Protocol

Protocol for: Regev-Yochay G, Gonen T, Gilboa M, et al. Efficacy of a fourth dose of Covid-19 mRNA vaccine against omicron. N Engl J Med. DOI: 10.1056/NEJMc2202542

This trial protocol has been provided by the authors to give readers additional information about the work.

Supplement

This supplement contains the following items:

1. Original protocols:
    a. Protocol 8980-21 version 2 (Version 1 is the Hebrew version)
    b. Protocol 8980-21 version 4
    c. Protocol 9035-21 version 1
    d. Protocol 9035-21 version 2
    e. Final combined protocol
    f. Summary of changes
2. Original statistical analysis plan, summary of additional statistical analysis

The original study design was a 2-intervention arm open labeled, non randomized trial assessing immunogenicity of a fourth dose of BNT162b2 or mRNA1273 with a matched control group. In Israel, vaccine rollout was almost solely carried out with BNT162b2 and mixing of different vaccine types was discouraged. Initially, in order to get a rapid approval from the regulatory authorities, we submitted a single intervention arm protocol with BNT162b and only later, we submitted the second intervention arm protocol.

The final protocol, is the combined protocol of the two protocols: of study 8980-21 (version 4) and study 9035-21 (version 2), which were approved separately.

The original protocol, Protocol 8980-21, version 1, dated Nov. 29, 2021, included only the BNT162b2 arm, compared to the matched control group was in Hebrew and version 2 (Dated Dec 15, 2021) is its translation to English.

Protocol 8980-21 version 2, Dec 15, 2021, is thus attached. Changes from version 2 to version 4 are summarized below.

The original protocol, Protocol 9035-21 (version 1), Dated Dec 30, 2021, included only the mRNA1273 arm, compared to the matched control group, is attached. Changes from version 1 to 2 are summarized.

At the end of this supplement the summary of changes from the separate two protocols to the combined protocol are noted.

Fourth COVID-19 vaccine dose

Research Protocol – Appendix A

Background: The Omicron variant of concern (VOC) is currently rapidly spreading worldwide, with extremely high transmission rates, with an estimated R of >3 (Barnard et al., n.d.; Khoury et al., n.d.) . While the virulence of this VOC is yet unknown, even with a much lower virulence than the delta strain, with such high transmission, once again the pandemic will have severe effects on healthcare capacity. Thus, further effort to contain this VOC is required. Currently, the only successful key in containment of the SARS-CoV-2 pandemic was vaccination. Following the first two doses, extremely high vaccine effectiveness was reported from many real-world observational studies (Amit et al., 2021; Dagan et al., 2021; Haas et al., 2021). Several vaccines were reported with high vaccine efficacy and effectiveness, mostly, the mRNA vaccines, of which the BNT162b2 was rolled out in Israel. Yet, with time, waning of this effect was reported, both in immunogenicity as well as in vaccine effectiveness against infections and hospitalizations (Bar-On et al., 2021; Levin et al., 2021)

Following these reports, Israel initiated rollout of the third dose of BNT162b2 on July 29, initially to adults 60 years and older and within a few weeks expanded it to the whole population if at least 5 months have passed from the second dose. We have recently reported the early effects of the 3rd dose(Gilboa et al., 2021) , showing significant increase in IgG and Neutralizing antibodies, as well as increased T-cell activity in a small group of non-responders to first two doses. We have also submitted further data, showing the superiority of the third dose in aspects of immunogenicity, with significantly higher amounts of antibodies as well as improved quality as measured by higher avidity and neutralizing capacity (see yet unpublished data Appendix A1).

We now have preliminary, yet unpublished data, showing slow waning of the third dose immune response within 4 months after the third dose (Appendix A2). Simultaneously, Milo R. et al presented yet unpublished data on early waning of vaccine effectiveness of the third dose. While these data would not have been worrisome in the Delta VOC era, this may be different with the emergence of the Omicron VOC.

It was recently reported, by us and others that vaccine effectiveness against the Omicron VOC is decreased. This was shown by reduced neutralization efficiency of Omicron VOC compared to that of the Delta VOC or the wild type (Wu-1), by sera of vaccinated individuals. We have just reported (Nemet et al., n.d.) , accepted to NEJM) that sera from BNT162b 2-dose vaccinated individuals does not neutralize Omicron at all, while it neutralizes to a low level the Delta VOC and more so the Wu-1. We compared this to 3-dose vaccinated, and also showed a significantly reduced neutralization of early 3-dose vaccinated as compared to the efficiency against Wu-1

or the delta VOC. Furthermore, data from Germany showed that neutralization of Omicron decreases within 3 months following the 3rd dose.

These data raise the question of when and will we need a 4th dose do cope with the emergence of Omicron. However, if we have reached the maximal effect of the current vaccine against Omicron, with a third dose, will a 4th dose have any added value? In this respect, several studies have demonstrated improved immunogenicity of 1 vaccine dose to SARS-CoV-2 recovered patients, yet a 2nd dose to these patients had very little added value. It is thus unclear whether a 4th dose of the current vaccine, which is not directed specifically at the Omicron VOC, can further improve our immunity and protection from this strain, and will it have any added value.

The Sheba HCW COVID Cohort was established on April 2020, when HCW were recruited to a large serology longitudinal follow up study. Since then, over 7000 HCW who were vaccinated by BNT162b2 are being followed monthly with blood sampling for immune responses, including mostly IgG, but also Neutralizing ab, avidity, microneutralization, IgA and cellular activation. Through this cohort we found the correlation between neutralizing ab levels and SARS-CoV-2 infections (Bergwerk et al., 2021) , the waning of immunity (Levin et al., 2021) and more.

Here, we will study the potential immunogenicity of a 4th dose, together with assessing safety and effectiveness in preventing infections.

Study Aims:

To assess the immunogenicity of a 4th dose, and its durability. This will be measured by following IgG, IgA, Pseudoneutralization assays, microneutralization, avidity, T-cell activity and B-cell repertoire. We will also assess safety and vaccine effectiveness by following incidence of SARS-CoV-2 infections.

Study design:

This is a prospective intervention study, to test the effect of a 4th dose, by comparing the immune response before and after the 4th dose, given to 150-200 volunteers, as well as comparing their responses with a control group of individuals vaccinated with 3 doses but without the 4th. For this study, we will recruit 150-200 volunteers, who received the 3rd dose at least 4 months previously, and have a known serology history (showing an immune response (even if just a low response) to the three previous doses, but with a recent relatively low IgG (below 700 BAU, or lower than 512 IU PNT50)). Volunteers will tested before and after vaccination with a 4th dose, and followed for 180 days. As controls, a sub-cohort of similar HCW, who are not receiving the 4th dose will be followed similarly.

On recruitment, volunteer will:

1. Receive a detailed explaination and sign the informed consent form (appendix ICF)
2. Fill a general comorbidity questionnaire (Appendix A-Q1)
3. Have up to 40cc blood drawn for all serology and cellular immunity tests.
4. Perform a PCR for SARS-CoV-2 test
5. Receive the 4th dose of BNT162b2 30µg.

Inclusion Criteria:

- Adults over age 18 y
- Received the 3rd BNT162b2 dose at least 4 months previously.
- Have a serology test within the previous 3 months of 700 BAU or less.
- Responded to the previous vaccine doses, i.e. at least one IgG>100.
- Agreed to attend all visits and signed the informed consent

Exclusion Criteria:

- Had previous SARS-CoV-2 infection (detected by either PCR, anti-S IgG before the 1st vaccine dose, anti-N IgG at any stage).
- Had an allergic response to a previous BNT162b2 dose.
- Have history of myopericarditis.

Control group: Will consist of a subgroup of the Sheba HCW COVID Cohort, who will match the volunteers for the 4th dose, by age, gender, time since 3rd dose and serology status (i.e. <700 IgG but at least one measure >100). This sub-cohort consists of >700 HCW.

