levels at day 28 following homologous and heterologous two-dose primary vaccine series.
Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. BNT/BNT = two-dose 30µg BNT162b2 regimen, BNT/NVX = 30µg BNT162b2 followed by NVXCoV2373 regimen, ASC = antibody secreting cells. PBMC = peripheral blood mononuclear cells.

Similarly, 28 days following booster vaccination, median spike IgG-ASC levels in the heterologous NVXCoV2373 study group were 383.3 ASC/10^6 PBMCs (IQR 172.5-653.3; n = 15) compared with 355.0 ASC/10^6 PBMCs (IQR 184.2-486.7, n = 19) in the homologous 30µg BNT162b2 group. Although higher spike IgG-ASC levels were observed in the NVXCoV2373 group, the difference was not statistically significant, **Table 4.5** and **Figure 4.9**. However, most participants in the NVXCoV2373 group were seropositive at baseline, prior to vaccination, which may have contributed to this finding.

In the control arm (participants who received 4CMenB at day 0), median spike IgG-ASC levels were 252.5 ASC/10^6 PBMCs (IQR 94.6-419.8; n = 12) at day 28. Although both the homologous and heterologous study groups demonstrated higher spike IgG-ASC than the control arm at day 28, neither difference reached statistical significance, **Figure 4.9**.

Compared with baseline (pre-vaccination) levels, spike-specific IgG-ASC levels were significantly increased at day 28 following (homologous) third dose 30µg BNT162b2 (p = 0.0067), while no significant increase occurred following third dose NVXCoV2373, **Figure 4.9**. However, baseline spike IgG-ASC levels were also highest in the NVXCoV2373 group, which may have impacted the ability to detect any significant rise in spike-specific IgG-ASC in this group. Additionally, spike-specific IgG-ASC were not significantly increased in the control group at day 210, 28 days following receipt of the bivalent Original/Omicron BA.1 vaccine, **Figure 4.9**.

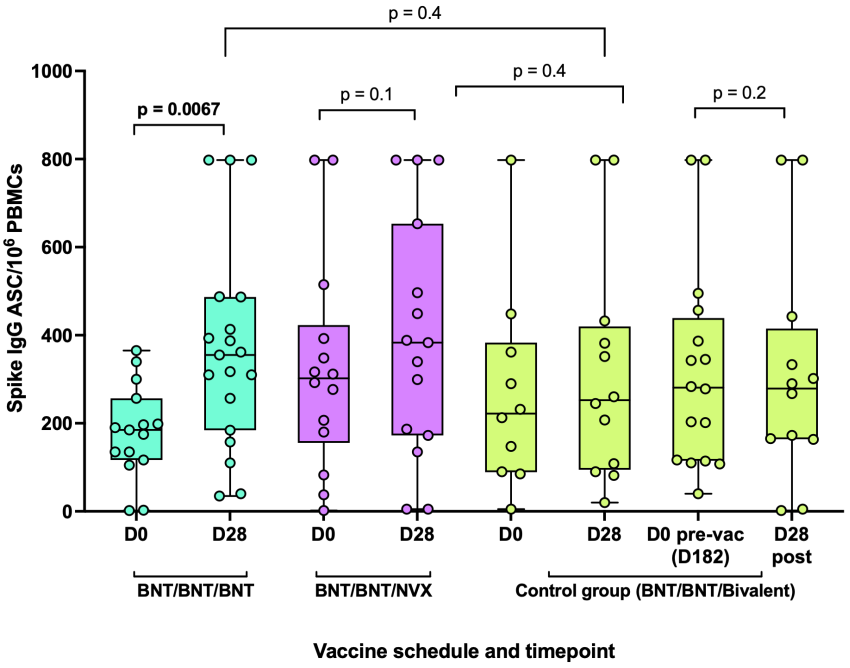


Figure 4.9 SARS-CoV-2 spike-specific IgG-ASC levels at day 28 following homologous and heterologous third (booster) dose vaccination in all participants.
Participants received their third (booster) dose at day 0 (D0). Control group participants received their third (booster) dose at day 182 (D182), as indicated in the figure. IgG-ASC frequencies are displayed as median with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells, BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1).

However, when participants with SARS-CoV-2 infection were excluded from the analysis, a nadir in spike-IgG ASC levels was observed at day 28 in the control group, **Figure 4.10**. Spike IgG-ASC levels at day 28 were significantly higher after third dose BNT162b2 compared with the control group ($p = 0.04$). Although spike IgG-ASC were also markedly higher in the NVX-CoV2373 group relative to the control group at day 28, this difference did not reach statistical significance, likely due to the limited sample size. Similarly, in control group participants without SARS-CoV-2 infection, spike IgG-ASC levels at day 210 were increased compared with (pre-vaccination) levels at day 182, though this difference was not statistically significant. However, this finding may be attributed to the small sample size.

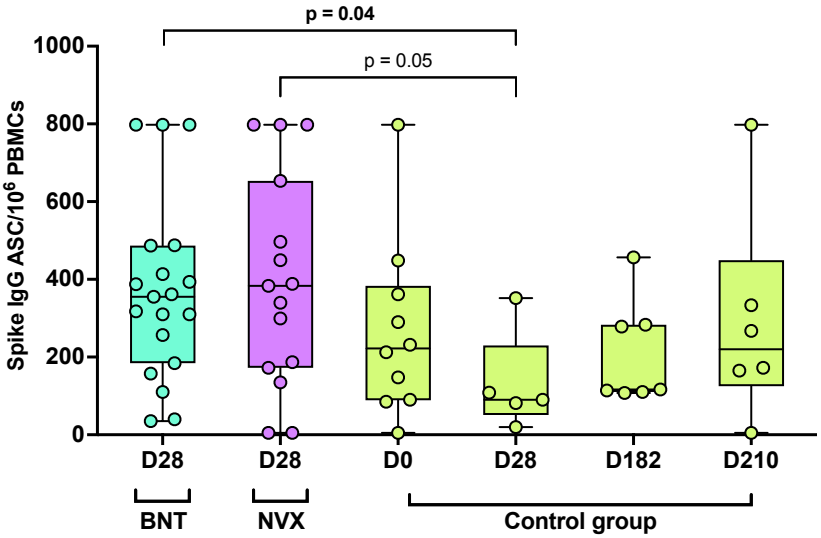


Figure 4.10 SARS-CoV-2 spike-specific IgG-ASC levels at day 28 following homologous and heterologous third (booster) dose vaccination in participants without breakthrough infection.
Control group participants received their third (booster) dose at day 182 (D182). IgG-ASC frequencies are displayed as median with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells, BNT = BNT162b2, NVX = NVXCoV2373. Only statistically significant results are displayed.

Similar to the BMEM response pattern to wild-type SARS-CoV-2 spike, Delta-specific IgG-ASC levels were also significantly higher 28 days after a third (booster) dose of BNT162b2 relative to pre-vaccination levels, **Figure 4.11**. Delta-specific IgG-ASC levels were also substantially higher at day 28 following NVXCoV2373, however this difference did not reach statistical significance. In addition, Delta-specific IgG-ASC levels 28 days following receipt of the bivalent vaccine in the control group were not significantly higher compared with (pre-vaccination) levels at day 182, reflective of the pattern observed in the spike-specific BMEM response.

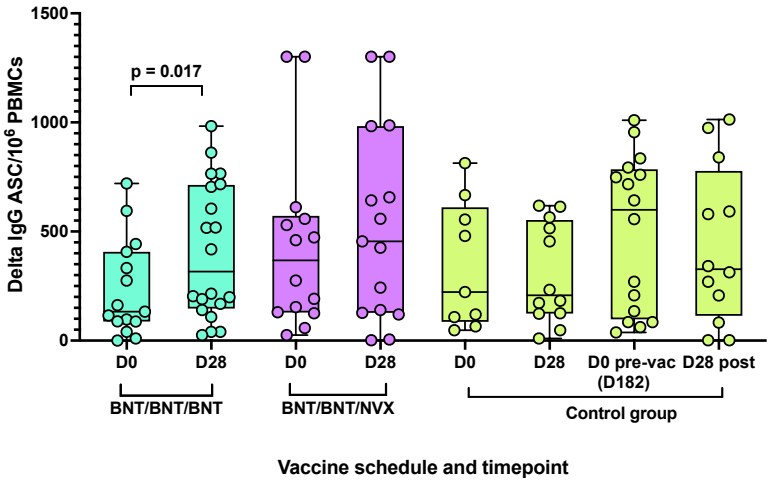


Figure 4.11 Delta-specific IgG-ASC levels following homologous and heterologous third dose vaccination in all participants.
Participants received their third (booster) dose at day 0 (D0). Control group participants received their third dose (bivalent vaccine) at day 182 (D182), as indicated in the figure. The median frequency of IgG-ASC specific for SARS-CoV-2 spike are shown with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells. Only statistically significant results are displayed.

To assess the impact of boosting through natural infection on the BMEM response to the Delta-variant following booster immunisation, participants with SARS-CoV-2 infection were excluded from the analysis. Although Delta spike-specific IgG-ASC levels were higher at day 28 in both the BNT162b2 and NVXCoV2373 groups compared with the control arm, these differences were not statistically significant. Vaccination with the bivalent Original/Omicron BA.1 vaccine did not lead to an increase in Delta-specific IgG-ASC levels, although this finding may be influenced by the very limited sample size, **Figure 4.12.**

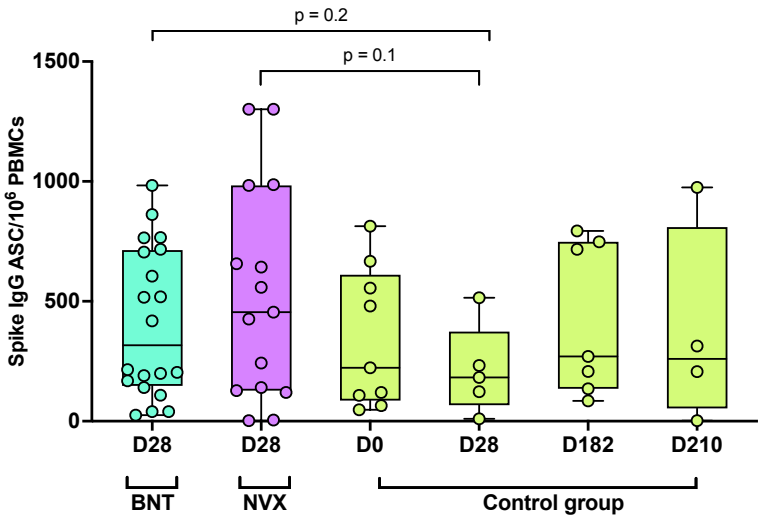


Figure 4.12 Delta spike-specific IgG-ASC levels following homologous and heterologous third dose vaccination in participants without SARS-CoV-2 infection.
IgG-ASC frequencies are displayed as median with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells, BNT = BNT162b2, NVX = NVXCoV2373.

Persistence of the spike-specific IgG BMEM response was assessed up to day 182 following booster vaccination, **Table 4.6**. In the BNT162b2 group, spike-specific IgG-ASC levels were significantly higher at day 182 compared with pre-vaccination levels, **Figure 4.13**. However, this increase was not observed in the NVXCoV2373 group, potentially due to the presence of detectable pre-vaccination spike-specific IgG-ASC in this cohort which may have confounded the analysis. In the control arm, spike-IgG ASC frequencies remained stable between day 0 and day 182 in the absence of COVID-19 vaccination. However, as shown in **Figure 4.10**, SARS-CoV-2 infection contributed substantially to the maintenance of spike-specific IgG-ASC levels in this group.

Table 4.6 SARS-CoV-2 spike-specific and Delta-specific IgG-ASC levels at day 182 following homologous and heterologous third dose vaccination.
Spike-specific IgG-ASC responses are shown as median values with interquartile ranges (IQR).

Day 182 post-boost	**BNT162b2 Homologous 3rd dose**	**NVXCoV2373 Heterologous 3rd dose**	**Control arm**
Wild-type SARS-CoV-2			
Cohort B (all participants)	270.4 (136.7-359.8)	195.0 (115.0-346.7)	280.8 (114.8-439.2)
Baseline seronegative	327.9 (140.6-372.5)	262.5 (155.0-341.3)	159.2 (92.50-413.8)
Baseline seropositive	250.0 (136.7-350.4)	168.3 (95.0-355.0)	312.9 (181.0-542.0)

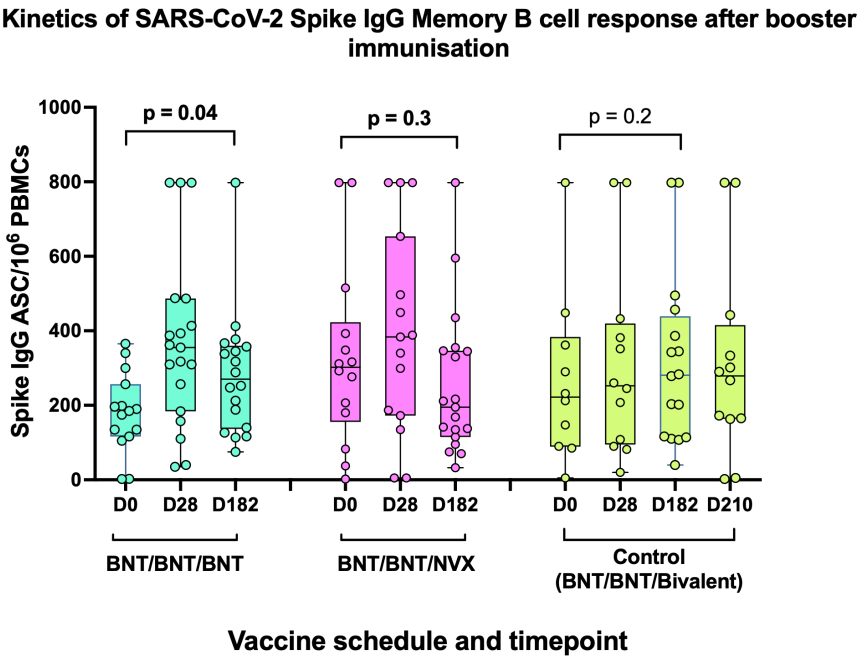


Figure 4.13 Persistence of SARS-CoV-2 IgG-specific memory B cell response following third dose immunisation.
Participants received their third (booster) dose at day 0 (D0). Control group participants received their third (booster) dose at day 182 (D182). Memory B cell frequencies are displayed as median values with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells, MBC = memory B cell, BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1).

There was also no significant difference in spike IgG-ASC levels between the COVID-19 vaccine arms and the control arm at day 182 and between the homologous BNT162b2 and heterologous NVXCoV2373 arms at day 182, [270.4 ASC/10^6 PBMCs (IQR 136.7-359.8; n = 18 vs 195.0 ASC/10^6 PBMCs (IQR 115.0-346.7; n = 19), respectively], **Table 4.6** and **Figure 4.14**.

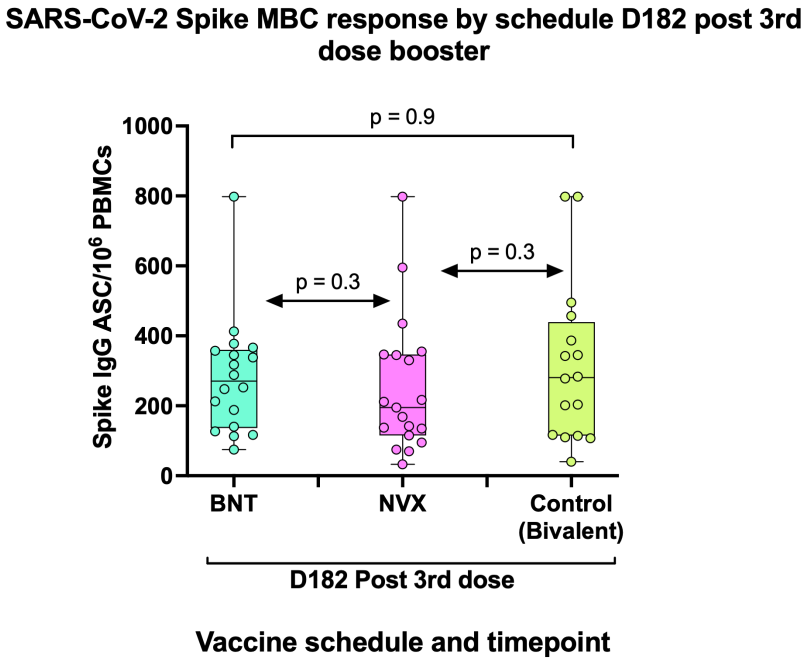


Figure 4.14 SARS-CoV-2 spik-specific IgG-ASC levels at day 182 after a third (booster) dose. *Participants received their third (booster) dose at day 0. Control group participants received their third dose (bivalent vaccine) at day 182 as indicated in the figure. Memory B cell frequencies are displayed as median values with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells, MBC = memory B cell, BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1).*

4.3.2 Third dose vaccination significantly enhances Spike- and Delta-specific memory B cell responses compared to the primary series

In seronegative participants, significantly higher spike-specific IgG-ASC levels were observed 28 days after a booster dose compared with the primary series, (p = 0.0052), **Figure 4.15, Table 4.7**. When this analysis was repeated in all participants, spike-specific IgG-ASC levels remained significantly higher 28 days after a booster dose compared with levels observed following the primary

series. These findings suggest that, irrespective of SARS-CoV-2 infection history, peak spike-specific IgG-ASC levels are significantly enhanced following a third (booster) vaccine dose.

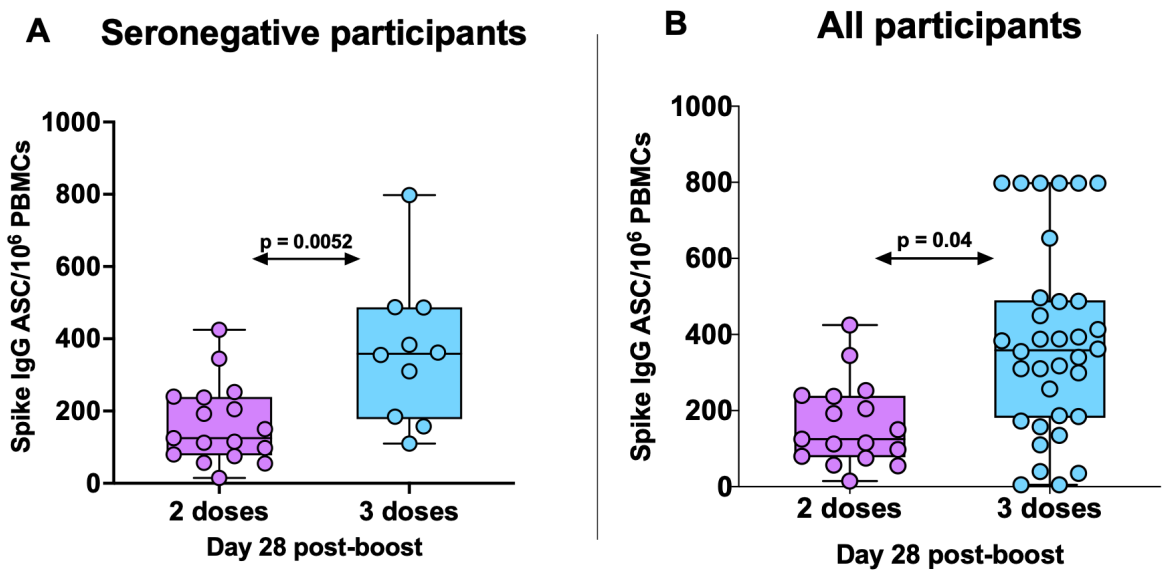


Figure 4.15 SARS-CoV-2 spike-specific IgG-ASC levels at day 28 following a two-dose primary vaccine series and third (booster) dose vaccination.
Day 28 refers to day 28 following a second or third dose. IgG-ASC frequencies are displayed as median with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. Two doses = receipt of either BNT162b2 or NVXCoV2373 as the second dose; three doses = receipt of either BNT162b2 or NVXCoV2373 as the third dose. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells,

Table 4.7 SARS-CoV-2 spike-specific and Delta-specific IgG-ASC levels at day 28 following a two-dose primary vaccine series and third dose vaccination. *Data displayed represent combined data from each cohort. Two doses = participants who received either BNT162b2 or NVXCoV2373 as the second dose. Three doses = participants who received either BNT162b2 or NVXCoV2373 as the third dose. Spike-specific and Delta-specific IgG-ASC responses are shown as median values with interquartile ranges (IQR).*

Day 28 post-boost	Two doses (Cohort A)	Three doses (Cohort B)	Control group (Cohort B only)
Wild-type SARS-CoV-2			
All participants	125.0 (77.50-238.8)	358.3 (181.3-489.8)	252.5 (94.6-419.8)
Baseline Seronegative	125.0 (77.50-238.8)	358.3 (177.5-486.9)	-
Baseline Seropositive	N/A	363.8 (176.0-614.2)	260.0 (108.3-381.7)

Delta-variant specific BMEM (Cohort B only)			
All participants	140.0 (75.0-313.8)	425.8 (140.0-716.7)	207.5 (123.7-552.5)
Baseline Seronegative	140.0 (75.0-313.8)	485.8 (202.3-720.2)	-
Baseline Seropositive	N/A	418.3 (114.2-740.8)	455.0 (125.0-565.0)

Day 28: day 28 following a second or third vaccine dose. BMEM = memory B cells.

In seropositive Cohort B participants, spike-specific IgG-ASC levels at day 28 were not significantly higher compared to the control group who had received only two doses, **Figure 4.16 (A)**. This analysis was conducted in seropositive participants to reflect the current landscape of near-universal SARS-CoV-2 seropositivity and to evaluate the impact of booster immunisation on BMEM generation. A similar pattern was observed when the analysis was repeated in all Cohort B participants, with no significant difference in spike-specific IgG-ASC observed between the COVID-19 vaccine arms and the control group at day 28 post-boost, **Figure 4.16 (B)**.

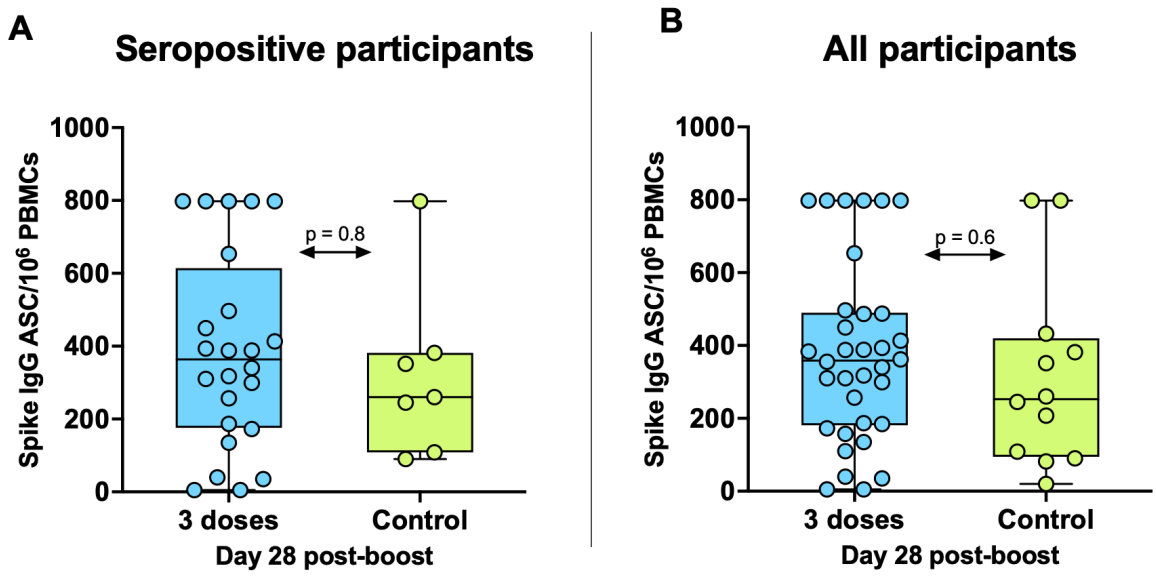


Figure 4.16 Spike-specific IgG-ASC levels at day 28 following a third (booster) vaccine dose in (A) seropositive participants and (B) all study participants.
Three doses = participants who received either BNT162b2 or NVXCoV2373 as the third dose; Control = participants who received meningococcal B vaccine at day 0 and day 84 and the Original/Omicron BA.1 vaccine at day 182.

Similar to the response observed against wild-type SARS-CoV-2, Delta-specific IgG ASC were significantly elevated 28 days after a third dose compared to levels observed following the primary series in seronegative participants, despite booster vaccination targeting wild-type virus, **Figure 4.17**. Similarly, when all participants were included, Delta-specific IgG-ASC levels at day 28 were substantially higher following a third dose compared with the primary series, however this difference was not statistically significant.

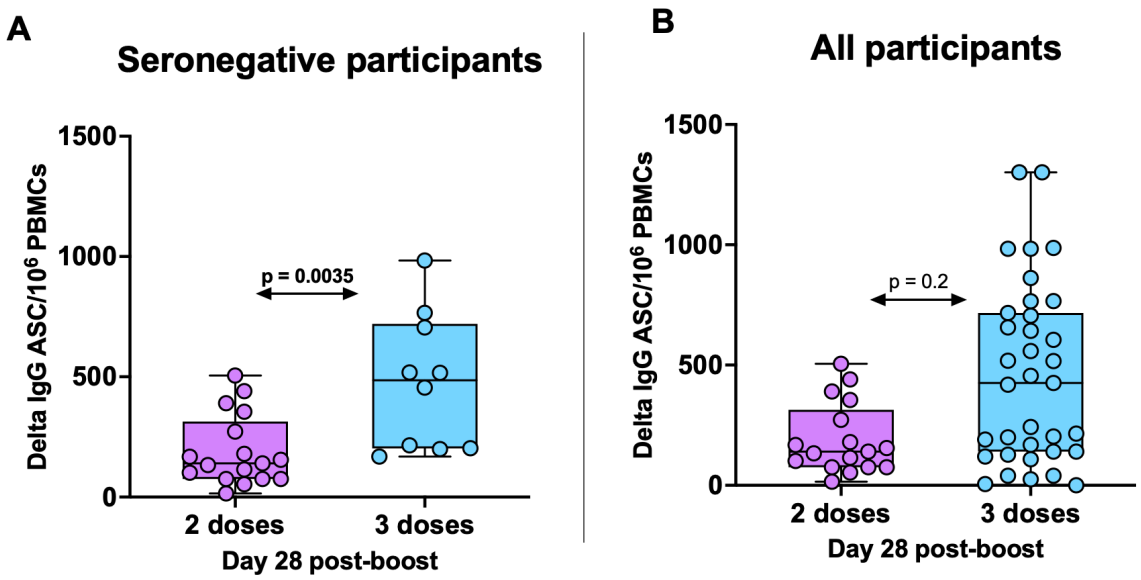


Figure 4.17 Delta spike-specific IgG-ASC levels at day 28 following two-dose primary vaccine series and third dose vaccination in (A) seronegative participants and (B) all participants.
Day 28 refers to day 28 following a second or third dose. IgG-ASC frequencies are displayed as median with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. Two doses = receipt of either BNT162b2 or NVXCoV2373 as the second dose; three doses = receipt of either BNT162b2 or NVXCoV2373 as the third dose. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells,

In seropositive participants, Delta-specific IgG ASC levels at day 28 were similar between the participants who had received a third dose and control group participants, (**Figure 4.18**). However, when all participants were included in the analysis, Delta-specific IgG-ASC levels were higher at day 28 after a third dose compared with levels in the control group. These results suggest that SARS-CoV-

2 infection exerts a significant effect on BMEM responses to variant strains with no significant difference in Delta-specific IgG-ASC levels found between vaccinated and unvaccinated seropositive participants. Furthermore, when all participants were included in the analysis (including seronegative participants), Delta-specific IgG-ASC levels were notably higher after a booster dose compared with levels in unvaccinated participants, suggesting that prior infection may exert an "imprinting" effect on the immune response on subsequent antigen encounter.

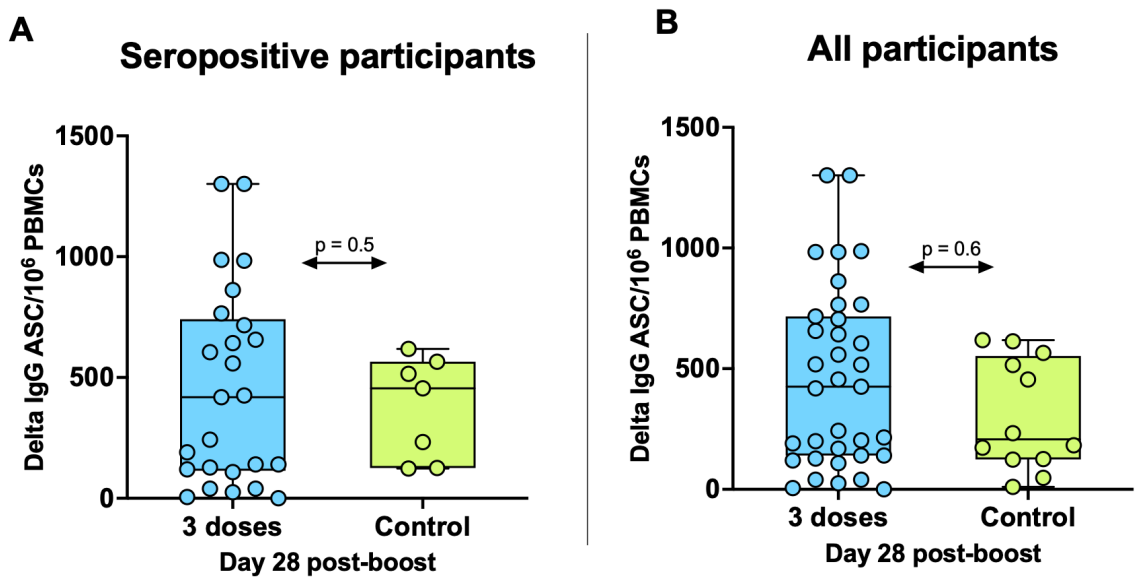


Figure 4.18 Delta-specific IgG-ASC levels at day 28 following a third vaccine dose in (A) seropositive participants and (B) all participants.
Three doses = participants who received either BNT162b2 or NVXCoV2373 as the third dose; Control = participants received meningococcal B vaccine at day 0 and day 84 and the Original/Omicron BA.1 vaccine at day 182.

4.3.3 Effect of prior SARS-CoV-2 infection on memory B Cell responses following COVID-19 vaccination

Although hybrid immunity is associated with enhanced immunogenicity following vaccination,[177] baseline spike-specific IgG-ASC levels were not significantly higher in seropositive participants

compared to seronegative Cohort B participants, (p = 0.6), **Table 4.8, Figure 4.19**. Similarly, spike-specific IgG-ASC levels at day 28 did not differ significantly between the two groups, [median 363.8 ASC/10^6 PBMCs, IQR 176.0-614.2 n=24 in seropositive participants vs. median 358.3 ASC/10^6 PBMCs, IQR 177.5-486.9, n=10 in seronegative participants] (p = 0.5). As all Cohort A participants were seronegative prior to vaccination, this analysis could not be performed using Cohort A data, **Figure 4.19**.

Table 4.8 SARS-CoV-2 spike-specific and Delta-specific IgG-ASC levels at day 0 and day 28 following a two-dose primary vaccine series and third dose vaccination.
Participants received their third dose at day 0. Spike- and Delta-specific IgG-ASC responses are shown as median values with interquartile ranges (IQR).

Cohort B	Seronegative	Seropositive
Wild-type SARS-CoV-2 spike		
Day 0	193.3 (112.5-257.1)	256.7 (125.8-344.2)
Day 28	358.3 (177.5-486.9)	363.8 (176.0-614.2)
Delta-variant specific BMEM		
Day 0	203.8 (54.2-318.1)	191.7 (101.7-544.2)
Day 28	485.8 (202.3-720.2)	418.3 (114.2-740.8)

BMEM = memory B cells.

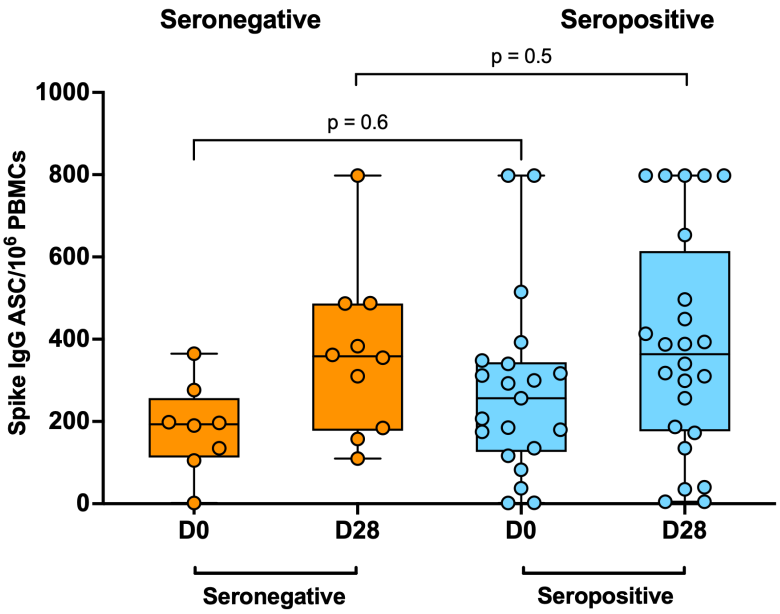


Figure 4.19 Cohort B spike-specific IgG-ASC levels at day 0 and day 28 post-third dose immunisation according to baseline anti-nucleocapsid IgG serostatus.
The analysis includes Cohort B participants who received either NVXCoV2373 or BNT162b2 as their third dose. Participants received their third vaccine dose at day 0. The median frequency of IgG-ASC specific for SARS-CoV-2 spike are shown with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells.

Similar to the SARS-CoV-2 spike BMEM response observed in seronegative and seropositive Cohort B participants, Delta-specific IgG-ASC frequencies were not significantly higher in seropositive participants at either day 0 or day 28 post-boost compared with seronegative participants (p = 0.3 and p = 0.26, respectively), **Figure 4.20**. These results suggest that broader cross-reactive BMEM responses were not elicited following vaccination in individuals with hybrid immunity, although this finding may be confounded by the limited sample size.

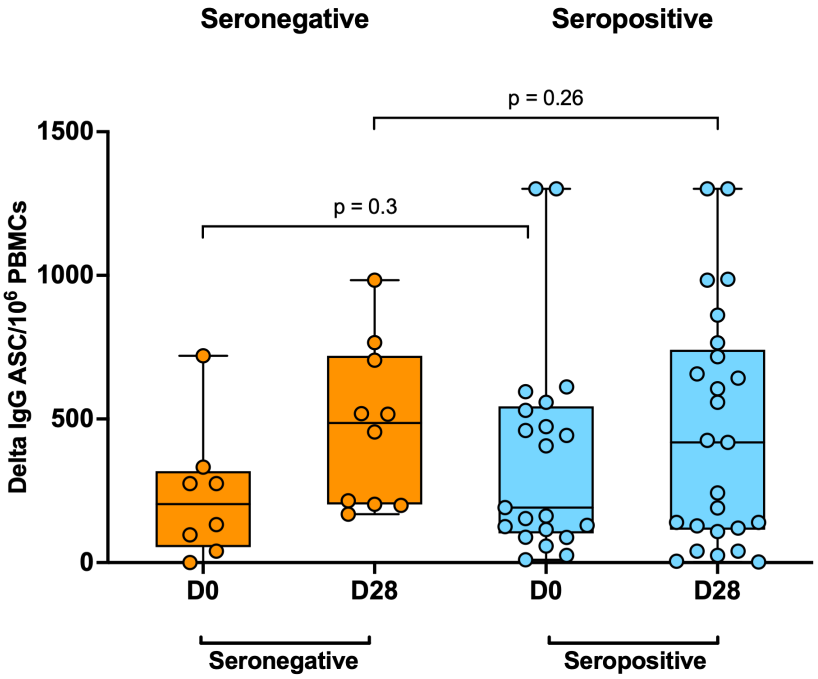


Figure 4.20 Cohort B Delta-specific IgG-ASC levels at day 0 and day 28 post-third dose immunisation according to baseline anti-nucleocapsid IgG serostatus.
The analysis includes Cohort B participants who received either NVXCoV2373 or BNT162b2 as their third dose. Participants received their third vaccine dose at day 0. The median frequency of IgG-ASC specific for SARS-CoV-2 spike are shown with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells.

4.3.4 Spike-specific memory B cell responses at day 182 are significantly higher following SARS-CoV-2 infection than after booster vaccination

To assess the impact of SARS-CoV-2 infection on spike-specific BMEM responses following booster vaccination (i.e., breakthrough infection), spike-specific IgG-ASC levels at day 182 were compared between participants with and without SARS-CoV-2 infection across both the COVID-19 vaccine and the control groups, **Figure 4.21**. Although higher spike-specific IgG-ASC levels were observed in participants with SARS-CoV-2 infection in both groups, these differences were not statistically

significant. Notably, spike-specific IgG-ASC levels at day 182 were significantly higher in (unvaccinated) control group participants with infection compared with vaccinated participants without infection, highlighting the important role of SARS-CoV-2 infection in driving BMEM responses.

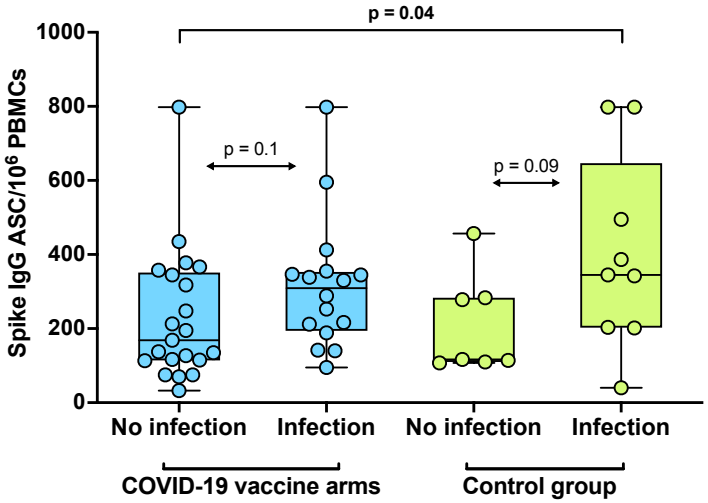


Figure 4.21 Spike-specific IgG-ASC levels at day 182 post-third dose immunisation in participants with and without evidence of SARS-CoV-2 breakthrough infection in the COVID-19 vaccine study arms and the control group.
Participants received their third dose at day 0 (D0). Control group participants received their third (booster) dose at day 182 (D182). Spike-specific IgG-ASC levels are displayed as median values with upper and lower inter-quartile ranges. Boxplots display the median values (horizontal line) with the first and third quartile; the whiskers represent the highest and lowest values. COVID-19 vaccine arms = participants who received either BNT162b2 or NVXCoV2373 as the third dose, ASC = antibody secreting cells, PBMCs = peripheral blood mononuclear cells.

4.3.5 COVID-19 booster vaccination elicits cross-reactive memory B cells targeting seasonal hCoVs and Delta variant

Cross-reactivity has been proposed as a key contributor to the development of humoral immunity.[105,178] To investigate the impact of COVID-19 vaccination on the generation of cross-

reactive immune responses to seasonal hCoV, a correlation analysis between spike-specific and OC43-specific BMEM responses was conducted, **Figure 4.22**. A significant positive correlation was observed between spike-specific and OC43-specific IgG-ASC levels at day 28 following a third dose (Spearman r = 0.4, p = 0.0007), suggesting that COVID-19 booster immunisation may enhance BMEM responses to antigenically related hCoVs.

A correlation analysis was also performed to evaluate whether a significant correlation exists between alpha coronavirus NL63 and beta-coronavirus spike-specific BMEM responses following booster vaccination. The results revealed a positive, though not statistically significant correlation, between NL63 and spike-specific IgG-ASC at day 28 post-booster vaccination (Spearman r = 0.3, p = 0.3), **Figure 4.23**. The lack of statistical significance however may be due to the limited sample size or the relatively low sequence homology between SARS-CoV-2 and NL63, compared to the greater homology shared with beta-coronaviruses OC43 and HKU1.

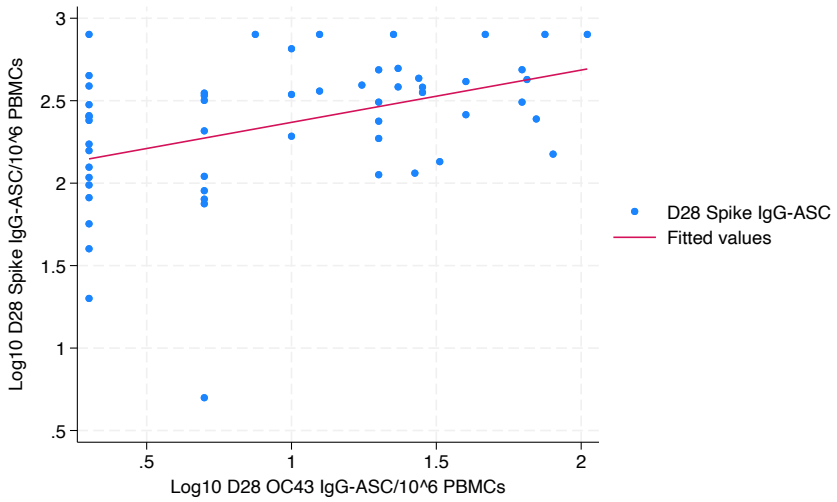


Figure 4.22 Correlation between spike-specific IgG-ASC and hCoV OC43-specific IgG-ASC levels at day 28 following a third (booster) dose.
Correlation was assessed using Spearman's rank correlation coefficient on Log10 transformed memory B cell frequencies (Spearman r = 0.4, p = 0.0007). ASC = antibody secreting cells, PBMC = peripheral blood mononuclear cells. D28: day 28 post-vaccination. Third dose vaccination refers to participants in Cohort B who received either NVXCoV2373 or 30µg BNT162b2.

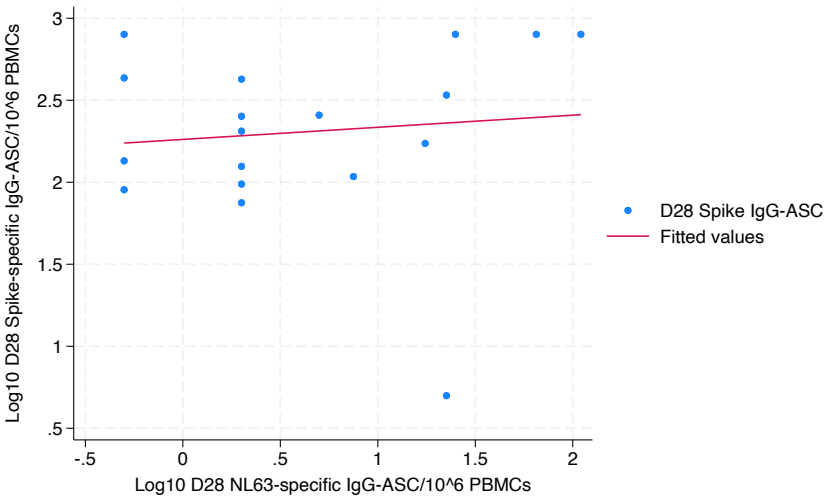


Figure 4.23 Correlation between spike-specific IgG-ASC and hCoV NL63-specific IgG-ASC levels at day 28 following a third (booster) dose.
Correlation was assessed using Spearman's rank correlation coefficient on Log10 transformed memory B cell frequencies (Spearman r = 0.3, p = 0.3). ASC = antibody secreting cells, PBMC = peripheral blood mononuclear cells. D28: day 28 post-vaccination. Third dose vaccination refers to participants in Cohort B who received either NVXCoV2373 or 30µg BNT162b2.

Similarly, significant cross-reactivity was detected between SARS-CoV-2 (wild-type) and the Delta-variant following COVID-19 booster vaccination. As shown in **Figure 4.24**, spike and Delta-specific IgG-ASC responses at day 28 were highly correlated ($r = 0.7$, $p < 0.00001$) following a third dose, indicating substantial cross-reactivity in the BMEM response following immunisation with vaccine targeting wild-type SARS-CoV-2.