Primary outcomes:

Serology tests as detailed below, including IgG, pseudoneutralization, microneutralization, avidity and IgA.

Adverse event reporting of vaccinated individuals by an electronic survey that will be filled from visit 2. This will be compared to adverse event reporting of individuals previously reporting after the 3rd dose from the Sheba HCW COVID Cohort, matched by gender and age. Adverse event questionnaire – Appendix A-Q2

Secondary outcomes:

T-cell activity and B-cell repertoire as described below.

SARS-CoV-2 incidence and specifically Omicron VOC incidence.

Detailed Serological and immunological assays:

1. **IgG antibody assay** – Using a commercially available test (SARS-CoV-2 IgG II Quant (Abbott, IL, USA)) we will measure the quantity of IgG antibodies against the SARS-CoV-2 receptor binding domain (RBD). This commercial test will be performed according to manufacturer's instructions
2. **Avidity –** to measure the quality of IgG antibodies we will use urea as a chaotropic reagent and test the strength of interaction between the IgG and the viral antigen (the RBD). Specifically, a 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4°C with 50µl per well of 1µg/ml of RBD antigen. After blocking with 5% skimmed milk at 25°C for 60 minutes, serum samples will be diluted 1:100, 1:400 and 1:1000 with 3% skimmed milk and added to antigen coated wells. The plate will be incubated at 25°C for 120 minutes, and following washing each sample,incubated either with the addition of 6M urea or PBS for 10 min. After washing, a goat anti-human IgG horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, PA, USA Code: 109-035-088) (diluted 1:15000) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will be measured at 450nm using a micro-plate reader (Sunrise, Tecan). Avidity index will be calculated as the ratio (in percentage) between sample OD with 6M urea and sample OD with PBS.
3. **SARS-CoV-2 Pseudovirus (psSARS-2) Neutralization Assay –** to test the overall neutralizing ability of each serum against the WT virus and specifically to compare with neutralizing levels of the SHEBA HCW following one, two and three vaccine doses we will use Pseudovirus (psSARS-2) Neutralization. SARS-CoV-2 Pseudo-virus (psSARS-2) Neutralization Assay will be performed using a propagation-competent VSV-spike which was shown to be highly correlative to authentic SARS-CoV-2 virus micro-neutralization assay. Following titration, 100 focus forming units (ffu) of psSARS-2 will be incubated with 2-fold serial dilution of heat inactivated (56°C for 30 min) tested sera. After incubation for 60 min at 37°C, virus/serum mixture will be transferred to Vero E6 cells that have been grown to confluency in 96-well plates and incubated for 90 min at 37°C. After the addition of 1% methyl cellulose in dulbecco's modified eagle's medium (DMEM) with 2% of fetal bovine serum (FBS), plates will be incubated for 24hr and 50% plaque reduction titer will be calculated by counting green

fluorescent foci using a fluorescence microscope. Sera not capable of reducing viral replication by 50% at 1 to 16 dilution or below will be considered non- neutralizing.

4. **SARS-CoV-2 micro-neutralization assay-** to compare the neutralizing capacity of omicron and delta variants following the forth vaccine dose a SARS-CoV-2 micro-neutralization assay with live virus will be performed. VERO-E6 cells at concentration of 20*103/well will be seeded in sterile 96-wells plates with 10% FCS MEM-EAGLE medium, and stored at 37^{o}C for 24 hours. One hundred TCID50 of Wild Type, Beta, Delta and Omicron SARS-CoV-2 isolates will be incubated with inactivated sera diluted 1:10 to 1:16,384 in 96 well plates for 60 minutes at 33ºC. Virus-serum mixtures will be added to the Vero E-6 cells and incubated for five days at 33ºC after which Gentian violet staining (1%) will be used to stain and fix the cell culture layer. Neutralizing dilution of each serum sample will be determined by identifying the well with the highest serum dilution without observable cytopathic effect. A dilution equal to 1:10 or above will be considered neutralizing.
5. **Memory immune response -** To investigate the memory response we will isolate peripheral blood mononuclear cell (PBMC) using Ficoll density gradient centrifugation and analyze T and B cells for T cell activation and antibody repertoire, respectively:
    a. T cell activation will be assessed by IFN-γ ELISpot assay. Specifically, IFN- γ -secreting cells will be enumerated using Elispot IFN-γ kits (IFN-γ kit, AID Autoimmun Diagnostika GmbH, Strassberg, Germany) according to manufacturer instructions. For antigen stimulation, 50 µl of SARS-CoV-2 peptide pools (S-complete, Miltenyi Biotech) will be used. Test medium will be used as negative control and Phytohaemagglutinin (PHA) will be used as positive control. IFN-γ-secreting cells frequency will be quantified using the AID ELISpot Reader (Strassberg, Germany). The unspecific background (mean SFU from negative control wells) will be subtracted from experimental readings.
    b. We will stimulate T cells by SARS-CoV-2 peptides to analyze the specific activation of CD4 and CD8 T cells in each cohort by flow cytometry using fluorescent probes.
    c. The frequency and specificity of SARS coV-2 specific memory B cells will be tested by flow cytometry using fluorescent probes corresponding to spike protein from different variants. Probe-binding B cells will be sorted by flow cytometry and single-cell immunoglobulin and whole transcriptome

sequencing will be performed. From the sequencing reactions, constructs will be made to express and test functionality of SARS-CoV-2- specific monoclonal antibodies, including binding, neutralization and affinity.

Benefits of participating in the study:

The potential benefit of improved protection from SARS-CoV-2. Currently, according the effectiveness of the previous doses, and in the absence of effective drug therapy, it is expected that an additional dose will raise the level of antibodies and thus raise the protection from SARS-CoV-2.

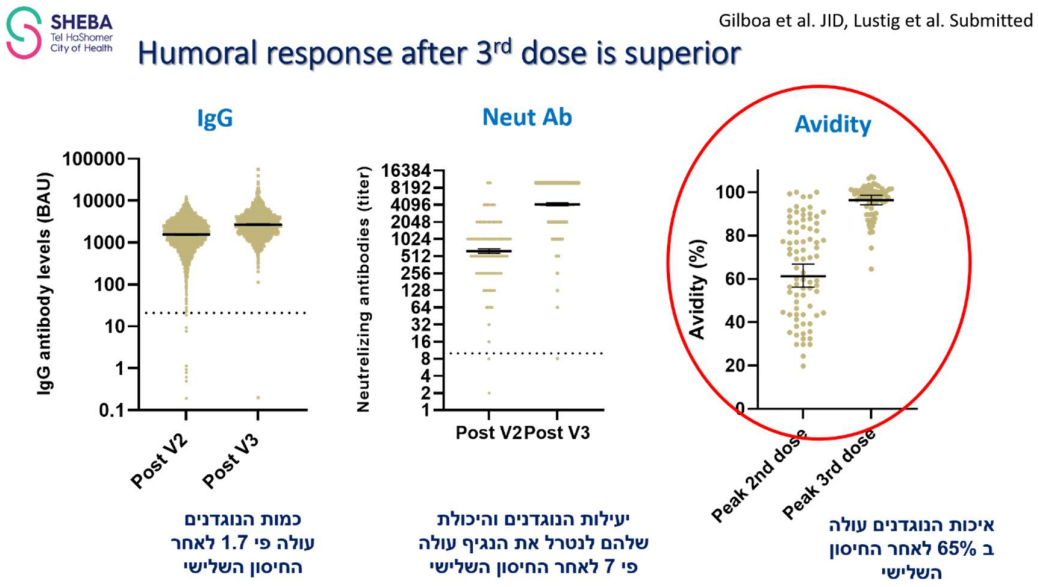
Risks in participating in the study:

The known previous adverse events are expected to occur, these included frequent local reactions (mostly local pain), as well as systemic reactions including fatigue, fever, lymphadenopathy, myalgia, these have typically lasted up to 48 hours. Rarely, myocarditis has occurred (1:6,000-24,000, mostly in young men). It is yet unknown if a 4th dose will have additional adverse reactions.