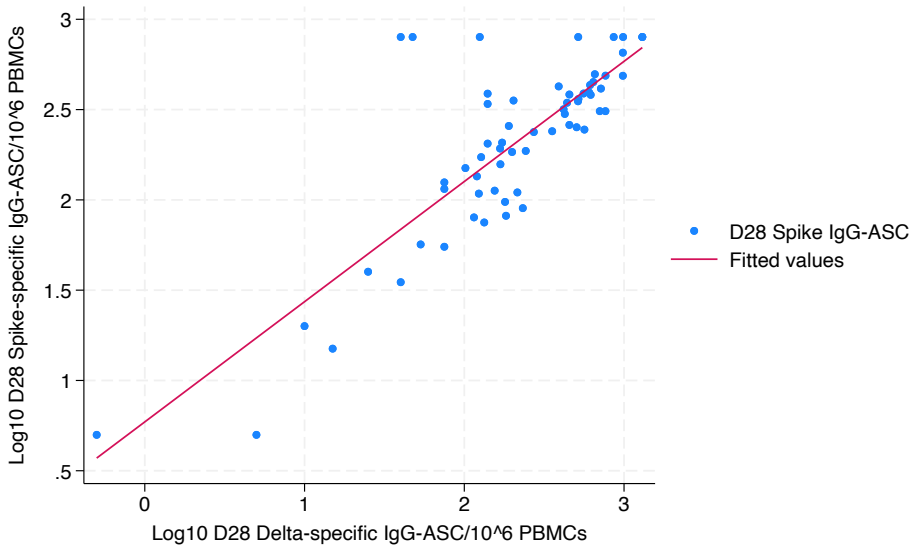


Figure 4.24 Correlation between spike-specific IgG-ASC and Delta-specific IgG-ASC levels at day 28 following a third (booster) dose.
Correlation was assessed using Spearman's rank correlation coefficient on Log10 transformed memory B cell frequencies (Spearman r = 0.7, p < 0.00001). ASC = antibody secreting cells, PBMC = peripheral blood mononuclear cells. D28: day 28 post-vaccination. Third dose vaccination refers to participants in Cohort B who received either NVXCoV2373 or 30µg BNT162b2.

4.3.6 Peak memory B cell responses following booster vaccination are predictive of long-term memory B cell persistence

To assess whether spike-specific BMEM responses at day 28 are predictive of longer-term BMEM responses following booster immunisation, a correlation analysis was performed. This revealed a significant positive correlation between spike-specific IgG-ASC levels at day 28 and day 182 post-booster vaccination (r = 0.6, p = 0.0001, **Figure 4.25 (A)**.

SARS-CoV-2 breakthrough infections were next excluded from the analysis to assess whether the relationship observed between day 28 and day 182 spike-specific IgG BMEM responses remained significant in the absence of additional antigen exposure through natural infection (r = 0.5, p = 0.01). As shown in **Figure 4.25 (B)**, this correlation remained statistically significant even in the absence of immune boosting through natural infection.

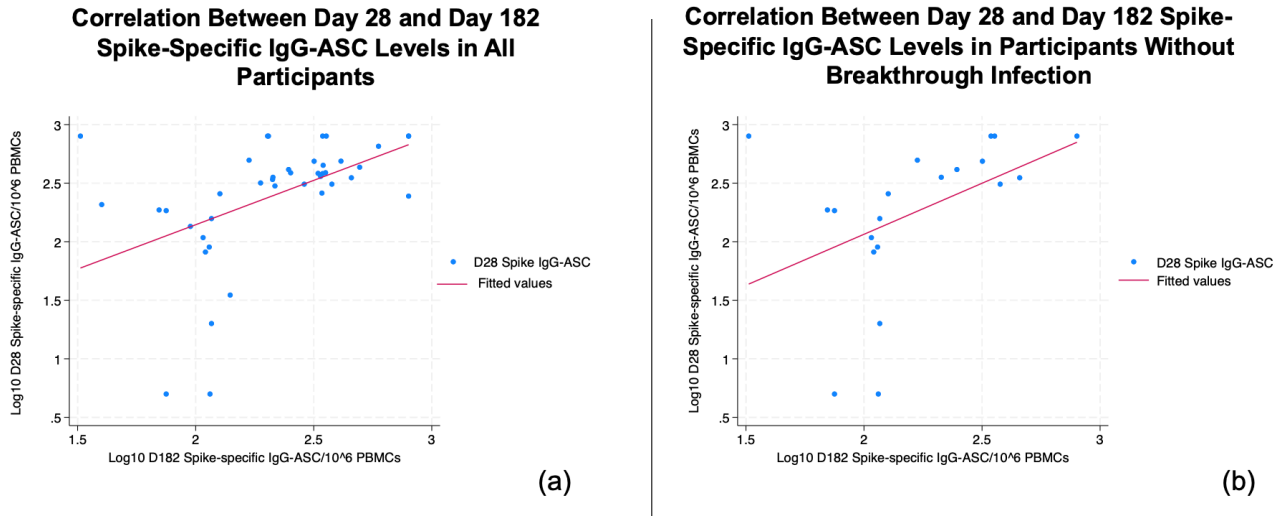


Figure 4.25 (A) Correlation between spike-specific IgG-ASC levels at day 28 and day 182 in all participants, (B) Correlation between spike-specific IgG-ASC levels at day 28 and day 182 in participants without breakthrough infection.
Correlation was assessed using Spearman's rank correlation coefficient on Log10 transformed memory B cell frequencies [(A) Spearman r = 0.6, p = 0.0001 (B) Spearman r = 0.5, p= 0.01]. ASC = antibody secreting cells, PBMC = peripheral blood mononuclear cells. D28: day 28 post-vaccination. Third dose vaccination refers to participants in Cohort B who received either NVXCoV2373 or 30µg BNT162b2 at day 0.

4.4 Discussion

This is the first study to investigate BMEM responses in a paediatric population following primary and booster homologous and heterologous COVID-19 vaccination while also examining these responses in the context of hybrid immunity and breakthrough infection. The results demonstrate that, when administered as a third dose, 30µg BNT162b2 or NVX-CoV2373 induced a substantial increase

in the spike-specific IgG BMEM response. Seven months after administration of a third 30µg dose of BNT162b2, spike–specific BMEM levels remained significantly higher than baseline (pre-vaccination) levels. Delta-specific BMEM responses were also significantly elevated following third dose BNT162b2 and a significant correlation was observed between spike-specific and Delta-specific BMEM responses at day 28 post-booster vaccination. Furthermore, a significant correlation between vaccine-induced spike-specific BMEM levels at day 28 and those at day 182 following a third booster dose was found, consistent with ongoing germinal centre B cell activation.[179,180]

4.4.1 Spike-specific memory B cell responses are significantly elevated following a homologous third dose of BNT162b2

Stable levels of SARS-CoV-2 spike-specific germinal centre-derived B cells have been identified in draining lymph nodes for up to 12 weeks following second dose mRNA vaccination, with peak responses observed approximately one week post-vaccination.[181] In parallel, SARS-CoV-2 spike-specific BMEM have been detected in peripheral blood up to 6 months after a second dose of BNT162b2.[182] In this study, SARS-CoV-2 spike-specific IgG BMEM responses were detectable in all participants four weeks after the second dose of either BNT162b2 or NVX-CoV2373. This is the first study in an adolescent population to examine spike-specific BMEM responses following homologous BNT/BNT or heterologous BNT/NVX regimens where we have demonstrated higher spike-specific BMEM levels following a heterologous boost, though this was not statistically significant. One other study was identified which examined BMEM responses in Thai adolescents who received a first dose of CoronaVac followed by a second dose of BNT162b2, administered either 3 or 6 weeks later. This

study reported no significant difference in BMEM responses based on dosing interval, with a median RBD-specific BMEM response of 8-25 SFU/1/10^6 PBMCs following the second dose.[176]

To our knowledge, this is also the first study to directly compare peak BMEM responses between primary and booster immunisation regimens in a paediatric population. In this study, a third (booster) dose induced substantially higher spike-specific BMEM responses compared with the primary vaccination series. Third dose BNT162b2 following a two-dose mRNA primary series has already been shown to elicit a significantly enhanced spike and RBD-specific BMEM response in adults[183] [median interval between the booster dose and the final dose of the primary immunisation series was 215 days (range: 135 – 271 days), similar to Com-COV3: mean 245 days (range 91 – 501 days)].

Third dose mRNA vaccination (following a homologous mRNA primary vaccine series) has been shown to result in increased RBD-specific BMEM responses and enhanced potency of antibodies expressed by these cells.[184] In line with previous reports, third dose BNT162b2 in this study induced a significant increase in spike-specific BMEM levels at day 28 post-boost.[184,185] While heterologous third dose immunisation has been shown to elicit a superior RBD-specific BMEM response compared with a homologous three dose mRNA-vaccine schedule,[186] this finding was not reflected in this study. Although third dose NVXCoV2373 elicited higher spike-specific BMEM frequencies at day 28 post-boost compared with baseline levels, this increase was not statistically significant. However, high baseline (pre-vaccination) spike-specific BMEM frequencies in the third dose NVX-CoV2373 group may have made any rise in BMEM levels difficult to detect. In seronegative participants, spike-specific IgG-ASC levels at day 28 were also significantly higher in both the BNT162b2 and NVXCoV2373 groups compared with the Control group. Consistent with previous studies, these findings suggest that booster immunisation enhances spike-specific BMEM responses.[183,187]

When comparing heterologous to homologous booster immunisation, third dose NVXCoV2373 was found to induce higher spike-specific IgG-ASC levels at day 28 post-boost compared with BNT162b2. Only one other study was identified which directly compares NVXCoV2373 and

BNT162b2 in terms of vaccine-induced BMEM. Conducted in adults, this study reported no significant difference in BMEM levels at day 0 and day 28 following a second dose of either vaccine.[188] Although the current study in adolescents suggests that vaccine platform may influence spike-specific BMEM responses after a third dose, further studies are needed to test this hypothesis.

There is also evidence to suggest that bivalent vaccines targeting SARS-CoV-2 ancestral strain and circulating variant strains induce a more robust and broadly neutralising antibody response compared with a monovalent booster.[189,190] When compared with a monovalent ancestral strain-containing vaccine (i.e., mRNA-1273 or BNT162b2), the bivalent ancestral/Omicron BA.1 vaccine as a third (booster) dose has been shown to induce significantly higher BMEM frequencies specific for Omicron BA.1, while both vaccines elicited significantly enhanced BMEM responses to ancestral strain and the Delta variant.[191] In the present study, however, a significant increase in spike-specific IgG-ASC levels was not observed at day 28 following receipt of the bivalent ancestral/Omicron BA.1 vaccine. There are three factors which may explain this unexpected finding.

Firstly, it has been shown that, when challenged by SARS-CoV-2 infection, BMEM are activated, and respond to infection by differentiating into antibody-secreting cells, producing large quantities of antibodies to defend against infection.[40] In this instance, there may not be evidence of waning immunity despite breakthrough infection.[40] Although participants in the control arm did not receive their COVID-19 vaccine until day 182, elevated spike-specific BMEM levels were detected throughout the study in this arm. In the absence of COVID-19 vaccination, the persistently elevated BMEM levels observed may indicate ongoing BMEM activation in response to environmental exposure to SARS-CoV-2. This theory is supported by the finding that, when participants with SARS-CoV-2 infection were excluded, spike-specific IgG-ASC levels were substantially higher 28 days after receipt of the bivalent vaccine. Secondly, it has been suggested that a high baseline level of antigen-specific BMEM may hinder the magnitude of post-vaccination responses.[192] Elevated levels of antibody binding can inhibit B cell responses by blocking epitope access, thereby preventing effective B cell engagement and response on re-exposure to the same or related antigens.[193]

Finally, immunological imprinting through previous antigenic exposure may affect subsequent vaccine-induced responses. It has been shown that 'boosting' with either a monovalent or BA.5 bivalent mRNA vaccine resulted in higher neutralising antibody titres against ancestral SARS-CoV-2 than against Omicron BA.5, suggesting that immunological imprinting may be difficult to overcome.[194] This important dynamic between SARS-CoV-2 infection and vaccination is discussed in detail in the next section.

4.4.2 Limited impact of hybrid immunity on memory B cell response to booster dose in adolescents

Immunity conferred through a combination of SARS-CoV-2 vaccination and prior infection - referred to as "hybrid immunity" - has been shown to enhance immunogenicity following subsequent vaccination, including more broadly cross-reactive humoral and cellular immune responses, and greater protection against SARS-CoV-2 infection.[168,195,196] However, in this study, both spike-specific and Delta-specific IgG-ASC levels were not significantly higher in participants with hybrid immunity compared with seronegative participants, either at baseline or following booster vaccination. However, the lack of statistical significance may also be attributed to the limited sample size.

In adults with prior SARS-CoV-2 infection, mRNA primary series vaccination has been shown to induce a significantly greater RBD-specific BMEM response and enhanced variant-neutralising antibody activity compared with infection-naïve individuals. However, consistent with the findings reported here, administration of a third dose did not lead to any further significant expansion of RBD-specific BMEM in either group. Furthermore, no difference in RBD-specific BMEM responses was observed between previously infected and infection-naïve individuals two weeks after the booster dose. The authors hypothesised that this finding may be attributed to rapid antigen clearance mediated

by antigen-specific memory present in both groups.[42] Similarly, in children aged 5 to 12 years who received a two-dose 10μg BNT162b2 primary series, no significant difference in spike-specific BMEM responses was observed between infection-naïve children and those with hybrid immunity up to 6 months following a booster dose.[82]

Additionally, spike-specific BMEM responses at day 182 were not significantly higher in participants with SARS-CoV-2 infection compared to those without infection following booster vaccination. A similar pattern was observed in the control group in the absence of COVID-19 vaccination. In children aged 5 – 12 years, spike-specific BMEM responses were significantly elevated six months after primary series vaccination (with a two-dose 10μg BNT162b2 regimen) in those with SARS-CoV-2 infection compared to baseline levels. However, no significant difference in spike-specific BMEM responses was observed between children with hybrid immunity who did not receive a third (booster) dose and children with SARS-CoV-2 infection who did receive a third dose.[82] In adults, it has been suggested that breakthrough infection leads to the expansion of BMEM and the production of high levels of antibodies in response to antigenic challenge. [40] Indeed, consistent with the findings reported here, this study in adults showed that spike-specific BMEM levels were not significantly higher in adult participants with breakthrough infection compared to those without at both 3- and 6-months following two doses of BNT162b2.[40] Similarly, comparable frequencies of spike-specific (wild-type) and BA.1-specific (Omicron) BMEM have been observed in both uninfected adults and those with breakthrough infection following (mRNA-based) primary series and booster immunisation.[197]

However, spike-specific IgG-ASC levels at day 182 were significantly higher in previously infected unvaccinated participants compared with infection naïve participants who had received a booster dose. These findings suggest that SARS-CoV-2 infection potently influences the generation of BMEM. Consistent with these findings, SARS-CoV-2 infection following primary series vaccination in children aged 5-12 years elicited a spike-specific BMEM response comparable to that observed after booster immunisation in the absence of infection.[82]

4.4.3 Booster immunisation induces significant cross-reactive Memory B Cell responses to seasonal coronaviruses and Delta

Pre-existing immunity to SARS-CoV-2 has been detected in pre-pandemic samples, attributed to the high degree of homology which exists between SARS-CoV-2 and seasonal hCoV.[178] Higher levels of cross-reactivity between hCoV and SARS-CoV-2 have, additionally, been reported in children and adolescents compared with adults due to more recent hCoV infection in this age group.[105] The hCoV OC43 (a betacoronavirus, similar to SARS-CoV-2) shares 50.5% sequence homology with SARS-CoV-2 while the alpha coronavirus NL63 shares 47.9% homology.[198,199] In line with the shared homology among the hCoV family, hCoV OC43-specific and spike-specific BMEM responses were found to be positively and significantly correlated following COVID-19 booster vaccination in this study. Cross-recognition of the spike S2 subunit by antibodies between seasonal hCoV and SARS-CoV-2 has been previously demonstrated.[200] Notably, this prior study also reported preferential antibody binding to hCoV epitopes, underscoring the significant influence of prior hCoV exposure on the immune response to SARS-CoV-2.[200] In the present study, a positive, although not statistically significant, correlation was observed between NL63-specific and SARS-CoV-2 spike-specific BMEM responses following booster immunisation. This lack of statistical significance may be attributed to the small sample size, particularly given that cross-reactivity between NL63 and SARS-CoV-2 has been detected in pre-pandemic samples.[201]

The phenomenon of immunological imprinting may also play a contributory role in these findings. Immune imprinting refers to the influence a first exposure to a viral antigen (via infection or

vaccination) has on the subsequent immune response generated on re-exposure to related strains or variants of the original antigen.[172] The findings reported here are consistent with previous reports highlighting cross-reactive immunity to hCoVs and the role of immune imprinting in memory B cell activation in response to antigenically similar viruses. [202-204] Similarly, a significant correlation was observed between spike-specific and Delta-specific BMEM responses following booster immunisation with vaccine targeting wild-type virus. This finding is supported by spike protein sequence homology analysis which revealed a high degree of similarity (99.37%) between wild-type SARS-CoV-2 and the Delta variant, consistent with the cross-reactive BMEM response observed in this study.[199]

The effects of immune imprinting have also been shown to persist despite repeated exposure to variant strains through vaccination and infection.[205] As illustrated by Dowell et al., previous primary infection with an Omicron strain followed by vaccination (targeting wild-type SARS-CoV-2) resulted in increased neutralising antibody titres targeting Omicron subvariant strains.[206] The importance of previous SARS-CoV-2 infection history as a determinant of the immune response to vaccination has been highlighted in several studies.[174,207,208] In the present study, third dose immunisation with vaccine targeting wild-type SARS-CoV-2 induced a Delta-specific BMEM response similar to that observed for SARS-CoV-2 spike. A beneficial aspect of immune imprinting is the generation of broadly reactive antibodies capable of recognising conserved epitopes between ancestral and variant strains. However, this mechanism of protection may hinder de novo B-cell activation, enabling immune evasion by newer emerging SARS-CoV-2 variants if preferential targeting of the immune response against conserved epitopes occurs.[173,174]

4.4.4 Peak memory B cell responses following booster vaccination are predictive of long-term memory B cell persistence

A paucity of data exists relating to the durability of vaccine-induced BMEM responses to SARS-CoV-2 vaccination, especially in the paediatric population. Memory B cells have been shown to persist up to 9 months after second dose mRNA-based vaccination in adults,[40,209] while a three-dose homologous NVXCoV2373 regimen in rhesus macaques has been shown to induce antigen-specific BMEM responses up to 7 months.[210] The data in this study show spike-specific BMEM response following a third dose were detectable up to 7 months after vaccination and, in the case of BNT162b2, were significantly higher at 7 months post-boost compared with pre-vaccination levels. However, the frequency of day 182 spike specific memory B cells did not differ significantly between study arms.

Current evidence suggests that B cell germinal centre activity is responsible for the durability of antibody responses and the induction of broadly reactive memory B cells. A pre-requisite for persistent germinal centre reactions is the sustained presence of antigen, which facilitates the ongoing selection of affinity-enhanced B cells.[211] This hypothesis has been validated by studies which have demonstrated presence of vaccine-derived spike protein in lymph nodes following vaccination and durable memory B cell responses.[212] Furthermore, strong correlations have been reported between BMEM responses, antigen-specific antibody concentrations, and functional antibody responses following vaccination.[213]

In the present study, a significant positive correlation was observed between (ancestral) spike-specific BMEM responses at day 28 and day 182 following a third (booster) dose, suggesting that long-term BMEM responses are sustained through enhanced antigen recognition and persistence in the germinal centre. Furthermore, this correlation remained significant in the absence of boosting through natural

infection, suggesting that the durability of the BMEM response is potently influenced by vaccination. These findings are consistent with previous findings in adults by Terreri et al.,[40] suggesting that BMEM are active during SARS-CoV-2 infection. However, further studies are needed to confirm this observation.

4.4.5 Limitations and Conclusions

This study has several limitations. An important limitation is the small sample size which restricts the generalisability of the findings reported. As only a subset of participants from each cohort was included in this exploratory analysis, this resulted in limited participant numbers once data were stratified by infection status, vaccine schedule and/or study timepoint. The results of this study should therefore be interpreted with caution. However, every effort has been made to interpret the findings of this study in the context of current literature and to highlight any notable similarities or differences.

This analysis was also performed using BMEM derived from PBMCs, providing insight into circulating antigen-specific BMEM activity following vaccination. However, a more accurate assessment of BMEM activity post-vaccination may have been achieved by analysing samples taken from a secondary lymphoid organ where B cell differentiation and somatic hypermutation occurs. Additionally, while the FluoroSpot assay is highly sensitive and enables the detection of multiple antigen-specific B cells, it is difficult to reliably ascribe antibody functionality based on the results of this assay. It is also difficult to accurately interpret the vaccine-induced antigen-specific BMEM response in the context of immune imprinting without knowledge of the primary infecting SARS-CoV-2 strain.

The study described in this chapter demonstrates that both homologous and heterologous primary COVID-19 vaccine schedules elicit BMEM responses specific for the SARS-CoV-2 spike protein, and

that these responses are enhanced by a third (booster) dose. Furthermore, the findings suggest that booster immunisation induces cross-reactive BMEM responses to both seasonal hCoV and the Delta variant. Consistent with previous studies, these results suggest that peak vaccine-induced BMEM responses are predictive of longer-term BMEM persistence, even in the absence of SARS-CoV-2 infection. The results from this study provide supportive evidence for the important role of COVID-19 vaccination in generating lasting BMEM responses which may provide a mechanism to reduce waning of vaccine-induced immunity over time.

Chapter 5: Mucosal Immune Responses Following Homologous and Heterologous Primary and Booster COVID-19 Vaccination in Adolescents

5.1 Introduction

The primary aim of the initial COVID-19 vaccine campaign, including subsequent booster doses, was to reduce severe disease and hospitalisations and this was achieved successfully. However, a deeper understanding of the mucosal immune response elicited by these vaccination strategies is essential to assess their potential impact on viral transmission and reinfection.

As the upper respiratory tract is the site of SARS-CoV-2 entry and replication, the mucosal antibody response is essential to the prevention of viral entry. A rapid and effective sterilising mucosal humoral immune response would have the potential to prohibit both initiation of viral invasion and eliminate transmission.[93] A greater understanding of mucosal immunity may therefore help to determine protection against SARS-CoV-2. Specifically, determination of the durability of mucosal IgA and IgG following vaccination may provide insight into the mechanisms underlying protection against reinfection, particularly as systemic antibody responses wane over time. Recent studies have suggested that mucosal antibodies (particularly IgA) may act as a correlate of protection against SARS-CoV-2 and high titres of mucosal IgA in the respiratory tract following natural infection have been associated with protection against reinfection.[92,214,215] The persistence of antibody in mucosal tissue and the mechanisms by which vaccination could induce an effective immune response in the mucosa have not been determined.

Upon antigen exposure, the mucosal antibody response is initially comprised of IgM, IgA and IgG, which have been shown to correlate with serum antibody levels.[94] Following infection, a more rapid decline in mucosal secretory IgA compared with mucosal IgG levels has been observed, although some studies in adults have reported the presence of mucosal IgA up to nine months post-infection.[95,96] Notably, mucosal IgA has been identified as the predominant mediator of neutralising activity against SARS-CoV-2, underscoring its critical role in defence against infection.[97] However, most studies examining the mucosal immune response to COVID-19 vaccination suggest that vaccination alone induces minimal mucosal secretory IgA in the absence of prior SARS-CoV-2 infection.[98]

Several studies have also demonstrated age-dependent differences in the systemic and mucosal immune response to SARS-CoV-2 infection.[216,217] In a study of unvaccinated individuals aged from 1 week to 83 years, distinctive immune responses to SARS-CoV-2 infection were observed in the upper respiratory tract (URT) and peripheral blood of children and adolescents compared with adults, with greater expression of innate and adaptive immune responses detected in the URT of children and adolescents.[218] Additionally, a more robust innate immune response to SARS-CoV-2 infection has been observed in the URT of children and adolescents compared with adults.[219] However, a significant knowledge gap remains in our understanding of the mucosal immune response in the paediatric population, particularly adolescents, to COVID-19 vaccination.

In children aged 5 to 11 years, spike-specific IgA levels were significantly increased 10 days following completion of a two-dose BNT162b2 primary vaccine series. However, when stratified by SARS-CoV-2 infection history, salivary spike-specific IgA levels were significantly higher in previously infected children both (before and after vaccination) compared with infection naïve children. Notably, salivary spike-specific IgA levels three weeks after the first SARS-CoV-2 positive test in unvaccinated children were not significantly different from levels observed in vaccinated children 11 days after their second dose. Furthermore, previously infected unvaccinated children demonstrated significantly higher salivary anti-RBD IgA2 levels, while vaccinated children showed

significantly elevated levels of IgA1.[99] A similar response has also been observed in adult studies.[220] Mucosal IgA2 predominates in mucosal secretions and is believed to play a key role in mediating the mucosal inflammatory response, while IgA1 is prevalent in serum and associated with immune homeostasis.[87] These findings suggest that SARS-CoV-2 infection influences the quality and magnitude of the mucosal immune response and enhances mucosal IgA secretion following vaccination in children, consistent with observations in adult studies.[215] However, to date, no study has investigated the mucosal immune response to both primary and booster COVID-19 vaccination in adolescents, nor examined the impact of SARS-CoV-2 infection on these responses.

Prior exposure to hCoV can generate cross-reactive immunity to SARS-CoV-2 following vaccination or infection. Of the seven coronaviruses which can infect humans, four are endemic seasonal strains: OC43 and HKU1 (beta-coronaviruses), and 229E and NL63 (alpha-coronaviruses). The remaining three recently emerged coronaviruses have been derived through zoonotic transmission: SARS-CoV, MERS-CoV (Middle East respiratory syndrome), and SARS-CoV-2 .[221] All hCoVs share similar structural proteins (spike, envelope, membrane, nucleocapsid), as well as similarity between non-structural proteins.[198] The degree of sequence homology across hCoVs – particularly among beta-coronaviruses – is notable and is illustrated in **Figure 5.1**.[199,222] Cross-reactive immune responses to SARS-CoV-2 have been detected in pre-pandemic samples, supporting the presence of pre-existing immunity.[223,224] In children and adolescents, higher levels of cross-reactivity to SARS-CoV-2 have been detected, attributed to more recent coronavirus infection.[225] Neutralising antibody responses to SARS-CoV-2 have also been found in children prior to SARS-CoV-2 exposure.[105]

Age-related differences in mucosal and systemic hCoV-specific antibody responses have been observed following SARS-CoV-2 infection and/or vaccination. In a study comparing children (aged 4-14 years) and adults with prior SARS-CoV-2 infection, mucosal hCoV-specific IgA titres were significantly lower in children and adolescents (median levels up to 6.5 times higher in adults).[103] However, only half of the children in this study were vaccinated, while all adult participants had received at least two vaccine doses. In vaccinated study participants, mucosal spike-specific IgA

levels were 1.7 times lower in children than in adults, while systemic spike-specific IgA levels were similar between children and adults.[103]

In the paediatric population, increased OC43- and HKU1-specific IgG titres have been reported following vaccination against SARS-CoV-2.[106] In adults, serum IgG responses to hCoV (OC43, HKU1, NL63 and 229E) were significantly reduced three weeks after a second vaccine dose but were significantly elevated following a third (booster) dose.[226] However, further studies in adults have shown that systemic NL63-specific IgG levels did not increase significantly following a third mRNA booster, and no association was observed between baseline NL63 immunity and SARS-CoV-2 vaccine-induced antibody responses.[227] Furthermore, serum hCoV IgG and SARS-CoV-2 spike IgG antibodies were poorly correlated following both a second and third dose in adults.[226] Whether age-dependent differences in cross-reactive adaptive immunity to hCoVs contribute to the age-dependent variation observed in the immune response to SARS-CoV-2 remains unknown.

This chapter describes results relating to the following objectives:

1. To investigate mucosal SARS-CoV-2 spike-specific IgG and IgA responses and their kinetics following homologous and heterologous primary and booster COVID-19 vaccination schedules in adolescents.
2. To investigate the impact of prior SARS-CoV-2 infection on the mucosal SARS-CoV-2 spike-specific IgG and IgA response following COVID-19 vaccination.
3. To assess the impact of breakthrough infection on mucosal spike-specific IgG and IgA responses after vaccination.
4. To examine the relationship between pre-existing seasonal hCoV immunity and the systemic immune response to SARS-CoV-2 following a two-dose primary vaccine series and a booster dose.

100.00	56.93	56.61	50.44	51.04	47.43	48.10	MERS
56.93	100.00	80.04	50.49	51.59	47.73	48.66	CoV2
56.61	80.04	100.00	49.90	50.19	47.35	47.98	CoV
50.44	50.49	49.90	100.00	73.38	52.71	53.90	OC43
51.04	51.59	50.19	73.38	100.00	53.23	54.94	HKU1
47.43	47.43	47.35	52.71	53.23	100.00	68.31	229E
48.10	48.66	47.98	53.90	54.94	68.31	100.00	NL63
MERS	CoV2	CoV	OC43	HKU1	229E	NL63	

Figure 5.1 Percentage identity matrix displaying percentage sequence homology shared among human coronaviruses.
The matrix has been adapted from Cicaloni et al. A Bioinformatics Approach to Investigate Structural and Non-Structural Proteins in Human Coronaviruses. Front Genet. 2022.14;13:891418. CoV2 = SARS-CoV-2; CoV = SARS-CoV.

5.2 Methods

5.2.1 Sample selection

To compare the systemic and mucosal immune response to COVID-19 vaccination, paired serum and mucosal samples from Cohorts A and Cohort B were analysed. Cohort A included participants who received 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as a second dose after a 30µg BNT162b2 prime. Cohort B included those who received 30µg BNT162b2 or NVXCoV2373 as a third (booster) dose. A control group, who received two doses of 4CMenB at days 0 and 84, followed

by a COVID-19 vaccine (Comirnaty Original/Omicron BA.1 vaccine) at day 182, served as the reference. Study designs are shown in **Figure 5.2** and **Figure 5.3**. An outline of the terminology used in this chapter is shown in **Figure 5.4**.

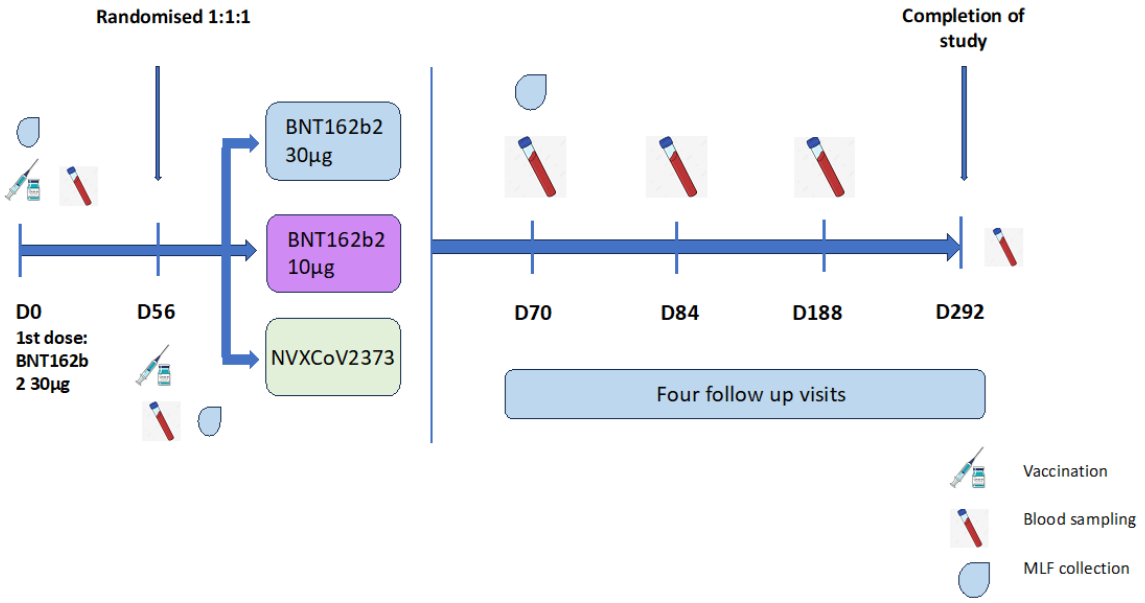


Figure 5.2 Com-COV3 Cohort A study design schematic.
Serum samples were collected at all study timepoints (as indicated in the figure). Mucosal samples were collected at day 0, day 56 and day 70. Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56. MLF = mucosal lining fluid.

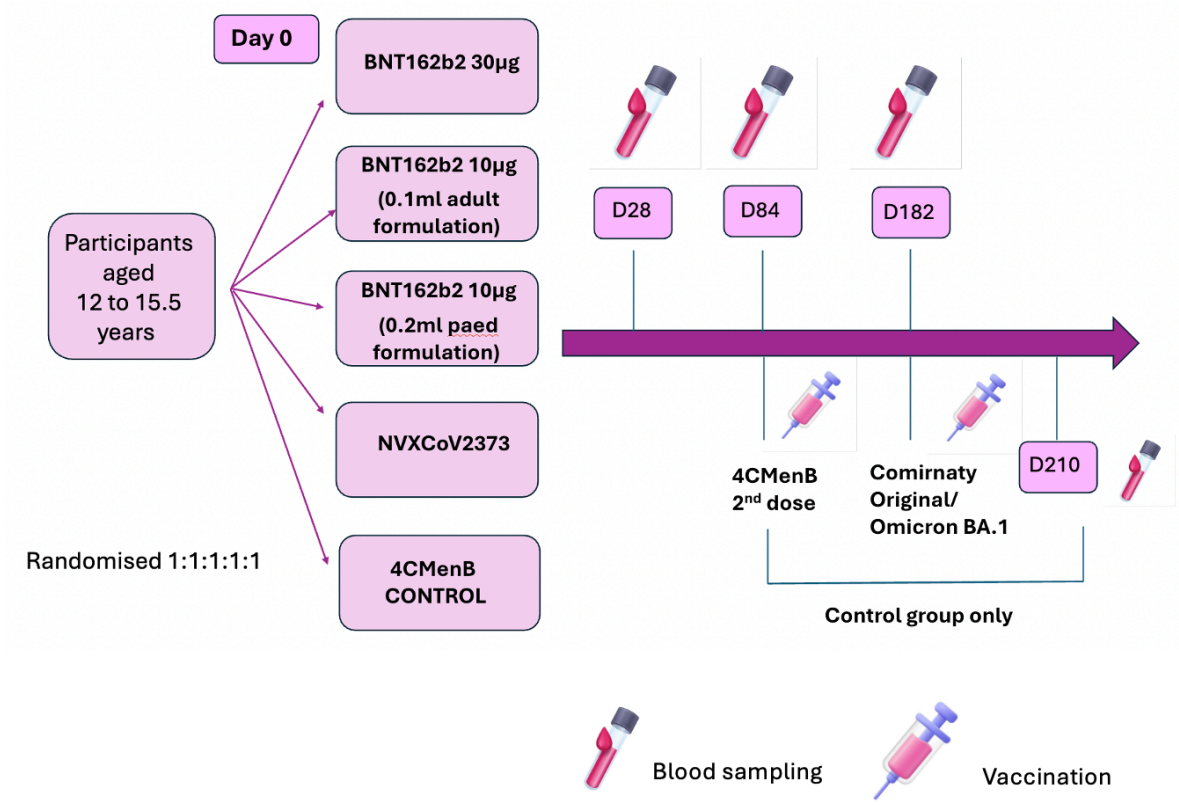


Figure 5.3 Com-COV3 Cohort B study design schematic.
Mucosal samples (not indicated here) were collected at all study timepoints. Serum samples were also collected at all study timepoints. Participants received their third dose at day 0, at least 90 days after the two-dose primary vaccine series. Control group participants received two doses of the meningococcal B vaccine (4CMenB), the first dose at day 0 and the second dose at day 84. Control group participants received the bivalent Comirnaty Original/Omicron BA.1 vaccine at day 182.

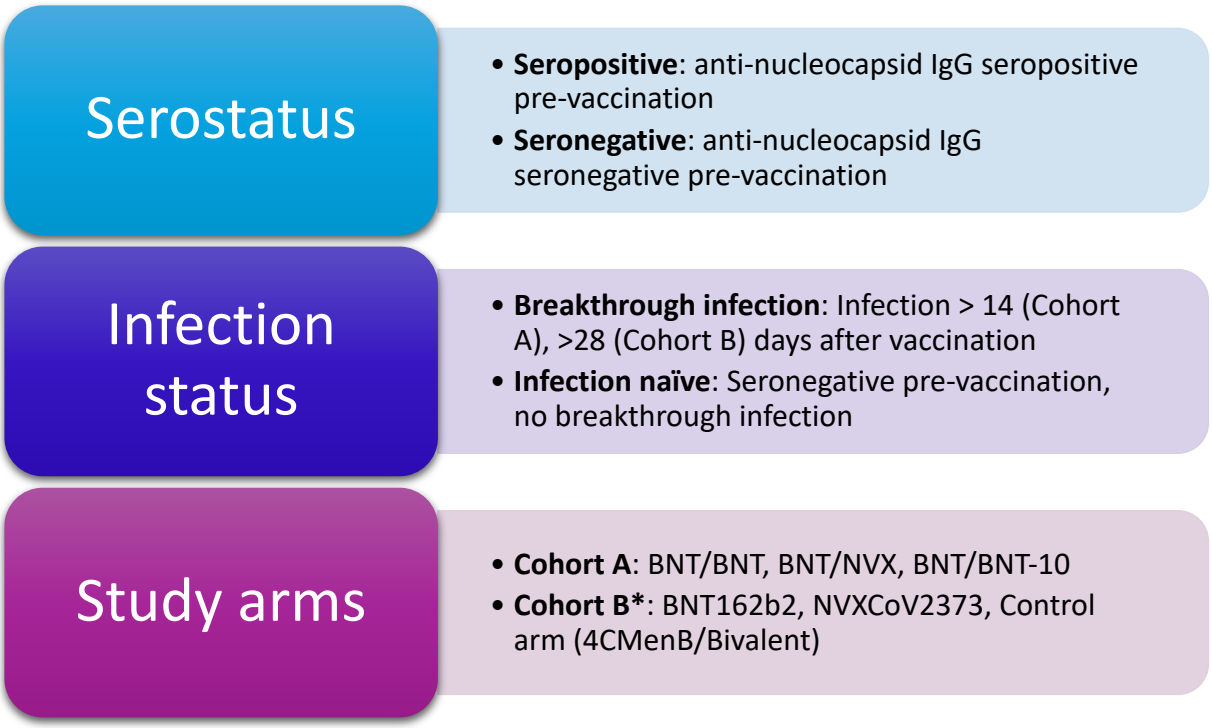


Figure 5.4 A summary of terminology used in this chapter.
*All Cohort A participants were primed with a two-dose 30µg BNT162b2 primary vaccine series. *Cohort B study groups referred to as BNT/BNT/BNT; BNT/BNT/NVX, and BNT/BNT/Bivalent in figures for clarity. BNT = 30µg BN162b2; NVX = NVXCoV2373; BNT-10 = 10µg BNT162b2; 4CMenB = meningococcal group B vaccine; bivalent vaccine = Original/Omicron BA.1 vaccine. A 'breakthrough infection' was defined as either: a self-reported SARS-CoV-2 infection >14 days after a second dose or >28 days after a third dose, a two-fold rise in anti-nucleocapsid IgG, a two-fold rise in anti-spike IgG antibodies, or seroconversion of anti-nucleocapsid IgG serostatus. Participants with evidence of SARS-CoV-2 infection within 14 days after a second dose or 28 days after a third vaccine dose were excluded from this analysis.*

As illustrated in **Figure 5.5** and **Figure 5.6**, samples were chosen and analysed according to the following categories: Cohort, study group, and SARS-CoV-2 infection status. This study was exploratory in nature and sample selection was based on mucosal sample availability and practical limitations. Balanced sample representation across study groups was prioritized within the constraints of sample availability.

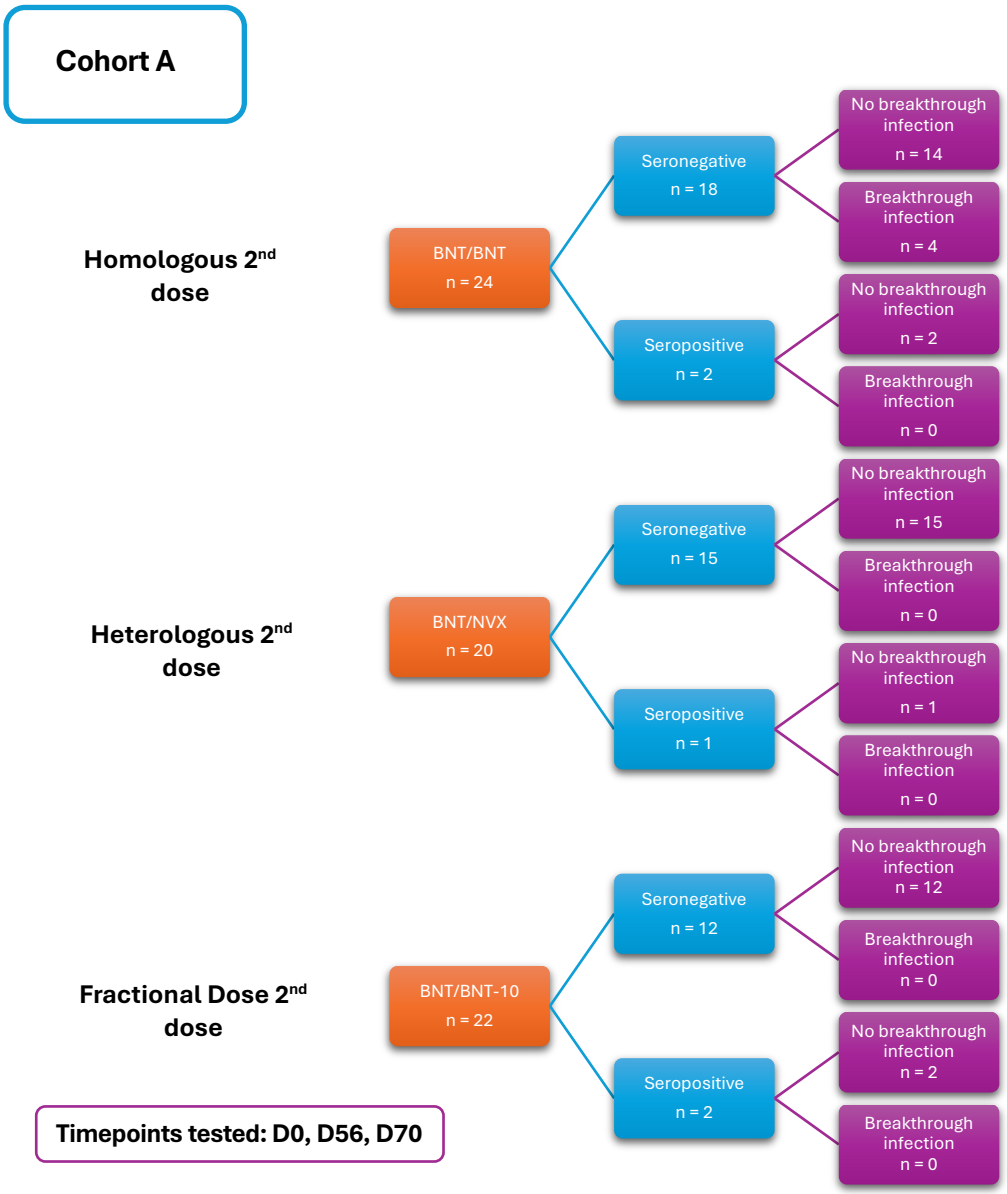


Figure 5.5 Cohort A sample analysis by study arm, serostatus and breakthrough infection status; n = number of participant samples analysed.
Seronegative refers to pre-first dose anti-nucleocapsid IgG seronegative; seropositive refers to pre-first dose anti-nucleocapsid IgG seropositive. Samples were tested at day 0, day 56, and day 70. Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56. BNT = 30µg BNT162b2; BNT-10 = 10µg BNT162b2; NVX = NVXCoV2373.

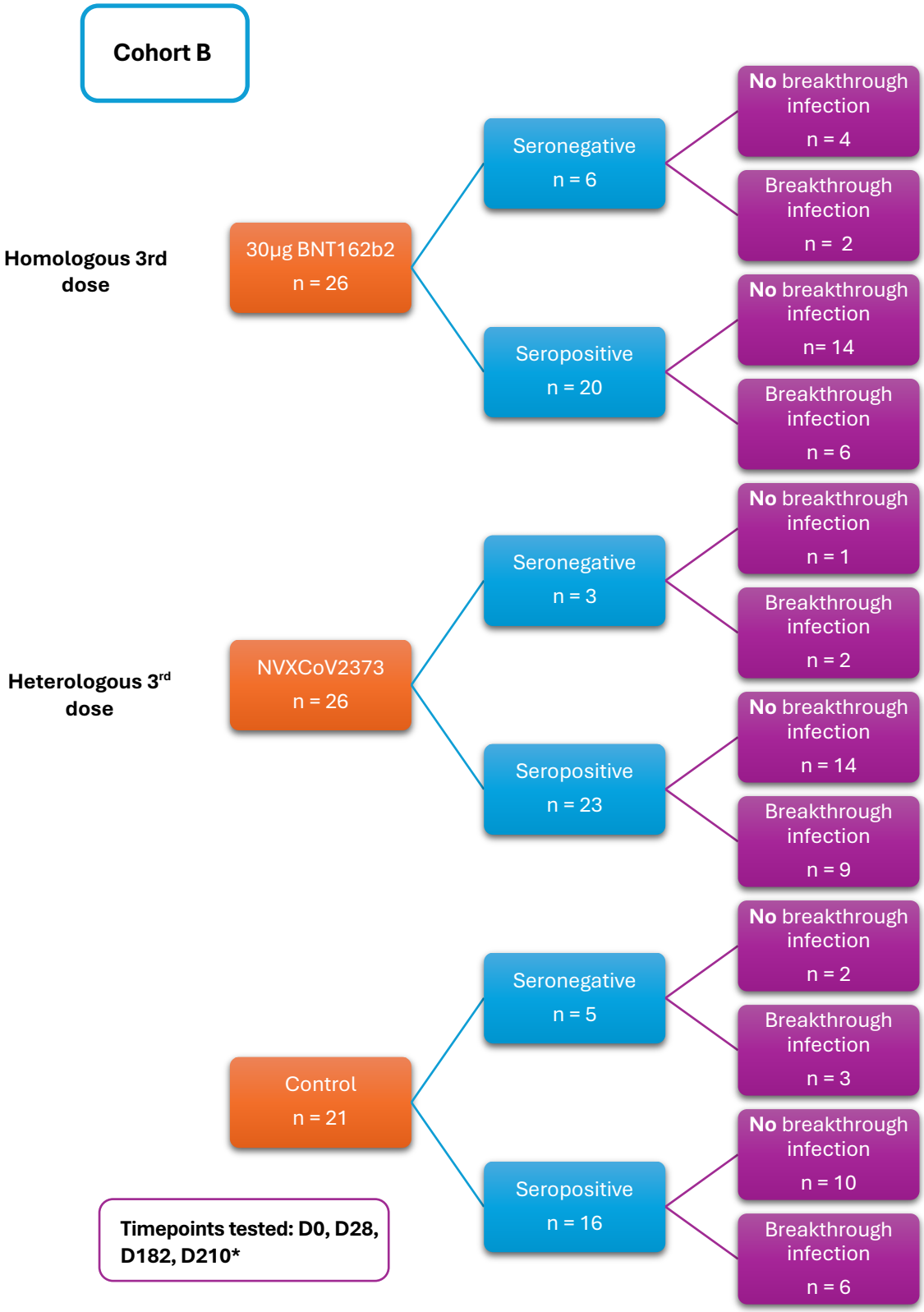


Figure 5.6 Cohort B sample analysis by study arm, serostatus and breakthrough infection status; n = number of participant samples analysed.
Seronegative/seropositive refers to pre-third dose anti-nucleocapsid IgG serostatus. Participants received their third (booster) dose at day 0. Samples were tested at day 0, day 28, and day 182.
**Only control group samples were tested at day 210 (28 days following receipt of the bivalent vaccine at day 182 in this group).*

5.2.2 Sample collection and storage

Serum and mucosal fluid samples were collected at the study timepoints outlined in **Figure 5.2** and **Figure 5.3.** See further details on serum sample processing and mucosal fluid collection, extraction and processing in **Chapter 2: sections 2.4.1** and **2.4.4**

5.2.3 Immunoassays

Paired serum and mucosal samples were analysed for IgA and IgG antibody responses to the following antigens: SARS-CoV-2 spike (ancestral), SARS-CoV-1 spike, receptor binding domain (RBD), nucleocapsid, MERS-CoV, hCoV OC43 (hCoV-OC43), NL63 (hCoV-NL63), HKU1 (hCoV-HKU1), and 229E (hCoV-229E) using a multiplex assay: the V-PLEX SARS-CoV-2 Coronavirus Panel 3, Meso Scale Diagnostics (MSD), Maryland, USA. Full details of this assay can be found in **Chapter 2**, **section 2.4.3**.

5.2.4 Statistical analysis

Data were log-transformed prior to testing for statistical significance. A p-value < 0.05 was considered significant. Correlation coefficients were calculated using Pearson's rank correlation. With respect to the correlation matrices, a conservative approach to this analysis was adopted and Spearman's rank correlation coefficient was used. Please refer to **Chapter 2: section 2.7** for full details.

5.3 Results

5.3.1 Mucosal SARS-CoV-2 spike IgG responses are enhanced following homologous and heterologous two-dose primary COVID-19 vaccination in adolescents (Cohort A)

As shown in **Figure 5.7**, mucosal spike-specific IgG titres were significantly increased by day 70, 14 days after a homologous (BNT/BNT), heterologous (BNT/NVX) and fractional (BNT/BNT-10) second dose compared with pre-second dose (day 56) levels. Mucosal spike-specific IgG titres were also significantly higher at day 70 compared with baseline (day 0) levels following both BNT/BNT and BNT/BNT-10 vaccine regimens. Although spike-specific IgG titres were substantially higher at day 70 following BNT/NVX compared with baseline levels, this increase did not reach statistical significance. However, this finding may be attributed to the small sample size.

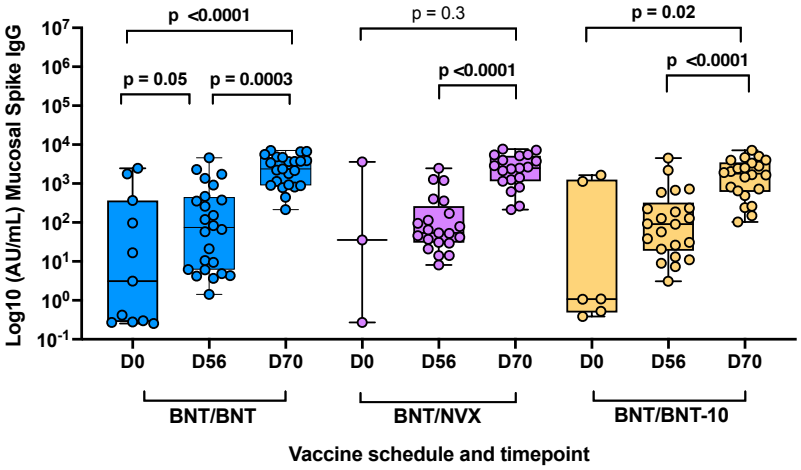


Figure 5.7 Mucosal SARS-CoV-2 spike-specific IgG titres following homologous and heterologous 2-dose primary vaccine series (Cohort A).
Results are presented as median with interquartile range (IQR). All participants received 30µg BNT162b2 as their first dose at day 0. Participants received their second dose at day 56. Day 70 refers to 14 day post-second dose vaccination. BNT = BNT162b2; NVX = NVXCoV2373; BNT-10 = 10µg BNT162b2. AU = Arbitrary Units.

5.3.2 Mucosal SARS-CoV-2 spike IgG responses are enhanced following mRNA booster vaccination but not after NVX-CoV2373 booster vaccination (Cohort B)

A third (booster) dose resulted in significantly higher mucosal spike-specific IgG titres at day 28 in the 30µg BNT162b2 study group compared with levels at both baseline (day 0) ($p = 0.03$) and those at day 28 in the control group ($p = 0.0017$), **Figure 5.8**. Mucosal spike-specific IgG titres were also significantly elevated at day 210, 28 days following receipt of the bivalent vaccine in the control arm, $p = 0.0026$, compared with pre-vaccination (day 182) levels. No statistically significant increase in mucosal spike-specific IgG titres was observed following a third dose of NVXCoV2373 vaccination.

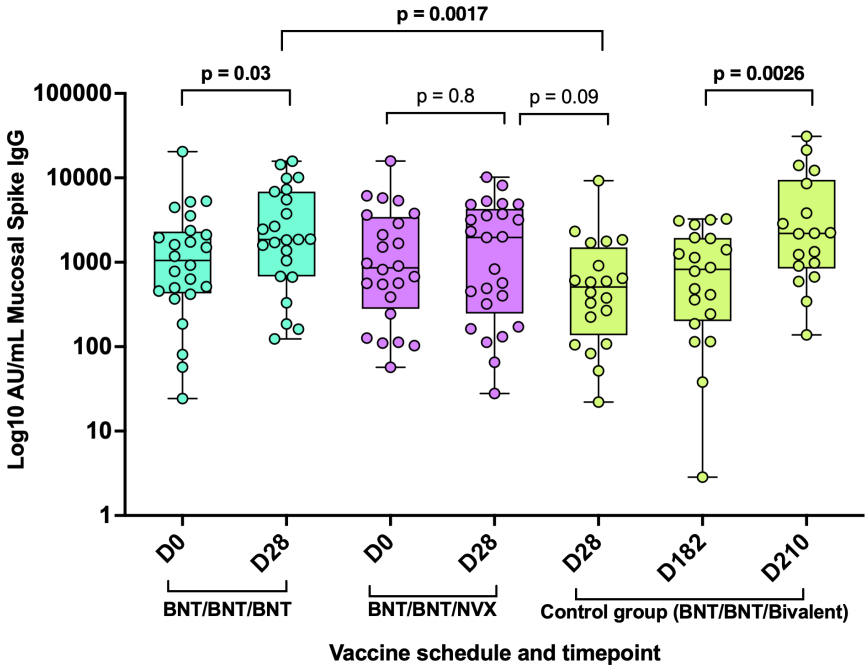


Figure 5.8 Mucosal SARS-CoV-2 spike-specific IgG titres following homologous and heterologous third dose vaccination (Cohort B) in all study participants.
Results are presented as median with interquartile range (IQR). All participants received two-dose 30µg BNT162b2 as their primary vaccine series. Participants received their third (booster) dose on day 0. Control group participants received their first dose of the 4CMenB vaccine at day 0 and the bivalent vaccine at day 182. Only participants in the control group were tested at day 210 (i.e., 28 days after receiving their third dose). BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.

The persistence of the mucosal spike-specific IgG response was assessed by comparing titres at day 182 with both baseline (day 0) and those at day 182 in the control arm. As shown in **Figure 5.9**, mucosal spike-specific IgG responses at day 182 were not significantly higher compared with baseline (day 0) levels in participants who received either a BNT162b2 or NVXCoV2373 booster dose ($p = 0.6$ and $p = 0.5$, respectively). Although participants in the control arm did not receive their COVID-19 vaccine until day 182, no significant decline in mucosal spike-specific IgG titres was observed over this period ($p = 0.7$). Furthermore, a significant increase in mucosal spike-specific IgG titres was observed in the control group at day 210 compared with baseline (day 0) levels, following administration of the bivalent vaccine at day 182.

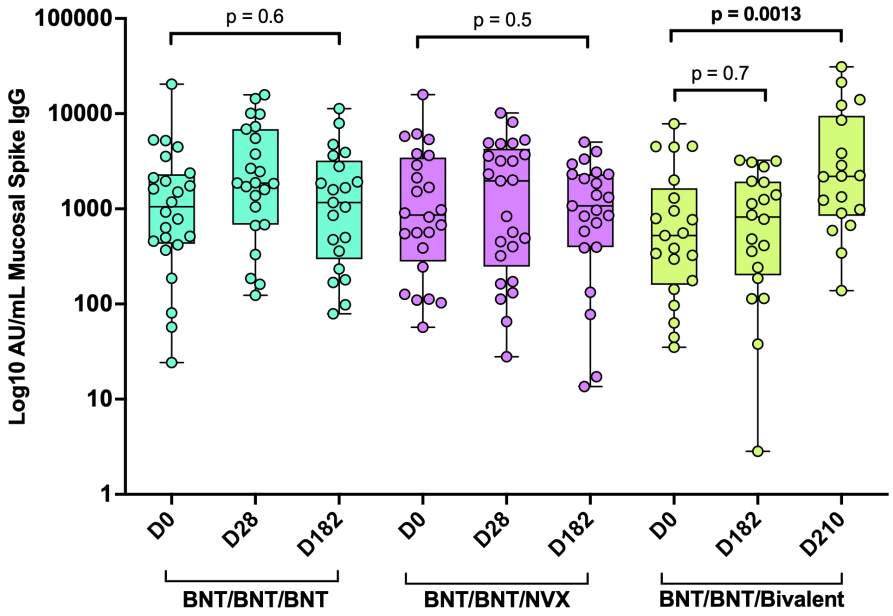


Figure 5.9 Persistence of mucosal SARS-CoV-2 spike-specific IgG response following homologous and heterologous third dose vaccination in all study participants.
Results are presented as median with interquartile range (IQR). All participants received two-dose 30µg BNT162b2 as their primary vaccine series. Participants received their third (booster) dose on day 0. Control group participants received their first dose of the 4CMenB vaccine at day 0 and the bivalent vaccine at day 182. Only participants in the control group were tested at day 210 (i.e., 28 days after receiving their third dose). BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.

When mucosal spike-specific IgG titres at day 182 were directly compared between the study groups, no significant differences in mucosal spike-specific IgG titres measured at day 182 were detected, **Figure 5.10 (A)**. Day 182 mucosal spike-specific IgG titres did not differ significantly between participants who received BNT162b2 and those who received NVXCoV2373 as a booster dose. To assess immune persistence in the absence of boosting through natural infection, participants with SARS-CoV-2 infection were excluded from the analysis **Figure 5.10 (B)**. However, even in the absence of SARS-CoV-2 infection, no significant differences in mucosal spike-specific IgG titres were detected across the study groups.

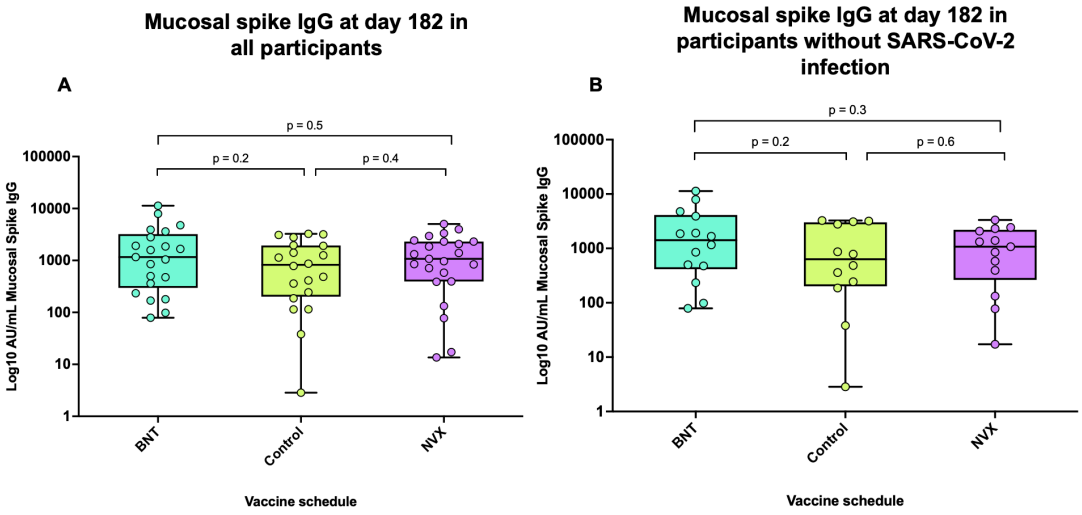


Figure 5.10 (A) Mucosal spike-specific IgG titres at day 182 following a third dose in all participants. (B) Mucosal spike-specific IgG titres at day 182 following a third dose in participants without SARS-CoV-2 infection (Cohort B).
Participants received their third (booster) dose on day 0. Control group participants received their first dose of the 4CMenB vaccine at day 0 and the bivalent vaccine at day 182. BNT = BNT162b2; NVX = NVXCoV2373. Results are presented as median with interquartile range (IQR). BNT = BNT162b2, NVX = NVXCoV2373, AU = Arbitrary Units.

5.3.3 Mucosal SARS-CoV-2 spike IgG responses are not enhanced by additional COVID-19 vaccine doses: Comparison of primary and booster vaccination

No significant difference in peak mucosal spike-specific IgG titres was detected between participants who received a third dose and those who received only two doses,

Figure 5.11. Similarly, mucosal spike-specific IgG titres measured 14 days after a second dose and titres measured at day 210 (28 days after receipt of the bivalent vaccine) were not significantly different (p = 0.7). Notably, no significant difference was found between mucosal spike-specific IgG titres at day 28 following a third (booster) dose and those at day 210 in the bivalent vaccine group,

despite the longer vaccine dose interval. These findings suggest additional doses following the primary vaccine series do not induce a significant increase in mucosal spike-specific IgG titres.

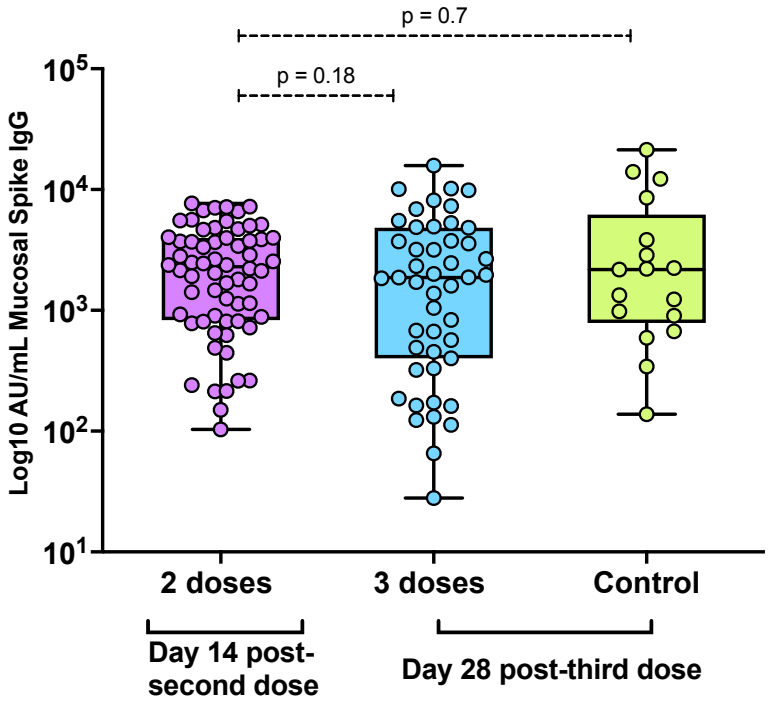


Figure 5.11 Peak mucosal SARS-CoV-2 IgG responses measured at day 14 after the second dose, day 28 after the third (booster) dose, and day 210 (corresponding to day 28 after administration of the bivalent vaccine).

In the case of the control group, mucosal SARS-CoV-2 IgG titres at day 210 (i.e., day 28 post-boost) are presented separately due to the longer interval between vaccine doses. Results are presented as median with interquartile range (IQR). Two doses = participants who received BNT162b2 or NVXCoV2373 as the second dose; three doses = participants who received BNT162b2 or NVXCoV2373 as the third dose. AU = Arbitrary Units.

5.3.4 Positive Correlation Between Serum and Mucosal Spike IgG Responses Following the First but Not the Second COVID-19 Vaccine Dose

Higher spike-specific IgG levels were observed in serum compared to mucosal fluid following the primary vaccine series [**Appendix: Supplementary Figure 6 (A)**]. Serum spike-specific IgG levels increased significantly across all study groups after both first and second vaccine doses (mirroring the mucosal spike-specific IgG response), as shown in [**Appendix: Supplementary Figure 6 (B)**].. A significant positive correlation between serum and mucosal spike-specific IgG titres was detected following the first vaccine dose (r = 0.5, p = 0.0002), but not after the second dose (r = -0.09, p = 0.5), **Figure 5.12 (A)** and **(B),** respectively. This finding may be due to the substantially higher spike-specific IgG titres observed in serum compared with mucosal fluid following vaccination.

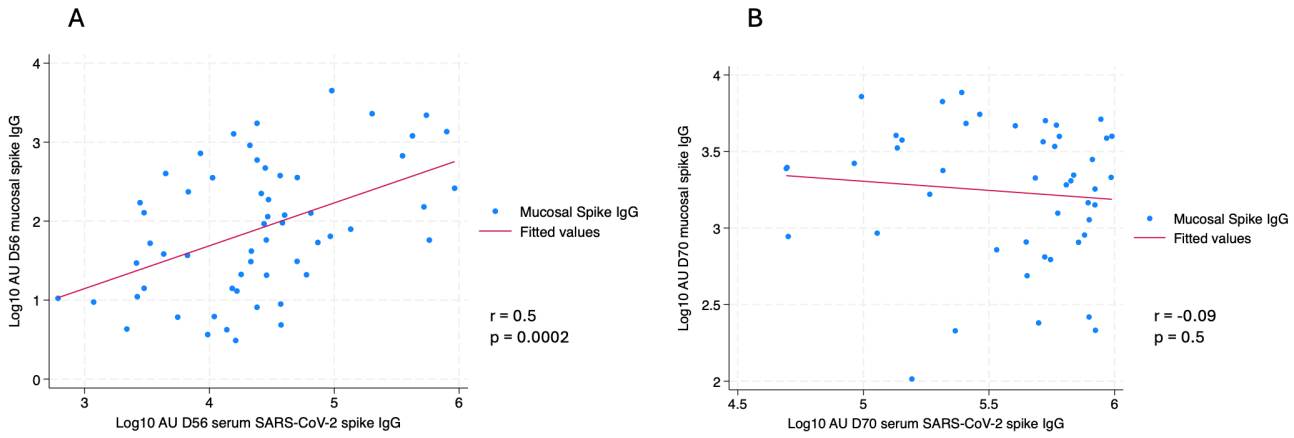


Figure 5.12 (A) Correlation between mucosal and serum spike-specific IgG titres at day 56 following the first dose (B) Correlation between mucosal and serum spike-specific IgG titres at day 70 after the first dose (14 days after the second dose).
Pearson's correlation coefficient and corresponding p-value are shown. AU = Arbitrary Units.

Like the systemic response observed following the primary vaccine series, serum spike-specific IgG levels were substantially higher than levels detected in mucosal fluid after a third dose (**Appendix, Supplementary Figure 7**). Serum spike-specific IgG titres were also significantly elevated 28 days post-booster across all vaccine groups (**Appendix, Supplementary Figure 7**). A significant positive correlation ($r = 0.5$, $p < 0.0001$) was also observed between mucosal and serum spike-specific IgG titres at this timepoint, supporting the hypothesis of transudation of spike-specific antibodies from the systemic to the mucosal compartment, **Figure 5.13**.

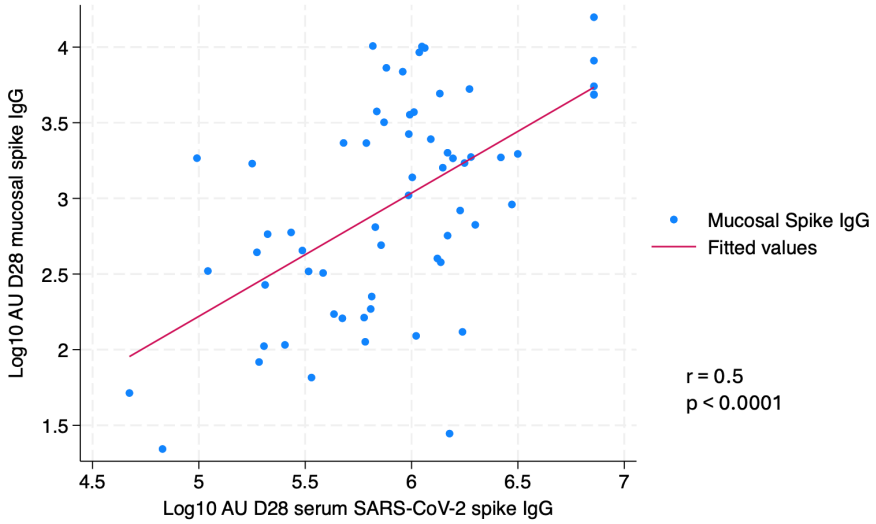


Figure 5.13 Correlation between mucosal and serum spike-specific IgG titres at day 28 following a third (booster) dose.
Pearson's correlation coefficient and corresponding p-value are shown. AU = Arbitrary Units.

5.3.5 Enhanced mucosal SARS-CoV-2 spike-specific IgG responses in adolescents with hybrid immunity following primary, but not booster, COVID-19 Vaccination

In participants with hybrid immunity, mucosal spike-specific IgG titres were significantly higher following both the prime and second vaccine doses compared with baseline and pre-second dose levels, respectively, **Figure 5.14**. Compared with SARS-CoV-2 infection naïve participants, mucosal spike-specific IgG titres were significantly higher at day 56 post-prime in seropositive participants. However, spike-specific IgG titres were not significantly different between seronegative participants and those with hybrid immunity by day 70 (day 14 post-second dose), (p = 0.6).

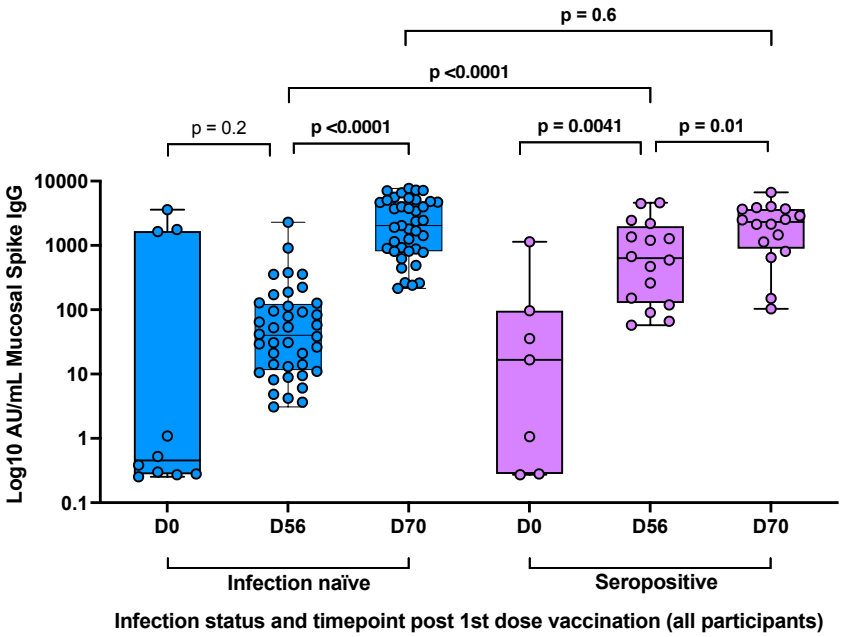


Figure 5.14 Mucosal SARS-CoV-2 spike IgG titres in SARS-CoV-2 infection naïve and seropositive participants following the two-dose primary vaccine series (Cohort A).

Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56. D70: day 70 (14 days post- second dose). Results are presented as median with interquartile range (IQR). AU = Arbitrary Units.

In contrast to the enhanced mucosal spike-specific IgG response observed in adolescents with hybrid immunity following the primary vaccine series, no significant difference in mucosal spike-specific IgG titres was observed between seronegative participants and those with hybrid immunity at either day 0 or day 28 following a third dose, **Figure 5.15**. These results suggest that the immunological advantages conferred by hybrid immunity may be diminished following completion of the primary vaccine series.

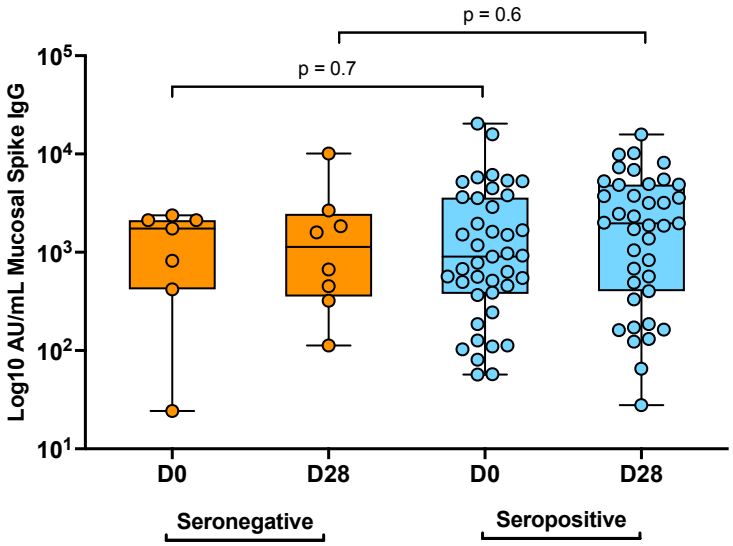


Figure 5.15 Mucosal SARS-CoV-2 spike IgG titres in seronegative and seropositive participants following third (booster) dose vaccination.
Participants received their third (booster) vaccine dose at day 0. Seronegative: anti-nucleocapsid IgG seronegative; seropositive: anti-nucleocapsid IgG seropositive. Results are presented as median with interquartile range (IQR). AU = Arbitrary Units.

5.3.6 Mucosal SARS-CoV-2 spike-specific IgA responses are significantly increased following homologous and heterologous two-dose primary vaccination, but not further enhanced by booster doses

Mucosal spike-specific IgA titres were significantly increased at day 70 (14 days after the second dose) following a homologous (BNT/BNT), heterologous (BNT/NVX) and fractional (BNT/BNT-10) second dose, **Figure 5.16.** However, mucosal spike-specific IgA titres at day 56 post-prime were not significantly elevated compared with baseline (day 0) levels across all vaccine groups.

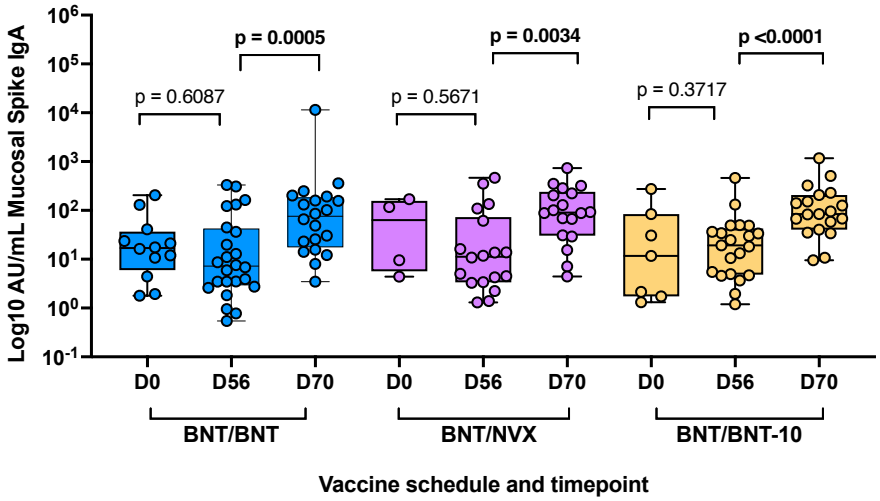


Figure 5.16 Mucosal SARS-CoV-2 spike IgA response following homologous and heterologous 2-dose primary series (Cohort A).
All participants received 30µg BNT162b2 as their first dose. Results are presented as median with interquartile range (IQR). BNT = 30µg BNT162b2, NVX = NVXCoV2373, BNT-10 = 10µg BNT162b2. AU = Arbitrary Units.

In contrast to the mucosal spike-specific IgA response observed following the primary vaccine series, no significant increase in mucosal spike IgA titres was detected after a booster dose, **Figure 5.17**. Mucosal spike-specific IgA levels at day 28 following either a third dose of BNT162b2 or NVXCoV2373 were not significantly elevated compared with baseline levels. Similarly, no significant increase in mucosal spike-specific IgA was observed at day 210 in the control group, 28 days following receipt of the bivalent vaccine. Additionally, mucosal spike-specific IgA titres at day 28 post-booster were comparable between NVXCoV2373 and BNT162b2 and the control group. These finding suggest that the mucosal spike IgA response is not further enhanced by additional (booster) COVID-19 vaccine doses.

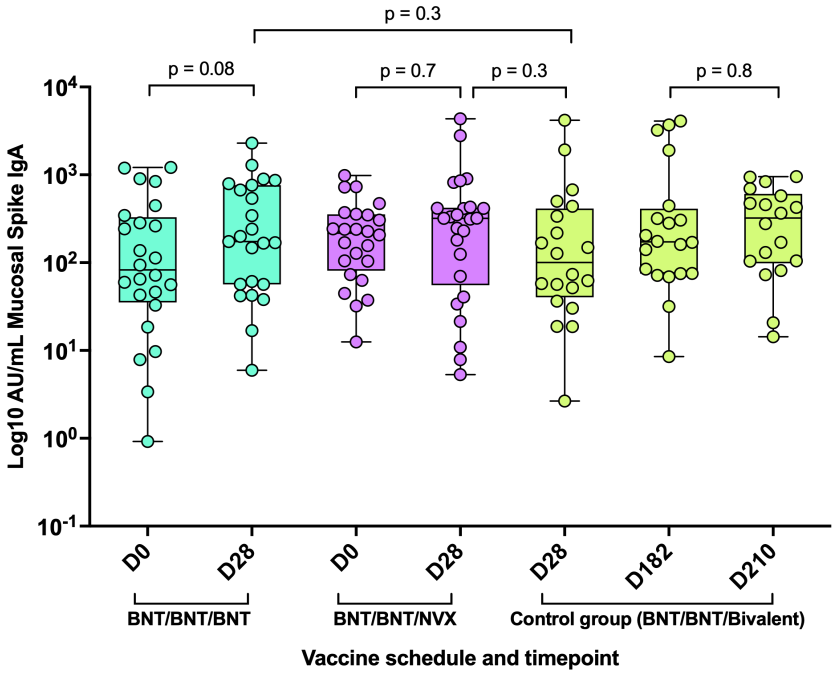


Figure 5.17 Mucosal SARS-CoV-2 spike IgA following homologous and heterologous third dose vaccination in all participants.
Participants received their third (booster) dose at day 0 (D0). Control group participants received their third dose (bivalent vaccine) at day 182 (D182). Results are presented as median with interquartile range (IQR). BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.

Although higher mucosal spike-specific IgA titres were detected at day 182 following a booster dose of either BNT162b2 or NVXCoV2373, these increases were not significantly different compared to

baseline levels, **Figure 5.18**. A similar pattern was observed in the control group, with no significant change in mucosal spike-specific IgA titres at day 182 (in the absence of vaccination) nor at day 210 (28 days after administration of the bivalent vaccine) compared with baseline levels.

5.3.7 Kinetics of SARS-CoV-2 spike IgA mucosal response after booster immunisation

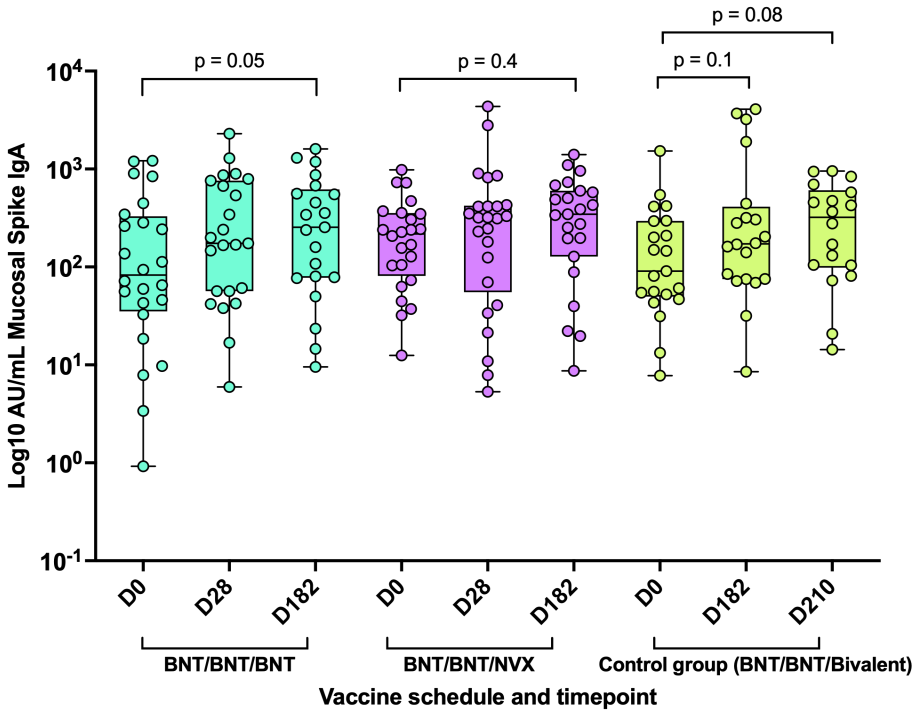


Figure 5.18 Persistence of mucosal SARS-CoV-2 IgA response following homologous and heterologous third dose vaccination in all participants.
Results are presented as median with interquartile range (IQR). BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.

No significant difference in mucosal spike-specific IgA titres was observed at day 182 between the COVID-19 vaccine groups and the control group. To assess mucosal immune persistence in the

absence of boosting through natural infection, participants with SARS-CoV-2 infection were excluded from the analysis. As shown in

Figure 5.19, mucosal spike-specific IgA titres remained comparable between the groups at day 182 even in the absence of SARS-CoV-2 infection following vaccination.

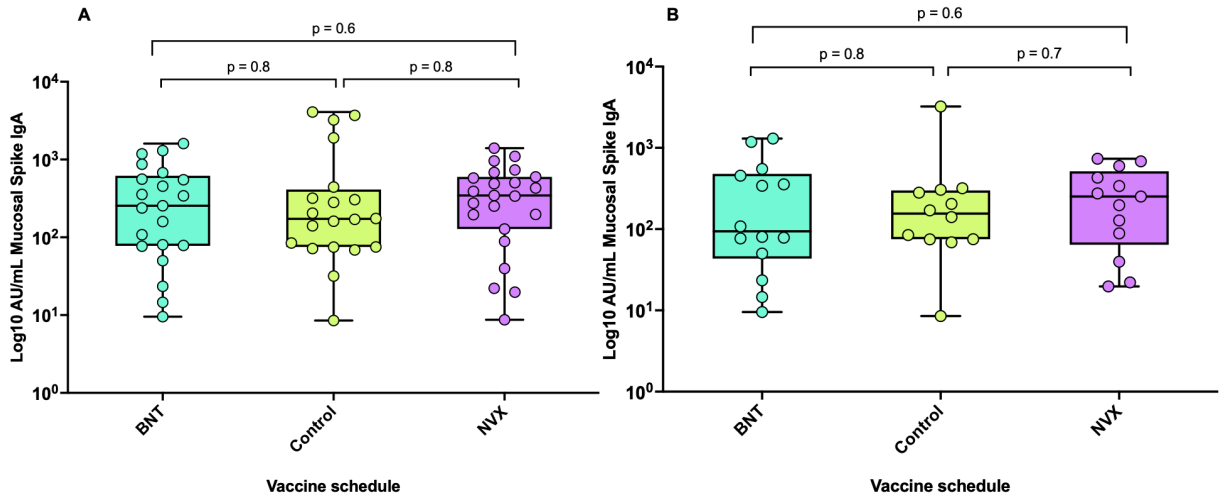


Figure 5.19 (A) Mucosal SARS-CoV-2 IgA response at day 182 following homologous and heterologous third dose vaccination and in the control group at day 182 in all participants. (B) Mucosal SARS-CoV-2 IgA titres at day 182 in participants without SARS-CoV-2 infection.
Participants received their third (booster) dose at day 0 (D0). Control group participants received their third (booster) dose at day 182 (D182). Results are presented as median with interquartile range (IQR). BNT = BNT162b2, NVX = NVXCoV2373. AU = Arbitrary Units.

5.3.8 Mucosal SARS-CoV-2 spike IgA levels are significantly increased following a booster dose

Mucosal spike-specific IgA titres were significantly higher at day 28 following a third dose compared with titres at 14 days after the second dose, (p = 0.0093),

Figure 5.20. Similarly, spike-specific IgA titres were significantly elevated 28 days following receipt of the bivalent vaccine as a booster dose in the control group compared with levels measured 14 days

after the second dose. Notably, despite the longer interval between vaccine doses, no significant difference in mucosal IgA titres was observed between titres measured at day 28 following a booster dose and day 210 (28 days after the bivalent booster).

It has been suggested that prior SARS-CoV-2 infection exerts a priming effect on the mucosal IgA response to vaccination.[228] In light of this and given that participants in Cohort A (the two-dose group) were seronegative at the time of vaccination, Cohort B (three-dose group) participants with SARS-CoV-2 infection were next excluded from the analysis. As shown in **Figure 5.21**, when participants with SARS-CoV-2 infection were excluded, no significant difference in mucosal IgA titres was found between the bivalent vaccine group and participants who had received only two doses. However, the lack of statistical significance may also be attributed to the limited sample size.

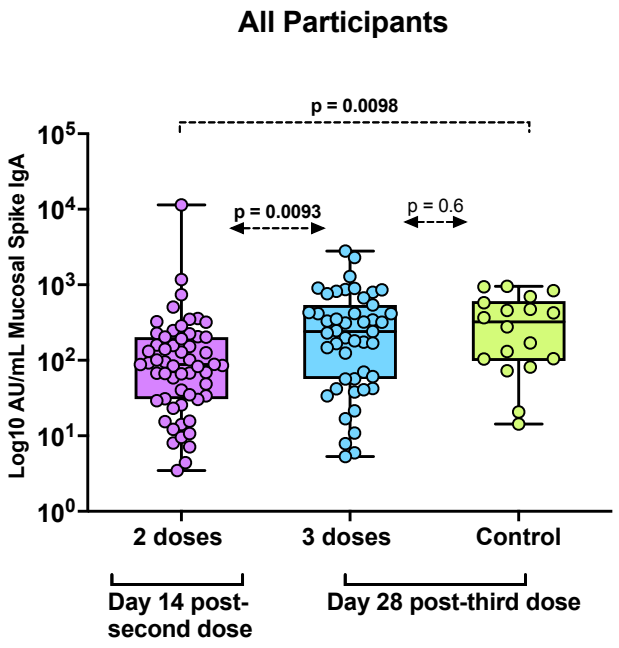


Figure 5.20 Peak mucosal SARS-CoV-2 IgA responses at day 14 after the second dose, day 28 after the third dose, and day 210 (corresponding to day 28 after administration of the bivalent vaccine).
In the case of the control group, mucosal SARS-CoV-2 IgA titres at day 210 (i.e., day 28 post-boost) are presented separately due to the longer interval between vaccine doses. Results are presented as median with interquartile range (IQR). Bivalent = bivalent vaccine (Original/Omicron BA.1). Two doses = participants who received either BNT162b2 or NVXCoV2373 as the second dose; three doses = participants who received either BNT162b2 or NVXCoV2373 as the third dose. AU = Arbitrary Units.