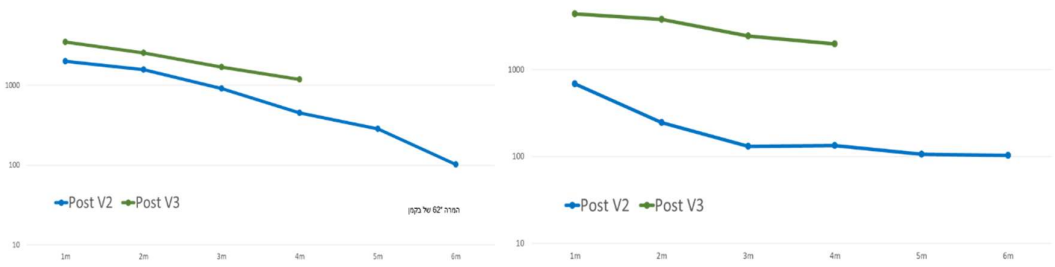
Research Timeline:

Visit number		1	2	3	4	5	6	7
	Day	0	7	14	21	60	90	180
Vaccine	BNT162b2 30 µg	X						
Blood sample	IgG	X	X	X	X	X	X	X
	PNT	X	X	X	X	X	X	X
	microneutralization	X	X	X	X	X	X	X
	IgA	X	X	X	X	X	X	X
	Tcell activity	X		X		X		X
	Bcell repertoir	X		X		X		X
Nasal/NP swab	SARS-CoV-2 PCR	X	X	X	X	X	X	X
Questionnaires	Background comorbidity	X						
	Adverse events			X		X		

Appendix A-1 – superiority of 3rd dose (submitted for publication)



Appendix A2 – Waning immune response after the third dose (preliminary unpublished data)



IgG Neutralizing Ab

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Fourth BNT162b2 COVID-19 vaccine dose

Research Protocol

Background: The Omicron variant of concern (VOC) is currently rapidly spreading worldwide, with extremely high transmission rates, with an estimated R of >3 (Barnard et al., n.d.; Khoury et al., n.d.) . While the virulence of this VOC is yet unknown, even with a much lower virulence than the delta strain, with such high transmission, once again the pandemic will have severe effects on healthcare capacity. Thus, further effort to contain this VOC is required. Currently, the only successful key in containment of the SARS-CoV-2 pandemic was vaccination. Following the first two doses, extremely high vaccine effectiveness was reported from many real-world observational studies(Amit et al., 2021; Dagan et al., 2021; Haas et al., 2021). Several vaccines were reported with high vaccine efficacy and effectiveness, mostly, the mRNA vaccines, of which the BNT162b2 was rolled out in Israel. Yet, with time, waning of this effect was reported, both in immunogenicity as well as in vaccine effectiveness against infections and hospitalizations (Bar-On et al., 2021; Levin et al., 2021)

Following these reports, Israel initiated rollout of the third dose of BNT162b2 on July 29, initially to adults 60 years and older and within a few weeks expanded it to the whole population if at least 5 months have passed from the second dose. We have recently reported the early effects of the 3rd dose(Gilboa et al., 2021) , showing significant increase in IgG and Neutralizing antibodies, as well as increased T-cell activity in a small group of non-responders to first two doses. We have also submitted further data, showing the superiority of the third dose in aspects of immunogenicity, with significantly higher amounts of antibodies as well as improved quality as measured by higher avidity and neutralizing capacity (see yet unpublished data Appendix A1).

We now have preliminary, yet unpublished data, showing slow waning of the third dose immune response within 4 months after the third dose (Appendix A2). Simultaneously, Milo R. et al presented yet unpublished data on early waning of vaccine effectiveness of the third dose. While these data would not have been worrisome in the Delta VOC era, this may be different with the emergence of the Omicron VOC.

It was recently reported, by us and others that vaccine effectiveness against the Omicron VOC is decreased. This was shown by reduced neutralization efficiency of Omicron VOC compared to that of the Delta VOC or the wild type (Wu-1), by sera of vaccinated individuals. We have just reported (Nemet et al., n.d.) , accepted to NEJM) that sera from BNT162b 2-dose vaccinated individuals does not neutralize Omicron at all, while it neutralizes to a low level the Delta VOC and more so the Wu-1. We compared this to 3-dose vaccinated, and also showed a significantly reduced neutralization of early 3-dose vaccinated as compared to the efficiency against Wu-1

or the delta VOC. Furthermore, data from Germany showed that neutralization of Omicron decreases within 3 months following the 3rd dose.

These data raise the question of when and will we need a 4th dose to cope with the emergence of Omicron. However, if we have reached the maximal effect of the current vaccine against Omicron, with a third dose, will a 4th dose have any added value? In this respect, several studies have demonstrated improved immunogenicity of 1 vaccine dose to SARS-CoV-2 recovered patients, yet a 2nd dose to these patients had very little added value. It is thus unclear whether a 4th dose of the current vaccine, which is not directed specifically at the Omicron VOC, can further improve our immunity and protection from this strain, and will it have any added value.

The Sheba HCW COVID Cohort was established on April 2020, when HCW were recruited to a large serology longitudinal follow up study. Since then, over 7000 HCW who were vaccinated by BNT162b2 are being followed monthly with blood sampling for immune responses, including mostly IgG, but also Neutralizing ab, avidity, microneutralization, IgA and cellular activation. Through this cohort we found the correlation between neutralizing ab levels and SARS-CoV-2 infections (Bergwerk et al., 2021) , the waning of immunity (Levin et al., 2021)and more. In our study, the median neutralizing ab titer of cases was 190, while it was significantly higher (530) among controls. Furthermore, (*Immune Correlates Analysis of the MRNA-1273 COVID-19 Vaccine Efficacy Clinical Trial*, n.d.) report that titer of 1000 or more are correlated with 96% vaccine efficacy and a titer of 100 is correlated with 91%, but this is for the era before Omicron. We and others have shown a decreased efficacy for Omicron of 8-fold to 24-fold compared to WT.

Here, we will study the potential immunogenicity of a 4th dose, together with assessing safety and effectiveness in preventing infections.

Study Aims:

To assess the immunogenicity of a 4th dose, and its durability. This will be measured by following IgG, IgA, Pseudoneutralization assays, microneutralization, avidity, T-cell activity and B-cell repertoire and comparing them to a matched control group, who are participating in the Sheba COVID Cohort study. We will also assess safety and vaccine effectiveness by active surveillance of adverse events and by following incidence of SARS-CoV-2 infections.

Study design:

This is a prospective intervention study, to test the effect of a 4th dose, by comparing the immune response before and after the 4th dose, given to 150-200 volunteers, as well as comparing their responses with a control group of individuals vaccinated with

3 doses but without the 4th. All study participants would be health care workers from Sheba medical Center, who are participating in the Sheba COVID Cohort study and have a serology test from the previous 3 months. Participation in the study will be confidential and will not be disclosed to the worker's direct supervisor. For this study, we will recruit 150-200 volunteers, who received the 3rd dose at least 4 months previously, and have a known serology history (showing an immune response (even if just a low response) to the three previous doses, but with a recent relatively low IgG (below 700 BAU, or lower than 512 IU PNT50). The justification for chosing this cutoff is explained in the introduction. Volunteers will tested before and after vaccination with a 4th dose, and followed for 180 days.