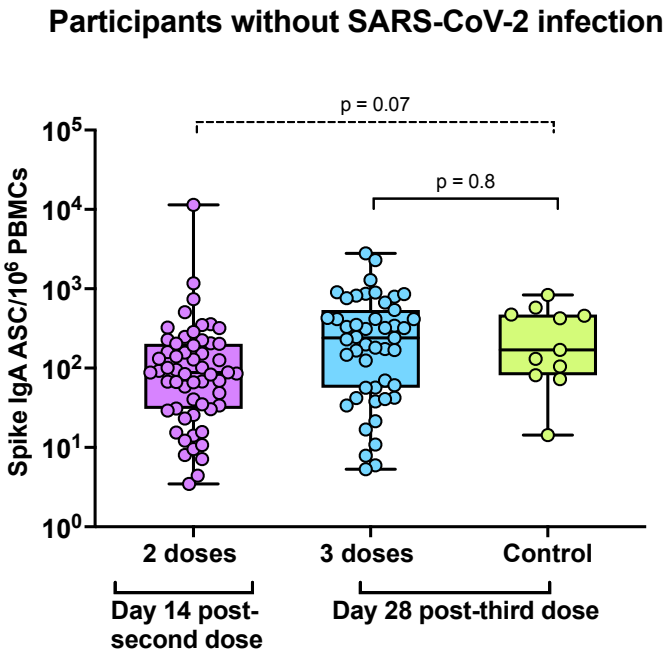


Figure 5.21 Peak mucosal SARS-CoV-2 IgA responses measured at day 14 after the second dose, day 28 after the third dose, and day 210 (corresponding to day 28 after administration of the bivalent vaccine) in participants without SARS-CoV-2 infection following vaccination.
In the case of the control group, mucosal SARS-CoV-2 IgA titres at day 210 (i.e., day 28 post-boost) are presented separately due to the longer interval between vaccine doses. Results are presented as median with interquartile range (IQR). Bivalent = bivalent vaccine (Original/Omicron BA.1).). Two doses = participants who received either BNT162b2 or NVXCoV2373 as the second dose; three doses = participants who received either BNT162b2 or NVXCoV2373 as the third dose. AU = Arbitrary Units.

5.3.9 Significant correlation between mucosal and serum SARS-CoV-2 spike-specific IgA responses following first, second, and third vaccine doses

Similar to the serum anti-spike IgG response, anti-spike IgA titres in serum were markedly higher compared with those observed in mucosal samples following both the primary vaccine series and the booster dose (**Appendix: Supplementary Figure 8**). Serum spike-specific IgA titres were significantly elevated across all study groups at day 70 (i.e., 14 days post-second dose) (**Appendix: Supplementary Figure 8**). Compared to baseline titres, serum spike-specific IgA titres were significantly increased at day 56 following 30µg BNT162b2 prime vaccination in the BNT/BNT and

BNT/NVX groups, though this increase was not observed in the BNT/BNT-10 study group. A significant positive correlation was observed between mucosal and serum spike-specific IgA titres following both the first ($r = 0.5$, $p < 0.00001$) and second vaccine doses ($r = 0.6$, $p < 0.00001$), suggesting translocation of spike-specific IgA between the systemic and mucosal compartments, **Figure 5.22**.

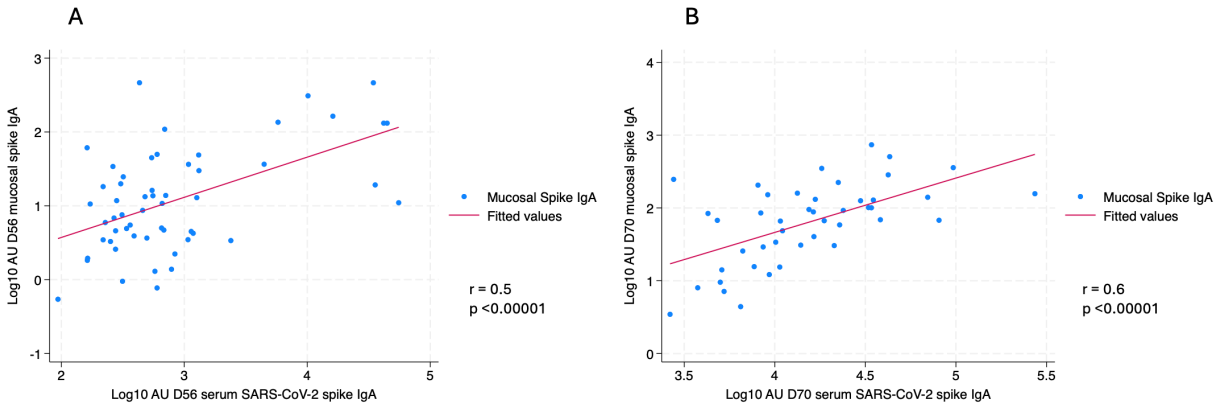


Figure 5.22 (A) Correlation between mucosal and serum SARS-CoV-2 spike IgA responses at day 56 after the first dose, (B) Correlation between mucosal and serum SARS-CoV-2 spike IgA responses at day 70 after the first dose (day 14 post-second dose vaccination).

Pearson's correlation coefficient and associated p-value are presented. AU = Arbitrary Units.

Similarly, serum spike-specific IgA titres were also significantly elevated at day 28 following a third dose of either BNT162b2 or NVXCoV2373 [**Appendix: Supplementary Figure 9 (B)**]. Furthermore, in the control group, serum spike-specific IgA levels were significantly increased at day 210, 28 days following receipt of the bivalent vaccine [**Appendix: Supplementary Figure 9 (B)**]. A significant positive correlation ($r = 0.4$, $p = 0.0013$) was again observed between mucosal and serum spike-specific IgA responses following booster vaccination, **Figure 5.23**.

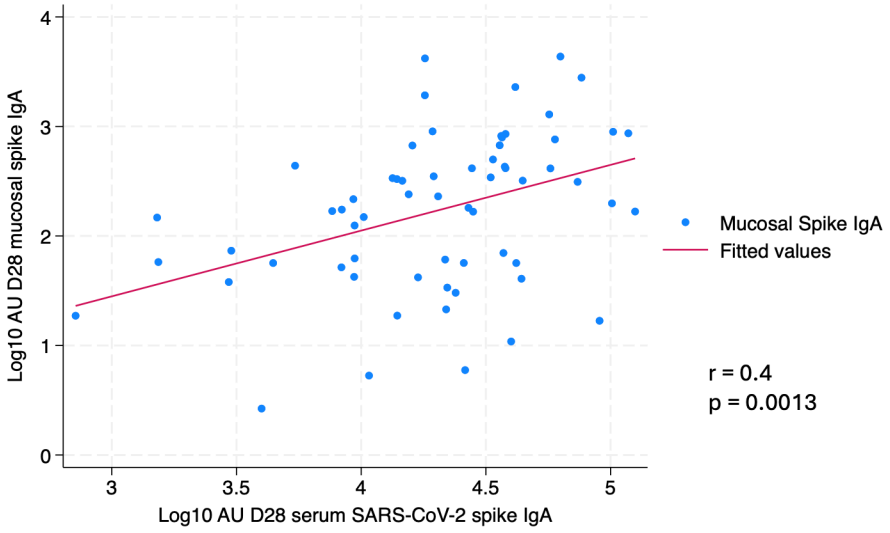


Figure 5.23 Correlation between mucosal and serum SARS-CoV-2 spike IgA responses at day 28 post-third dose vaccination.
Pearson's correlation coefficient and associated p-value presented. AU = Arbitrary Units.

5.3.10 Enhanced mucosal SARS-CoV-2 spike-specific IgA responses following primary series and third dose vaccination in adolescents with hybrid immunity

Mucosal spike-specific IgA titres were significantly elevated at day 70 (14 days post-second dose) in both seropositive and infection naïve individuals relative to pre-second dose levels. Mucosal spike-specific IgA levels at day 70 were also significantly higher in seropositive compared with infection naïve participants, $p = 0.04$. Although IgA titres were not markedly increased at day 56 in either group relative to baseline (day 0) levels, seropositive participants also demonstrated significantly higher titres than infection naïve participants at this timepoint, $p < 0.0001$,

Figure 5.24. These findings suggest that mucosal spike-specific IgA responses following a first and second dose are enhanced in individuals with hybrid immunity.

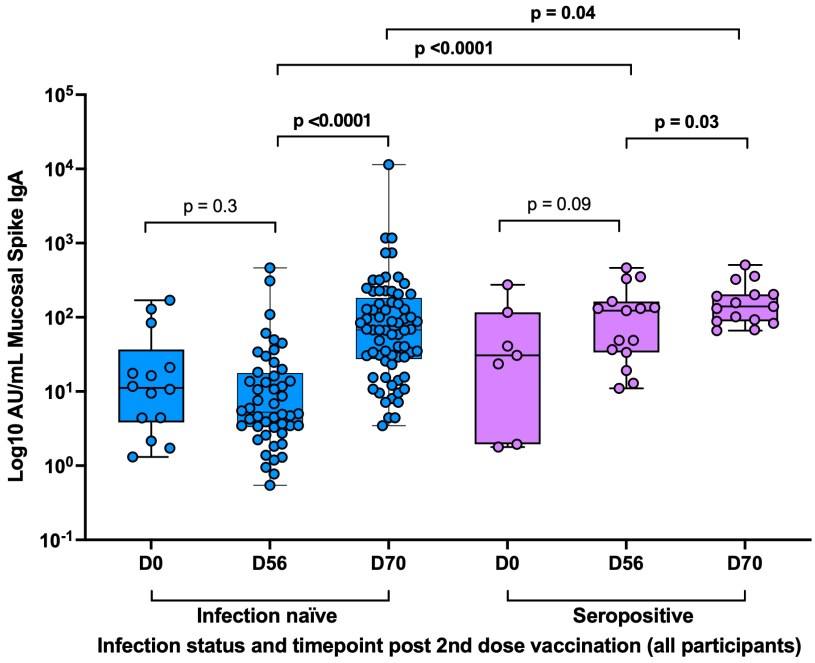


Figure 5.24 Mucosal SARS-CoV-2 IgA response following two-dose primary vaccine series in infection naïve and seropositive participants.
Results are presented as median with interquartile range (IQR). First dose administered at day 0 and second dose at day 56. Day 70 = 14 days post-second dose; infection naïve = anti-nucleocapsid IgG seronegative prior to vaccination; anti-nucleocapsid IgG seropositive prior to vaccination. AU = Arbitrary Units.

Similarly, mucosal spike-specific IgA titres were also substantially higher 28 days after a third dose in seropositive participants compared with baseline (pre-vaccination) levels. No significant change in mucosal IgA titres was observed in seronegative participants following a booster dose, **Figure 5.25**. Additionally, day 28 post-booster spike-specific IgA titres were significantly higher in seropositive participants compared with levels in seronegative participants. These findings mirror the mucosal IgA response following the primary vaccine series and are consistent with findings in adult studies which show that prior SARS-CoV-2 infection enhances mucosal IgA responses following COVID-19 vaccination.[215]

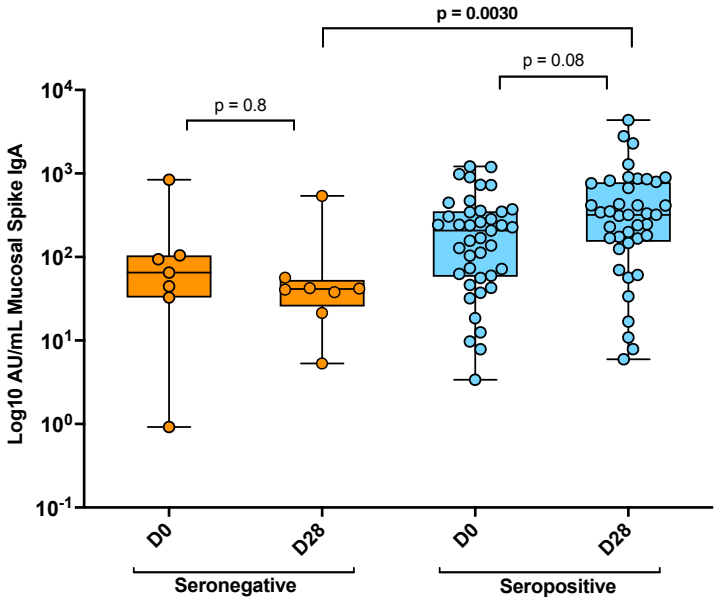


Figure 5.25 Mucosal SARS-CoV-2 IgA response following a third (booster) dose in seronegative and seropositive participants.
Results are presented as median with interquartile range (IQR). First dose administered at day 0 and second dose at day 56. Infection naïve = anti-nucleocapsid IgG seronegative prior to vaccination and no evidence of SARS-CoV-2 infection after vaccination; anti-nucleocapsid IgG seropositive prior to vaccination. AU = Arbitrary Units.

5.3.11 Pre-existing hCoV-specific serum IgG positively correlates with SARS-CoV-2 vaccine-induced spike IgG following primary series, but not after booster immunisation

To evaluate the relationship between pre-existing immunity to seasonal hCoV and the humoral immune response following SARS-CoV-2 vaccination, correlation analyses were performed on pre-vaccination serum IgG titres against hCoV OC43, HKU1, NL63 and 229E and serum SARS-CoV-2 spike-specific IgG titres measured 14 days after the second vaccine dose and 28 days after the third (booster) dose. As shown in **Figure 5.26 (A)** and detailed in **Table 5.1**, statistically significant positive correlations were observed between pre-second dose hCoV-specific IgG titres and day 14 (post-

second dose) serum spike-specific IgG titres across all hCoVs tested. In contrast, no significant correlation was detected between baseline (day 0) hCoV-specific IgG and serum anti-spike IgG at day 28 following a third (booster) dose, **Figure 5.26 (B)**. Results of significance testing for the correlations depicted in **Figure 5.26** are summarised in **Table 5.1**, which also presents the corresponding 95% confidence intervals. These findings suggest that pre-existing hCoV-specific IgG levels may influence the systemic immune response to the primary COVID-19 vaccine series, but this effect was not sustained following booster immunisation.

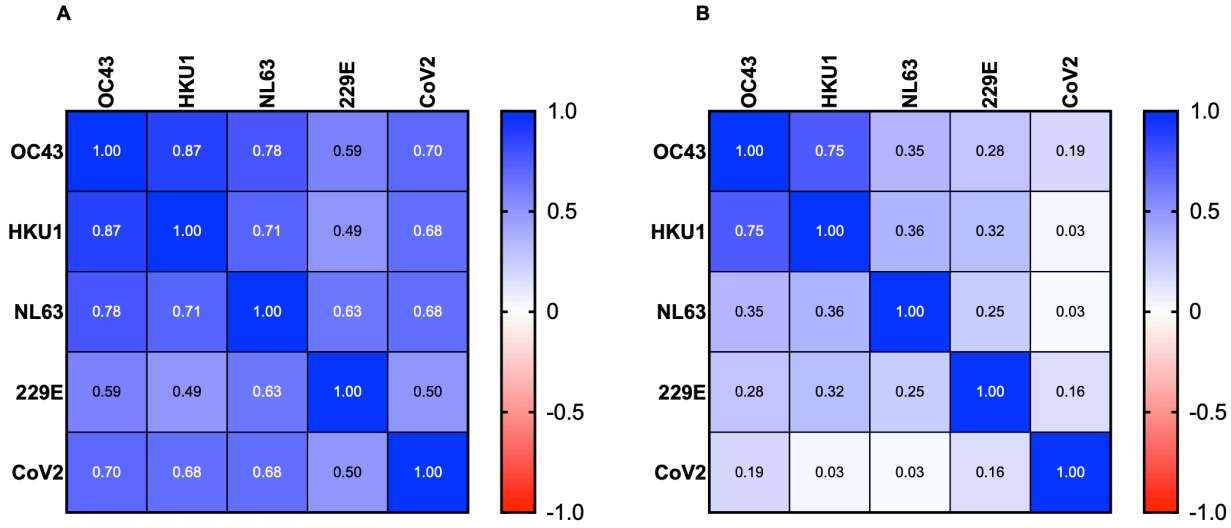


Figure 5.26 Correlation matrices demonstrating the relationship between serum pre-vaccination hCoV IgG titres and SARS-CoV-2 spike IgG titres at (A) day 14 post-second vaccine dose and (B) day 28 post-booster (third) dose.
Correlation coefficients calculated using Spearman's rank correlation are displayed.

Table 5.1 Results of statistical significance testing for Figure 5.26.
P values and 95% confidence intervals for correlation coefficients (Spearman) are presented in ***Figure 5.26.*** *Correlation coefficients were calculated between pre-vaccination hCoV IgG titres and anti-spike IgG titres at day 14 post-second dose (Cohort A) and day 28 post-third dose (Cohort B) vaccination. 95% confidence intervals are presented in parentheses in this table.*

Cohort A					
	OC43	**HKU1**	**NL63**	**229E**	**CoV2 spike**
OC43		**<0.0001** (0.79 - 0.92)	**<0.0001** (0.65 - 0.86)	**<0.0001** (0.38 - 0.74)	**<0.0001** (0.51- 0.82)
HKU1	**<0.0001** (0.79 - 0.92)		**<0.0001** (0.56-0.82)	**0.00007** (0.26-0.67)	**<0.0001** (0.48 - 0.81)
NL63	**<0.0001** (0.65- 0.86)	**<0.0001** (0.56 - 0.82)		**<0.0001** (0.44 -0.77)	**<0.0001** (0.48 - 0.81)
229E	**0.000001** (0.38 - 0.74)	**0.00007** (0.26-0.67)	**<0.0001** (0.44-0.77)		**0.0003** (0.25 to 0.70)
Cohort B					
	OC43	**HKU1**	**NL63**	**229E**	**CoV2 spike**
OC43		**<0.0001** (0.58-0.85)	**0.016** (0.06-0.58)	0.05 (-0.01 - 0.52)	0.21 (-0.12 - 0.46)
HKU1	**<0.0001** (0.58 - 0.85)		**0.015** (0.06-0.59)	0.027 (0.03-0.55)	0.84 (-0.27 - 0.33)
NL63	**0.016** (0.061-0.58)	**0.015** (0.06 -0.59)		0.09 (-0.05-0.50)	0.86 (-0.28 - 0.33)
229E	0.05 (0.00 - 0.52)	0.02 (0.02 -0.55)	0.09 (-0.05-0.50)		0.29 (-0.14 - 0.44)

5.3.12 Serum and mucosal hCoV IgG and IgA responses are significantly elevated following primary COVID-19 vaccination but not after a booster dose

Mucosal IgG titres specific for hCoV OC43, HKU1, NL63, and 229E were significantly elevated 14 days after a second vaccine dose, **Figure 5.27 (A)**. In contrast, no significant change in mucosal hCoV-specific IgA titres was observed across any of the hCoVs tested following completion of the primary vaccine series, **Figure 5.27 (B)**. Serum IgG titres against hCoVs OC43, HKU1, and 229E were significantly increased 14 days after the second dose, **Figure 5.27 (C)**.

Following third (booster) dose vaccination, no significant change in mucosal hCoV-specific IgG or IgA titres was detected across all hCoVs examined, **Figure 5.27 (D and E)**. However, serum IgG titres specific for hCoV OC43, HKU1, and 229E were significantly elevated at day 28 post-booster, **Figure 5.27 (F)**. These findings suggest that systemic COVID-19 vaccination induces back-boosting of serum hCoV-specific IgG responses, while mucosal cross-reactive IgG responses to hCoV were only evident after completion of the primary vaccine series.

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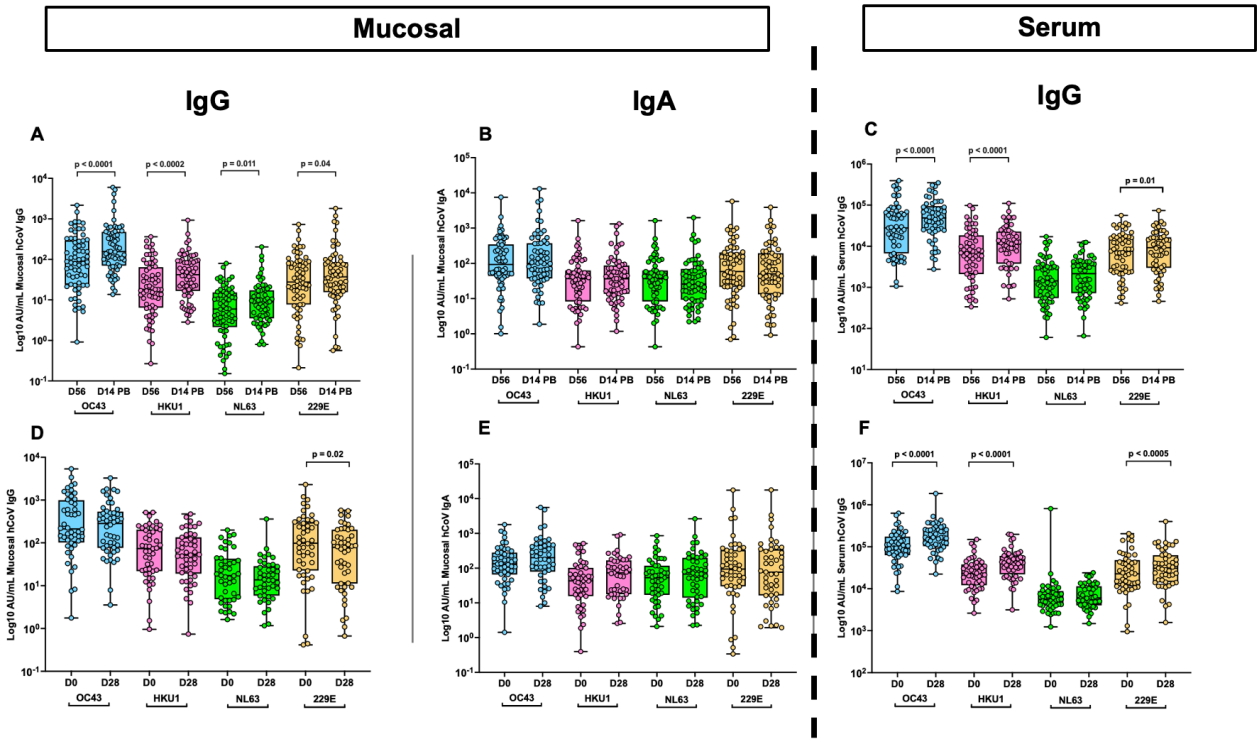

Figure 5.27 hCoV-specific mucosal and serum IgG and IgA responses following two-dose primary vaccine series (Cohort A) and a third (booster) dose (Cohort B).
(A) mucosal hCoV IgG, (B) mucosal hCoV IgA titres and (C) serum hCoV IgG titres pre-second dose (D56) and 14 days post-second dose (PB); (D) mucosal hCoV IgG, (E) mucosal hCoV IgA, and (F) serum hCoV IgG titres pre-third dose (day 0) and day 28 post-third dose. hCoV-specific mucosal and serum IgG and IgA responses for hCoV OC43, HKU1, NL63, and 229E are shown. Only significant ($p < 0.05$) results are shown. AU = arbitrary unit, PB = post-boost (second dose).

5.4 Discussion

Overall, the findings presented demonstrate that, compared to baseline (pre-vaccination) levels, mucosal spike-specific IgG and IgA responses were significantly increased following both a homologous and heterologous primary two-dose vaccine series. While significantly increased mucosal spike-specific IgG titres were observed following a third (booster) dose of an mRNA vaccine, no significant increase in mucosal spike-specific IgG titres was observed following NVXCoV2373 as a booster dose. Notably, both primary and booster immunisation schedules elicited significantly greater

mucosal spike-specific IgG and IgA responses in participants with hybrid immunity compared with infection naïve individuals. Serum and mucosal IgG and IgA antibody responses were found to be significantly correlated following both primary and booster vaccination, providing supportive evidence for antibody transudation between the systemic and mucosal compartments. Possible explanations for these findings are considered below.

5.4.1 Mucosal SARS-CoV-2 spike-specific IgG responses are significantly enhanced following homologous and heterologous primary two-dose and mRNA booster immunisation

Few studies have investigated the mucosal immune response to COVID-19 vaccination in the paediatric population. Although most available data relating to vaccine-induced SARS-CoV-2 mucosal immunity have been derived from studies involving adult participants, significant age-related differences in the mucosal immune response to SARS-CoV-2 infection have been described.[216] SARS-CoV-2 vaccination and infection have also been shown to induce significantly more robust immune responses in children and adolescents compared with adults.[229]

In this study, significantly higher mucosal spike-specific IgG titres were detected 14 days after both a homologous and a heterologous primary two-dose vaccine series. These findings are consistent with a study in healthcare workers which demonstrated a significant rise in salivary spike-specific IgG levels 14 days after completion of a two-dose BNT162b2 primary vaccine series.[98] Similarly, in children aged 5-11 years, spike-specific salivary antibody levels were significantly elevated 10 days after a second dose of BNT162b2.[230] This is also the first study to investigate the mucosal immune response

in adolescents to second dose NVXCoV2373 following BNT162b2 prime and which demonstrated substantially higher mucosal spike-specific IgG titres following vaccination. To date, no other study in children or adults has investigated mucosal immunity following NVXCoV2373 as a second dose.

Mucosal spike-specific IgG titres were also significantly higher 28 days after a booster dose of both 30μg BNT162b2 and the bivalent Original/Omicron BA.1 vaccine compared to baseline (pre-vaccination) levels. Although mucosal spike-specific IgG titres rose 28 days after NVXCoV2373, this result was not statistically significant compared with baseline levels. Previous studies in adults have also demonstrated increased mucosal anti-spike IgG titres following booster mRNA vaccination.[231] However, to our knowledge, no study has examined mucosal immune responses in children or adolescents following a third (booster) dose. Only one study was found which examined the mucosal immune response to booster vaccination with NVXCoV2373. Data from this study in nonhuman primates showed RBD-specific IgG antibody responses in mucosal fluid (derived from nasal, throat, and lung lavage sampling) following administration of a third dose of NVXCoV2373 were similar to responses observed following a third dose of the Moderna SARS-CoV-2 vaccine (mRNA-1273).[232] Unlike the mucosal spike-specific IgG response observed following a third dose of the bivalent vaccine in this study, Lasrado et al. found no significant increase in mucosal IgG titres against (wild-type) RBD 21 days following an XBB.15 mRNA booster, though mucosal IgG titres against XBB.15 were significantly increased post-boost.[233]

The effect of multiple booster doses on the mucosal immune response to SARS-CoV-2 is uncertain. A study by Bladh et al. in adults demonstrated a significant rise in nasal spike-specific IgG titres 7 days after a fourth (booster) mRNA vaccine dose.[234] Similarly, Azzi et al. detected an increase in salivary spike-specific IgG and IgA titres after a third dose in adults compared with levels detected following the primary series.[231] This is the first study in adolescents to directly compare peak mucosal spike-specific IgG responses between a two-dose primary regimen and a booster dose. However, no significant difference in mucosal IgG titres was observed between the primary and booster schedules in adolescents. It is worth noting that peak mucosal spike-specific IgG titres in this study were

measured 14 days after the second dose and 28 days after the third (booster) dose. Any differences in immunogenicity may therefore reflect differences in the timing of peak mucosal antibody secretion following vaccination.

Variability in timing of peak mucosal antibody responses has been demonstrated following SARS-CoV-2 infection and vaccination. Peak secretory IgA responses have been detected in sputum and throat swabs two to three weeks following SARS-CoV-2 infection.[235] Similarly, peak mucosal IgA responses have been shown to occur two to three weeks following breakthrough infection after two or three vaccine doses.[174] Peak salivary IgG responses have been detected two weeks after a second dose[98] while IM AZD-1222 (AstraZeneca) as a two-dose primary series induced a peak nasal IgG response between two and four weeks after vaccination.[236] Unlike the mucosal IgG response observed in the present study however, Azzi et al. found significantly higher spike mucosal IgG titres in adults two weeks after a third dose compared with the response observed two weeks after the second dose however, this analysis was based on samples collected at the same time point following both second and third vaccine doses unlike the mucosal samples collected in Cohorts A and B.[231]

5.4.2 Significant correlation between serum and mucosal SARS-CoV-2 spike-specific IgG following the first and third doses, and spike-specific IgA across all doses

In this study, serum and mucosal spike-specific IgG responses were significantly correlated following both prime and third dose vaccine regimens. It has been suggested that SARS-CoV-2 spike IgG detected in mucosal fluid is the result of transudation from the systemic circulation into the mucosa.[237]

The significant relationship between mucosal and serum spike IgG observed in this study provides further supportive evidence for this hypothesis. This finding has also been observed in adult studies following administration of a third dose .[92] However, in the present study, a negative correlation between mucosal and serum spike-specific IgG titres was detected 14 days after the second dose. Although this observation is not consistent with previous findings suggestive of "spillover" of spike-specific IgG from the systemic circulation into mucosal tissues, this inconsistency may be due to a disproportionate increase in serum anti-spike IgG relative to mucosal spike-specific IgG titres following vaccination. Indeed, spike-specific IgG titres detected following the second dose were substantially lower in mucosal fluid samples than in serum. This finding has also been observed in other studies with 100-fold lower spike-specific IgG titres detected in mucosal fluid relative to serum in one study.[98,212]

A significant positive correlation between mucosal and serum spike-specific IgA titres was observed at day 56 post-first dose, day 14 after the second dose and 28 days after the third dose. Unlike the results reported in this study, Puhach et al. found that mucosal and serum spike-specific IgA responses were poorly correlated in adults who had received up to 3 doses of a COVID-19 vaccine. The authors hypothesised that this finding was consistent with "compartmentalisation" of IgA responses in the mucosa and serum.[214] The findings of this study in adolescents, however, suggest a stronger association between mucosal and serum spike-specific IgA responses following vaccination.

5.4.3 Mucosal SARS-CoV-2 spike IgA responses are significantly increased following a two-dose primary vaccine series but not following a third dose

SARS-CoV-2 spike-specific mucosal IgA responses were significantly enhanced (compared to baseline) following both a BNT/BNT and BNT/NVX two-dose primary vaccine series but not following a single dose (prime vaccination) of 30μg BNT162b2. This finding is likely reflective of the SARS-CoV-2 infection status of Cohort A – a mostly infection naïve population. Previous studies in adults have shown that a single COVID-19 vaccine dose in seropositive individuals elicits an immune response comparable to that observed following two doses in infection naïve individuals. However, a single dose in infection naïve individuals has been shown to induce a significantly weaker immune response compared with the response observed following two doses in those with prior SARS-CoV-2 infection.[98,238]

Consistent with the findings reported in this study in adolescents, spike-specific salivary IgA levels were significantly enhanced day 57 following completion of a two-dose BNT162b2 primary regimen in children aged 5 to 11 years .[238] Conti et al. also demonstrated significantly increased spike-specific salivary IgA responses following two doses of BNT162b2 as the primary series in 5 to 11 year olds.[230] This is the first study to examine mucosal spike-specific IgA responses in adolescents following both a homologous and a heterologous second and third (booster) dose. Consistent with studies in younger children, mucosal spike-specific IgA titres in the present study were significantly higher 14 days after the second dose across all vaccine schedules. In contrast, findings from adult studies suggest that, in the absence of prior SARS-CoV-2 infection, minimal increases in spike-specific IgA have been observed following mRNA vaccination, suggesting an age-related difference in the mucosal spike-specific IgA response to vaccination.[239] Similarly, a weak mucosal IgA response was observed in

adults 14 days after a second dose of BNT162b2, while a significantly enhanced mucosal IgA response was observed in seropositive adults.[98]

Mucosal spike-specific IgA responses in this study were not significantly increased at day 28 following a third dose compared with baseline levels. These findings are consistent with those from adult studies. In one such study, salivary spike-specific IgA levels measured 14 days after a third (booster) dose of BNT162b2 were not significantly elevated compared with baseline (pre-vaccination) levels. [231] Similarly, a fourth booster dose was not associated with a significant increase in mucosal spike-specific IgA levels in adults. [234] However, other studies in adults have demonstrated significantly elevated salivary spike-specific IgA levels 14 days after a third dose relative to levels observed 14 days after the second dose. [231] Although Azzi et al. also found that a third dose elicited higher mucosal spike IgA titres compared with the primary series, this increase was not significant.[231] These findings are consistent with those observed in this study in adolescents where significantly higher peak spike-specific IgA titres were detected following a third dose compared with the two-dose primary vaccine series.

Peak mucosal spike-specific IgA levels were also significantly higher following receipt of the bivalent vaccine as a booster compared with those observed after the second dose. However, this observation may be attributed to the longer interval between doses in the control group and the occurrence of intercurrent SARS-CoV-2 infection, which may have primed local mucosal spike-specific IgA production prior to vaccination. Indeed, this difference in mucosal spike-specific IgA titres was no longer significant for bivalent vaccine recipients once participants with SARS-CoV-2 infection were excluded. This lack of statistical significance, however, may also be due to the limited sample size.

No published study was identified which examines the mucosal SARS-CoV-2 immune response following a third (booster) dose in children or adolescents. However, one preprint was found which investigated the mucosal IgA response in adolescents following a third dose of either the NVX-CoV2601vaccine (targeting Omicron XBB.1.5) or the bivalent NVX-CoV2373/NVXCoV2601

vaccine (targeting ancestral strain and Omicron XBB.1.5). Adolescents in this study had received two doses of an mRNA vaccine as their primary vaccine series.[240] In this study, mucosal spike-specific IgA titres against XBB.15 were significantly elevated following both vaccine schedules, in contrast to the findings of the current study where booster vaccination with NVXCoV2373 (targeting wild-type SARS-CoV-2) did not induce a similarly significant mucosal IgA response.

5.4.4 Enhanced mucosal spike-specific IgG and IgA responses following vaccination in individuals with hybrid immunity

Spike-specific mucosal IgG titres were significantly elevated at day 56 after a single dose compared with the response observed in infection naïve participants. Similar findings have also been observed in adult studies.[98] However, by day 14 after the second dose, mucosal spike-specific IgG titres were comparable between seropositive and infection naïve participants. Adult studies have also reported significantly increased mucosal spike-specific IgG titres following a single dose in participants with hybrid immunity relative to infection naïve participants while no significant difference in the mucosal response was observed following two doses.[214,238]

However, no significant difference in the peak mucosal spike-specific IgG response was observed between participants with prior SARS-CoV-2 infection and seronegative participants following a third dose. Studies in adults have demonstrated that a fourth (booster) dose elicits significantly increased nasal spike-specific IgG responses in both participants without prior infection and those with hybrid immunity.[234] However, the findings in the present study may reflect the significantly smaller infection naïve subpopulation used for this analysis.

Spike-specific mucosal IgA responses were also significantly higher 14 days after a second dose in both infection naïve and seropositive participants. Mucosal spike-specific IgA titres at both day 56 post-prime and 14 days after the second dose were also significantly higher in seropositive compared with infection naïve participants. Similar findings have been reported in adult studies following prime-boost vaccination with BNT162b2. Gorochov et al. demonstrated significantly higher spike-specific salivary IgA levels in adults with a prior history of SARS-CoV-2 infection compared with infection naïve participants.[238] Notably, seropositive participants in this study received only a single dose while infection naïve participants had received two vaccine doses.[238]

Spike-specific IgA titres were also significantly higher in seropositive participants compared with infection naïve participants at day 28 after the third dose. Unlike the findings reported here following a third (booster) dose, a study in adults reported no significant increase in mucosal spike-specific IgA titres following a fourth dose irrespective of previous infection status.[234] However, Havervall et al. in their study (in adults) found mucosal spike-specific IgA titres in seropositive individuals were significantly higher compared with levels in infection naïve participants at baseline [pre-booster (third) dose]. Similarly, at 5 weeks following an mRNA booster (third) dose in adults, mucosal spike-specific IgA titres were not significantly different between infection naïve and previously infected participants.[92] The findings of the present study in adolescents are consistent with previous studies in adults which suggest that prior local antigen exposure stimulates mucosal secretory IgA production and, in turn, enhances the mucosal IgA response following vaccination in individuals with prior SARS-CoV-2 infection.[239,241]

It has also been suggested that tissue-resident B cells generated in response to prior SARS-CoV-2 infection and subsequently triggered by circulating antigen derived from IM vaccination are responsible for the robust IgA spike-specific mucosal response observed following vaccination in individuals with a history of infection.[242,243] The results of this study are consistent with this hypothesis as well as findings from previous studies which suggest that in individuals with prior SARS-CoV-2 infection, vaccination leads to an enhanced mucosal spike-specific IgA response.[244]

5.4.5 Pre-existing serum hCoV IgG correlates with vaccine-induced anti-spike IgG at day 14 post-primary series but not after a booster dose

A statistically significant positive correlation was observed between pre-existing hCoV-specific IgG titres and serum vaccine-induced anti-spike IgG titres 14 days after the second dose. Higher levels of cross-reactivity between hCoV and SARS-CoV-2 have been observed in children and young adults, likely due to more recent coronavirus infection in this age group.[105] Additionally, it has been shown that humoral and cellular immune responses to SARS-CoV-2 target the spike 2 (S2) domain (responsible for membrane fusion) which is highly conserved between coronaviruses. Cross recognition of the S2 domain following SARS-CoV-2 infection has been shown to enhance responses to seasonal hCoV in children and adults and back-boosting of hCoV antibody responses following SARS-CoV-2 infection has been shown to occur in children.[106] A similar effect has been observed in convalescent adult patients with back-boosting of antibodies against the S2 domain of betacoronaviruses OC43 and HKU1 observed following SARS-CoV-2 infection.

Prior to the pandemic, the prevalence of seasonal hCoV was highest in children and early in the pandemic, the lowest rates of SARS-CoV-2 infection occurred in this age group.[245] Previous studies have shown that children exhibit strong and durable cross-reactive immune responses to SARS-CoV-2, which may contribute to the observed differences in the immune response between children and adults following SARS-CoV-2 infection.[245] Unlike the findings in the present study in adolescents, a study in adults found little evidence to support cross-reactivity between pre-existing hCoV immunity and SARS-CoV-2 immunogenicity following vaccination or infection. These findings provide further evidence for the hypothesis that this relationship may be age-dependent.[246] The findings reported here are consistent with previous studies in children and provide further evidence that pre-existing hCoV immunity in the paediatric population modulates SARS-CoV-2 vaccine-induced immune responses.

However, no correlation was found between pre-existing hCoV immunity and serum anti-spike IgG 28 days after a third (booster) dose. To our knowledge, this is the first study in children or adolescents to examine the relationship between pre-existing hCoV IgG and systemic anti-spike IgG responses following a booster dose. One study in adults demonstrated a high prevalence of antibodies to hCoV in a Chinese population and significantly increased hCoV IgG titres following a third dose (CoronaVac), consistent with the results reported in this study in adolescents. [247] However, no relationship was found in this study in adults between baseline hCoV IgG levels and vaccine-induced anti-spike IgG levels, findings which are also consistent with the results reported in the present study in adolescents. [247] The absence of a significant correlation between pre-existing hCoV IgG and post-third dose anti-spike IgG in this study, however, may be due to a disproportionate increase in anti-spike IgG titres after the third dose, further amplified by SARS-CoV-2 infection between doses boosting antibody titres.

5.4.6 Systemic seasonal coronavirus antibody responses are boosted by COVID-19 vaccination

Several studies have demonstrated cross-reactivity between SARS-CoV-2 and the seasonal coronaviruses and that hCoV antibody responses are boosted by SARS-CoV-2 infection.[106,198,248] This has been attributed to the high degree of homology shared between members of the human coronavirus family including SARS-CoV-2.[198] Additionally, increased antibodies to hCoV have been detected in individuals with a history of prior SARS-CoV-2 infection. [224] A positive correlation between hCoV IgG and anti-spike IgG responses has also been reported in adults.[224] Findings in the present study in adolescents concur with results from previous studies and demonstrate that hCoV antibody titres (both mucosal and serum IgG) are boosted following COVID-19 vaccination.

Although serum hCoV IgG levels were also significantly elevated 28 days after a booster dose, no significant increase in mucosal hCoV-specific IgA was observed following either a second or a booster dose. In children, increased serum hCoV IgG levels have been observed following SARS-CoV-2 infection or vaccination however, minimal cross-reactivity between mucosal hCoV and SARS-CoV-2 IgA responses has been demonstrated following SARS-CoV-2 infection. [247] It has been proposed that the mucosal SARS-CoV-2 spike IgA response is due to priming by infection and re-activation of the response following vaccination.[239] As suggested by Dowell et al., the lack of cross-reactivity in the mucosal IgA response between hCoV and SARS-CoV-2 spike suggests that the mucosal spike-specific IgA response arises de novo, following vaccination.[103]

5.4.7 Limitations and Conclusions

This study has several limitations. Analyses in this study were performed using mucosal fluid samples collected using SAM strips. However, use of other sample types (e.g., BAL or saliva), alternative sampling methods and different immunoassays in other studies may have impacted the results observed in these studies. These factors should be considered when comparing the findings of this study with the current literature.[249] Additionally, although comparisons have been made between the mucosal and serum antibody response observed in Cohort A and Cohort B, mucosal fluid sampling took place at different timepoints in both studies (day 14 after the second dose in Cohort A and day 28 after the third dose in Cohort B). Results from each Cohort may therefore have been influenced by variations in the timing of peak mucosal antibody secretion. Previous studies have suggested that peak mucosal antibody secretion most likely occurs approximately 2 weeks after antigen exposure.[250]

This study also used a small sample size, and findings should be interpreted with caution. Additionally, the antigen-specific mucosal IgG and IgA results reported in this study have not been

normalised to total mucosal IgG and IgA antibody levels. The findings reported may therefore have been impacted by sampling technique and variability in antibody concentration in mucosal fluid samples. It has been shown however, that detection of SARS-CoV-2 in nasal samples is more accurate compared with salivary samples. Salivary antibody secretion is also influenced by multiple external factors including circadian rhythm, saliva flow rate, as well as the oral microbiome composition.[251-253] A similar degree of variability in antibody concentrations in nasal secretions has not been reported. The findings of this study are also consistent with those from previous studies, supporting the accuracy of the results reported here.

This study showed that mucosal spike-specific IgG and IgA responses were significantly enhanced following both a homologous and a heterologous second dose. An mRNA vaccine as a third (booster) dose induced a significant increase in mucosal spike-specific IgG titres, while no further enhancement of the mucosal spike-specific IgA response was observed following a booster dose. However, peak mucosal spike-specific IgA responses were significantly higher following a third dose compared with levels observed after two doses. A significant correlation was also found between pre-existing hCoV immunity and SARS-CoV-2 antibody responses following vaccination. Despite the successful development and global deployment of highly efficacious COVID-19 vaccines, re-infection is common, and current vaccines have not been shown to prevent virus transmission. Future research should continue to focus on the development of mucosal vaccines capable of inducing sterilising immunity and generating durable protective immunity at mucosal sites.

Chapter 6: An Exploration of Biomarkers of Protection against SARS-CoV-2 Infection, and the Kinetics of Antibody Avidity and Neutralising Antibody Responses.

6.1 Introduction

Affinity is defined as the strength of binding between an antibody and a specific target epitope on the surface of an antigen and avidity as the cumulative strength of binding between a multivalent antibody (i.e., an antibody with multiple binding sites) and multiple epitopes on the antigen surface.[108,109] Affinity is the result of multiple rounds of somatic hypermutation, within the BCR variable region genes which form the antigen binding site of antibody molecules, and occurs during the germinal centre reaction, resulting in the production of antibodies with greater affinity (stronger, more effective binding) for an antigenic epitope. The avidity of this interaction can correlate with increased antibody effector function.[114,115] Antibody avidity has been proposed as a marker of vaccine efficacy and has been associated with protection against infection and disease.[117,118,254] With respect to SARS-CoV-2, antibodies with higher avidity have been shown to correlate with greater protection against infection and severe disease.[255] High avidity antibodies have also been associated with greater protection against infection caused by SARS-CoV-2 variants and demonstrate enhanced cross-protective immune responses.[108]

Multiple factors have been identified which influence antibody avidity. Natural infection with SARS-CoV-2 has been shown to lead to antibody avidity maturation over time in vaccine naïve individuals and to correlate with disease severity.[256] Vaccine platform has also been shown to influence the kinetics of antibody avidity with a significantly greater antibody avidity response observed following

a two-dose BNT162b2 vaccine regimen compared with a two-dose CoronaVac regimen (Sinovac COVID-19 vaccine, using whole inactivated virus) and no significant difference in avidity observed between mRNA-1273 (COVID-19 Moderna vaccine) and BNT162b2 following a three-dose regimen.[127,257] Most studies to date however, have focused on the antibody avidity response to mRNA-based vaccination with only one study in rhesus macaques identified which examined antibody avidity maturation following a three dose NVXCoV2373 regimen and which demonstrated increased antibody avidity responses following vaccination.[210] Authors assert that the presence of the saponin-based adjuvant, Matrix-M, will augment antibody avidity and affinity maturation when administered as part of booster vaccine campaigns.[258] Avidity has also been shown to vary according to age and gender though few studies have focussed on antibody avidity responses following COVID-19 vaccination in the paediatric population.[259] Additionally, increasing affinity maturation has been shown to occur with increasing vaccine doses.[125] A two-dose BNT162b2 vaccine regimen in adults has been shown to induce high avidity antibodies,[125] while a third dose of an mRNA vaccine elicited antibodies with greater avidity and neutralising capacity against both wild-type and Omicron variant SARS-CoV-2 strains.[108] It has also been observed that antibody avidity responses continued to increase up to 6 months after a two-dose mRNA-based primary regimen while avidity levels remained stable between 3 and 6 months following third dose vaccination.[127]

Although scant data exist regarding antibody avidity maturation following COVID-19 vaccination in children and adolescents, data from non-COVID-19 vaccine related studies reveal increased antibody avidity following vaccination with the *Haemophilus influenzae* type b (Hib) conjugate vaccine, despite waning antibody titres in the period after vaccination.[260] Similarly, in children vaccinated with the 7-valent pneumococcal capsular polysaccharide, CRM197 conjugate vaccine (PCV7) at 2, 4, 6, and 12 months of age, a durable antibody response associated with high avidity was found 4 years after vaccination.[261] Following vaccination with Adjuvant System 03 (AS03)-adjuvanted monovalent H1N1 Influenza vaccine (Arepanrix; GlaxoSmithKline, Laval, Quebec, Canada; two doses, 21-day dosing interval) in children aged 6 to 35 months, avidity was observed to rise significantly after each

dose, though a weak response was found in a small subset of children previously unexposed to wild-type virus.[262]

Hybrid immunity has also been shown to enhance antibody avidity, highlighting the importance of antigenic exposure through infection and vaccination in driving antibody avidity maturation.[263] Similar findings were also observed following SARS-CoV-2 breakthrough infection with studies in adults showing significantly enhanced antibody avidity following infection compared to vaccination alone and a corresponding increase in neutralising antibody activity against wild-type and SARS-CoV-2 variants.[264]

In addition to antibody avidity, other immunological markers of protection against SARS-CoV-2 infection have been identified. Neutralising antibody titres represent the most well researched and promising correlate of protection identified to date and are strongly predictive of protection against SARS-CoV-2 infection.[265] Although quantitative thresholds of protection against infection with respect to neutralising antibodies have been identified, these have been significantly diminished by the Omicron variant due to its significant immune evasive properties.[266] The presence of hybrid immunity has also been shown to influence neutralising antibody activity against SARS-CoV-2 variants with greater potency and a broader antibody repertoire observed in individuals with hybrid immunity compared to individuals vaccinated without prior infection.[266-268] Other potential biomarkers include total binding antibodies, which have been shown to correlate with protection against disease, and to mediate protection via FcR-mediated antibody effector functions (e.g., antibody dependent cellular cytotoxicity, phagocytosis).[269,270] Mucosal SARS-CoV-2 specific IgA antibodies have also been identified as a potential marker of protection against infection and disease, attributed to the potent neutralising capacity of dimeric secretory IgA and its enhanced binding avidity.[89,92,271,272] Memory B cells induced following vaccination have also been associated with protection against SARS-CoV-2 infection in both children and adults.[166,273]

Heterologous vaccine schedules in adults have been shown to be highly immunogenic and to demonstrate greater vaccine efficacy compared with homologous vaccine schedules.[36,274] Correspondingly, significantly greater antibody avidity has been observed in adults following heterologous vaccine regimens compared with homologous schedules.[275] However, no study to date has investigated antibody avidity responses following homologous and heterologous two-dose primary vaccine series and third (booster) dose vaccination in adolescents nor explored COVID-19 biomarkers of vaccine-induced protection in an adolescent population.

This chapter describes results relating to the following objectives:

1. To assess the antibody avidity response to homologous and heterologous primary and booster COVID-19 vaccine schedules in adolescents.
2. To determine the effect of prior SARS-CoV-2 infection and breakthrough infection on antibody avidity maturation following primary and booster COVID-19 vaccination in adolescents.
3. To examine the correlation between antibody avidity and neutralising antibody responses to ancestral SARS-CoV-2 and SARS-CoV-2 variants following primary and booster COVID-19 vaccination, and whether this association varies by vaccine schedule.
4. To determine whether levels of BMEM, mucosal antibodies, neutralising antibodies or total binding antibodies to SARS-CoV-2 induced following primary and booster COVID-19 immunisation in adolescents represent immune biomarkers of vaccine-induced protection against SARS-CoV-2.

6.2 Methods

6.2.1 Sample selection

Cohort A samples at day 56 (pre-second dose), day 84 (28 days post-second dose), and day 236 (day 180 post-second dose) were selected for analysis of antibody avidity towards ancestral SARS-CoV-2. Cohort B samples taken at day 0, day 28, and day 182 following a third (booster) dose were selected. These timepoints were chosen to represent baseline (pre-vaccination), peak vaccine-induced antibody avidity (day 28 post second/third dose) and avidity maturation over time (day 180 post-second dose/day 182 post-third dose) following vaccination. Cohort A included participants who received 30µg BNT162b2 or NVX-CoV2373 as a second dose after a 30µg BNT162b2 prime. Cohort B included those who received 30µg BNT162b2 or NVX-CoV2373 as a third dose. The control group, which received two doses of 4CMenB at days 0 and 84, followed by a COVID-19 vaccine at day 182, served as the reference. One additional timepoint was measured in the Control group: day 210, 28 days post-third dose. The study designs for Cohort A and Cohort B are shown in **Figure 6.1** and **Figure 6.2**, respectively.

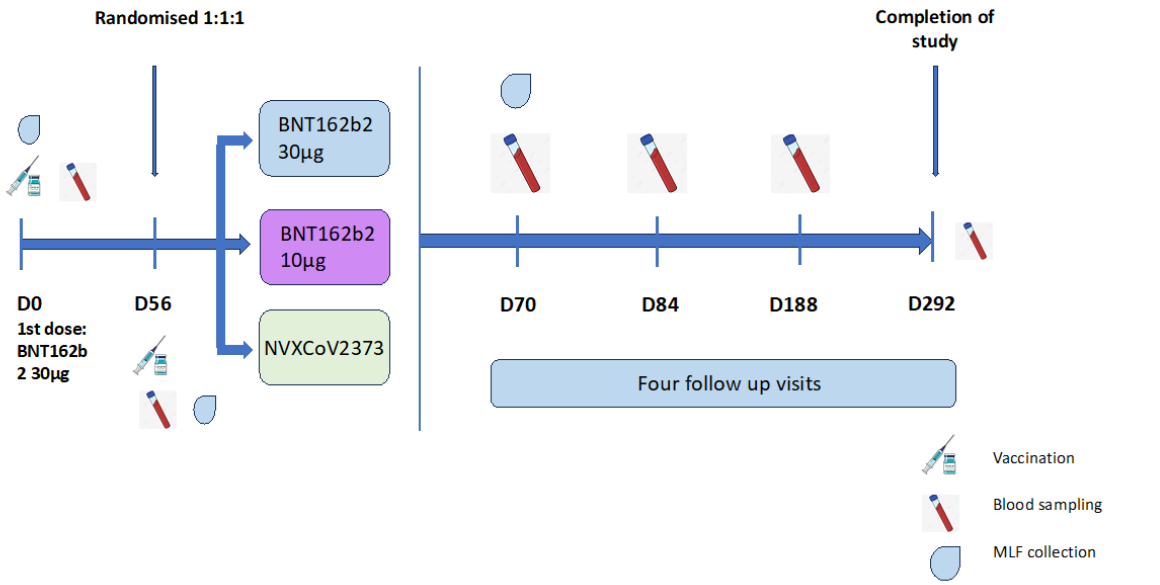

Figure 6.1 Com-COV3 Cohort A study design schematic.
Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56.

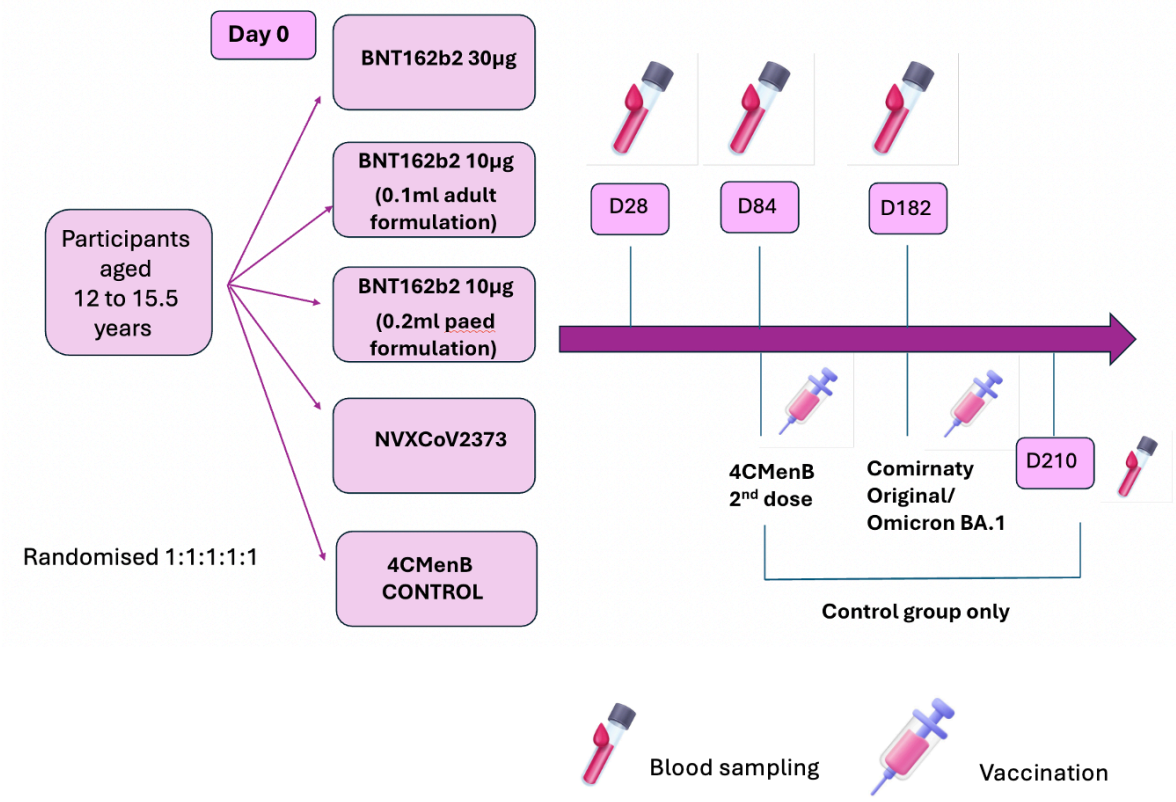

Figure 6.2 Com-COV3 Cohort B study design schematic.
Mucosal samples (not indicated here) were collected at all study timepoints. Participants received their third dose at day 0. Control group participants received two doses of the Meningococcal B vaccine

(4CMenB), the first dose at day 0 and the second dose at day 84. Control group participants received the Comirnaty Original/Omicron BA.1 vaccine at day 182.

An outline of the terminology used in this chapter is shown in

Figure 6.3. Samples were also analysed according to participant 'SARS-CoV-2 infection status' as illustrated in **Figure 6.4** and **Figure 6.5**.

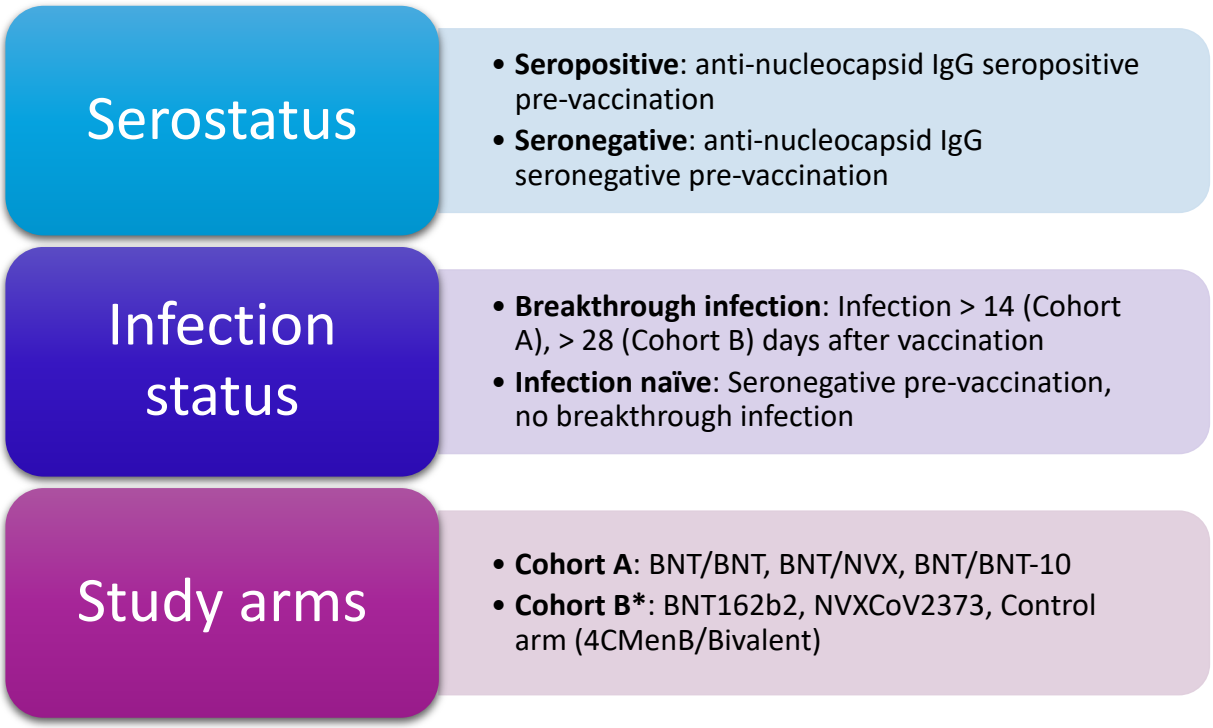

Figure 6.3 A summary of terminology used in this chapter.
*All Cohort A participants were primed with a two-dose 30µg BNT162b2 primary vaccine series. *Cohort B study groups referred to as BNT/BNT/BNT; BNT/BNT/NVX, and BNT/BNT/Bivalent in figures for clarity. BNT = 30µg BN162b2; NVX = NVXCoV2373; BNT-10 = 10µg BNT162b2; 4CMenB = meningococcal group B vaccine; bivalent vaccine = Original/Omicron BA.1 vaccine. A 'breakthrough infection' was defined as either: a self-reported SARS-CoV-2 infection >14 days after a second dose or >28 days after a third dose, a two-fold rise in anti-nucleocapsid IgG, a two-fold rise in anti-spike IgG antibodies, or seroconversion of anti-nucleocapsid IgG serostatus. Participants with evidence of SARS-CoV-2 infection within 14 days after a second dose or 28 days after a third vaccine dose were excluded from this analysis.*

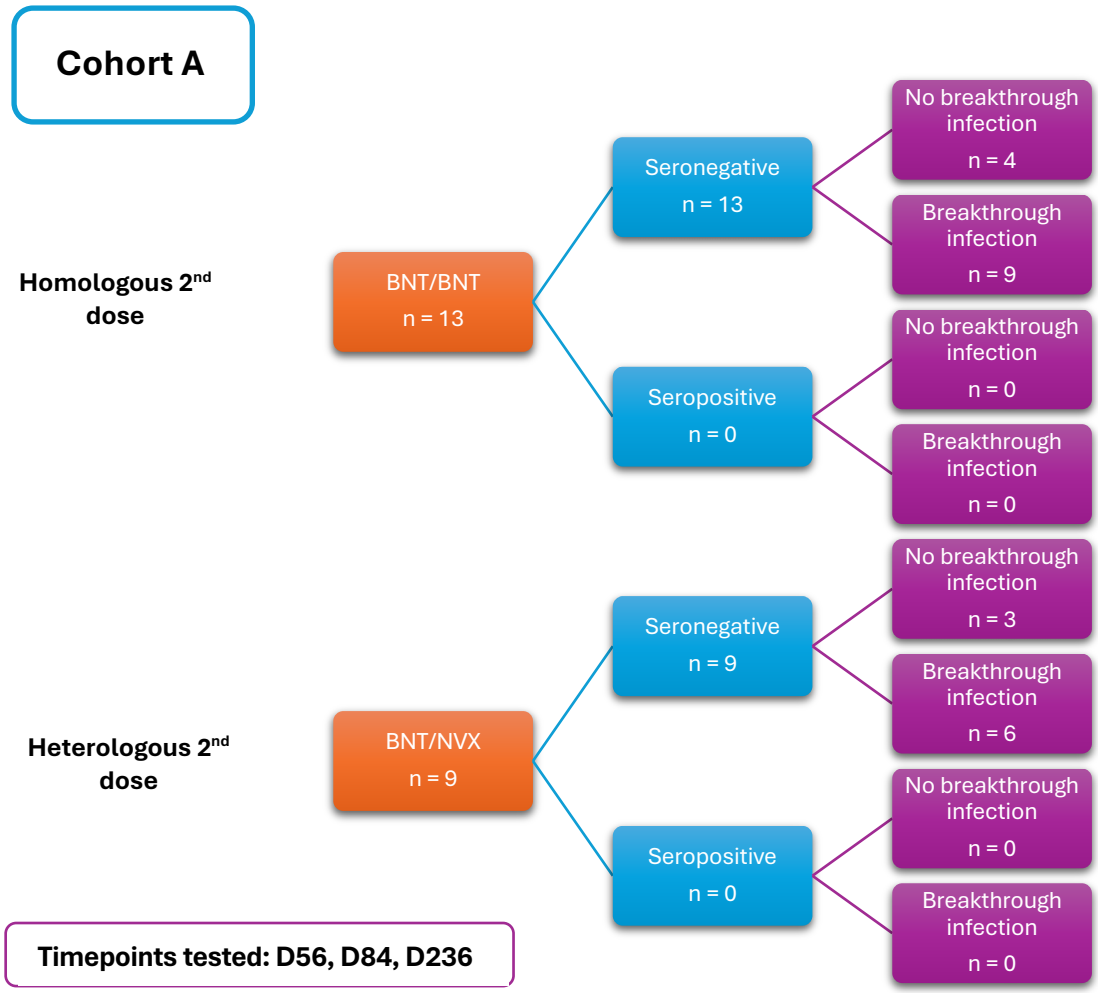

Figure 6.4 Cohort A sample analysis by study arm, serostatus and breakthrough infection status.
n = number of participant samples analysed. Seronegative refers to pre-first dose anti-nucleocapsid IgG seronegative; seropositive refers to pre-first dose anti-nucleocapsid IgG seropositive. Samples were tested at day 56 (pre-second dose), day 84 (day 28 post-second dose), and day 236 (day 180 post-second dose). Participants received their first dose (30µg BNT162b2) at day 0 and their second dose at day 56. BNT = 30µg BNT162b2;NVX = NVXCoV2373.

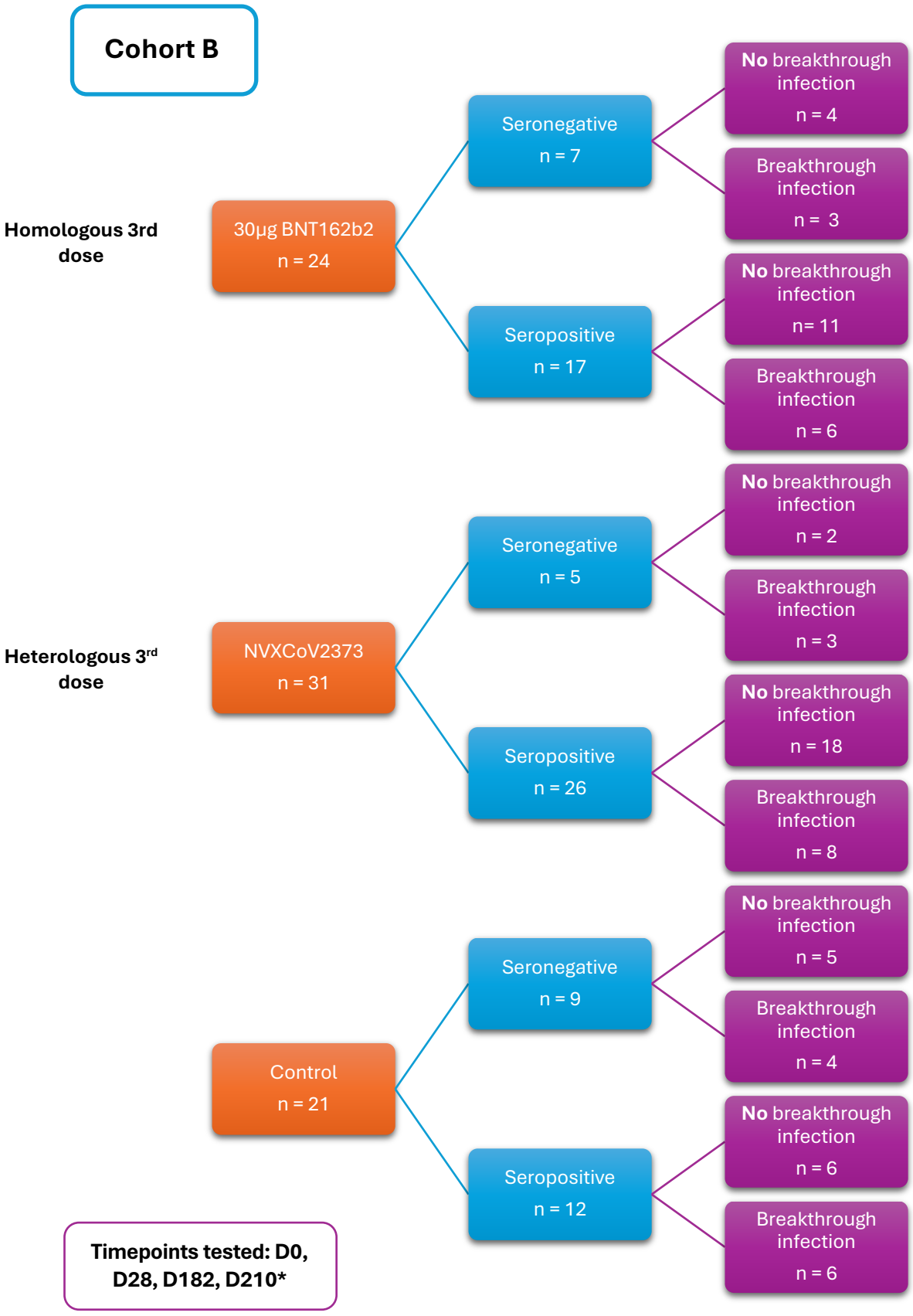

Figure 6.5 Cohort B sample analysis by study arm, serostatus and breakthrough infection status.

n = number of participant samples analysed. Seronegative/seropositive refers to pre-third dose anti-nucleocapsid IgG serostatus. Participants received their third (booster) dose at day 0. Samples were tested at day 0, day 28, and day 182.
Only control group samples were tested at day 210 (28 days following receipt of the bivalent vaccine at day 182 in this group).

6.2.2 Immunoassays

To determine antibody avidity, samples were diluted to give an ELISA Unit (EU) of 1.0. Serum anti-SARS-CoV-2 spike IgG concentrations were measured using an in-house ELISA to determine the dilution required to yield an EU of 1.0. This facilitated standardisation of antibody levels and minimised the influence of antibody titres on antibody avidity. The results of the SARS-CoV-2 spike total IgG ELISA avidity assay are reported in this chapter as percentage binding, referring to the proportion of antibodies bound to antigen which remain following the dissociation step. For full details regarding the assay procedure, please refer to the following sections:

- Anti-SARS-CoV-2 spike glycoprotein IgG ELISA: **Chapter 2: section 2.5**.
- SARS-CoV-2 spike total IgG avidity ELISA, **Chapter 2: section 2.6**.

6.2.3 Statistical analysis

A two-sample T-test was used to compare anti-spike IgG antibody avidity results between groups. Significance testing was conducted using paired t-tests within each group and unpaired t-tests between groups. Data were log-transformed prior to statistical testing. For full details regarding the statistical analyses used, please refer to **Chapter 2, section 2.7**.

6.3 Results

6.3.1 Anti-Spike IgG avidity is enhanced following homologous and heterologous two-dose primary vaccine (Cohort A) and booster (Cohort B) immunisation

In Cohort A, following 30µg BNT162b2 prime vaccination, both a homologous (BNT162b2) and a heterologous (NVXCoV2373) second dose resulted in significantly increased anti-spike IgG avidity 28 days after the second dose, **Figure 6.6**. A sustained increase in avidity was observed throughout the follow-up period, leading to a significantly elevated avidity response by day 180 in both the BNT/BNT and BNT/NVX groups compared to day 28 ($p = 0.0051$ and $p = 0.0083$, respectively).

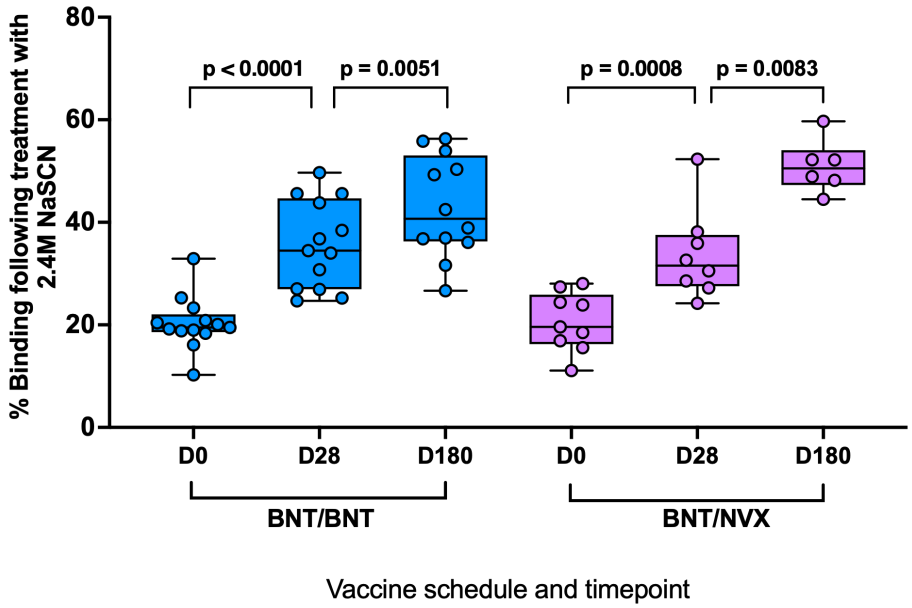

Figure 6.6 Antibody avidity response over time following homologous and heterologous two-dose primary vaccine series (Cohort A).
Results are presented as median with interquartile range (IQR). All participants received their first dose (30µg BNT162b2) at day 0. Day 28 = 28 days post-second dose; D180 = 180 days post-second dose. BNT = 30µg BNT162b2; NVX = NVXCoV2373; NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

In Cohort B, anti-spike IgG avidity was significantly greater at day 28 following both a homologous (30µg BNT162b2) and heterologous (NVXCoV2373) third dose compared with baseline (day 0) levels and those at day 28 in the control arm, **Figure 6.7**. In the control group, following receipt of the bivalent vaccine at day 182, anti-spike IgG avidity was also significantly elevated at day 210 (28 days post-boost in the control group; p = 0.0025).

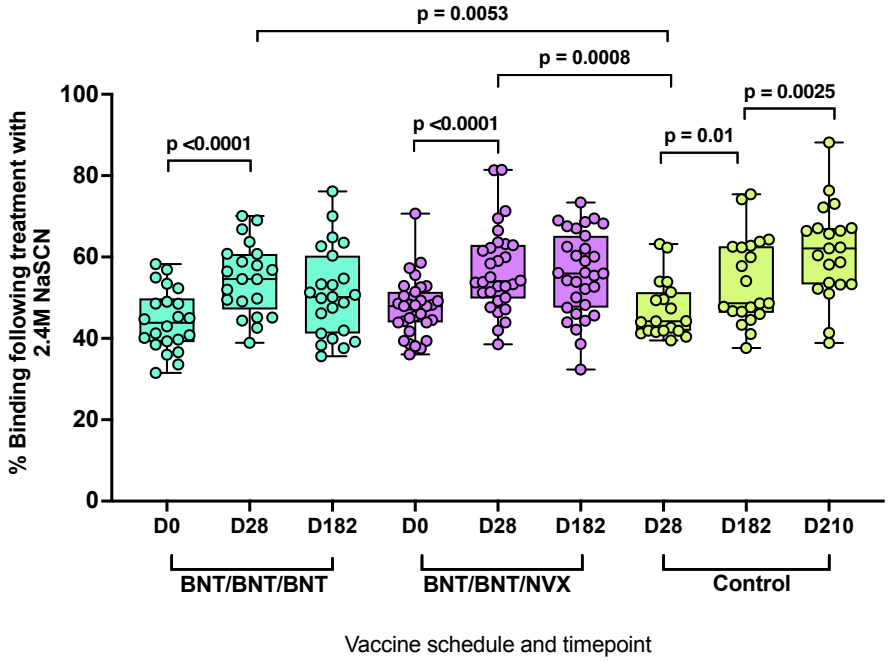

Figure 6.7 Antibody avidity response over time following homologous and heterologous third dose vaccination (Cohort B).
Results are presented as median with interquartile range (IQR). All participants received two-dose 30µg BNT162b2 as their primary vaccine series. Participants received their third (booster) dose at day 0. Control group participants received their first dose of the 4CMenB vaccine at day 0 and received the bivalent vaccine at day 182. Only participants in the control group were tested at day 210 (i.e., 28 days after receiving their third dose). Only statistically significant results are shown. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity). BNT = 30µg BNT162b2; NVX = NVXCoV2373

Unlike the response observed in Cohort A, antibody avidity in Cohort B participants did not increase over time in either the homologous or heterologous third dose groups, **Figure 6.7**. In the control group, despite the absence of COVID-19 immunisation at day 28, avidity was significantly higher at day 182 compared to day 28. However, no significant difference in avidity was found at day 182 between the control group and either the homologous (p = 0.3) or heterologous (p = 0.60) groups.

6.3.2 Anti-spike IgG avidity is significantly enhanced following a booster dose (Cohort B) compared with the primary series (Cohort A)

Anti-spike IgG avidity levels at day 28 day following a third dose were significantly higher compared with levels measured 28 days after the second dose ($p < 0.0001$), **Figure 6.8**. Similarly, avidity levels at day 210 in the control group (28 days following administration of the bivalent vaccine) were significantly higher compared to those observed at day 28 after two doses, despite the longer interval between doses in the control group ($p < 0.0001$).

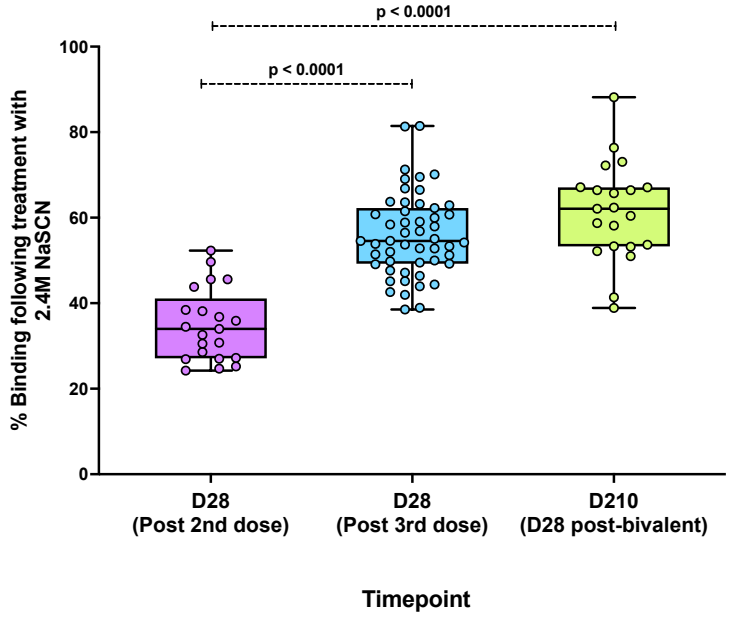

Figure 6.8 Peak anti-spike antibody avidity following second and third (booster) doses.
'D28' denotes day 28 post-second or third dose vaccination; '2nd dose' refers to combined data from Cohort A (participants who received either 30µg BNT162b2 or NVXCoV2373 as the second dose); '3rd dose' refers to combined data from Cohort B (participants who received either 30µg BNT162b2 or NVXCoV2373 as a third dose). Bivalent refers to participants in the control group who received the bivalent Original/Omicron BA.1 vaccine at day 182. Peak avidity response in this group was measured at day 210, 28 days after receiving the bivalent vaccine). NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

Considering the longer interval between doses in the control group, SARS-CoV-2 infection during this period may have contributed to the significant difference in the avidity response observed between the Control group at day 210 and the two-dose (Cohort A) group at day 28. To account for this, participants with SARS-CoV-2 infection between day 0 and day 210 were removed from the analysis. However, the anti-spike IgG avidity response remained significantly elevated in the Control group at day 210 compared with the response observed at day 28 in the two-dose group ($p < 0.0001$), despite the absence of immune boosting through SARS-CoV-2 infection, **Figure 6.9**.

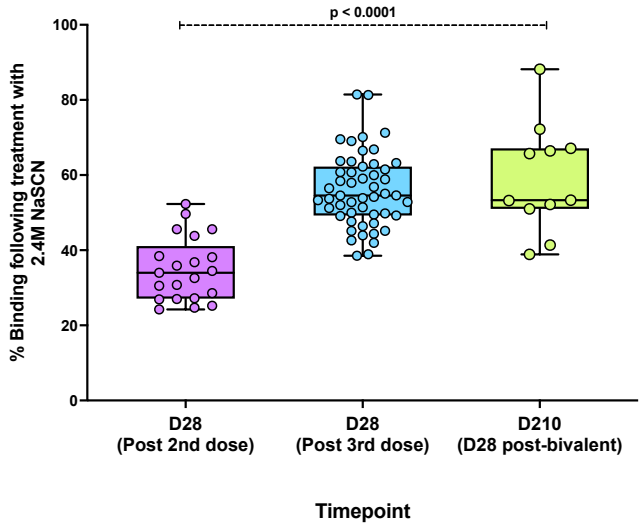

Figure 6.9 Peak anti-spike antibody avidity following a second (Cohort A) and third (booster; Cohort B) dose. Participants with SARS-CoV-2 infection in the Control group have been excluded from the analysis.
'D28' denotes day 28 post-second or third dose vaccination; '2nd dose' refers to combined data from Cohort A (participants who received either 30µg BNT162b2 or NVXCoV2373 as the second dose); '3rd dose' refers to combined data from Cohort B (participants who received either 30µg BNT162b2 or NVXCoV2373 as a third dose). Bivalent refers to participants in the control group who received the bivalent Original/Omicron BA.1 vaccine at day 182. Peak avidity response in this group was measured at day 210, 28 days after the booster dose. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

6.3.3 Antibody avidity is not significantly enhanced following booster vaccination in those with hybrid immunity

The effect of hybrid immunity on avidity was assessed by comparing the avidity response between seropositive and seronegative participants in Cohort B, as shown in **Figure** 6.10. This analysis could not be performed using Cohort A samples as all participants in this cohort were seronegative prior to vaccination.

In Cohort B seronegative participants, antibody avidity was significantly increased at day 28 post-boost compared with baseline (day 0, pre-boost) levels, $p = 0.0026$. Similarly, in seropositive participants, avidity was also significantly increased at day 28, $p < 0.0001$. Baseline (day 0) avidity levels were also substantially higher in seropositive participants than in seronegative participants, $p = 0.04$. However, no significant difference in avidity was observed between seropositive and seronegative participants at days 28 and 182 post-boost. For clarity, only statistically significant results are shown in **Figure** 6.10. These results suggest that avidity is not enhanced following booster immunisation in participants with hybrid immunity. However, this finding may be due to the limited sample size.

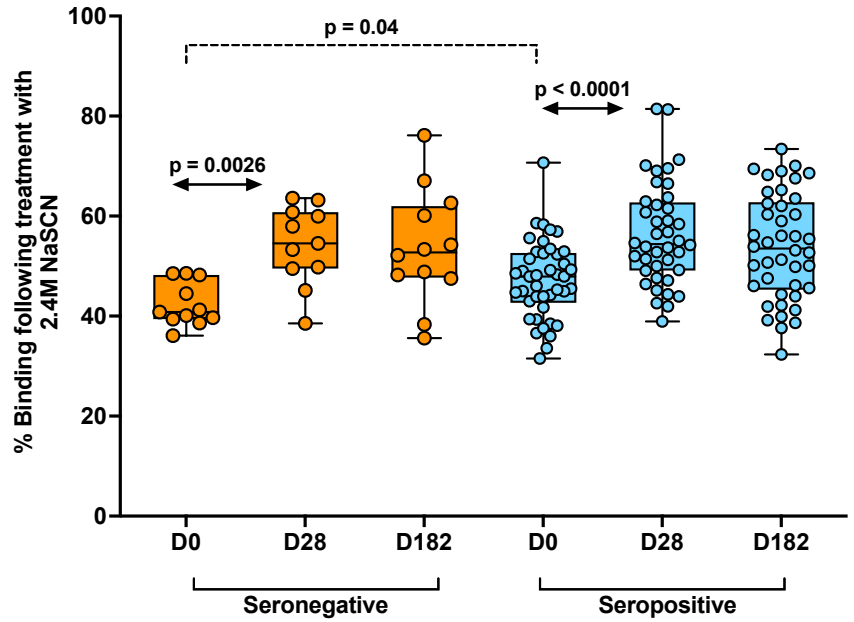

Figure 6.10 Antibody avidity following third dose vaccination according to anti-nucleocapsid IgG serostatus pre-third dose (Cohort B)
Participants with SARS-CoV-2 breakthrough have been excluded from the analysis. 'Seronegative' refers to anti-nucleocapsid IgG seronegative; 'seropositive' refers to anti-nucleocapsid IgG seropositive; '3rd dose' refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose following a two-dose 30µg BNT162b2 primary vaccine series. D0 = pre-third dose vaccination; D28 = 28 days post-third dose; D182 = 182 days post-third dose. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity). Only statistically significant results are shown.

6.3.4 Breakthrough infection enhances antibody avidity post-primary vaccination, but not post-booster

To assess the influence of SARS-CoV-2 infection on the avidity response to vaccination, the results were further analysed according to breakthrough infection status (evidence of SARS-CoV-2 infection occurring >14 days after the second dose and >28 days after the third dose). The results are shown in **Figure 6.11.** No change in avidity between 28 and 180 days after the second dose (Cohort A) was found in participants without breakthrough infection, (p = 0.5). However, in Cohort A participants

with breakthrough infection, avidity was significantly increased by day 180 post-boost (p = 0.0010). However, when avidity levels at 180 days post-boost were compared between participants with and without breakthrough infection, no significant difference in avidity was observed, p = 0.2, **Figure 6.11**. However, this finding may be due to very low sample numbers.

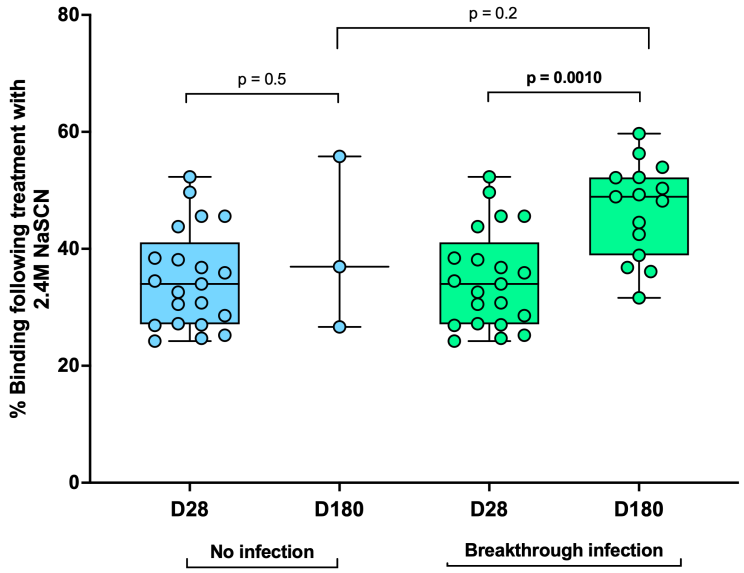

Figure 6.11 Antibody avidity response over time following two-dose primary vaccine series in participants with no breakthrough infection ('no infection') and in participants with breakthrough infection ('breakthrough infection') following the second dose (Cohort A).
Participants who received 30µg BNT162b2 or NVXCoV2373 as the second dose are included in the analysis. D28 = 28 days post-second dose; day 180 = 180 days post-second dose. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

To explore the effect of breakthrough infection on anti-spike antibody avidity following a third dose (Cohort B), participants were stratified by breakthrough infection status during the study follow-up period, as shown in **Figure 6.12**.

In vaccinated Cohort B participants with breakthrough infection, avidity was not significantly increased by day 182, p = 0.8. Similarly, no significant decline in avidity was observed by day 182 in participants without breakthrough infection, (p = 0.1). Avidity levels at day 182 were also not

significantly different between participants with and without breakthrough infection post-boost (p = 0.2).

In contrast, in the absence of vaccination at day 28 in the Control group, SARS-CoV-2 infection resulted in significantly enhanced avidity by day 182 (p = 0.03). However, in Control group participants without SARS-CoV-2 infection, avidity levels were not significantly different at day 182 compared with day 28, (p = 0.1). These findings suggest a limit to the avidity response achievable through infection or vaccination.

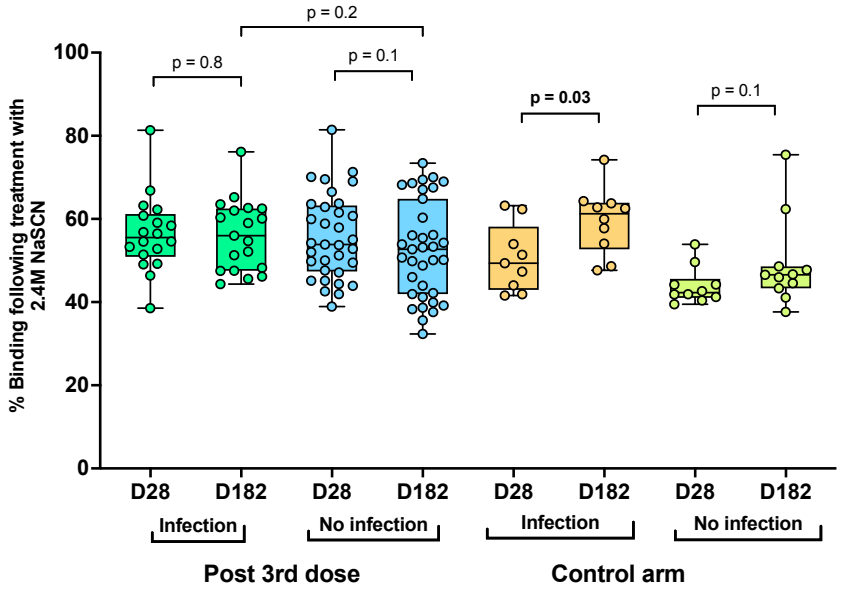

Figure 6.12 Antibody avidity following third dose vaccination (Cohort B) in participants without breakthrough infection ('no infection') and with breakthrough infection ('infection') compared to the Control arm.