As controls, a sub-cohort of similar HCW, who are recruited to the Sheba COVID Cohort study (IRB 8008-20) and are followed monthly with serology tests, and are not receiving the 4th dose. The control group, all of whom signed an informed consent and allowed blood samples to be used for further immunologic studies, will be matched by age, gender, time from 3rd vaccine dose and IgG titers, and will be followed similarly, as by the original 8008-20 protocol.

On recruitment, volunteer will:

6. Receive a detailed explanation and sign the informed consent form (appendix ICF)
7. Fill an initial inclusion/ exclusion criteria questionnaire.
8. Fill a general comorbidity questionnaire (Appendix Q1), additionally they will be screened for COVID-19 symptoms such as fever, cough, anosmia
9. Have up to 40cc blood drawn for all serology and cellular immunity tests.
10. Perform a PCR for SARS-CoV-2 test
11. Receive the 4th dose of BNT162b2 30µg.
12. Will have a physician checkup and followup for 15 minutes after receiving the dose.

Six additional visits will follow as described in the research timeline:

Research Timeline:

Visit number		1	2	3	4	5	6	7
	Day	0	7	14	21	60	90	180
Vaccine	BNT162b2 30 µg	X						
Blood sample	IgG	X	X	X	X	X	X	X
	PNT	X	X	X	X	X	X	X
	microneutralization	X	X	X	X	X	X	X
	IgA	X	X	X	X	X	X	X
	Tcell activity	X		X		X		X
	Bcell repertoir	X		X		X		X
Nasal/NP swab	SARS-CoV-2 PCR	X	X	X	X	X	X	X
Questionnaires	Background comorbidity	X						
	Adverse events			X		X		

Inclusion Criteria:

1. Age: Volunteer must be at least 18 years of age, at the time of signing the informed conset.
2. Sex: Male or Female. All female volunteers of reproductive age will be requested to use contraceptive measures for the two months following enrolment.
3. Received 3 doses of BNT162b2 with the 3rd dose at least 4 months previously.
4. Have a serology test within the previous 3 months of 700 BAU or less.
5. Responded to the previous vaccine doses, i.e. at least one IgG>100.
6. Medical Conditions: Volunteers with any medical condition are allowed, as long as they adhere to the criteria above.
7. Agreed to attend all visits and signed the informed consent

Exclusion Criteria:

1. Had previous SARS-CoV-2 infection (detected by either PCR, anti-S IgG before the 1st vaccine dose, anti-N IgG at any stage).
2. Had an allergic response to any of the previous BNT162b2 doses.
3. Has history of myopericarditis.
4. Report that they do not feel well or have a fever on the day of vaccination.
5. Pregnant on day of recruitment.

Criteria for premature cessation of the study:

- Increased rates of immediate adverse events, higher than those reported in previous studies (11 per million) https://www.cdc.gov/mmwr/volumes/70/wr/mm7002e1.htm)

Insurance: This is an investigator initiated trial and therefore is insured by Inbal.

Funding: This study is funded by the Sheba Medical Center, without external funding of any pharmaceutical company. All costs of the tests conducted by the virological unit will funded by the virological unit and all tests conducted in the laboratories of the Sheba medical center will be funded by them.

Control group: Will consist of a subgroup of the Sheba HCW COVID Cohort, who will match the volunteers for the 4th dose, by age, gender, time since 3rd dose and serology status (i.e. <700 IgG but at least one measure >100). This sub-cohort consists of >700 HCW that have already signed informed consent to participate in studies regarding immunogenicity of the BNT162b2 vaccine (IRB – 8008-20), including performing additional immunology tests on samples obtained. Any

samples sent out are de-identified. All parameters and their trajectories will be compared between the Study participants and the Control group.

Vaccine storage and transport: Vaccines will be delivered by thermal shipping and will be stored in 2-8° C.

Statistical analysis: Sample size was determined to identify a 3-fold difference before and after the fourth dose, 190 participants are needed.

Matching of a control group, will follow the algorithm used in our study (Bergwerk et al, NEJM), attached below (Appendix A3). The Cohort from which the controls are picked includes >2000 HCW, with known serology result below 700.

Breakthrough infections will be defined as occurring after at least 8 days since 4th dose. Infection incidence will be compared between the intervention and the control group. Statistical analysis would be conducted in collaboration between the Gertner Institute for health policy and epidemiology and Sheba infection prevention and control unit using accepted methods for comparison between two groups in a case-control study and with SAS statistical program. All data regarding participants would be stored in secured computers and would be available only for this specific study purpose. Any data transferred to a third party will be de-identified, the identification key will be kept by the PI.

Primary outcomes:

Serology tests as detailed below, including IgG, pseudoneutralization, microneutralization, avidity and IgA. These outcomes will be compared between pre- and post- 4th dose as well as with those outcomes in the control group.

Adverse event reporting of vaccinated individuals by an electronic survey that will be filled from visit 2. This will be compared to adverse event reporting of individuals previously reporting after the 3rd dose from the Sheba HCW COVID Cohort, matched by gender and age. Adverse event questionnaire – Appendix Q2.

Serious adverse events will be defined as any adverse event that resulted in death, hospitalization, permanent damage, required treatment in the emergency room or was life threatening.

Secondary outcomes:

T-cell activity and B-cell repertoire as described below.

SARS-CoV-2 incidence and specifically Omicron VOC incidence.

Detailed Serological and immunological assays:

6. **IgG antibody assay** – Using a commercially available test (SARS-CoV-2 IgG II Quant (Abbott, IL, USA)) we will measure the quantity of IgG antibodies against the SARS-CoV-2 receptor binding domain (RBD). This commercial test will be performed according to manufacturer's instructions
7. **IgA antibody assay-** To test if The forth vaccine dose generates an additive IgA response which may be important in neutralizing SARS-CoV-2 in addition to IgG we will measure IgA levels in serum. A 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4ºC with 50µl per well of 2µg/ml for detection of IgA antibodies. After blocking with 5% skimmed milk at 25ºC for 60 minutes, serum samples will be added to antigen coated wells. The plate will be incubated at 25ºC for 120 minutes, washed and a HRP-conjugated isotype specific antibody (anti-human IgA HRP conjugate (Abcam, MA, USA, product number: ab7383) (diluted 1:2000)) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will be measured at 450nm using a micro-plate reader (Sunrise, Tecan). ELISA index value below 1.1 negative and equal or above 1.1, positive.

8. **Avidity –** to measure the quality of IgG antibodies we will use urea as a chaotropic reagent and test the strength of interaction between the IgG and the viral antigen (the RBD). Specifically, a 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4°C with 50µl per well of 1µg/ml of RBD antigen. After blocking with 5% skimmed milk at 25°C for 60 minutes, serum samples will be diluted 1:100, 1:400 and 1:1000 with 3% skimmed milk and added to antigen coated wells. The plate will be incubated at 25°C for 120 minutes, and following washing each sample,incubated either with the addition of 6M urea or PBS for 10 min. After washing, a goat anti-human IgG horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, PA, USA Code: 109-035-088) (diluted 1:15000) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will be measured at 450nm using a micro-plate reader (Sunrise, Tecan). Avidity index will be calculated as the ratio (in percentage) between sample OD with 6M urea and sample OD with PBS.
9. **SARS-CoV-2 Pseudovirus (psSARS-2) Neutralization Assay –** to test the overall neutralizing ability of each serum against the WT virus and specifically to compare

with neutralizing levels of the SHEBA HCW following one, two and three vaccine doses we will use Pseudovirus (psSARS-2) Neutralization. SARS-CoV-2 Pseudo-virus (psSARS-2) Neutralization Assay will be performed using a propagation-competent VSV-spike which was shown to be highly correlative to authentic SARS-CoV-2 virus micro-neutralization assay. Following titration, 100 focus forming units (ffu) of psSARS-2 will be incubated with 2-fold serial dilution of heat inactivated (56°C for 30 min) tested sera. After incubation for 60 min at 37°C, virus/serum mixture will be transferred to Vero E6 cells that have been grown to confluency in 96-well plates and incubated for 90 min at 37°C. After the addition of 1% methyl cellulose in dulbecco's modified eagle's medium (DMEM) with 2% of fetal bovine serum (FBS), plates will be incubated for 24hr and 50% plaque reduction titer will be calculated by counting green fluorescent foci using a fluorescence microscope. Sera not capable of reducing viral replication by 50% at 1 to 16 dilution or below will be considered non- neutralizing.