'3rd dose' refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose; Control arm refers to participants who received the bivalent Original/Omicron BA.1 vaccine at day 182; breakthrough infection = evidence of SARS-CoV-2 infection > 28 days after vaccination; D28 = 28 days post-third dose; D182 = 182 days post-third dose. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

6.3.5 Antibody avidity significantly correlates with neutralising antibodies against wild-type SARS-CoV-2 after homologous, but not heterologous, two-dose primary vaccine series (Cohort A)

Neutralising antibodies against SARS-CoV-2 have been shown to correlate with antibody avidity following infection and vaccination.[108] Antibodies with greater avidity have also been associated with broader neutralising antibody activity against SARS-CoV-2 variants.[276] To investigate whether a correlation exists between avidity and VNA induced following COVID-19 vaccination in adolescents, a correlation analysis was performed. A statistically significant correlation was found between avidity and VNA against wild-type SARS-CoV-2 following BNT/BNT, (r = 0.6, p = 0.04). but not following BNT/NVX, (r = 0.06, p = 0.88),

Figure 6.13.

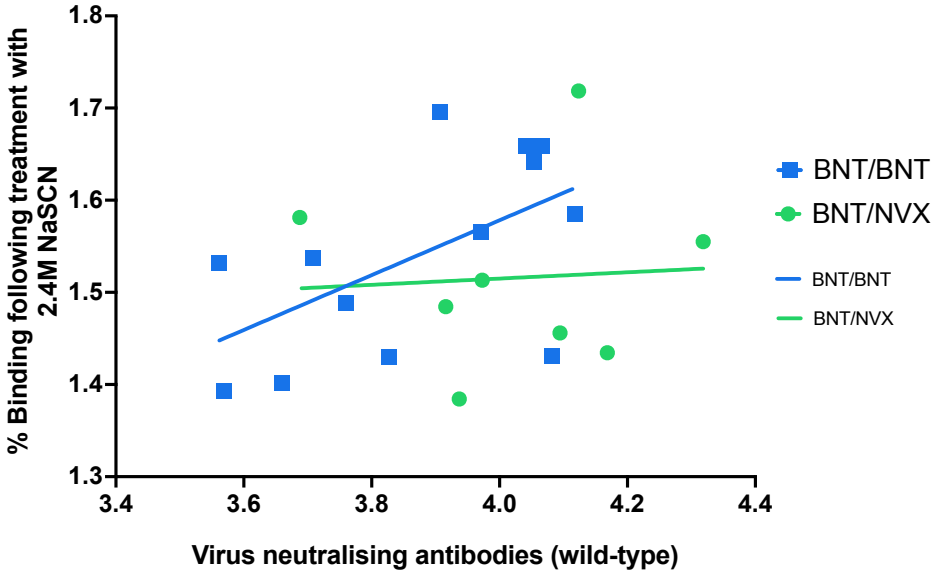

Figure 6.13 Correlation between day 28 anti-spike IgG antibody avidity and day 28 virus neutralising antibodies against wild-type SARS-CoV-2 by study group following two-dose primary vaccine series (Cohort A).
Day 28 = day 28 post-second dose. Data were log-transformed before analysis. Correlation coefficients were calculated using Pearson's correlation. Second dose 30µg BNT162b2: r = 0.6, p = 0.04, second dose NVXCoV2373: r = 0.06, p = 0.88. BNT = 30µg BNT162b2; NVX = NVXCoV2373; NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

A significant correlation was observed between antibody avidity and neutralising antibodies against Omicron BA.1 in participants who received 30µg BNT162b2 as their second vaccine dose (r = 0.7, p = 0.0064). A positive correlation was also detected in the heterologous BNT/NVX group but this was not statistically significant, r = 0.6, p = 0.15,

Figure 6.14.

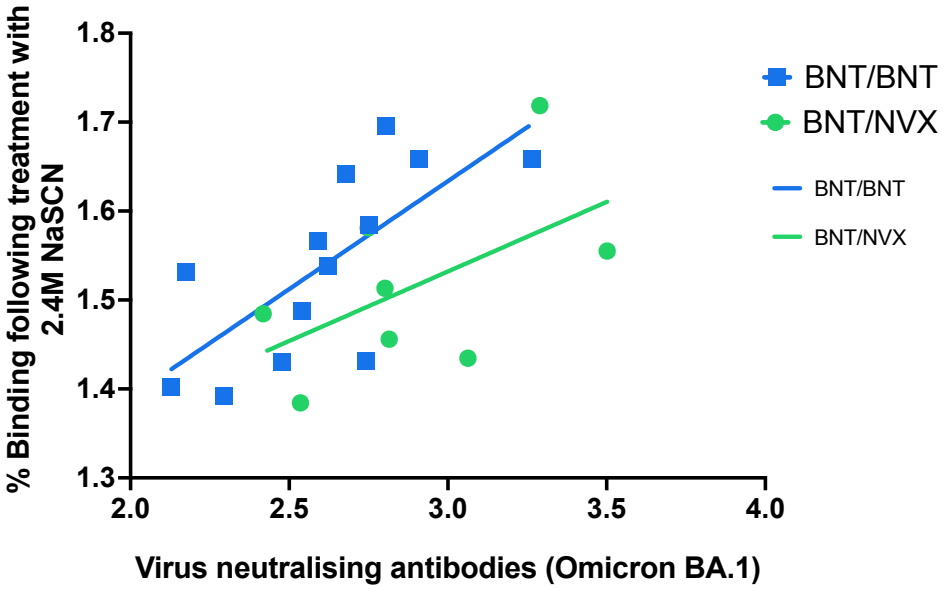

Figure 6.14 Correlation between day 28 anti-spike IgG antibody avidity and day 28 virus neutralising antibodies against Omicron BA.1 by study group following two-dose primary vaccine series (Cohort A).
Day 28 = day 28 post-second dose. Data were log-transformed before analysis. Correlation coefficients were calculated using Pearson's correlation: second dose 30µg BNT162b2: r = 0.7, p = 0.0064, second dose NVXCoV2373: r = 0.6, p = 0.15. BNT = 30µg BNT162b2; NVX = NVXCoV2373; NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

6.3.6 Significant correlation between avidity and neutralising antibodies against Omicron BA.5 following third dose NVXCoV2373 but not mRNA vaccination (Cohort B)

No correlation was found between avidity and neutralising antibodies against wild-type SARS-CoV-2 28 days after the third dose across the study groups: r = 0.3, p = 0.24 in the 30µg BNT162b2 group; r = 0.3, p = 0.05 in the NVXCoV2373, and r = 0.3, p = 0.17 in the Control group,

Figure 6.15.

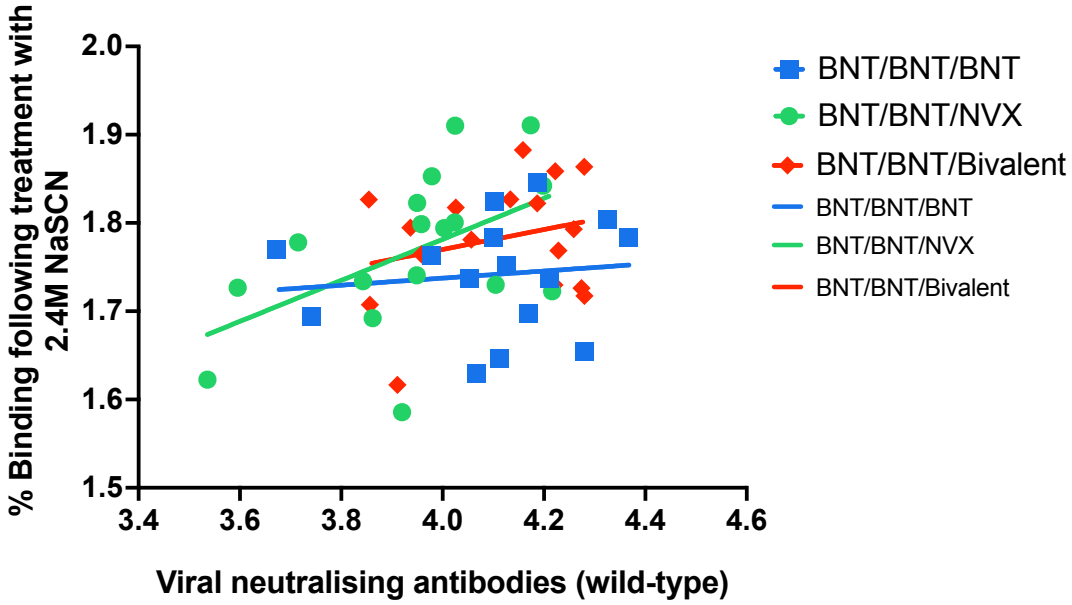

Figure 6.15 Correlation between day 28 anti-spike IgG antibody avidity and day 28 virus neutralising antibodies targeting wild-type SARS-CoV-2 by study group following a third (booster) dose (Cohort B). *Day 28 = day 28 post-third dose. The Control group received the bivalent (Original/Omicron BA.1) vaccine at day 182. Data included in the figure are taken from day 210 (28 days post-third dose in the Control group). Data were log-transformed before analysis. Correlation coefficients were calculated using Pearson's correlation: 28 post-boost): third dose 30µg BNT162b2: r = 0.3, p = 0.24, third dose NVXCoV2373: r = 0.3, p = 0.05; bivalent vaccine: r = 0.3, p = 0.17.BNT = 30µg BNT162b2; NVX = NVXCoV2373; bivalent = bivalent Original/Omicron BA.1 vaccine. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).*

A statistically significant correlation was found between avidity and neutralising antibodies against Omicron BA.5 at day 28 following third dose NVXCoV2373, r = 0.5, p = 0.03,

Figure 6.16, but not for either third dose BNT162b2 (r = 0.08, p = 0.7) nor the Control group (r = -0.009, p = 0.9).

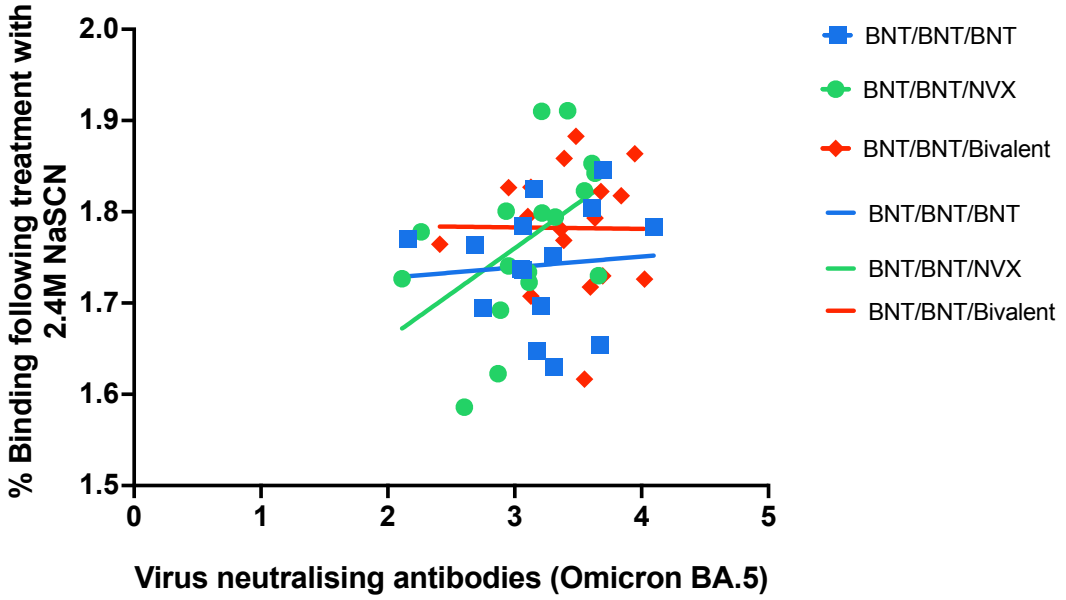

Figure 6.16 Correlation between day 28 anti-spike IgG antibody avidity and day 28 virus neutralising antibodies targeting Omicron BA.5 by study group following a third (booster) dose (Cohort B).
Day 28 = day 28 post-third dose. The Control group received the bivalent (Original/Omicron BA.1) vaccine at day 182. Data included in the figure are taken from day 210 (28 days post-third dose in the Control group). Data were log-transformed before analysis. Correlation coefficients were calculated using Pearson's correlation: 28 post-boost): third dose 30µg BNT162b2 r = 0.08, p = 0.7, third dose NVXCoV2373: r = 0.5, p = 0.03; bivalent vaccine r = -0.009, p = 0.9. BNT = 30µg BNT162b2; NVX = NVXCoV2373; bivalent = bivalent Original/Omicron BA.1 vaccine. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

6.3.7 Exploring biomarkers of protection following COVID-19 vaccination: antibody avidity, mucosal immunity, memory B cell responses, total binding and neutralising antibodies

Several immunogenicity markers of COVID-19 vaccination were explored in this thesis as potential biomarkers of vaccine-induced protection against SARS-CoV-2 infection. Several correlates and determinants of protection against SARS-CoV-2 have been proposed.[266,277,278] The results from the exploratory immunoassays undertaken as part of this thesis were assessed as possible determinants of protection against SARS-CoV-2 and the results of this analysis are presented in detail in the following sections. This analysis was performed separately for each Cohort of the trial. This approach was adopted due to the presence of variables in each cohort which may have significantly affected the interpretation of the results attained, specifically, the SARS-CoV-2 variants in circulation during each study period, the number of vaccine doses received, infection history (Cohort A was a largely infection naïve cohort while most participants in Cohort B had hybrid immunity), and exposures/infection risk (Cohort A participants were recruited during the pandemic when self-isolation practices and social distancing were still in place to minimise spread of the virus while Cohort B participants had returned to school and regular social activities).

6.3.7.1 Mucosal SARS-CoV-2 spike IgA is associated with protection against infection in vaccinated individuals with hybrid immunity

Studies in adults have shown that mucosal SARS-CoV-2 spike IgA may be associated with increased protection against infection.[279] To assess whether mucosal spike IgA is a marker of protection against SARS-CoV-2, mucosal spike IgA responses at 14 days and 28 days after a second and third dose respectively, were compared between participants who developed infection and those who did not during study follow up after vaccination. As shown in

Figure 6.17 **(A)**, mucosal spike-specific IgA levels at day 14 after the second dose were not significantly different between participants with and without breakthrough infection. Similar results were observed at day 28 after a third dose with no significant difference in mucosal spike-specific IgA levels observed between participants,

Figure 6.17 (B).

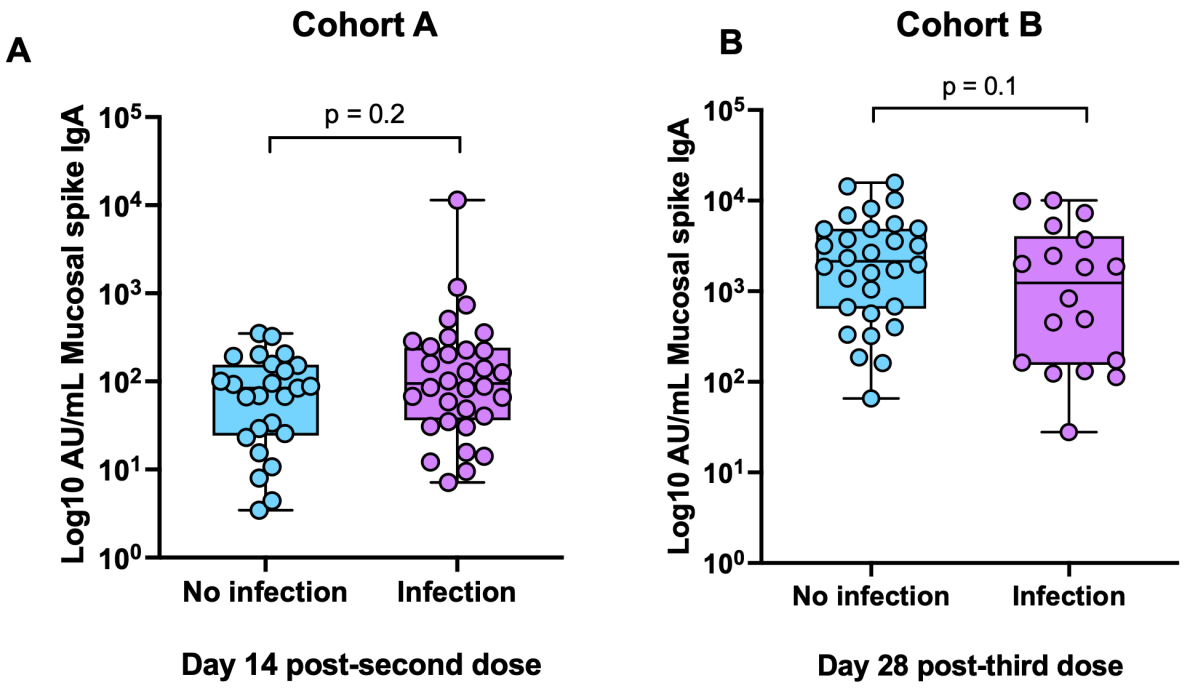

Figure 6.17 Mucosal SARS-CoV-2 spike IgA titres 14 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.
Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. AU = arbitrary units.

It has been shown that individuals with hybrid immunity are at lower risk of SARS-CoV-2 infection following vaccination.[278] In this study, while mucosal spike-specific IgA levels measured 14 days after the second dose were not associated with protection against breakthrough infection in individuals with hybrid immunity, significantly higher mucosal spike-specific IgA levels at day 28 following a booster dose were observed in seropositive Cohort B participants who did not develop infection during study follow up compared with participants who did ($p = 0.02$),

Figure 6.18. These findings highlight the significant influence of prior SARS-CoV-2 infection in the mucosal IgA response following COVID-19 vaccination.

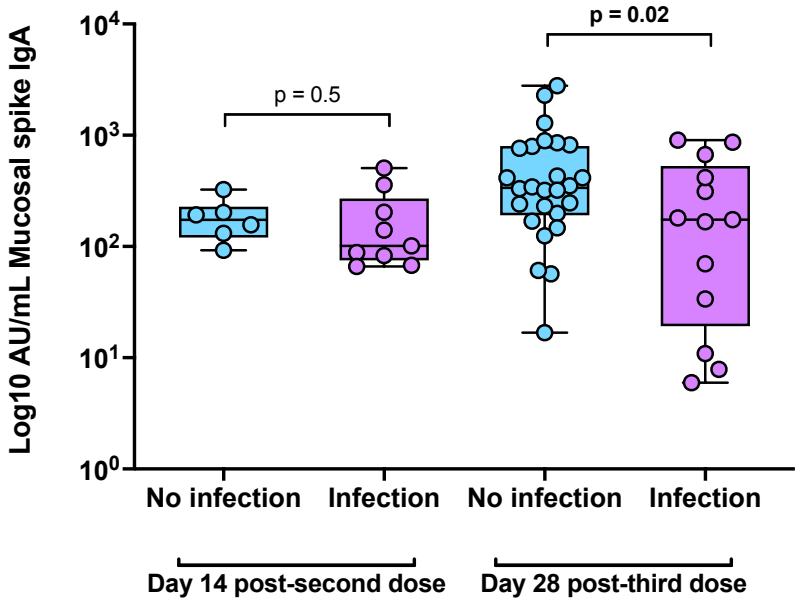

Figure 6.18 Mucosal SARS-CoV-2 spike IgA titres 14 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination in individuals with hybrid immunity.
Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. Only pre-vaccination anti-nucleocapsid IgG seropositive participants (i.e., with evidence of previous SARS-CoV-2 infection) were included in the analysis. AU = arbitrary units.

6.3.7.2 Neutralising antibodies following two-dose primary and booster immunisation are associated with protection against SARS-CoV-2

It is well established that a strong correlation exists between VNA and protection against wild-type SARS-CoV-2 and variant strains.[265] Booster vaccination has been shown to bolster waning immunity, strengthen neutralising antibody titres and enhance protection against infection.[36] To assess whether higher VNA titres following vaccination are associated with increased protection against breakthrough

infection, VNA responses at day 28 following a second and third (booster) dose were compared between participants who developed infection during study follow up and those who did not.

Hybrid immunity has been associated with greater protection against SARS-CoV-2 infection and VNA in individuals with hybrid immunity have been shown to be a more reliable determinant of protection against SARS-CoV-2.[280] In healthy vaccinated 5- to 12-year-olds with a prior history of SARS-CoV-2 infection, VNA titres were associated with protection against infection.[273] During Cohort A recruitment, the Delta variant (B.1.617.2) was the dominant variant in circulation while the Omicron variant was the most prevalent during study follow up. The emergence of the Omicron variant (B.1.1.529) in December 2021 precipitated a sharp increase in SARS-CoV-2 infection rates in the United Kingdom during study follow up, **Figure 6.19**. Omicron sub-lineages BA, BQ.1 and XBB were the predominant circulating strains during the Cohort B study, **Figure 6.20**.

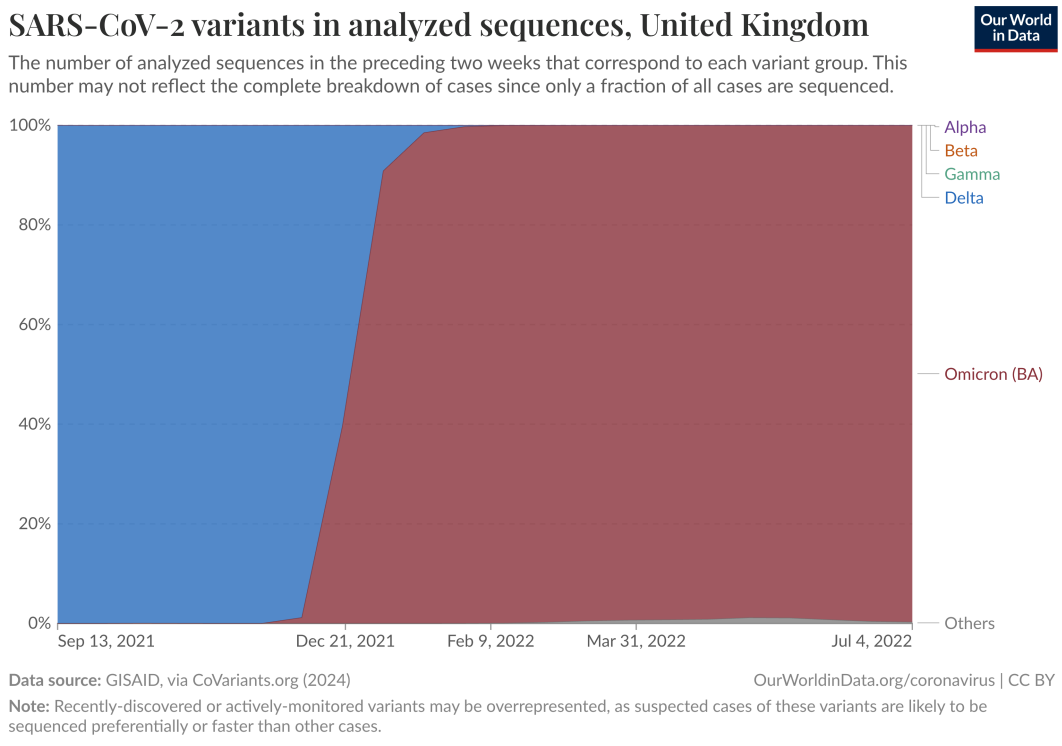

Figure 6.19 SARS-CoV-2 variants in circulation during Cohort A study recruitment (September 2021 to November 2021) and study follow up.
During study recruitment, the Delta variant was the dominant variant in circulation while the Omicron variant was the most prevalent variant strain during the follow up study period. GISAID, via CoVariants.org (2024) – with major processing by Our World in Data. GISAID, via CoVariants.org, "COVID-19, sequencing" [original data].

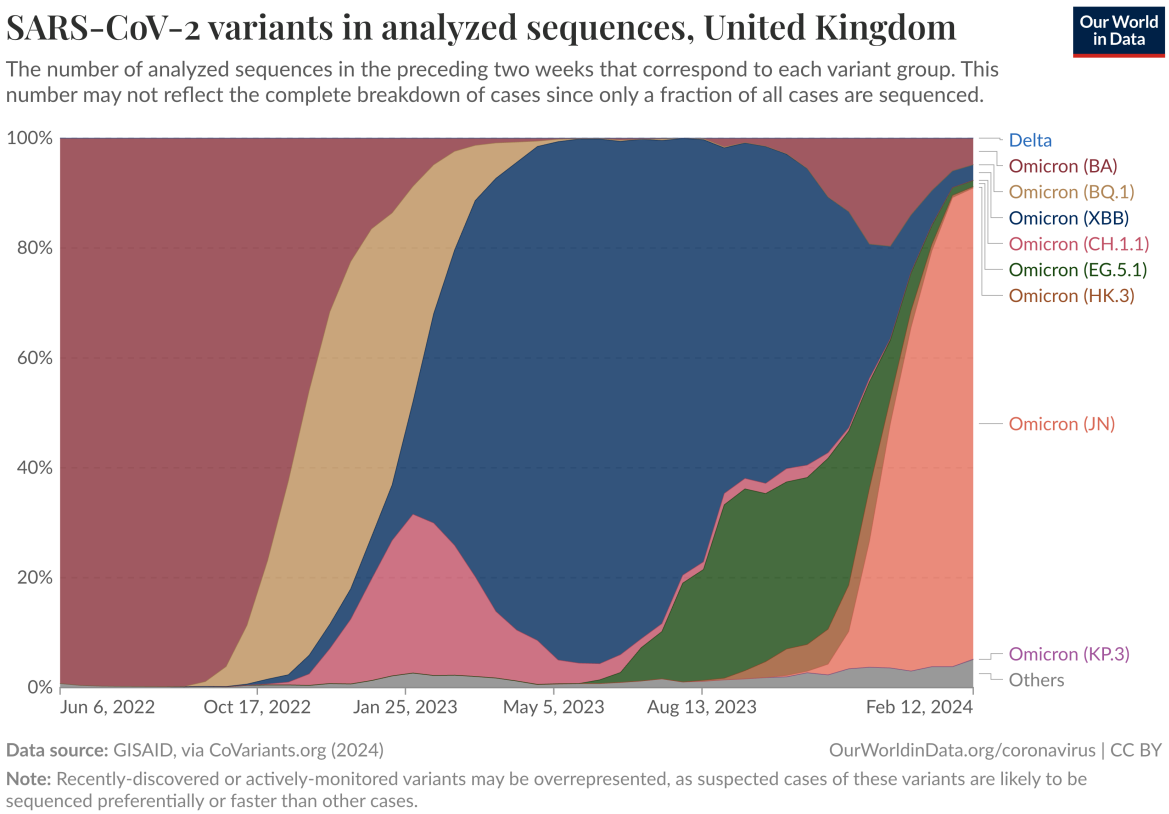

Figure 6.20 SARS-CoV-2 variants in circulation during Cohort B study recruitment (June 2022 to June 2023) and during study follow up.
The Omicron variant was the predominant variant in circulation with Omicron sub-lineages BA, BQ.1 and XBB the most prevalent. GISAID, via CoVariants.org (2024) – with major processing by Our World in Data. GISAID, via CoVariants.org, "COVID-19, sequencing" [original data].

VNA against Omicron BA.1 at day 28 after a second dose (Cohort A) were not significantly different between participants who developed breakthrough infection and those who did not during study follow up. VNA titres against Omicron BA.5 at day 28 after a third dose (Cohort B) however, were significantly higher in participants without breakthrough infection, suggesting an association between VNA titres and protection against infection following a third (booster) dose, p = 0.03, **Figure 6.21**.

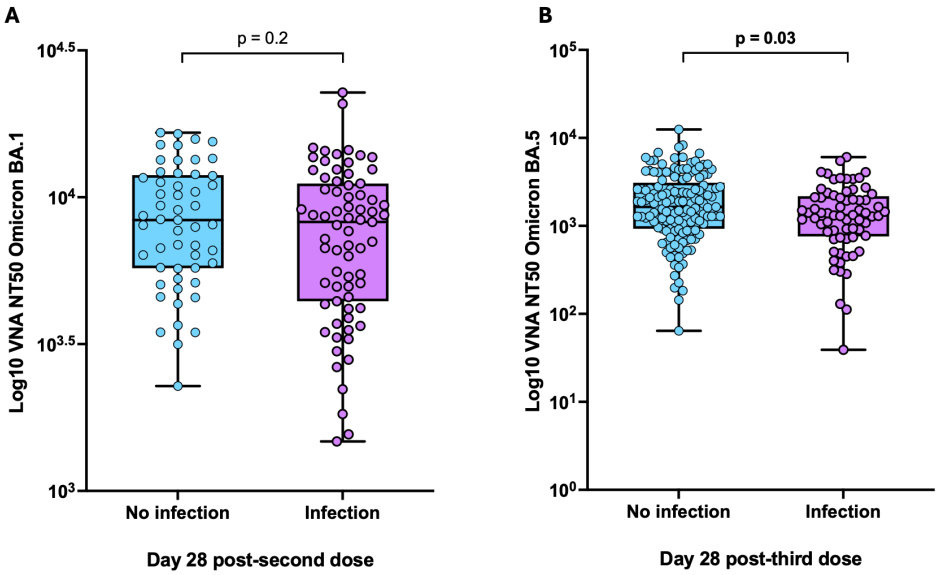

Figure 6.21 Virus neutralising antibody titres against Omicron BA.1 14 days after a two-dose primary vaccine series (Cohort A) and virus neutralising antibody titres against Omicron BA.5 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.
Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. AU = arbitrary units.

In participants with hybrid immunity, Omicron BA.1 VNA titres at day 28 after a second dose (Cohort A) were also significantly higher in participants who did not develop breakthrough infection, p = 0.01. However, no significant difference in Omicron BA.5 VNA titres was observed at day 28 following a third dose (Cohort B) between participants who developed breakthrough infection and those who did not during study follow up, p = 0.2, **Figure 6.22**.

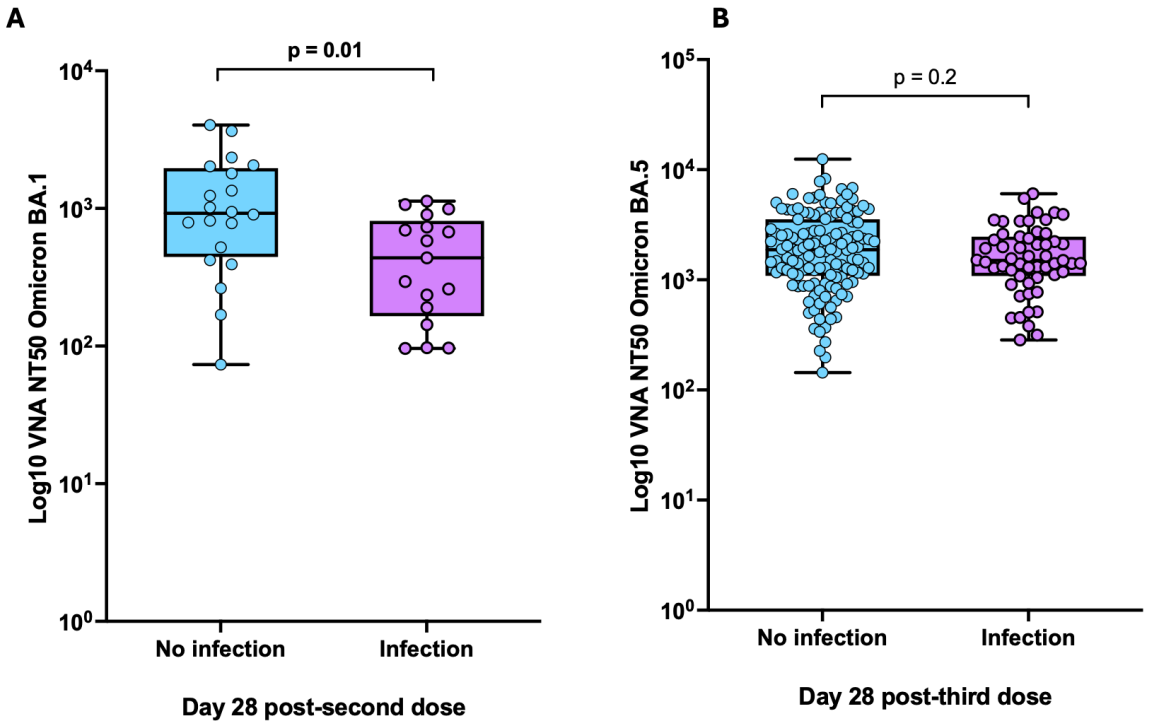

Figure 6.22 Virus neutralising antibody titres against Omicron BA.1 14 days after a two-dose primary vaccine series (Cohort A) and virus neutralising antibody titres against Omicron BA.5 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination in individuals with hybrid immunity .

Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2, NVXCoV2373, or 10µg BNT162b2 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. Only pre-vaccination anti-nucleocapsid IgG seropositive participants (i.e., with evidence of previous SARS-CoV-2 infection) were included in the analysis. AU = arbitrary units.

VNA titres against XBB.15 at day 28 following a third dose were significantly higher in participants who did not develop breakthrough infection, p = 0.03, **Figure 6.23 (A)**. In seropositive participants however, no significant difference in day 28 XBB.15 VNA titres was observed between participants who developed infection and those who did not following vaccination, **Figure 6.23 (B)**.

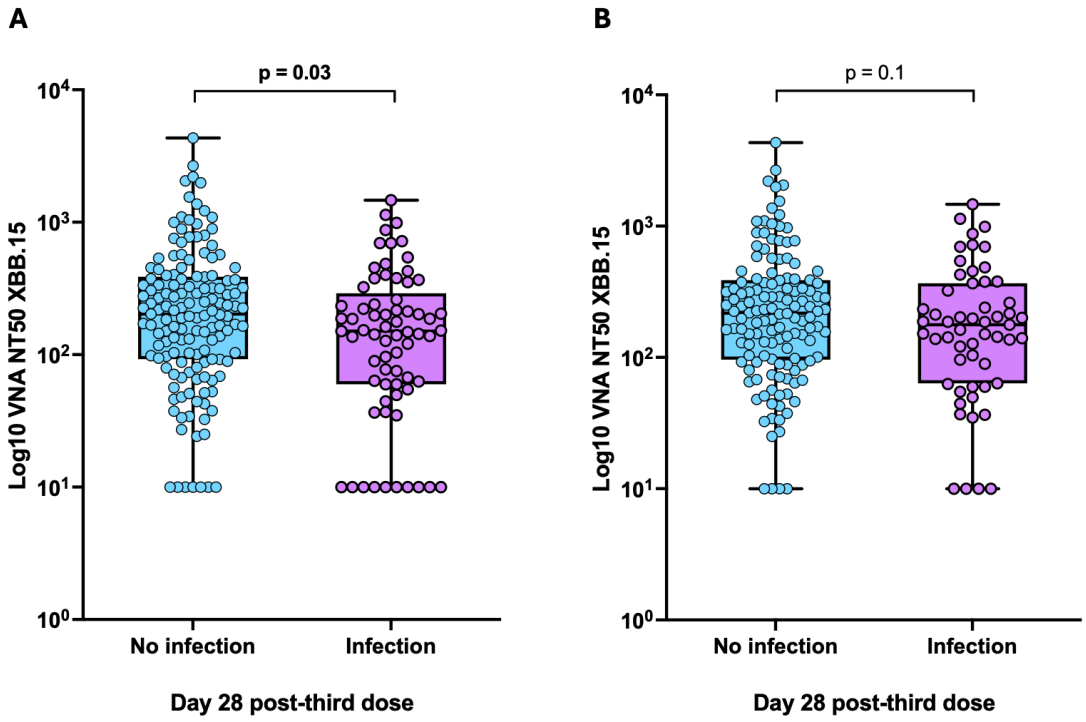

Figure 6.23 Virus neutralising antibody titres against XBB.15 at day 28 following a third dose in (A) all participants and (B) participants with hybrid immunity.
Third dose vaccination refers to participants who received either 30µg BNT162b2, NVXCoV2373 or 10µg BNT162b2 as their third dose. Infection = participants with evidence of SARS-CoV-2 infection >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. In (A): All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination, (B): Only pre-vaccination anti-nucleocapsid IgG seropositive participants (i.e., with evidence of previous SARS-CoV-2 infection) were included in the analysis. AU = arbitrary units.

Anti-spike IgG antibody avidity, mucosal spike-specific IgG, mucosal hCoV OC43 IgA, SARS-CoV-2 spike-specific BMEM, total binding antibodies to (wild-type) SARS-CoV-2, and VNA against wild-type SARS-CoV-2 were also tested as biomarkers of vaccine-induced protection against SARS-CoV-2. However, none of these immunological markers demonstrated potential as biomarkers of protection against infection. The results of this analysis can be found in the **Appendix**, page 318-323.

6.4 Discussion

6.4.1 Antibody avidity is significantly enhanced following a two-dose primary (Cohort A) and booster (Cohort B) immunisation series

This is the first study to examine antibody avidity in adolescents following a two-dose heterologous primary vaccine series and third dose using both homologous and heterologous vaccine schedules. This is also the first study to assess avidity following vaccination using NVXCoV2373 as the second and third dose in an adolescent population. In this study, avidity was significantly enhanced following both a homologous and a heterologous second dose. Avidity continued to increase following both homologous and heterologous second dose regimens and was significantly greater at day 180 compared with baseline (pre-vaccination) levels. Studies in adults have also demonstrated significantly enhanced antibody avidity following both a homologous and heterologous two-dose primary vaccine series.[125,281] A study in adolescents aged 11 to 17 years also demonstrated significantly increased antibody avidity following a two-dose BNT162b2 primary vaccine series with a sustained increase in avidity observed up to 5 months after the second dose.[282]

A significant rise in avidity was also observed at day 28 following a third (booster) dose with either homologous 30µg BNT162b2 or heterologous NVXCoV2373. However, avidity did not continue to increase after the third dose and avidity levels at day 182 were not significantly greater than levels observed at day 28 (post-boost). These findings are consistent those of a study by Mu et al. which demonstrated a significant increase in avidity following third dose 30µg BNT162b2 in adolescents.[282] A significant rise in avidity was also observed in adults following either BNT162b2 or mRNA-1273 (Moderna COVID-19 vaccine) given as a third dose.[127] However, no further increase in avidity was

found beyond one-month post-booster immunisation in the 30µg BNT162b2 third dose vaccine group in adults while a significant decline in avidity was observed in the third dose mRNA-1273 vaccine group during study follow up, consistent with the results reported in this study in adolescents.[127] Only one study was identified which examined avidity maturation following NVXCoV2373 as a second or a third vaccine dose. In this study, conducted in rhesus macaques, a three-dose NVXCoV2373 vaccine series resulted in significantly increased avidity following the booster vaccine series, in line with the results reported here.[210]

In this study, avidity was substantially enhanced after a third dose booster with either an mRNA (BNT162b2) or a protein-adjuvanted vaccine (NVXCoV2373). However, it has been suggested that vaccine platform may significantly influence avidity maturation. A study in adults comparing homologous two-dose BNT162b2 and homologous two-dose ChAdOx1-S (AstraZeneca [AZ]) schedules with a heterologous AZ/BNT162b2 regimen demonstrated a significantly greater antibody avidity response following heterologous AZ/BNT162b2 vaccination compared with either of the homologous regimens studied. Furthermore, avidity was also significantly higher following AZ/AZ compared with BNT162b2/BNT162b2 leading the authors to conclude that a vaccine schedule incorporating at least one dose of a viral vector-based vaccine platform induces a greater avidity response compared with an mRNA-based regimen.[275]

Antibody avidity has also been shown to positively correlate with the number of vaccine doses received.[108] In this study, three doses of a COVID-19 vaccine using either a homologous or a heterologous schedule induced a significantly enhanced avidity response at 28 days after immunisation compared with the response observed at day 28 following completion of the primary vaccine series. When breakthrough infections were excluded from this analysis, antibody avidity at 28 days post-boost remained significantly higher after the third dose compared with levels observed at 28 days after the primary vaccine series. These results are again consistent with those of Mu et al. which showed that avidity was significantly greater at 21 days after 30µg BNT162b2 as a third dose compared with the avidity response observed at 28 days after the second (30µg BNT162b2) dose.[282]

In adults, repeated vaccine doses have also been shown to elicit an enhanced avidity response with one study illustrating increased antibody avidity following either three doses of an mRNA vaccine or two doses of an adenovirus-based vaccine plus an mRNA vaccine as the third dose (compared to the primary series).[108] Additionally, a further study in healthcare workers by Oyebanji et al. showed avidity levels were significantly higher following an mRNA vaccine as a third dose compared with levels measured after the (two-dose BNT162b2) primary series.[283] However, receipt of additional vaccine doses following the third dose in the study were not associated with any further increase in antibody avidity. The study by Oyebanji et al. did however include "frail nursing home residents" and immunosensescence may have played a role in the results observed, as demonstrated in previous studies.[284] A limit to antibody avidity maturation following either vaccination and/or infection might also be possible. In this study in adolescents, no increase in avidity was observed during the study follow up period after the third dose despite the occurrence of breakthrough infection during this time. Most studies classify high avidity as an antibody binding capacity of >60% but studies to date have not commented on whether a limit to this response exists.[285,286]

SARS-CoV-2 infection has also been shown to play an important role in antibody avidity maturation.[264] In this study, avidity increased significantly between day 28 and day 180 after the second vaccine dose in participants with breakthrough infection. A similar rise in avidity was not observed in participants without infection during this time. In contrast, avidity did not increase significantly between day 28 and 182 after the third dose in participants with breakthrough infection. However, relatively fewer participants experienced breakthrough infection in the third dose cohort, and most participants were seropositive prior to vaccination. Notably, in the absence of a COVID-19 booster dose at day 28, a significant rise in avidity occurred between days 28 and 182 in control group participants with SARS-CoV-2 infection during this time. These findings suggest that avidity maturation, in the absence of vaccination, is significantly enhanced by SARS-CoV-2 infection. Similarly, adult studies have shown that avidity is significantly increased following infection compared with vaccination alone, highlighting the immune-enhancing effects of hybrid immunity.[264,287] Indeed, repeated antigenic exposures through infection or vaccination have been

shown to drive affinity and avidity maturation, enhance cross-variant immune responses, and contribute to the improved protection observed against SARS-CoV-2 variant strains following booster vaccine doses.[31,264,288]

In individuals with hybrid immunity, vaccination has been shown to induce a more potent avidity response, particularly against SARS-CoV-2 variants.[264] This is the first study to examine the effect of hybrid immunity on the avidity response following a third dose in adolescents. Two other studies were identified which investigated antibody avidity following COVID-19 vaccination in adolescents. However, neither study investigated the impact of SARS-CoV-2 infection on the avidity response to vaccination.[130,282] In the present study, avidity was significantly higher at 28 days after a third dose compared with levels at baseline (pre-vaccination), irrespective of participant serostatus pre-vaccination. Avidity levels at baseline were also significantly greater in seropositive participants compared with seronegative participants, while no significant difference in avidity was found at 28 days after the third dose between seronegative and seropositive participants. This analysis could not be performed in Cohort A as all Cohort A participants included in this analysis were seronegative at baseline.

6.4.2 Anti-spike IgG antibody avidity correlates with neutralising antibodies against variant strains after primary and booster vaccination

An association between high avidity antibodies and breadth of neutralising antibody activity against SARS-CoV-2 and variant strains following vaccination and/or SARS-CoV-2 infection has been demonstrated.[108,276] Avidity has also been shown to correlate with neutralising antibody activity following vaccination against other viruses.[289] A study in healthcare workers showed a significantly enhanced affinity and avidity response following a two-dose BNT162b2 primary vaccine series and a

corresponding substantial increase in neutralising antibodies.[125] Conversely, Dapporto et al. demonstrated that neutralising antibody activity following a two-dose mRNA vaccine schedule was associated with weak antibody avidity toward wild-type SARS-CoV-2.[108] In this study, a statistically significant correlation was found between avidity and neutralising antibodies against Omicron BA.1 at day 28 following BNT/BNT while no significant correlation was detected in the heterologous BNT/NVX group. This is the first study to examine the relationship between VNA activity and antibody avidity following NVXCoV2373 as a first or a second vaccine dose. Although a comparable VNA response has been demonstrated following either an mRNA or NVXCoV2373-based primary regimen[290], the results of this study suggest that unlike the correlation observed following BNT162b2 vaccination, the VNA response to second dose NVXCoV2373 was not associated with a corresponding increase in antibody avidity. The findings reported in this study are consistent with previous studies that show a significant association between avidity and VNA activity against variant strains following mRNA-based booster vaccination.[276]

No correlation was detected between VNA against ancestral SARS-CoV-2 and avidity after a third vaccine dose in this study, irrespective of the vaccine schedule received. In a study involving healthy adolescents, Mu et al. demonstrated that VNA activity and avidity were significantly enhanced following third dose BNT162b2 vaccination however, a direct correlation analysis was not performed.[282] Similarly, third dose NVXCoV2373 in rhesus macaques induced a potent VNA response and increased avidity after vaccination, however a direct correlation analysis was again not performed.[167] This is the first clinical study to investigate the relationship between avidity and VNA activity following NVXCoV2373 as a third dose and found a statistically significant correlation between VNA against Omicron BA.5 and anti-spike IgG antibody avidity following NVXCoV2373 booster immunisation.

6.4.3 Biomarkers of vaccine-induced protection against SARS-CoV-2

The identification of correlates of protection that predict protection against clinical disease is important in vaccine development and facilitates the deployment of more targeted and effective vaccine strategies. A correlate of protection can be mechanistic or non-mechanistic, i.e., can reflect the immune mechanism underlying protection or be statistically associated with the mechanism of protection.[79] Many immune markers have been identified as potential correlates of protection against SARS-CoV-2 infection and disease. Among these, neutralising antibodies and total binding antibodies against SARS-CoV-2 spike protein are among the most promising and have been strongly associated with protection against infection and severe disease.[280,291] Neutralising antibodies in particular are considered a reliable correlate of protection.[280] However, it has been postulated that protection against SARS-CoV-2 is multifactorial and arises from a conglomeration of various aspects of the cellular and humoral immune response akin to a "Swiss cheese model" of immunity, forming a stratified system of immune defence against SARS-CoV-2.[292] To investigate this further and, in an effort to identify potential vaccine-induced biomarkers of protection against SARS-CoV-2 infection in this study, immunogenicity markers measured during the study were compared between participants with and without 'breakthrough' infection after vaccination during study follow up. This was the first study to explore potential biomarkers of vaccine-induced immunity against SARS-CoV-2 in an adolescent population.

6.4.4 Mucosal spike-specific IgA is associated with protection against SARS-CoV-2 in individuals with prior SARS-CoV-2 infection

A correlation between mucosal spike IgA and protection against SARS-CoV-2 infection has been demonstrated in previous adult studies.[92,271] Havervall et al. showed that significantly higher mucosal (wild-type) spike-specific IgA levels (>75th centile) at study enrolment (5 weeks after the third vaccine dose) were associated with a substantially lower risk of Omicron-related breakthrough infection following vaccination (relative risk reduction of 65% following three vaccine doses). In individuals with hybrid immunity, the risk of infection was even lower (risk reduction of 79%).[279] Similarly Zuo et al. showed that salivary anti-RBD IgA levels (but not anti-RBD IgG levels) were significantly lower in participants with breakthrough infection compared to participants without infection following a second and a third mRNA vaccine dose.[278] Mucosal IgA has also been shown to correlate with virus neutralisation reinforcing its protective role in preventing viral infection.[95] However in this study, no significant difference in mucosal spike IgA was found at 14 days after a second dose nor at 28 days after a third vaccine dose between participants with and without infection following immunisation. However, in seropositive participants, mucosal spike-specific IgA levels at 28 days after a third dose were significantly higher in participants who did not develop infection during study follow up, consistent with findings previously reported in adults.[271]

Mucosal spike-specific IgG levels at day 14 and day 28 after a second and third vaccine dose, respectively, were not significantly different between participants who developed infection and those who did not following vaccination. Similar findings have been reported in adult studies.[95,278] Unlike the findings reported here, the CORSER 5 Case-Control Study demonstrated that salivary spike-specific IgG levels correlated with protection against infection caused by the Omicron variant. In the CORSER 5 study, cases of SARS-CoV-2 infection were matched to uninfected controls based on age

and number of vaccine doses received. The study found that salivary spike-specific IgG was significantly lower in participants with infection compared with uninfected controls.[293] However, although cases were matched to controls based on number of vaccine doses received and timing of study inclusion, any other differences which may have affected the participants' risk of infection were not taken into account at the time of enrolment. Additionally, salivary sampling of cases was performed within 5 days of symptom onset and was not related to time since vaccination, unlike the analysis performed in the current study.

The CORSER 5 Case-Control Study also showed that lower levels of salivary IgG against hCoV OC43 were detected in participants with infection compared to participants without breakthrough infection. However, as previously noted, salivary sampling of cases in this study took place within 5 days of symptom onset and analysis was not performed according to time elapsed since vaccination across cases and controls.[293] In Com-COV3 study participants, no significant difference in mucosal OC43 IgA after a second or third vaccine dose was detected between participants with and without infection after vaccination. Significant cross-reactivity has been demonstrated between hCoV and SARS-CoV-2 and studies report conflicting findings in relation to the hypothesis that pre-existing hCoV immunity confers protection against SARS-CoV-2.[294-296] Most studies focus on mucosal or serum OC43 IgG responses and their association with protection against SARS-CoV-2 infection. In this study, mucosal OC43 IgA was chosen for further investigation as no other study to date has investigated whether mucosal OC43 IgA is associated with protection against infection. Furthermore, as a respiratory pathogen, the primary site of OC43 infection and first exposure to this seasonal hCoV occurs at the mucosa (similar to SARS-CoV-2) rendering it an important and appropriate sample type for this investigation. In addition, mucosal secretory IgA is associated with a more potent neutralising antibody response compared with serum, making it a more likely site of cross-reactive immunity and potential protection against SARS-CoV-2 invasion.[89]

6.4.5 Antibody avidity as a biomarker of vaccine-induced protection against SARS-CoV-2

No significant difference in avidity at 28 days after a second or third vaccine dose was found between participants with and without infection following immunisation. A strong correlation between antibody avidity and protection against viral infection has been demonstrated in several studies.[297-299] Indeed, waning of antibody avidity has been associated with breakthrough infection.[289,300] In the context of SARS-CoV-2, high avidity antibodies have also been shown to provide enhanced long-lasting protection against SARS-CoV-2 variants.[301] Although in the current study, avidity levels at 28 days following a second or a third vaccine dose did not appear to predict protection against future infection, antibody avidity increased significantly following both second and third vaccine doses and as suggested by other authors, antibody avidity is a likely mechanism underlying protection against viral infections including SARS-CoV-2 infection.[255,286] These findings reinforce the importance of the quality of the humoral immune response in defence against infection.

6.4.6 Memory B cells and protection against SARS-CoV-2 infection following vaccination

Several studies have also demonstrated the important role of SARS-CoV-2-specific IgG BMEM in SARS-CoV-2 infection.[302,303] BMEM generally reside in a state of dormancy and are activated upon antigenic exposure through infection or vaccination. They play a powerful part in the defence against infection as both tissue resident memory B cells and as circulating memory B cells, in addition to their presence in secondary lymphoid organs.[78,304] However, it has been suggested that BMEM may also play a role as determinants of protection against SARS-CoV-2 infection.[277] As demonstrated by Byrne

et al, significantly lower RBD-specific BMEM were detected at study enrolment (prior to the third vaccine dose) and at 14 days post-boost in participants with breakthrough infection compared to participants who did not develop infection during the study.[277] Similarly, in solid organ transplant recipients, frequencies of SARS-CoV-2 specific BMEM prior to booster vaccination predicted protection against severe SARS-CoV-2 infection after vaccination.[303] In Com-COV3 participants, no significant difference in the frequency of spike specific BMEM derived IgG-ASC was found at 28 days after a second or third vaccine dose between participants who developed infection and those who did not during study follow up.

This is the first study to examine BMEM as a predictor of vaccine-induced protection against SARS-CoV-2 in adolescents. In children aged 5 to 12 years who received two doses of BNT162b2, Zhong et al. demonstrated that spike-specific BMEM responses were significantly lower at 3 months after the second vaccine dose in children who developed breakthrough infection in the first three months after vaccination compared with children who did not. Most of the children were SARS-CoV-2 infection naïve prior to vaccination.[82] The authors also theorised the existence of an 'onion model' of immune correlates of protection and that the function of these are influenced by whether immunity is derived through vaccination alone or through a combination of vaccination and infection, i.e., hybrid immunity. Like the 'Swiss cheese model of protection' suggested earlier, the 'onion model' adopts a similar view that protection is composed of a many-layered immune defence. In the onion model, high neutralising antibody titres and binding antibodies conceal the parts played by other components of the immune system.[292] When neutralising antibody levels fall to suboptimal levels, BMEM and cellular immunity provide protection against severe and symptomatic SARS-CoV-2 infection.[305] Although spike-specific BMEM in Com-COV3 participants were not predictive of protection against infection when analysed at one month after vaccination, these responses remain critical to sustaining protection against infection, as demonstrated in previous studies.[303,306]

6.4.7 Neutralising antibody response following two-dose primary and booster immunisation are predictive of protection against SARS-CoV-2

Neutralising antibodies correlate strongly with protection against SARS-CoV-2 infection and disease.[307,308] Indeed, a correlation between VNA titres and protection against SARS-CoV-2 has been developed into a predictive model of vaccine efficacy.[308] Immune evasion by variants has resulted in diminished protection against infection in the absence of booster vaccination which has been shown to restore neutralising antibody titres to protective levels and, in turn, has been shown to restore vaccine efficacy.[30,266,309,310] In this study, significantly higher VNA titres against Omicron BA.5 and Omicron XBB.15 were detected at 28 days after the third dose in participants who did not develop infection following vaccination. Previous studies have shown that Omicron subvariants possess a propensity to evade vaccine-derived immunity following the primary vaccine series and that optimisation of vaccine efficacy against Omicron-related infection is achieved through booster immunisation.[31,311] A study in 12- to 17-year-olds found that vaccine efficacy against Omicron-related infection was 73% at two months after a two-dose BNT162b2 regimen but quickly fell to 16% within 6 months of vaccination. A third vaccine dose was shown to significantly improve protection with vaccine efficacy against Omicron restored to 83% following the booster dose.[30] Consistent with these findings, the results reported here show that neutralising antibody activity against Omicron was not predictive of protection against infection following the primary vaccine series but a third (booster) dose was associated with greater protection against SARS-CoV-2 infection.

Hybrid immunity has also been shown to enhance the breadth and potency of neutralising antibodies against VOCs, particularly Omicron subvariant strains.[267,312] Prior infection and booster vaccination have also been associated with enhanced protection against infection.[313,314] In this study, higher VNA titres against Omicron BA.1 at 28 days after the second dose were associated with protection against

breakthrough infection in seropositive participants. In contrast, at day 28 following a booster dose, there was no significant difference in Omicron BA.5 or XBB.15 VNA titres between seropositive participants who developed a breakthrough infection during study follow up and those who did not. Fewer participants in Cohort B experienced breakthrough infection, which may have influenced the study findings.

6.4.8 Total binding antibodies against SARS-CoV-2 spike as a biomarker of protection

Multiple studies have demonstrated that SARS-CoV-2 antibodies are associated with protection against infection.[270,291,293,315] Notably, hybrid immunity has also been shown to grant greater protection against infection, particularly Omicron-related infection, following vaccination.[316] In children, total binding SARS-CoV-2 antibodies were shown to be predictive of protection against infection following a two-dose BNT162b2 vaccine series.[82] However in this study, no significant difference in anti-spike antibodies was found at 28 days after a second or third vaccine dose between infected and uninfected participants. However, unlike the studies previously referenced, this study included a heterogeneous mixture of vaccine platforms and doses which may have influenced the results observed. Most studies examining correlates of protection to date have used results from homogeneous mRNA-based vaccine schedules in their analysis.

6.4.9 Limitations and Conclusions

This study demonstrated that antibody avidity increased significantly following both a homologous and a heterologous primary two-dose vaccine series and third (booster) dose. Antibody avidity was significantly enhanced following breakthrough infection in two-dose (Cohort A) vaccine recipients but not after a third dose (Cohort B), suggesting a limit to antibody avidity achievable through vaccination or infection. A statistically significant correlation was observed between VNA against Omicron BA.1 and antibody avidity at day 28 following homologous BNT/BNT. A significant correlation was also found between Omicron BA.5 neutralising antibodies and antibody avidity following third dose NVXCoV2373. Mucosal spike-specific IgA and VNA levels following vaccination were identified as potential determinants of protection against SARS-CoV-2. However, protection against SARS-CoV-2 is likely multifactorial, involving multiple components of the immune response. However, booster immunisation is important in ensuring durable immunity against immune evasive SARS-CoV-2 variants.

The findings of this study were limited by a small sample size. All Cohort A participants included in this analysis were seronegative and, therefore, the effects of hybrid immunity on antibody avidity could not be evaluated in this cohort. With respect to the biomarker study, thresholds of protection against infection and whether markers were associated with protection against symptomatic or asymptomatic infection were not assessed. The biomarker analysis also included results from homologous as well as heterologous and fractional dose vaccine schedules. Studies investigating correlates of protection have traditionally utilised data involving participants who received homogeneous vaccine regimens. These additional variables may have influenced the results obtained making any significant associations that might have been present difficult to detect.

This is the first study to explore COVID-19 biomarkers of vaccine-induced protection in adolescents and the first study to investigate avidity following heterologous COVID-19 vaccination in adolescents. However, knowledge gaps remain with respect to the optimal vaccine dose interval required to promote greater antibody avidity. Additionally, future research should also focus on the effects of less well studied vaccine platforms such as adenoviral vector vaccines or different administration routes (e.g., intradermal vs. intramuscular vs intranasal) on the avidity response to vaccination. Most studies of avidity rely on the use of chaotropic-agent-based ELISAs (e.g., urea-treated assays) or a modified ELISA using a protein-denaturing agent that disrupts antibody-antigen complex formation as the gold standard.[317] Antibody avidity is an essential measure of vaccine efficacy and performance. Newer tools have become available such as biosensor platforms which offer improved assay precision.[318,319] However, standardisation is essential to enable accurate and reliable comparisons across vaccine studies, which will help guide optimal future vaccine strategies.

Chapter 7: Discussion

7.1 Summary of main findings

This thesis investigated COVID-19 vaccine-induced immunity in an adolescent population and characterised the systemic and mucosal humoral immune response to homologous and heterologous COVID-19 vaccination within both a primary two-dose and booster (third dose) immunisation series. In addition, this thesis explored immune markers of vaccine-induced protection against SARS-CoV-2 using these novel schedules.

7.1.1 Heterologous COVID-19 Vaccine Schedules Induce Robust Immunogenicity in Adolescents

This was the first RCT to evaluate the immunogenicity and reactogenicity of both homologous and heterologous second and third COVID-19 vaccine doses in adolescents. The study found that both homologous and heterologous schedules were highly immunogenic. In Cohort A (two-dose primary series), anti-spike antibody levels in the BNT/NVX group were at least as high as the homologous BNT/BNT group at day 28 after the second dose. Furthermore, neutralising antibody titres against the Omicron BA.1 and BA.2 variants were comparable between the homologous and heterologous primary vaccine regimens. While anti-spike antibody titres were similar across the study groups by day 236, more breakthrough infections were observed in the BNT/BNT-10 and BNT/BNT groups.

This was also the first RCT to evaluate the reactogenicity and immunogenicity of a third (booster) dose in adolescents using fractional doses of BNT162b2, the NVXCoV2373 vaccine and the Comirnaty Original/Omicron BA.1 (15/15µg) vaccine. A heterologous booster dose was shown to

elicit a robust immune response and was well tolerated. Furthermore, this was the first study to demonstrate that a one-third dose (10µg) of the adult BNT162b2 vaccine used as a booster induced a superior immune response compared with the same 10µg dose of the paediatric BNT162b2 vaccine.

Although the safety, immunogenicity, and tolerability of heterologous COVID-19 vaccination has been well established in adults, scant data exist to support the use of these schedules in the paediatric population. This study demonstrates that heterologous COVID-19 primary and booster vaccine schedules in adolescents are highly immunogenic and well tolerated, supporting their use in immunisation campaigns.

7.1.2 Enhanced Spike-Specific Memory B Cell Responses Following COVID-19 Vaccination

This was the first study to assess the BMEM response in adolescents following homologous and heterologous second and third dose vaccine schedules, and to evaluate the influence of SARS-CoV-2 infection. The findings showed that peak spike-specific IgG-ASC levels were significantly higher following a third dose compared to the primary series, irrespective of participant SARS-CoV-2 infection history prior to vaccination. Similarly, significantly higher Delta-specific IgG-ASC levels were observed at day 28 following a booster dose, despite immunisation with a vaccine targeting ancestral SARS-CoV-2. The study also showed that a booster dose elicited cross-reactive BMEM to seasonal hCoV and the Delta variant, with significant correlations detected between SARS-CoV-2 spike-specific BMEM and responses to both Delta and hCoV OC43 at day 28 post-boost. The spike-specific BMEM response at day 28 was also found to be a significant predictor of longer-term BMEM responses, even in the absence of SARS-CoV-2 infection.

The significant cross-reactivity detected between SARS-CoV-2 spike and both the hCoV OC43 and the Delta-variant are also suggestive of immune imprinting i.e., the influence of a prior antigen encounter in shaping the BMEM response, a phenomenon originally described in the setting of Influenza virus infection. This antibody 'recall effect' , which occurs on subsequent challenge with antigenically-related strains, has been shown to elicit a "back-boosting" effect on the immune response.[320] Similar effects have also been reported in adults with a significant correlation between antibodies to SARS-CoV-2 and hCoV beta-coronaviruses detected in individuals admitted to ICU with severe SARS-CoV-2 disease.[321] In the present study in adolescents, a similar response was also observed following both the primary vaccine series and booster dose with significantly increased antibody titres to hCoV observed following vaccination. It is unclear whether such cross-reactivity offers an immunological advantage. In adults, milder COVID-19 disease has been reported in individuals with previous hCoV infection.[322] In children, it has been suggested that their milder clinical course may be explained by greater cross-reactivity between hCoV and SARS-CoV-2 relative to adults.[105,323] In the current study, a significant correlation was found between pre-existing hCoV immunity and vaccine-induced SARS-CoV-2 spike IgG, suggesting that pre-existing hCoV immunity may also influence the systemic immune response to COVID-19 vaccination.

The results of this study are also consistent with those of adult studies demonstrating the importance of booster doses in enhancing spike-specific BMEM responses and in mitigating waning immunity.[324,325] Taken together with the antibody avidity results reported in Chapter 6, which also showed significantly increased antibody avidity following a booster dose, these results support the hypothesis that frequency of antigenic exposure is an important determinant of the quality of the immune response to vaccination. This observation may be explained by repeated antigenic exposure leading to clonal expansion of BMEM and antigen persistence in germinal centres, resulting in greater affinity and avidity maturation of the antibodies produced.[184]

7.1.3 Antibody Avidity is Significantly Increased Following both Primary and Booster Immunisation in Adolescents

This was the first study to assess the antibody avidity response to homologous and heterologous second and third (booster) doses in adolescents. The findings demonstrated that avidity was significantly higher at day 28 following both homologous and heterologous vaccine schedules, compared to baseline. Avidity continued to increase over time following the second dose and was significantly greater at day 180 compared with baseline (day 0) levels, though it is likely that this finding was significantly influenced by breakthrough infection. While no further increase in avidity was observed following a third dose, avidity levels at day 182 post-third dose were significantly higher in unvaccinated (infected) control group participants, highlighting the influence of SARS-CoV-2 infection on avidity maturation.

When avidity levels at day 28 were directly compared between second and third dose vaccine recipients, significantly higher antibody avidity levels were observed in participants who had received three doses, mirroring the BMEM response observed after a booster dose. Additionally, in participants who experienced breakthrough infection after the second dose, antibody avidity was significantly higher at day 180 compared with levels in participants who did not have a breakthrough infection. In contrast, no further increase in avidity was observed in participants with breakthrough infection following a third dose suggesting a limit to antibody avidity maturation achievable through infection and/or vaccination.

In adults, a substantial increase in anti-spike IgG antibody avidity has been observed following a booster dose.[108] Consistent with the findings of the present study in adolescents, SARS-CoV-2 infection has also been shown to significantly influence the avidity response in adults with enhanced

avidity observed following breakthrough infection.[287,326] The results of the current study support the theory that avidity maturation over time is driven by the persistence of antigen in the germinal centre fuelling ongoing affinity maturation.[276] However, findings from adult studies suggest there may be a limit to avidity maturation achievable through repeated vaccination, in line with the observations in third dose recipients in this study following breakthrough infection that suggest there may be a ceiling of exposure.[283]

7.1.4 The Mucosal SARS-CoV-2 Response is Significantly Increased following Homologous and Heterologous Primary and Booster Immunisation in Adolescents

This was the first study to investigate the mucosal immune response in adolescents following homologous and heterologous second and third (booster) vaccine doses and to evaluate the influence of SARS-CoV-2 infection. Mucosal spike-specific IgG levels were significantly increased at day 14 following both homologous and heterologous second doses. Mucosal IgG levels were similarly significantly increased at day 28 following a booster dose with either BNT162b2 or the bivalent vaccine. However, peak mucosal spike-specific IgG responses following a booster dose were not significantly higher than those observed after the primary series. Serum anti-spike IgG responses were also significantly increased following both second and third doses, and a statistically significant correlation detected between mucosal and serum spike-specific IgG levels at day 56 post-prime and day 28 post-booster. These findings are consistent with adult studies and support the hypothesis of transudation of SARS-CoV-2 spike-specific IgG from the systemic circulation into mucosal tissues.[237]

Although mucosal spike-specific IgA levels were significantly increased following either a homologous, heterologous, or fractional second dose, no significant increase in mucosal IgA levels was observed following a third (booster) dose, irrespective of the vaccine schedule received. However, mucosal spike-specific IgA levels were significantly enhanced in participants with hybrid immunity (prior SARS-CoV-2 infection) following both the primary vaccine series and booster immunisation. Additionally, following a third dose, peak mucosal IgA levels were significantly higher compared to those observed after a second dose. However, when SARS-CoV-2 infection was removed from the analysis, this difference was no longer significant for the Control group, which had a longer interval between vaccine doses. These results are consistent with those in adult studies and support the hypothesis that the mucosal spike-specific IgA response to COVID-19 vaccination is dependent on prior priming of tissue-resident B cells through SARS-CoV-2 infection, which are subsequently stimulated through antigen derived via IM vaccination.[242-244]

The significant correlation detected between serum and mucosal spike-specific IgA responses following both primary and booster immunisation suggests that translocation of mucosal spike-specific IgA may also occur following vaccination. This finding is supported by a previous study where IgA2 (abundant in serum) was detected in vaccinated children while IgA1 (predominant in mucosal tissues) was found in unvaccinated children with prior SARS-CoV-2 infection.[99]

This was also the first study to investigate whether a correlation exists between pre-existing hCoV immunity and systemic vaccine-induced immunogenicity to SARS-CoV-2. A significant correlation was detected between baseline (pre-second dose) serum hCoV-specific IgG and the anti-spike IgG response observed at day 14 after a second dose. Conversely, no correlation was found between baseline (pre-third dose) hCoV-specific IgG and antibody levels at day 28 after a third (booster) dose. These findings are consistent with previous studies in children and demonstrate a high degree of cross-reactivity among coronaviruses.[204] These findings also align with the BMEM response observed to hCoV OC43 and NL63 as well as to the Delta variant, described earlier. Furthermore, as discussed

in Chapter 4, these results suggest an age-dependent difference in hCoV cross-reactivity which may influence vaccine-induced immunity in children and adolescents.[327]

7.1.5 COVID-19 biomarkers of protection

This was the first study to explore immune biomarkers of vaccine-induced protection against SARS-CoV-2 in adolescents. The study identified mucosal SARS-CoV-2 spike-specific IgA and VNA activity against Omicron sublineages BA.1, BA.5, and XBB.15 as potential biomarkers of protection across both Cohort A and Cohort B. A positive correlation between mucosal spike-specific IgA and protection against SARS-CoV-2 has previously been demonstrated in adults following booster immunisation.[92,271] However, in contrast to findings in adults, this association was only observed in seropositive adolescents. Though not clearly stated in these studies, it is likely that the adults tested had a previous history of SARS-CoV-2 infection which may account for this finding.

Although VNA are an important correlate of protection, their ability to protect against infection has been attenuated by spike mutations in VOCs.[266,328] Booster vaccine doses (ancestral or variant-specific) have been shown to improve vaccine effectiveness by restoring VNA activity to protective levels.[329,330] In this study, significantly higher VNA titres against Omicron BA.5 and XBB.15 were found in participants who did not develop infection following a third (booster) vaccine dose. Similarly, significantly higher VNA titres against Omicron BA.1 were found in seropositive participants who did not experience breakthrough infection after a second vaccine dose.

These findings highlight the important role of frequency of antigen exposure in shaping the immune response and, consistent with previous studies, illustrate its protective effects against infection. [267] In the setting of hybrid immunity, the immune response induced by vaccination has previously been shown to generate enhanced protection and to induce a more robust immune response.[267]

7.2 Limitations

7.2.1 Estimation of SARS-CoV-2 infection

To evaluate the impact of SARS-CoV-2 infection on the immune response to vaccination, participants were stratified by infection status before vaccination. These categories were defined using anti-nucleocapsid serostatus as well as self-reported SARS-CoV-2 infection. However, anti-nucleocapsid antibodies wane over time and participants classified as “infection naïve” or seronegative, may have been miscategorised due to loss of anti-nucleocapsid seropositivity. Misclassification of participants may have made any differences in the immune response between participants based on their infection status difficult to discern. Additionally, lower levels of anti-nucleocapsid antibodies have been detected in children compared with adults and a more rapid decline in antibody levels observed in children.[331,332] Lower levels of anti-nucleocapsid antibodies have also been found in serum following infection in vaccinated individuals compared with individuals infected prior to vaccination.[333]

A higher number of Cohort A participants developed breakthrough infection. However, most participants in this cohort were seronegative prior to vaccination and infection may therefore have been more easily detected through seroconversion in this cohort. In contrast, the majority of Cohort B participants had hybrid immunity which, as discussed above, may have provided enhanced protection against infection following immunisation. These factors, taken together with the relatively low incidence of breakthrough infection in Cohort B, may have impacted the identification of immune biomarkers of protection in this cohort. Cohort A participants also received their second dose at the beginning of an Omicron wave and, due to the immune evasive nature of this variant, protection against infection following the primary vaccine series may have been reduced. This, in turn, may also have reduced the ability to detect a biomarker in this cohort.

Since self-testing kits (LFTs) were free and widely available during the Cohort A study follow-up period, participants in this cohort may also have been more likely to self-test than participants in Cohort B. Furthermore, although a greater number of BNT/BNT-10 recipients experienced breakthrough infection, these participants, once unblinded, may also have been more likely to self-test. In contrast, participants in Cohort B were less likely to self-test as social distancing measures were no longer in place and self-testing kits were no longer freely available (LFT kits were provided to Cohort B participants to mitigate this risk). Additionally, most SARS-CoV-2 infections identified in Cohort B were asymptomatic and detected through serological testing.

This thesis presents findings suggestive of immune imprinting and cross-reactivity due to the combined effects of prior infection and vaccination. However, several criteria need to be satisfied in order to demonstrate immune imprinting. These include: the detection of antibodies (and/or BMEM) specific to a previously encountered virus,; detection of shared epitopes between the "imprinted" virus and the new virus; presence of new (mutated) epitopes present on the new virus that are not recognised by the imprinted virus; and evidence that challenge with a new virus protein (through infection or vaccination) stimulates a BMEM response dominated by antibodies targeting epitopes shared by both viruses (i.e., the "imprinted" and the 'new' viral protein).[334] This has implications in terms of the testing necessary to determine whether imprinting has taken place and because not all of these criteria were fulfilled in this study, the findings reported here and their implications should therefore be interpreted with caution.

7.2.2 Participant population

Most participants in this study were Caucasian with few participants belonging to an ethnic minority. This limits the generalisability of the study findings. Additionally, as the study focused on the immune response in adolescents to COVID-19 vaccination, the results may not be applicable to younger

children. Only healthy volunteers were eligible to enrol in the study, further limiting the generalisability of the study findings. However, samples from this study have since been used in a separate study to evaluate the humoral and cellular immune response to a two-dose BNT162b2 primary vaccine series in immunocompromised children.[335]

The sample size of the study may also limit the reliability of the data generated. With respect to Cohort A, the target study sample size was based on practical constraints while for Cohort B, it was based on the number of participants required to achieve the co-primary non-inferiority outcome. The rationale for the sample size used in the exploratory work undertaken as part of this thesis is discussed in the following section.

7.2.3 Limitations of laboratory assays

The differences in sampling timepoints between Cohort A and Cohort B limit the comparisons which can be drawn between these cohorts. In Cohort A, mucosal samples were collected before the first dose, 56 days after the first dose and 14 days after the second dose, while sampling in Cohort B took place before the third dose and 28 days after the third dose. Furthermore, the timing of peak mucosal antibody responses following vaccination has not yet been established. These differing timepoints may have influenced the mucosal antibody results observed in each Cohort and consequently, the comparisons made between cohorts.

To determine the influence of SARS-CoV-2 infection, sample selection was also based on participant infection status. More samples were analysed from Cohort B as a greater number of participants in this cohort had evidence of hybrid immunity. Results from Cohort B were therefore considered more reflective of the current global status of near-universal SARS-CoV-2 seropositivity, and thus more relevant. However, the small sample size limits the accuracy and interpretation of the study findings,

and significant differences in the immune response between groups may not have been detected as a result.

It was not possible to undertake all assays necessary to conclusively demonstrate immune imprinting in this study. Antibody avidity was assessed against the ancestral SARS-CoV-2 strain. However, testing using variant strains may have provided greater insight into vaccine-induced cross-reactive immunity and potential imprinting effects. In addition, mucosal IgA subtype analysis, which may have helped to differentiate between infection-derived and vaccine-derived mucosal IgA, was not performed.

A BMEM ELISpot was chosen for this analysis as it is a highly sensitive assay, capable of detecting antigen-specific BMEM ASCs, even at very low frequencies (LLOD was one antigen-specific B cell per 250,000 PBMCs). The FluoroSpot/ELISpot used was a multiplex assay and facilitated the simultaneous detection of antibodies to multiple antigens on a single plate through fluorescent detection. Flow cytometry was an alternative for this study and would have enabled characterisation of B cell subsets and their surface markers. It would also have facilitated cell sorting and targeting of specific cell populations for further research. However, B-cell surface markers may not be a reliable indicator of B-cell function.[336] The same surface marker can be expressed by different cell types and surface expression can also be affected by cytokine secretion.[337] For example, CD27 is a marker of BMEM but CD27 negative BMEM have been identified, and CD27 can also be found on plasma cells as well as germinal centre B cells.[337,338]

7.2.4 Limitations of statistical analyses

As previously discussed, an important limitation of this work was the small sample size used. As small sample populations are susceptible to a higher degree of variability, all data were log-

transformed prior to statistical analysis to help address this issue. However, despite these efforts, the small sample size used represents a significant limitation and results reported should be interpreted with caution.

7.3 Contribution of this Study to COVID-19 vaccine research

This was the first study to evaluate the reactogenicity and immunogenicity of heterologous and fractional second and third vaccine doses in adolescents. The study was also the first to examine mucosal responses, BMEM, and antibody avidity in adolescents to these novel schedules, and to explore potential biomarkers of vaccine-induced protection in this age group. Prior to this study, fractional doses had already been studied in children and shown promise when compared with the immune response to full (standard) doses in adults. This study extends these findings to adolescents.

This was the first study to directly compare fractional doses of the paediatric and adult formulations of BNT162b2 when administered as a booster dose in adolescents. It demonstrated that a fractional dose of the adult BNT162b2 vaccine elicited a superior immune response compared to the same dose administered using the paediatric formulation. These findings may have important implications in terms of cost-effectiveness, vaccine availability and schedule flexibility if lower doses of the adult BNT162b2 vaccine can be used to optimise protection against VOCs in the paediatric population.

This study demonstrates that heterologous and fractional COVID-19 booster doses in adolescents are highly immunogenic and associated with favourable reactogenicity. The performance of NVXCoV2373 and the bivalent Original/Omicron BA.1 vaccines further supports their use in

heterologous immunisation schedules in this age group. This was also the first study to examine the impact of a longer dosing interval on immunogenicity in adolescents and demonstrated comparable immune responses between the bivalent vaccine and 30μg BNT162b2 booster schedules. Heterologous vaccine schedules offer multiple potential advantages including more flexible vaccine schedules, and more efficient deployment of global vaccine supplies. The data generated in this study are policy-relevant and may inform future pandemic preparedness strategies.

The results of the trial enhance our understanding of the immune response to COVID-19 vaccination in adolescents and provide a foundation for further research into vaccine-induced humoral immunity, specifically in relation to mucosal immunity, BMEM, and antibody avidity. This work showed that both homologous and heterologous COVID-19 vaccination in adolescents elicit robust immune responses. Notably, SARS-CoV-2 spike-specific Bmem responses were significantly higher 28 days after a third dose compared with both pre-vaccination levels and responses observed 28 days after the primary immunisation series. Similarly, antibody avidity was significantly increased 28 days after a booster dose compared with both pre-vaccination levels and post-primary vaccine series levels. These findings reflect repeated rounds of somatic hypermutation in B-cell germinal centres, leading to the generation of B cells – both plasma cells and Bmem - with increased antibody affinity and specificity. They also suggest that frequency of antigen encounter not only enhances B-cell responses but also drives antibody avidity maturation, consistent with previous reports.[276]

Bmem responses at day 182 (post-boost) were not significantly different between vaccinated participants and control group participants, despite the latter not receiving a COVID-19 vaccine at day 28. Similarly, antibody avidity levels did not differ significantly between the two groups at day 182. Avidity in the control group was also significantly higher at day 182 compared with day 28, even in the absence of vaccination. These findings suggest that Bmem are active during breakthrough infection and are continuously responding to environmental antigenic exposure (in line with findings previously reported).[40] This, in turn, promotes avidity maturation, even in the absence of vaccination.

Similar to the spike-specific Bmem response, mucosal spike-specific IgG levels were significantly elevated following both second and third vaccine doses. This may reflect either transudation of spike-specific IgG antibodies from the systemic circulation or local production by tissue-resident Bmem, as suggested in previous studies.[92,242] The parallel between the spike-specific Bmem and mucosal spike-specific IgG responses observed here supports this hypothesis.

Likewise, mucosal spike-specific IgA levels were significantly higher at day 28 following the third dose compared with day 28 after the second dose. This finding aligns with previous reports which suggest that priming with SARS-CoV-2 infection leads to the formation of tissue resident Bmem which secrete spike-specific IgA in response to vaccine-derived antigen.[272,304]

Additionally, significant cross-reactivity between SARS-CoV-2 spike-specific Bmem and hCoV OC43-specific Bmem was observed. This was mirrored by a significant increase in both serum and mucosal hCoV-specific IgG levels following vaccination, suggesting that cross-reactive adaptive immunity is generated in both the systemic (B cell) and mucosal immune compartments in response to SARS-CoV-2 vaccination in adolescents.

Finally, significant correlation between pre-existing serum hCoV-specific IgG and post-vaccination serum anti-spike IgG levels suggests that baseline (pre-existing) hCoV immunity may influence SARS-CoV-2 vaccine-induced immunity.

These findings provide valuable insight into the humoral immune response to homologous and heterologous COVID-19 vaccination in this age group and underscore the important immunogenicity results of the trial. As most studies focus on the immune response in adults to vaccination, this study highlights important age-related similarities and differences in the immune response to COVID-19 vaccination and contributes important data relevant to the adolescent population.

These findings provide policy-relevant data that may inform future vaccine strategies and contribute to pandemic preparedness strategies. The results of this study support the use of heterologous vaccine schedules and fractional doses as part of booster immunisation campaigns in adolescents. The study also highlights the potential for mucosal SARS-CoV-2 spike IgA as a biomarker of vaccine-induced protection in this age group.

7.4 Conclusions and Future research

Mucosal COVID-19 vaccines are currently in development, with many studies reporting promising results with respect to immunogenicity and reactogenicity in children, adolescents, and adults.[339-341] The findings from this study suggest that IM vaccination induces a weaker mucosal immune response compared with the systemic response observed and that future research should focus on the development of intranasal vaccines that will strengthen local mucosal immunity at the site of infection. Mucosal SARS-CoV-2 spike-specific IgA was also identified as a potential biomarker of vaccine-induced protection against infection, further highlighting the importance of strengthening mucosal defences against this pathogen. Future studies should also examine immunogenicity following heterologous IM and intranasal vaccination as this combined vaccination strategy may enhance protection against SARS-CoV-2 infection and produce a more durable immune response.

Most participants in this study were Caucasian and therefore not representative of the general population. These findings highlight the need for further research into the immune response to heterologous vaccination in children and those from diverse ethnic backgrounds to enhance the generalisability of the results of this study. Additionally, this study examined the immune response in

adolescents to vaccination. When combined with results from other COVID-19 vaccine studies, these results will yield informative data with respect to the association between age and vaccine-induced immunogenicity to COVID-19. A separate study examining age-dependent vaccine-induced immunogenicity and the impact of different vaccine schedules and platforms on this association is currently underway and the results will be reported separately.

This thesis also highlighted several areas where immune imprinting may help explain the immune response observed. Indeed, studies published to date suggest that immune imprinting plays a greater role in the immune response observed in children and adolescents than in adults. This may have implications with respect to future vaccine strategies. As imprinting appears to be a feature of immune-evasive viruses evolving and adapting over time, future vaccine strategies may need focus on designing vaccines to promote a de novo variant-specific immune response. Future immunisation with either vaccines containing antigenically distant virus strains to support a de novo response, or design of a universal vaccine capable of stimulating both variant-specific as well as cross-reactive adaptive immunity, may be most effective in shaping protective humoral immunity. However, other factors affecting vaccine efficacy may also need to be considered including age-related differences in immunogenicity, the effect of vaccine platform, and optimal dosing interval. As discussed earlier, a study investigating the impact of these factors on vaccine-induced immunogenicity is currently underway.

In conclusion, this study demonstrated that heterologous and fractional second and third dose COVID-19 vaccine schedules in adolescents are highly immunogenic and demonstrate favourable reactogenicity profiles. The findings provide greater insight into vaccine-induced humoral and mucosal immunity in adolescents, as well as the influence of SARS-CoV-2 infection. The results of this study also highlight age-dependent differences in the immune response to vaccination including those related to vaccine platform and dose number. These results provide a foundation for further research into optimal vaccine strategies to achieve sustained protection against SARS-CoV-2 and may inform future pandemic strategy planning.

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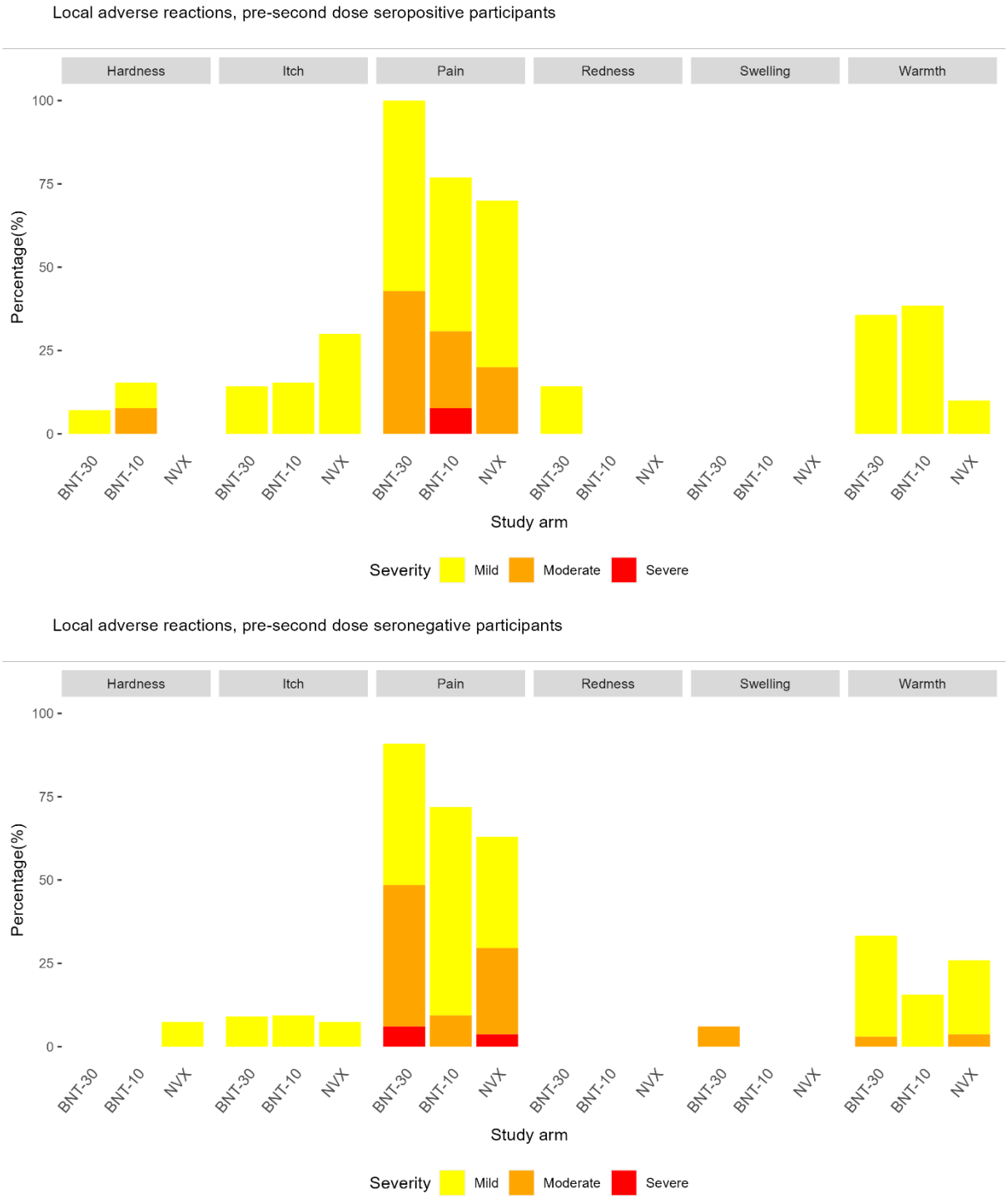
Appendix

Supplementary Figure 1. Severity of solicited adverse reactions in days 0-7 after second vaccination by study arm as self-reported in participant electronic diaries in the safety analysis population
BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373. The severity presented is the participant's highest severity across 7 days following vaccination for each solicited adverse event. Fever: Mild: 38·0°C to <38·5°C; moderate: 38·5°C to <39°C; severe: ≥39·0°C. Feverish: Self-reported feeling of feverishness. For systemic symptoms, grading was classified as: Mild – easily tolerated with no limitation on normal activity; Moderate – some limitation of daily activity; Severe – unable to perform normal daily activity. Figure taken from Kelly E, et al. (2023).[1]

[1] Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.