10. **SARS-CoV-2 micro-neutralization assay-** to compare the neutralizing capacity of omicron and delta variants following the forth vaccine dose a SARS-CoV-2 micro-neutralization assay with live virus will be performed. VERO-E6 cells at concentration of 20*103/well will be seeded in sterile 96-wells plates with 10% FCS MEM-EAGLE medium, and stored at 37^{o}C for 24 hours. One hundred TCID50 of Wild Type, Beta, Delta and Omicron SARS-CoV-2 isolates will be incubated with inactivated sera diluted 1:10 to 1:16,384 in 96 well plates for 60 minutes at 33ºC. Virus-serum mixtures will be added to the Vero E-6 cells and incubated for five days at 33ºC after which Gentian violet staining (1%) will be used to stain and fix the cell culture layer. Neutralizing dilution of each serum sample will be determined by identifying the well with the highest serum dilution without observable cytopathic effect. A dilution equal to 1:10 or above will be considered neutralizing.
11. **Memory immune response -** To investigate the memory response we will isolate peripheral blood mononuclear cell (PBMC) using Ficoll density gradient centrifugation and analyze T and B cells for T cell activation and antibody repertoire, respectively:
    a. T cell activation will be assessed by IFN-γ ELISpot assay. Specifically, IFN- γ -secreting cells will be enumerated using Elispot IFN-γ kits (IFN-γ kit, AID Autoimmun Diagnostika GmbH, Strassberg, Germany) according to manufacturer instructions. For antigen stimulation, 50 µl of SARS-CoV-2 peptide pools (S-complete, Miltenyi Biotech) will be used. Test medium will be used as negative control and Phytohaemagglutinin (PHA) will be used as

positive control. IFN-γ-secreting cells frequency will be quantified using the AID ELISpot Reader (Strassberg, Germany). The unspecific background (mean SFU from negative control wells) will be subtracted from experimental readings.

b. We will stimulate T cells by SARS-CoV-2 peptides to analyze the specific activation of CD4 and CD8 T cells in each cohort by flow cytometry using fluorescent probes.

c. The frequency and specificity of SARS coV-2 specific memory B cells will be tested by flow cytometry using fluorescent probes corresponding to spike protein from different variants. Probe-binding B cells will be sorted by flow cytometry and single-cell immunoglobulin and whole transcriptome sequencing will be performed. From the sequencing reactions, constructs will be made to express and test functionality of SARS-CoV-2- specific monoclonal antibodies, including binding, neutralization and affinity.

Benefits of participating in the study:

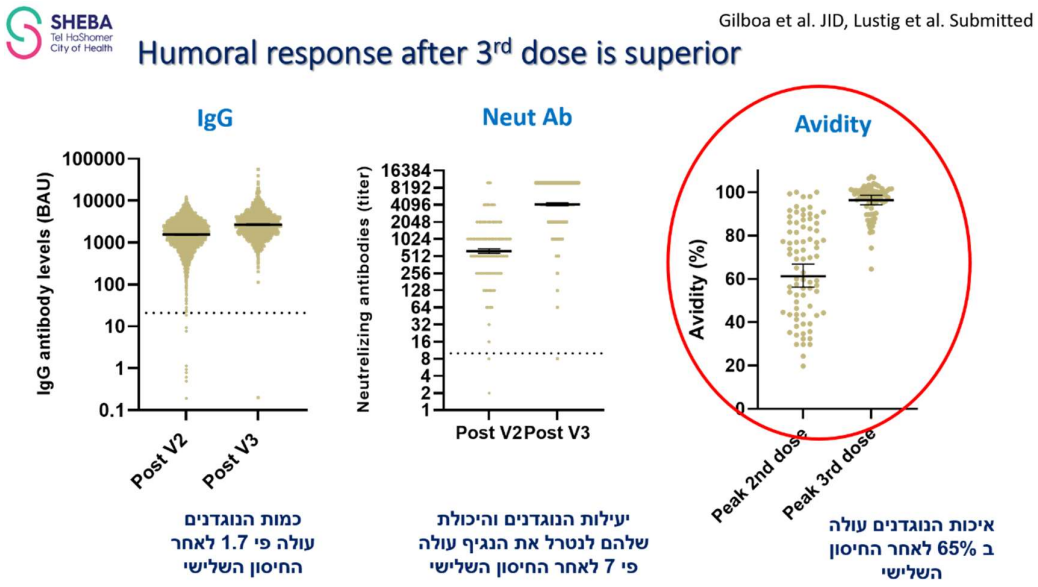
The potential benefit of improved protection from SARS-CoV-2. Currently, according the effectiveness of the previous doses, and in the absence of effective drug therapy, it is expected that an additional dose will raise the level of antibodies and thus raise the protection from SARS-CoV-2.

Risks in participating in the study:

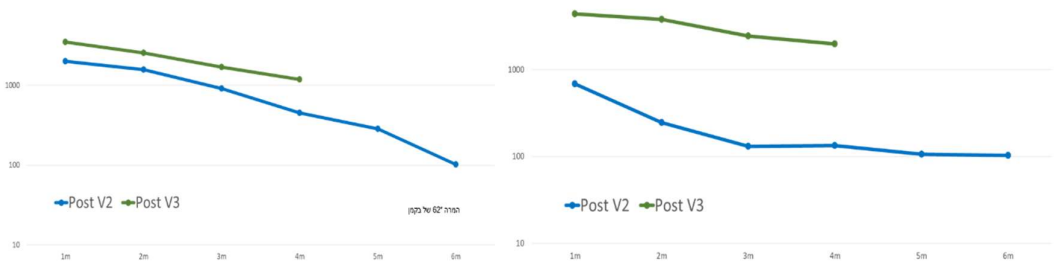
The known previous adverse events are expected to occur, these included frequent local reactions (mostly local pain), as well as systemic reactions including fatigue, fever, lymphadenopathy, myalgia, these have typically lasted up to 48 hours. Rarely, myocarditis has occurred (1:6,000-24,000, mostly in young men). It is yet unknown if a 4th dose will have additional adverse reactions.

Some concerns may be raised regarding potential affinity maturation of B cells specific for ancestral variant epitopes encoded by the vaccine, skewing of the T cell response and depletion of memory T cells against other pathogens. The latter point with respect to T cells is not supported by convincing evidence in humans to suggest that this may be the case in the long term nor that it may be detrimental to human health. Many different vaccines are administered at regular intervals in humans and significant detrimental effects to the immune system's response to specific and other pathogens have not been clearly documented.