Supplementary Figure 2. Severity of solicited local adverse reactions in days 0-7 after second vaccination by study arm and pre-second dose serostatus as self-reported in participant electronic diaries in the safety analysis population

BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373. The severity presented is the participant's highest severity across 7 days post vaccination for each solicited adverse event. Fever: Mild: 38·0°C to <38·5°C; moderate: 38·5°C to <39°C; severe: ≥39·0°C. Feverish: Self-reported feeling of feverishness. For systemic symptoms, grading was classified as: Mild – easily tolerated with no limitation

on normal activity; Moderate – some limitation of daily activity; Severe – unable to perform normal daily activity. Figure taken from Kelly E, et al. (2023).[1]

Supplementary Table 1. Summary of unsolicited adverse events up to 28 days post second dose by study arm

	BNT-30 (N=48)	**BNT-10 (N=47)**	**NVX (N=37)**	**Not randomised to receive second dose (N=16)**	**Overall (N=148)**
Number of adverse events	45	31	22	12	110
Number of unique participants with at least one adverse event*	24 (50.0%)	16 (34.0%)	13 (35.1%)	5 (31.3%)	58 (39.2%)
Timing					
Between first and second dose	25 (55.6%)	16 (51.6%)	4 (18.2%)	-	45 (40.9%)
Within 28 days after second dose	20 (44.4%)	15 (48.4%)	18 (81.8%)	-	53 (48.2%)
Did not receive a second dose	-	-	-	12 (100%)	12 (10.9%)
Severity					
Grade 1	24 (53.3%)	19 (61.3%)	11 (50.0%)	8 (66.7%)	62 (56.4%)
Grade 2	18 (40.0%)	9 (29.0%)	10 (45.5%)	4 (33.3%)	41 (37.3%)
Grade 3	3 (6.7%)	3 (9.7%)	1 (4.5%)	0 (0%)	7 (6.4%)
Causality					
No relationship	26 (57.8%)	22 (71.0%)	17 (77.3%)	4 (33.3%)	69 (62.7%)
Unlikely	12 (26.7%)	6 (19.4%)	2 (9.1%)	7 (58.3%)	27 (24.5%)
Possible	7 (15.6%)	1 (3.2%)	3 (13.6%)	1 (8.3%)	12 (10.9%)
Definite	0 (0%)	2 (6.5%)	0 (0%)	0 (0%)	2 (1.8%)

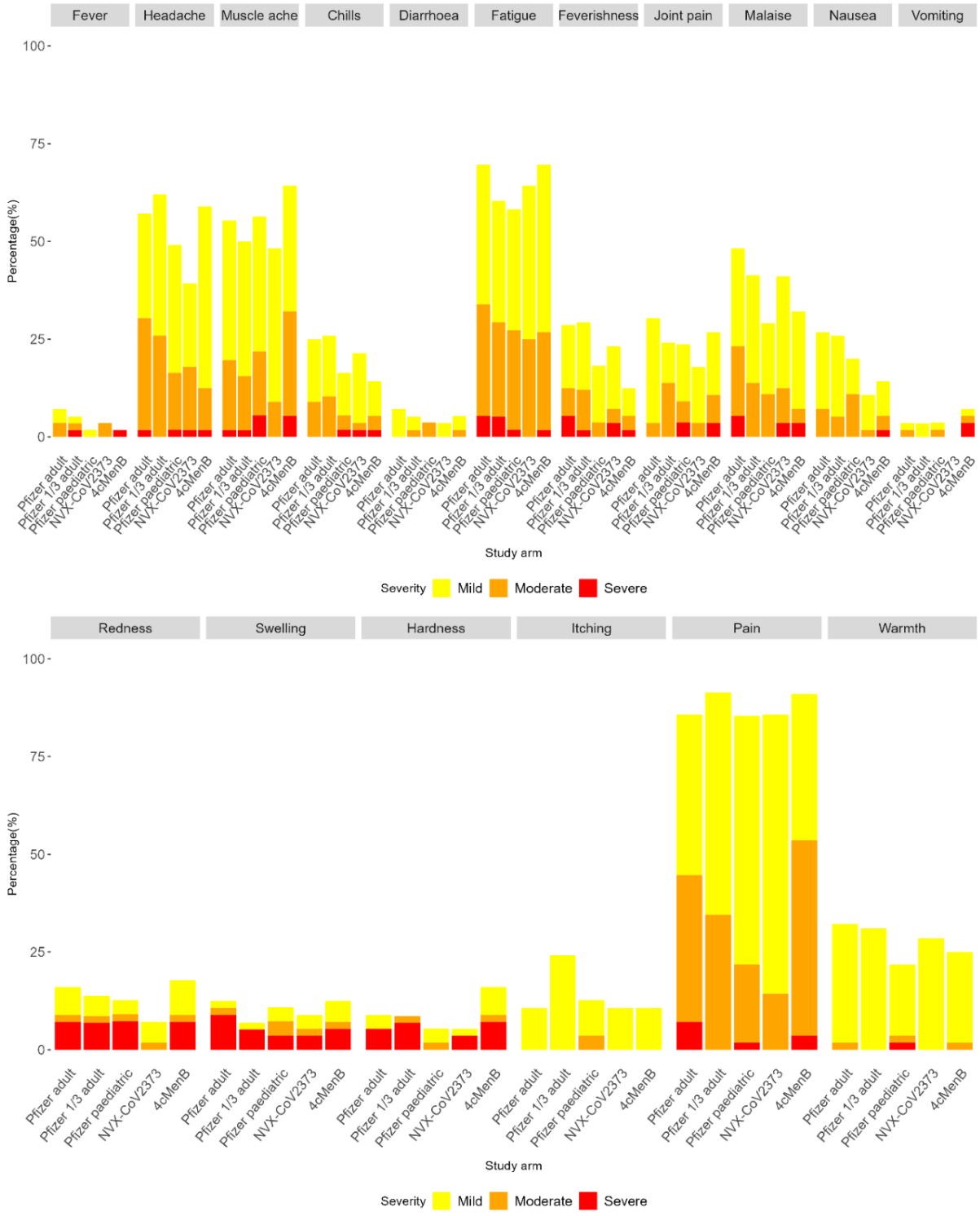
BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373.
Percentages are column percentages, and the denominator is the number of adverse events.
**Denominators are the number of participants in the study arm.*

Supplementary Table 2. Cohort A serious adverse events.

[1] Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.

Days since first dose	Days since second dose	Study arm	Description	Start date	Resolution date	Severity	Causality	SAE
177	121	BNT-30	Intentional self-harm	28/03/2022	29/03/2022	Grade 4	No relationship	Yes - hospitalisation
243*	185	NVX	Anorexia Nervosa	01/06/2022	Ongoing†	Grade 3	No relationship	Yes – important medical event

BNT-30: BNT162b2 30µg; NVX: NVXCoV2373.
First dose received in the community.
†Ongoing at end of study.

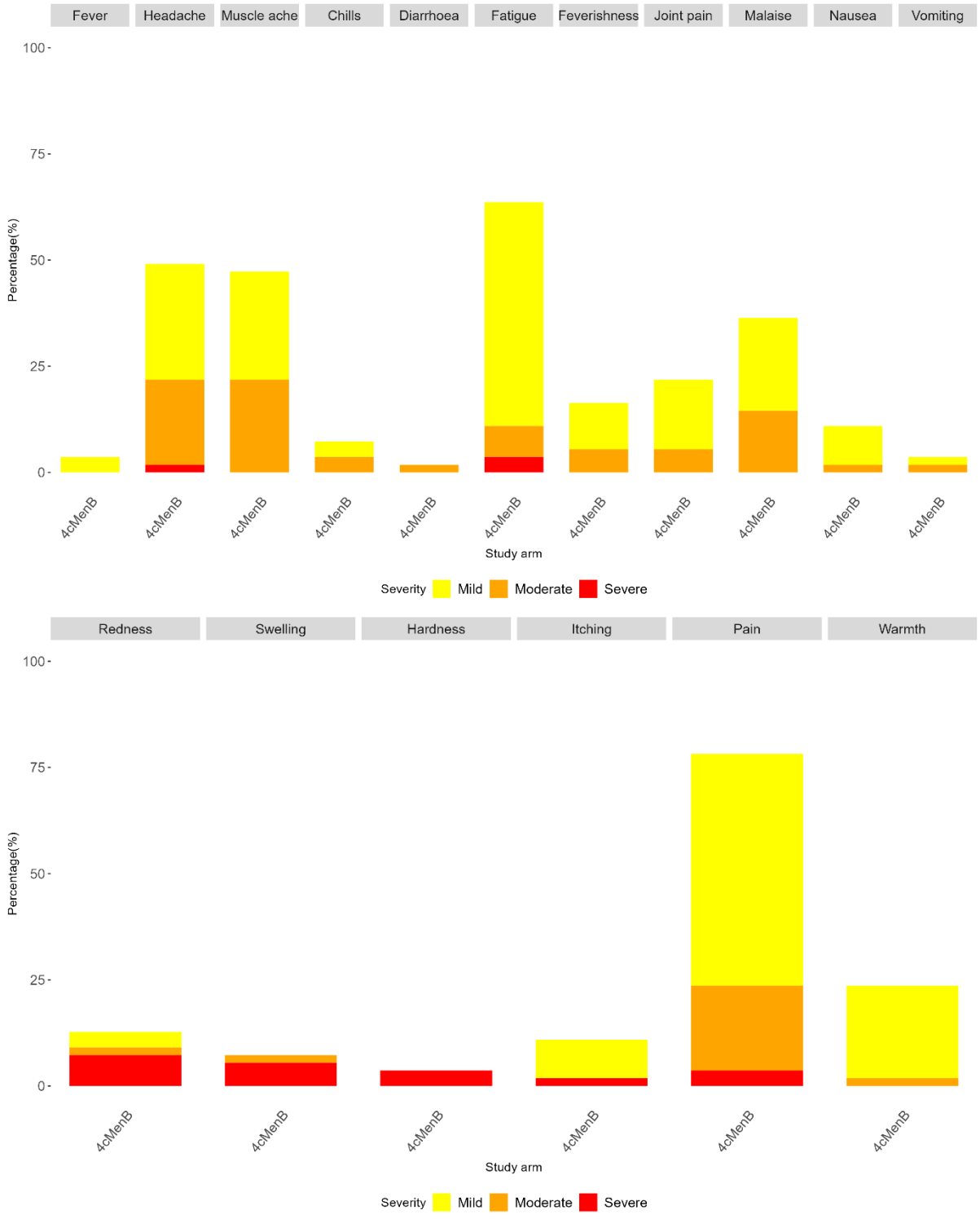


Supplementary Figure 3. Severity of solicited adverse reactions in days 0–7 after third vaccination by study arm as self-reported in participant electronic diaries in the safety analysis population

The severity presented is the participant's highest severity across 7 days following vaccination for each solicited adverse event. Fever: Mild: 38·0 °C to<38.5 °C; Moderate: 38.5 °C to<39 °C; Severe: ≥39.0 °C. Feverish: Self-reported feeling of feverishness. For systemic symptoms, grading was classified as Mild – easily tolerated with no limitation on normal activity; Moderate – some limitation of daily activity; Severe

– unable to perform the normal daily activity. There were no self-reported SARS-CoV-2 infections in days 0-7 after vaccination. Figure taken from Kelly E, et al. (2023).[1]

[1] Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.



Supplementary Figure 4. Severity of solicited adverse reactions in days 0-7 after bivalent COVID-19 vaccination in the 4CMenB study arm as self-reported in participant electronic diaries in the safety analysis population.

The severity presented is the participant's highest severity across 0-7 days post vaccination for each solicited adverse event. Fever: Mild: 38·0°C to <38·5°C; moderate: 38·5°C to <39°C; severe: ≥39·0°C. Feverish: Self-reported feeling of feverishness. There were no self-reported SARS-CoV-2 infections

reported within 0-7 days post vaccination for bivalent COVID-19 vaccination at day 182 visit. Figure taken from Kelly E, et al. (2023).[1]

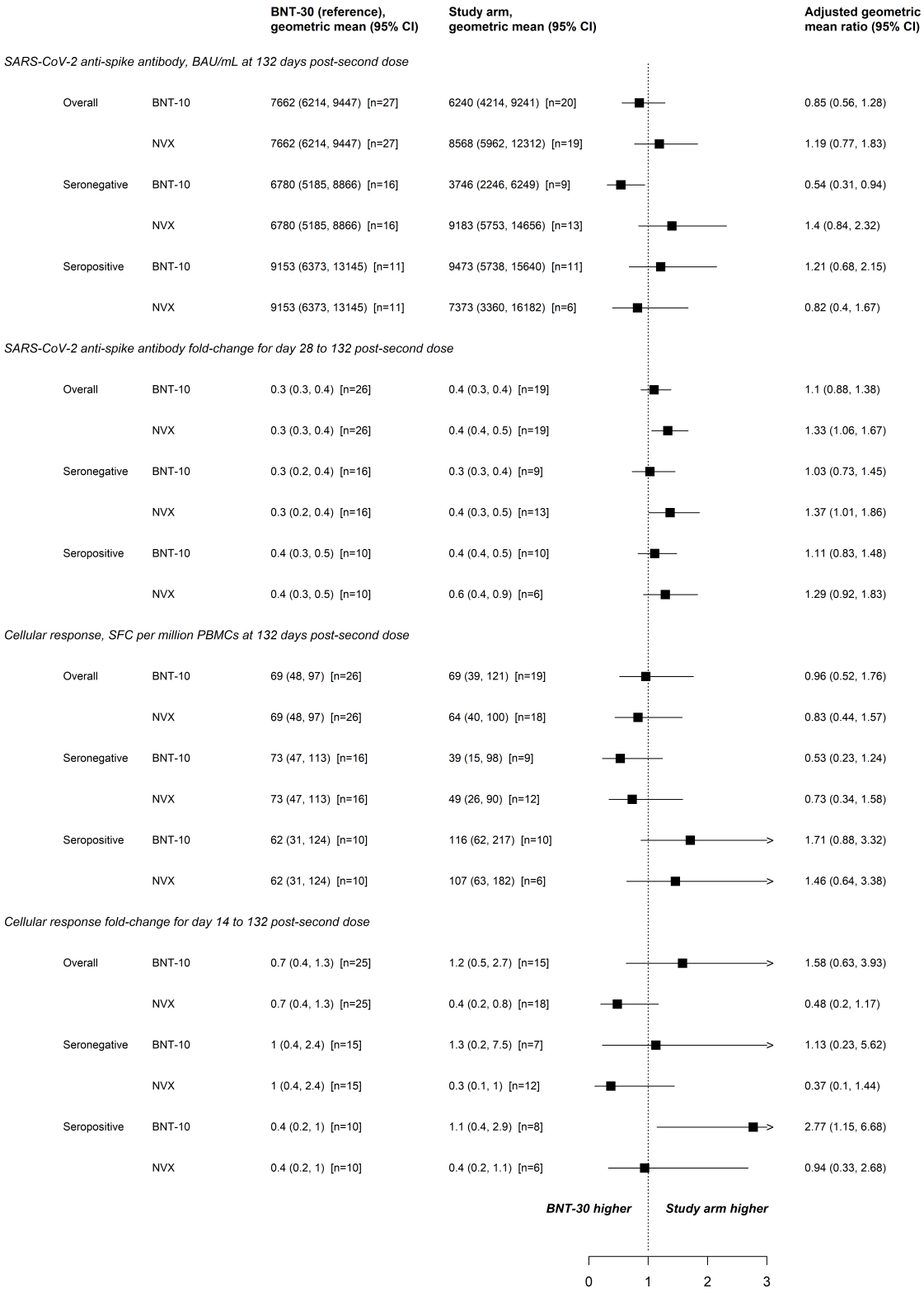
Supplementary Table 3. Summary of unsolicited adverse events up to 28 days post third vaccination by study arm in the safety analysis population

	Pfizer full dose adult formulation, N = 56	**Pfizer 1/3 dose adult formulation, N = 58**	**Pfizer full dose paediatric formulation, N = 55**	**NVX-CoV2373 full dose, N = 56**	**4CMenB, N = 56**	**Overall, N = 281**
Number of adverse events	29	37	47	23	46	182
Number of unique participants reporting at least one adverse event*	19	26	28	18	28	119
Days since boost vaccination, median (IQR)	13 (6, 21)	10 (3, 24)	16 (6, 25)	17 (9, 25)	28 (12, 185)	16 (7, 26)
Adverse event of special interest	2 (6.9%)	4 (11%)	5 (11%)	4 (17%)	8 (17%)	23 (13%)
Serious adverse event						
No	29 (100%)	36 (97%)	44 (94%)	23 (100%)	46 (100%)	178 (98%)
An important medical event	0 (0%)	0 (0%)	2 (4.3%)	0 (0%)	0 (0%)	2 (1.1%)
Hospitalisation	0 (0%)	1 (2.7%)	1 (2.1%)	0 (0%)	0 (0%)	2 (1.1%)
Outcome of serious adverse event						

[1] Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.

Ongoing	-	0 (0%)	1 (33%)	-	-	1 (25%)
Recovered	-	1 (100%)	2 (67%)	-	-	3 (75%)
Recovered with sequelae	-	0 (0%)	0 (0%)	-	-	0 (0%)
Severity						
Grade 1	17 (59%)	18 (49%)	26 (55%)	13 (57%)	20 (43%)	94 (52%)
Grade 2	11 (38%)	13 (35%)	11 (23%)	5 (22%)	22 (48%)	62 (34%)
Grade 3	1 (3.4%)	6 (16%)	10 (21%)	5 (22%)	4 (8.7%)	26 (14%)
Causality						
No relationship	17 (59%)	22 (59%)	28 (60%)	12 (52%)	21 (46%)	100 (55%)
Unlikely	9 (31%)	9 (24%)	11 (23%)	8 (35%)	18 (39%)	55 (30%)
Possible	1 (3.4%)	2 (5.4%)	6 (13%)	2 (8.7%)	3 (6.5%)	14 (7.7%)
Probable	2 (6.9%)	4 (11%)	2 (4.3%)	1 (4.3%)	4 (8.7%)	13 (7.1%)
Ongoing at end of study						
No	29 (100%)	37 (100%)	45 (96%)	23 (100%)	45 (98%)	179 (98%)
Yes	0 (0%)	0 (0%)	2 (4.3%)	0 (0%)	1 (2.2%)	3 (1.6%)

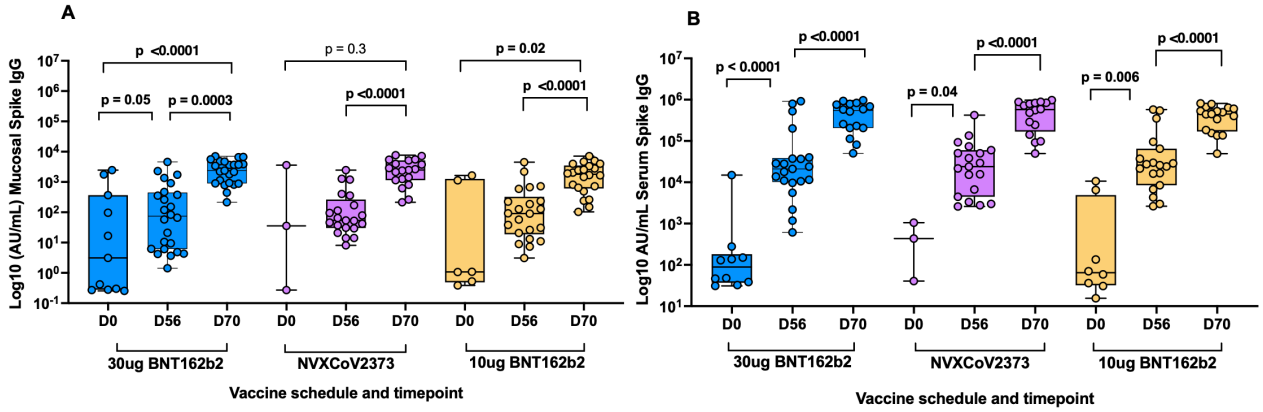
Percentages are column percentages, and the denominators are the number of adverse events.
**Denominators are the number of participants in the study arm. Adverse events include those occurring within 28 days of Bivalent COVID-19 vaccine at day 182 visit in the 4CMenB study group.*



Supplementary Figure 5. Immune responses by study arm and pre-second dose serostatus in the day 132 modified intention-to-treat populations excluding 'breakthrough infections' during follow-up

BNT-30: BNT162b2 30µg; BNT-10: BNT162b2 10µg; NVX: NVX-CoV2373; CI: confidence interval. Data presented are the geometric means, adjusted geometric mean ratios and their corresponding 95% confidence intervals. Fold-changes were calculated by dividing the immune response at either 132 or 236 days following second dose by that at 28 days following second dose. The boxes indicate the adjusted

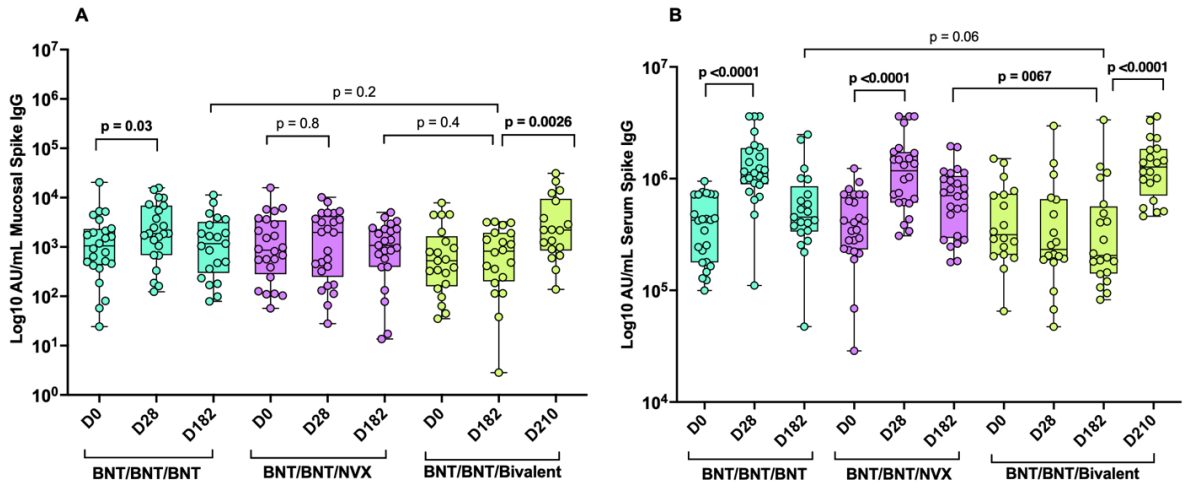
geometric mean ratio, and the horizontal lines indicate the corresponding 95% confidence intervals. The geometric mean ratios between BNT-30 and either BNT-10 or NVX are adjusted for study site as a fixed effect. The vertical dotted line refers to an adjusted geometric mean ratio of one and indicates the line of no difference. A confidence interval that lies completely to one side and not intersecting the line of no difference indicates a significant difference in the geometric mean concentrations between the study arm and the reference BNT-30 study arm. A 'breakthrough infection' between second dose and day 236 visit was defined as either: a self-reported SARS-CoV-2 infection >14 days after second dose, a two-fold rise in anti-nucleocapsid IgG from second dose to 132 days after second dose or from 132 to 236 days after second dose, a two-fold rise in anti-spike antibodies from 28 to 132 days after second dose or 132 to 236 days after second dose, or a seroconversion of anti-nucleocapsid IgG from second dose to day 132 days after second dose or 132 to 236 days after second dose. Figure taken from Kelly E, et al. (2023).[1]



Supplementary Figure 6. (A) Mucosal spike-specific IgG titres following homologous and heterologous second dose COVID-19 vaccination (B) Serum spike-specific IgG titres following homologous and heterologous second dose COVID-19 vaccination

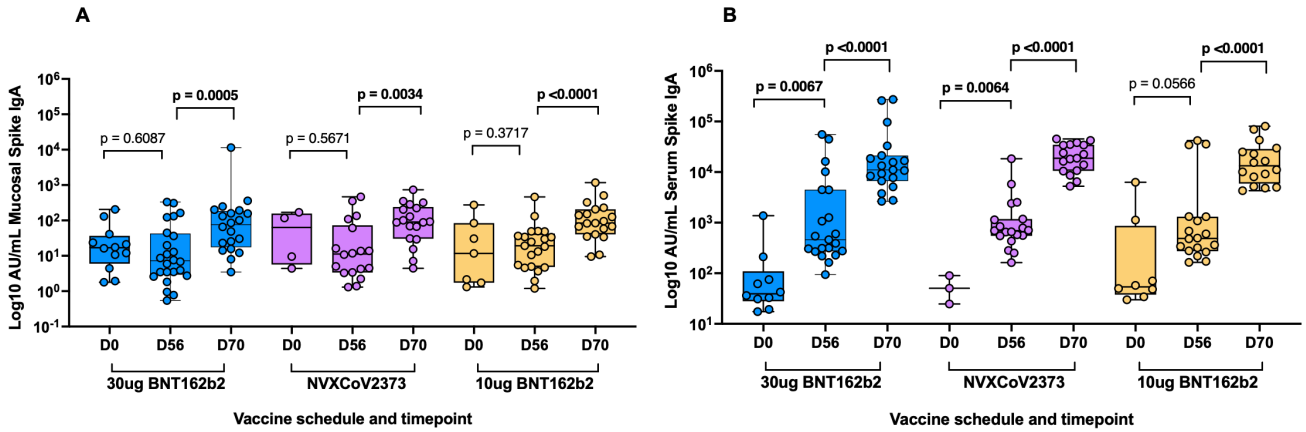
All participants received 30 µg BNT162b2 as the first dose; the second dose received is indicated in the figure. Mucosal spike-specific IgG levels were measured at baseline (Day 0), post-prime (Day 56), and post-second dose (Day 70). Data are presented as median with interquartile range. Data are presented as median with interquartile ranges. AU = Arbitrary Units.

[1] Kelly E, Greenland M, de Whalley PCS, et al. Reactogenicity, immunogenicity and breakthrough infections following heterologous or fractional second dose COVID-19 vaccination in adolescents (Com-COV3): A randomised controlled trial. *Journal of Infection* 2023; **87**(3): 230-41.



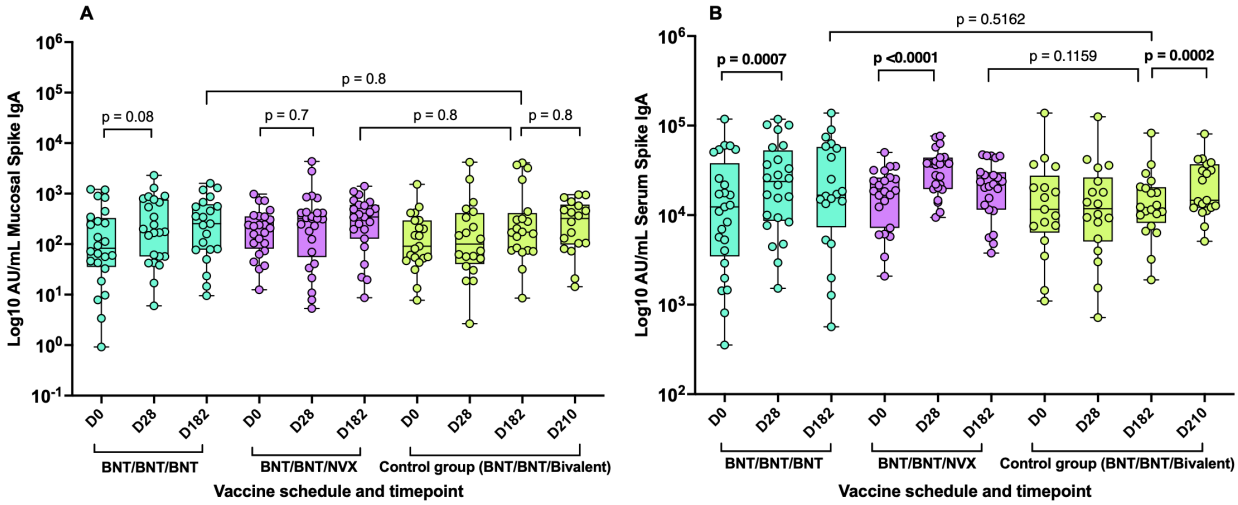
Supplementary Figure 7. (A) Mucosal SARS-CoV-2 spike IgG responses following homologous and heterologous third dose vaccination, (B) Serum SARS-CoV-2 spike IgG responses following homologous and heterologous third dose vaccination

BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.



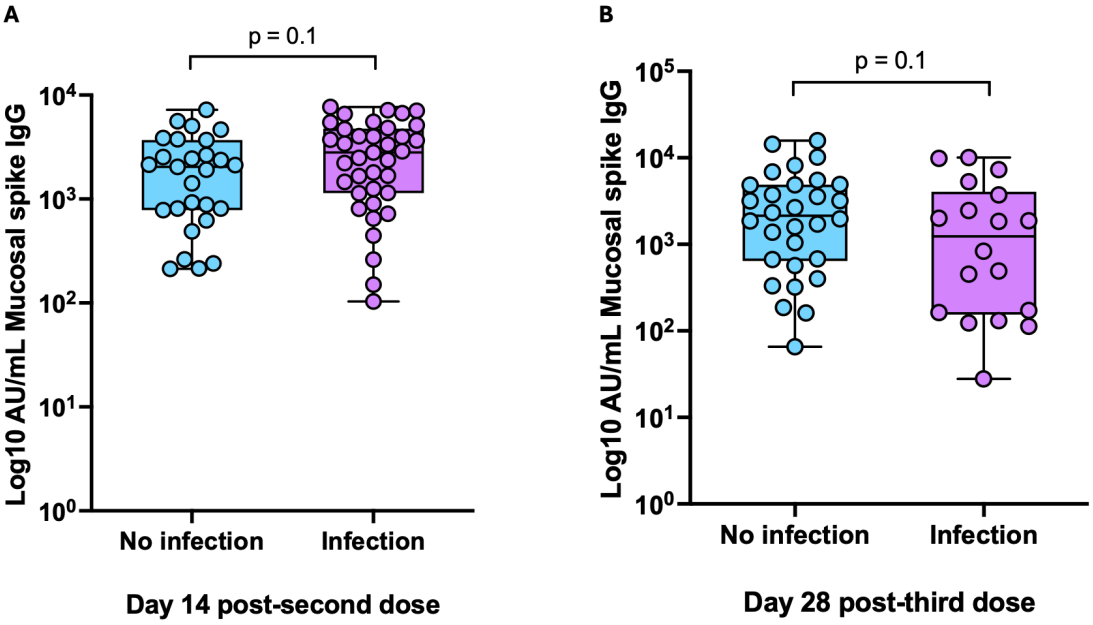
Supplementary Figure 8. Mucosal SARS-CoV-2 spike IgA response following homologous and heterologous second dose vaccination, (B) Serum SARS-CoV-2 spike IgA response following homologous and heterologous second dose vaccination

All participants received 30µg BNT162b2 as their first dose. AU = Arbitrary Units.



Supplementary Figure 9. (A) Mucosal SARS-CoV-2 spike IgA response following homologous and heterologous third dose vaccination, (B) Serum SARS-CoV-2 spike IgA response following homologous and heterologous third dose vaccination

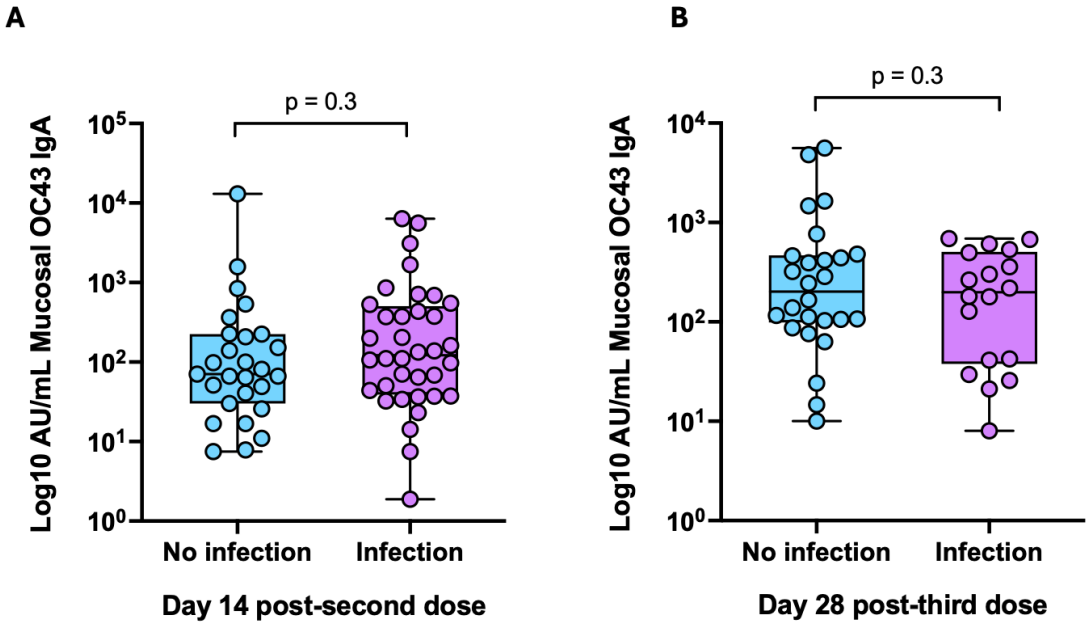
All participants received two-dose 30µg BNT162b2 as their primary vaccine series. BNT = BNT162b2, NVX = NVXCoV2373, Bivalent = bivalent vaccine (Original/Omicron BA.1). AU = Arbitrary Units.



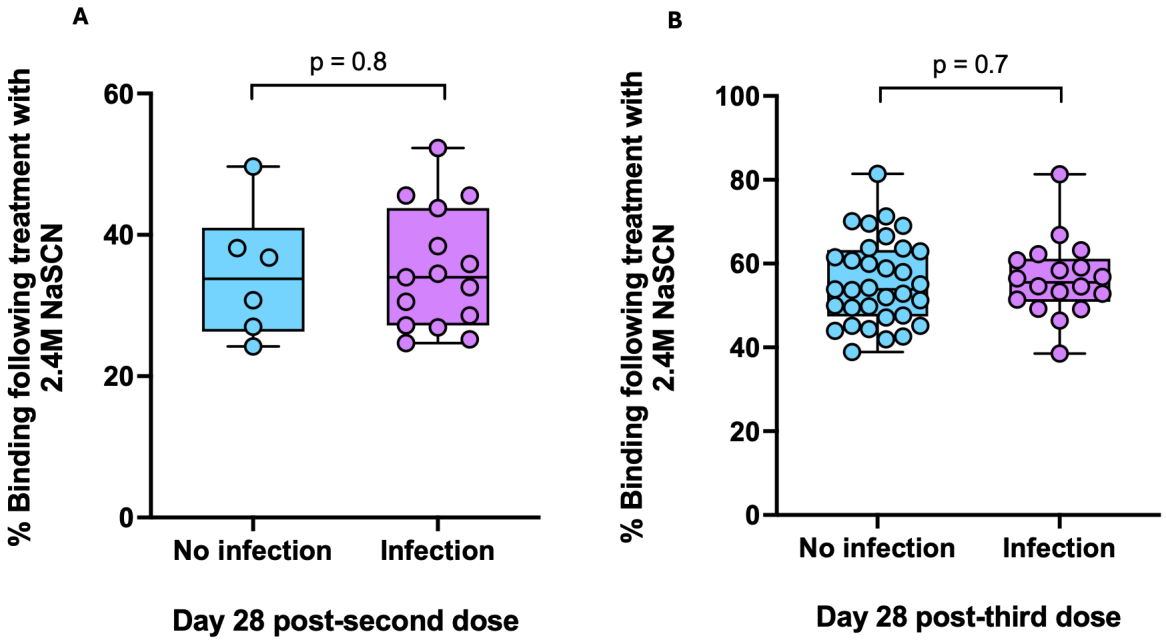
Supplementary Figure 10. Mucosal SARS-CoV-2 spike IgG titres 14 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.

Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included

in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. AU = arbitrary units.

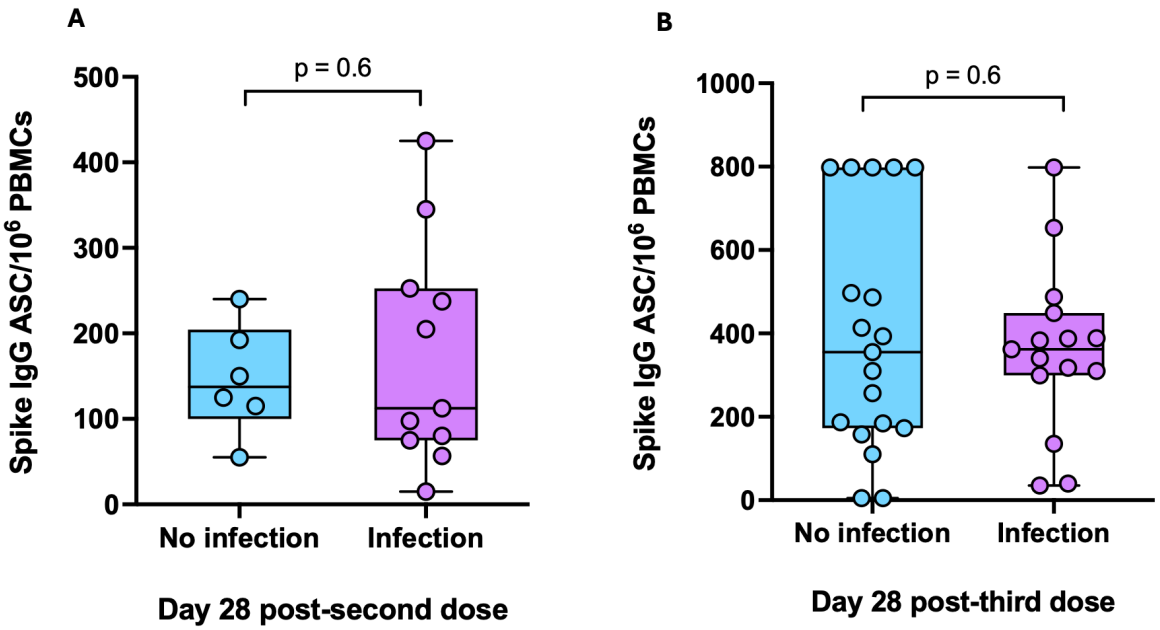


Supplementary Figure 11. Mucosal hCoV OC43 spike IgA titres 14 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.
Cohort A includes participants who received either 30µg BNT162b2, 10µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. AU = arbitrary units.



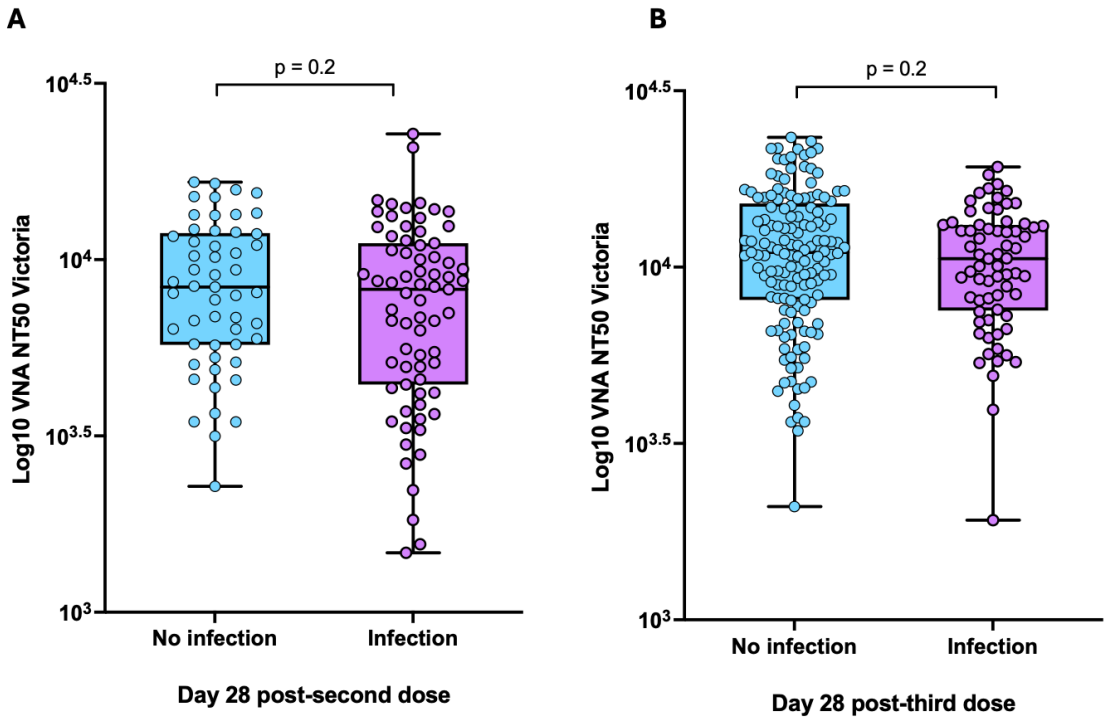
Supplementary Figure 12. Anti-spike IgG antibody avidity 28 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.

Cohort A includes participants who received either 30µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. NaSCN = sodium thiocyanate; M = molarity (concentration of sodium thiocyanate used to assess avidity).

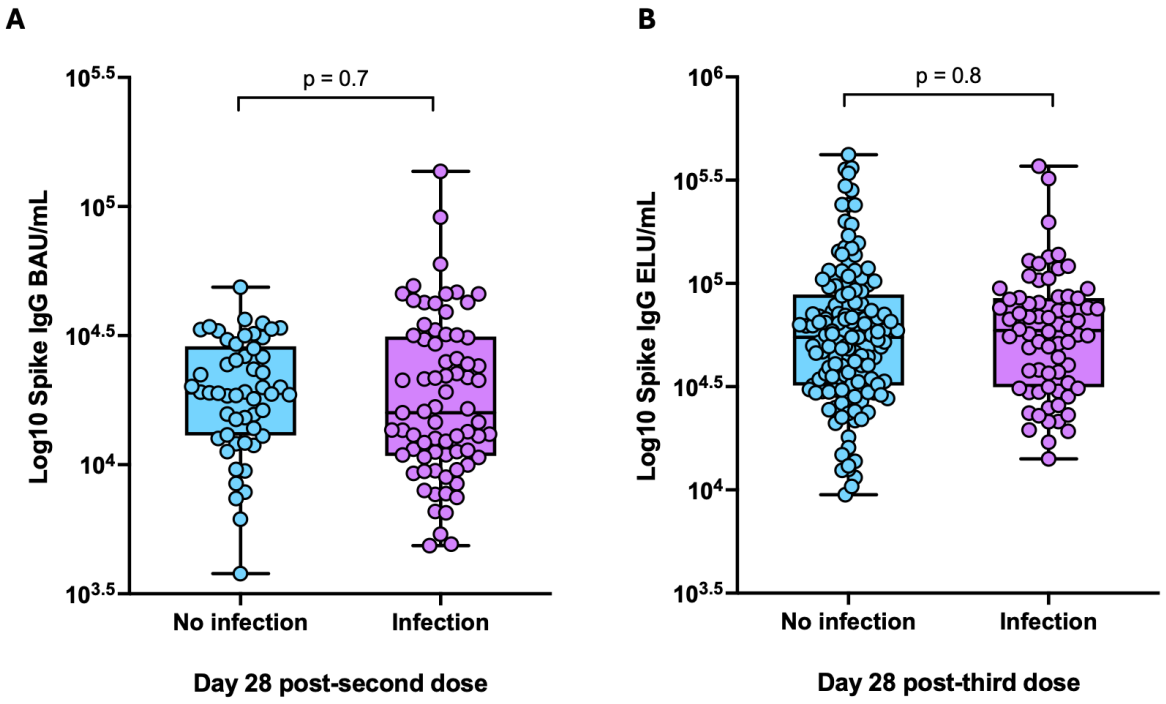


Supplementary Figure 13. SARS-CoV-2 spike-specific memory B cells 28 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.

Cohort A includes participants who received either 30µg BNT162b2 or NVXCoV2373 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2 or NVXCoV2373 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination. PBMCs = peripheral blood mononuclear cells.



Supplementary Figure 14. Neutralising antibodies against Victoria (wild-type) SARS-CoV-2 28 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.
Cohort A includes participants who received either 30µg BNT162b2, NVXCoV2373 or 10µg BNT162b2 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2, NVXCoV2373, or 10µg BNT162b2 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination.



Supplementary Figure 15. Total binding antibodies against SARS-CoV-2 spike protein (wild-type) 28 days after a two-dose primary vaccine series (Cohort A) and 28 days after a third (booster) dose (Cohort B) according to breakthrough infection status following vaccination.

Cohort A includes participants who received either 30µg BNT162b2, NVXCoV2373 or 10µg BNT162b2 as their second dose (A). Third dose vaccination refers to participants who received either 30µg BNT162b2, NVXCoV2373, or 10µg BNT162b2 as their third dose (B). Infection = participants with evidence of SARS-CoV-2 infection >14 days after the second dose (Cohort A) and >28 days after the third dose (Cohort B). No infection = Participants who did not develop SARS-CoV-2 infection after vaccination. All participants were included in the analysis, irrespective of baseline serostatus or SARS-CoV-2 infection history prior to vaccination.