Appendix A-1 – superiority of 3rd dose (submitted for publication)

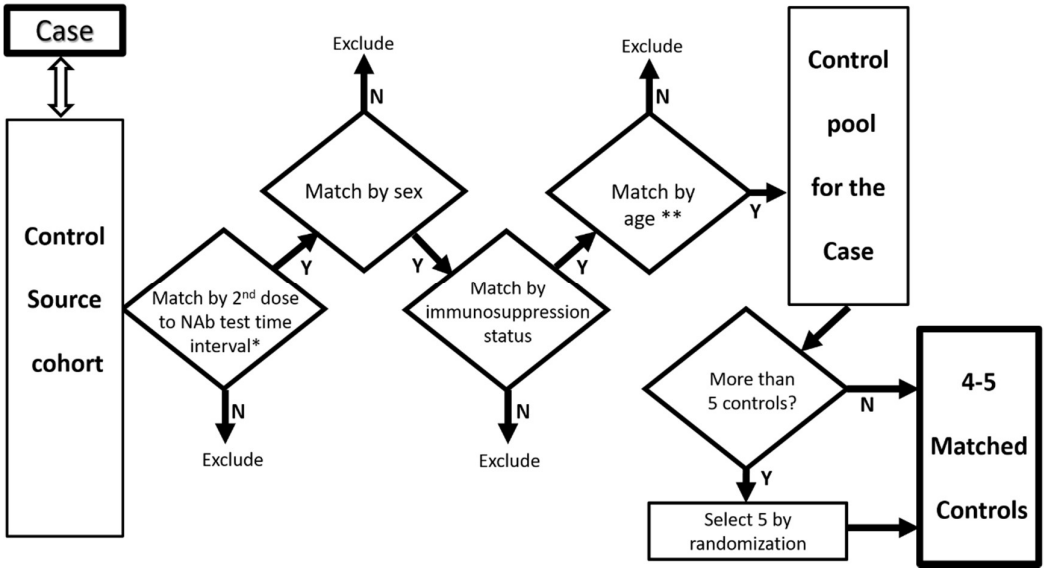


Appendix A2 – Waning immune response after the third dose (preliminary unpublished data)



IgG Neutralizing Ab

Appendix A3: Matching algorithm for picking controls from the SHEBA COVID Cohort.



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Fourth COVID-19 vaccine dose- mRNA1273

PI – Gili Regev-Yochay

Research Protocol

Background: The Omicron variant of concern (VOC) is currently rapidly spreading worldwide, with extremely high transmission rates, with an estimated R of >3 (Barnard et al., n.d.; Khoury et al., n.d.) . While the virulence of this VOC is yet unknown, even with a much lower virulence than the delta strain, with such high transmission, once again the pandemic will have severe effects on healthcare capacity. Thus, further effort to contain this VOC is required. Currently, the only successful key in containment of the SARS-CoV-2 pandemic was vaccination. Following the first two doses, extremely high vaccine effectiveness was reported from many real-world observational studies(Amit et al., 2021; Dagan et al., 2021; Haas et al., 2021). Several vaccines were reported with high vaccine efficacy and effectiveness, mostly, the mRNA vaccines, of which the BNT162b2 was rolled out in Israel. Yet, with time, waning of this effect was reported, both in immunogenicity as well as in vaccine effectiveness against infections and hospitalizations (Bar-On et al., 2021; Levin et al., 2021).

Following these reports, Israel initiated rollout of the third dose of BNT162b2 on July 29, initially to adults 60 years and older and within a few weeks expanded it to the whole population if at least 5 months have passed from the second dose. We have recently reported the early effects of the 3rd dose(Gilboa et al., 2021) , showing significant increase in IgG and Neutralizing antibodies, as well as increased T-cell activity in a small group of non-responders to first two doses. We have also submitted further data, showing the superiority of the third dose in aspects of immunogenicity, with significantly higher amounts of antibodies as well as improved quality as measured by higher avidity and neutralizing capacity (see yet unpublished data Appendix A1).

We now have preliminary, yet unpublished data, showing slow waning of the third dose immune response within 4 months after the third dose (Appendix A2). Simultaneously, Milo R. et al presented yet unpublished data on early waning of vaccine effectiveness of the third dose. While these data would not have been worrisome in the Delta VOC era, this may be different with the emergence of the Omicron VOC.

It was recently reported, by us and others that vaccine effectiveness against the Omicron VOC is decreased. This was shown by reduced neutralization efficiency of Omicron VOC compared to that of the Delta VOC or the wild type (Wu-1), by sera of vaccinated individuals. We have just reported (Nemet et al., n.d.) , accepted to NEJM) that sera from BNT162b 2-dose vaccinated individuals does not neutralize Omicron at all, while it neutralizes to a low level the Delta VOC and more so the Wu-

1. We compared this to 3-dose vaccinated, and also showed a significantly reduced neutralization of early 3-dose vaccinated as compared to the efficiency against Wu-1 or the delta VOC. Furthermore, data from Germany showed that neutralization of Omicron decreases within 3 months following the 3rd dose.

These data raise the question of when and will we need a 4th dose to cope with the emergence of Omicron. However, if we have reached the maximal effect of the current vaccine against Omicron, with a third dose, will a 4th dose have any added value? In this respect, several studies have demonstrated improved immunogenicity of 1 vaccine dose to SARS-CoV-2 recovered patients, yet a 2nd dose to these patients had very little added value. It is thus unclear whether a 4th dose of the current vaccine, which is not directed specifically at the Omicron VOC, can further improve our immunity and protection from this strain, and will it have any added value.

There is increasing data showing slight superiority of the Moderna mRNA1273 vaccine of the BNT162B2 vaccine(Khoury et al., n.d.-b). Boosting with mRNA1273 on previously BNT162b2 has not been reported. Yet, studies showing effectiveness of heterologous boosting (mostly of ChAdoX with BNT162b2) have been reported (Choi et al., 2021), including the large Cov-Boost (https://www.covboost.org.uk/). It is thus important to assess whether boosting with mRNA1273, of previously BNT162b2 fully vaccinated (3-doses) individuals, will be advantageous. Moreover, it is important to assess whether boosting with an Omicron modified vaccine will be advantageous over the original mRNA1273. While one may assume that it should be more efficient, since the immune system of previously vaccinated individuals has been primed towards the wild-type Spike protein, it is unclear whether there will be any advantage.

The Sheba HCW COVID Cohort was established on April 2020, when HCW were recruited to a large serology longitudinal follow up study. Since then, over 7000 HCW who were vaccinated by BNT162b2 are being followed monthly with blood sampling for immune responses, including mostly IgG, but also Neutralizing ab, avidity, microneutralization, IgA and cellular activation. Through this cohort we found the correlation between neutralizing ab levels and SARS-CoV-2 infections (Bergwerk et al., 2021) , the waning of immunity (Levin et al., 2021)and more. In our study, the median neutralizing ab titer of cases was 190, while it was significantly higher (530) among controls. Furthermore, (*Immune Correlates Analysis of the MRNA-1273 COVID-19 Vaccine Efficacy Clinical Trial*, n.d.) report that titer of 1000 or more are correlated with 96% vaccine efficacy and a titer of 100 is correlated with 91%, but this is for the era before Omicron. We and others have shown a decreased efficacy for Omicron of 8-fold to 24-fold compared to WT.

Here, we will study the potential immunogenicity of a 4th dose of COVID-19 vaccine, mRNA1273 (50ug), together with assessing safety and effectiveness in preventing infections.

Study Aims:

To assess the immunogenicity of a 4th dose, and its durability. This will be measured by following IgG, IgA, Pseudoneutralization assays, microneutralization, avidity, T-cell activity and B-cell repertoire and comparing them to a matched control group, who are participating in the Sheba COVID Cohort study. We will also assess safety and vaccine effectiveness by active surveillance of adverse events and by following incidence of SARS-CoV-2 infections.

Study design:

This is a prospective intervention study, to test the effect of a 4th dose mRNA1273 (Spikevax) 50 µcg , by comparing the immune response before and after the 4th dose, given to 150-200 volunteers, as well as comparing their responses with a control group of individuals vaccinated with 3 doses of BNT162b2 but without the 4th. All study participants would be health care workers from Sheba medical Center, who are participating in the Sheba COVID Cohort study and have a serology test from the previous 3 months. Participation in the study will be confidential and will not be disclosed to the worker's direct supervisor. For this study, we will recruit 150-200 volunteers, who received the 3rd dose at least 4 months previously, and have a known serology history (showing an immune response (even if just a low response) to the three previous doses, but with a recent relatively low IgG (below 700 BAU, or lower than 512 IU PNT50). The justification for choosing this cut-off is explained in the introduction. Volunteers will be tested before and after vaccination with a 4th dose of Spikevax , and followed for 180 days.

As controls, a sub-cohort of similar HCW, who are recruited to the Sheba COVID Cohort study (IRB 8008-20) and are followed monthly with serology tests, and are not receiving the 4th dose. The control group, all of whom signed an informed consent and allowed blood samples to be used for further immunologic studies, will be matched by age, gender, time from 3rd vaccine dose and IgG titers, and will be followed similarly, as by the original 8008-20 protocol.

On recruitment, volunteer will:

13. Receive a detailed explanation and sign the informed consent form (appendix ICF)
14. Fill an initial inclusion/ exclusion criteria questionnaire.
15. Fill a general comorbidity questionnaire (Appendix Q1), additionally they will be screened for COVID-19 symptoms such as fever, cough, anosmia
16. Have up to 40cc blood drawn for all serology and cellular immunity tests.
17. Perform a PCR for SARS-CoV-2 test
18. Receive the 4th dose of Spikevax (Moderna mRNA 1273) 50 µcg.

19. Will have a physician check-up and follow up for 15 minutes after receiving the dose.

Six additional visits will follow as described in the research timeline:

Research Timeline:

Visit number		1	2	3	4	5	6	7
	Day	0	7	14	21	60	90	180
Vaccine	Moderna-mRNA1273 50 µcg	X						
Blood sample	IgG	X	X	X	X	X	X	X
	PNT	X	X	X	X	X	X	X
	microneutralization	X	X	X	X	X	X	X
	IgA	X	X	X	X	X	X	X
	Tcell activity	X		X		X		X
	Bcell repertoire	X		X		X		X
Nasal/NP swab	SARS-CoV-2 PCR	X	X	X	X	X	X	X
Questionnaires	Background comorbidity	X						
	Adverse events			X		X		
MD checkup		X	**	**	**	**	**	**

*Visit day is allowed to be +/-2days of planned time.

** If any symptoms or AE will be reported, a physician checkup will be added to the visit.

Inclusion Criteria:

8. Has participated in the Sheba COVID Cohort serology study, and has history of IgG and Neutralizing ab in the recent year.
9. Age: Volunteer must be at least 18 years of age, at the time of signing the informed consent.
10. Sex: Male or Female. All female volunteers of reproductive age will be requested to use contraceptive measures for the two months following enrolment.
11. Received 3 doses of BNT162b2 with the 3rd dose at least 4 months previously.
12. Have a serology test within the previous 3 months of 700 BAU or less.
13. Responded to the previous vaccine doses, i.e. at least one IgG>100.
14. Medical Conditions: Volunteers with any medical condition are allowed, as long as they adhere to the criteria above.
15. Agreed to attend all visits and signed the informed consent

Exclusion Criteria:

6. Had previous SARS-CoV-2 infection (detected by either PCR, anti-S IgG before the 1st vaccine dose, anti-N IgG at any stage).

7. Had an allergic response to any of the previous BNT162b2 doses.
8. Has history of myopericarditis.
9. Report that they do not feel well or have a fever on the day of vaccination.
10. Pregnant on day of recruitment.

Criteria for premature cessation of the study:

- Increased rates of immediate adverse events, higher than those reported in previous studies (11 per million) https://www.cdc.gov/mmwr/volumes/70/wr/mm7002e1.htm)

Insurance: This is an investigator initiated trial and therefore is insured by Inbal.

Funding: This study is funded by the Sheba Medical Center, without external funding of any pharmaceutical company. All costs of the tests conducted by the virological unit will funded by the virological unit and all tests conducted in the laboratories of the Sheba medical center will be funded by them.

Control group: Will consist of a subgroup of the Sheba HCW COVID Cohort, who will match the volunteers for the 4th dose, by age, gender, time since 3rd dose and serology status (i.e. <700 IgG but at least one measure >100). This sub-cohort consists of >700 HCW that have already signed informed consent to participate in studies regarding immunogenicity of the BNT162b2 vaccine (IRB – 8008-20), including performing additional immunology tests on samples obtained. Any samples sent out are de-identified. All parameters and their trajectories will be compared between the Study participants and the Control group.

Vaccine storage and transport: Vaccines will be delivered by thermal shipping and will be stored in 2-8° C.

Statistical analysis: Sample size was determined to identify a 3-fold difference before and after the fourth dose, 190 participants are needed.

Matching of the control group, will follow the algorithm used in our stud (Bergwerk et al, NEJM). The Cohort from which the controls are picked included >2000 HCW, with known serology result below 700.

Breakthrough infections will be defined as occurring after at least 8 days since 4th dose. Infection incidence will be compared between the intervention and the control group. Statistical analysis would be conducted in collaboration between the Gertner Institute for health policy and epidemiology and Sheba infection prevention and control unit using accepted methods for comparison between two groups in a case-control study and with SAS statistical program. All data regarding participants would be stored in secured computers and would be available only for this specific study

purpose. Any data transferred to a third party will be de-identified, the identification key will be kept by the PI.

Primary outcomes:

Serology tests as detailed below, including IgG, pseudoneutralization, microneutralization, avidity and IgA. These outcomes will be compared between pre- and post- 4th dose of Spikevax 50 µcg as well as with those outcomes in the control group.

Adverse event reporting of vaccinated individuals by an electronic survey that will be filled from visit 2. This will be compared to adverse event reporting of individuals previously reporting after the 3rd dose from the Sheba HCW COVID Cohort, matched by gender and age. Adverse event questionnaire – Appendix Q2.

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SARS-CoV-2 incidence and specifically Omicron VOC incidence.

Detailed Serological and immunological assays:

12. **IgG antibody assay** – Using a commercially available test (SARS-CoV-2 IgG II Quant (Abbott, IL, USA)) we will measure the quantity of IgG antibodies against the SARS-CoV-2 receptor binding domain (RBD). This commercial test will be performed according to manufacturer's instructions
13. **IgA antibody assay-** To test if The forth vaccine dose generates an additive IgA response which may be important in neutralizing SARS-CoV-2 in addition to IgG we will measure IgA levels in serum. A 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4ºC with 50µl per well of 2µg/ml for detection of IgA antibodies. After blocking with 5% skimmed milk at 25ºC for 60 minutes, serum samples will be added to antigen coated wells. The plate will be incubated at 25ºC for 120 minutes, washed and a HRP-conjugated isotype specific antibody (anti-human IgA HRP conjugate (Abcam, MA, USA, product number: ab7383) (diluted 1:2000)) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will

be measured at 450nm using a micro-plate reader (Sunrise, Tecan). ELISA index value below 1.1 negative and equal or above 1.1, positive.

14. **Avidity –** to measure the quality of IgG antibodies we will use urea as a chaotropic reagent and test the strength of interaction between the IgG and the viral antigen (the RBD). Specifically, a 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4°C with 50µl per well of 1µg/ml of RBD antigen. After blocking with 5% skimmed milk at 25°C for 60 minutes, serum samples will be diluted 1:100, 1:400 and 1:1000 with 3% skimmed milk and added to antigen coated wells. The plate will be incubated at 25°C for 120 minutes, and following washing each sample,incubated either with the addition of 6M urea or PBS for 10 min. After washing, a goat anti-human IgG horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, PA, USA Code: 109-035-088) (diluted 1:15000) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will be measured at 450nm using a micro-plate reader (Sunrise, Tecan). Avidity index will be calculated as the ratio (in percentage) between sample OD with 6M urea and sample OD with PBS.
15. **SARS-CoV-2 Pseudovirus (psSARS-2) Neutralization Assay –** to test the overall neutralizing ability of each serum against the WT virus and specifically to compare with neutralizing levels of the SHEBA HCW following one, two and three vaccine doses we will use Pseudovirus (psSARS-2) Neutralization. SARS-CoV-2 Pseudo-virus (psSARS-2) Neutralization Assay will be performed using a propagation-competent VSV-spike which was shown to be highly correlative to authentic SARS-CoV-2 virus micro-neutralization assay. Following titration, 100 focus forming units (ffu) of psSARS-2 will be incubated with 2-fold serial dilution of heat inactivated (56°C for 30 min) tested sera. After incubation for 60 min at 37°C, virus/serum mixture will be transferred to Vero E6 cells that have been grown to confluency in 96-well plates and incubated for 90 min at 37°C. After the addition of 1% methyl cellulose in dulbecco's modified eagle's medium (DMEM) with 2% of fetal bovine serum (FBS), plates will be incubated for 24hr and 50% plaque reduction titer will be calculated by counting green fluorescent foci using a fluorescence microscope. Sera not capable of reducing viral replication by 50% at 1 to 16 dilution or below will be considered non- neutralizing.

16. **SARS-CoV-2 micro-neutralization assay-** to compare the neutralizing capacity of omicron and delta variants following the forth vaccine dose a SARS-CoV-2 micro-neutralization assay with live virus will be performed. VERO-E6 cells at concentration of 20*103/well will be seeded in sterile 96-wells plates with 10% FCS MEM-EAGLE medium, and stored at 37^{o}C for 24 hours. One hundred TCID50 of Wild Type, Beta, Delta and Omicron SARS-CoV-2 isolates will be incubated with inactivated sera diluted 1:10 to 1:16,384 in 96 well plates for 60 minutes at 33ºC. Virus-serum mixtures will be added to the Vero E-6 cells and incubated for five days at 33ºC after which Gentian violet staining (1%) will be used to stain and fix the cell culture layer. Neutralizing dilution of each serum sample will be determined by identifying the well with the highest serum dilution without observable cytopathic effect. A dilution equal to 1:10 or above will be considered neutralizing.
17. **Memory immune response -** To investigate the memory response we will isolate peripheral blood mononuclear cell (PBMC) using Ficoll density gradient centrifugation and analyze T and B cells for T cell activation and antibody repertoire, respectively:
    a. T cell activation will be assessed by IFN-γ ELISpot assay. Specifically, IFN- γ -secreting cells will be enumerated using Elispot IFN-γ kits (IFN-γ kit, AID Autoimmun Diagnostika GmbH, Strassberg, Germany) according to manufacturer instructions. For antigen stimulation, 50 µl of SARS-CoV-2 peptide pools (S-complete, Miltenyi Biotech) will be used. Test medium will be used as negative control and Phytohaemagglutinin (PHA) will be used as positive control. IFN-γ-secreting cells frequency will be quantified using the AID ELISpot Reader (Strassberg, Germany). The unspecific background (mean SFU from negative control wells) will be subtracted from experimental readings.
    b. We will stimulate T cells by SARS-CoV-2 peptides to analyze the specific activation of CD4 and CD8 T cells in each cohort by flow cytometry using fluorescent probes.
    c. The frequency and specificity of SARS coV-2 specific memory B cells will be tested by flow cytometry using fluorescent probes corresponding to spike protein from different variants. Probe-binding B cells will be sorted by flow cytometry and single-cell immunoglobulin and whole transcriptome sequencing will be performed. From the sequencing reactions, constructs will

be made to express and test functionality of SARS-CoV-2- specific monoclonal antibodies, including binding, neutralization and affinity.

Benefits of participating in the study:

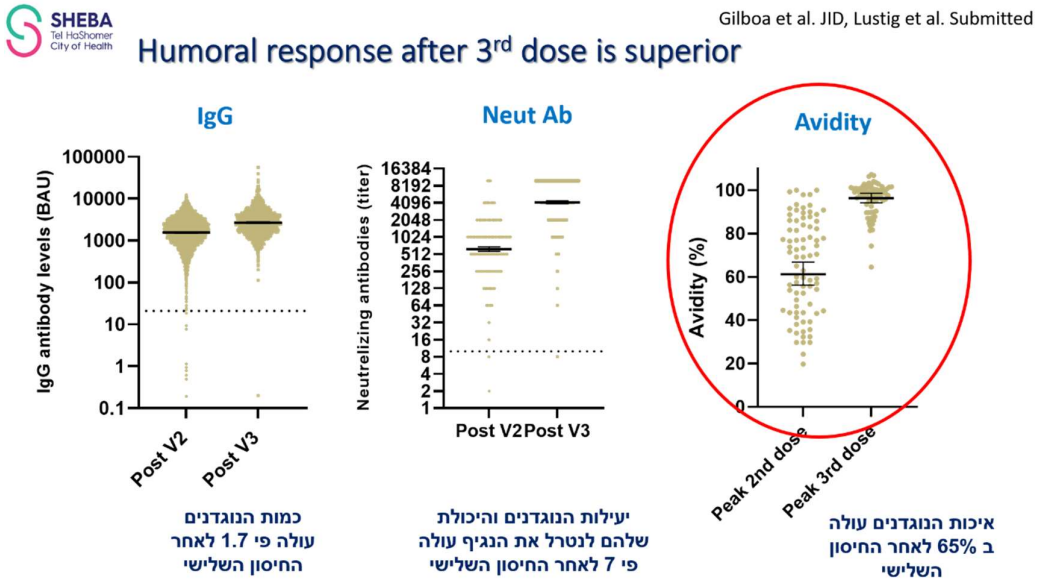
The potential benefit of improved protection from SARS-CoV-2. Currently, according the effectiveness of the previous doses, and in the absence of effective drug therapy, it is expected that an additional dose will raise the level of antibodies and thus raise the protection from SARS-CoV-2.

Risks in participating in the study:

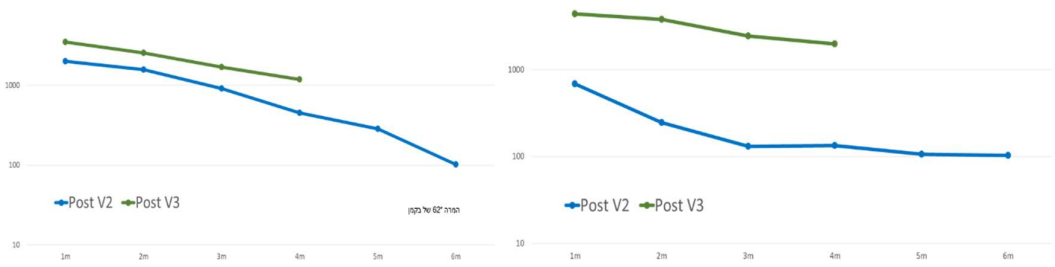
The known previous adverse events are expected to occur, these included frequent local reactions (mostly local pain), as well as systemic reactions including fatigue, fever, lymphadenopathy, myalgia, these have typically lasted up to 48 hours. Rarely, myocarditis has occurred (1:6,000-24,000, mostly in young men). It is yet unknown if a 4th dose will have additional adverse reactions.

Some concerns may be raised regarding potential affinity maturation of B cells specific for ancestral variant epitopes encoded by the vaccine, skewing of the T cell response and depletion of memory T cells against other pathogens. The latter point with respect to T cells is not supported by convincing evidence in humans to suggest that this may be the case in the long term nor that it may be detrimental to human health. Many different vaccines are administered at regular intervals in humans and significant detrimental effects to the immune system's response to specific and other pathogens have not been clearly documented.

Appendix A-1 – superiority of 3rd dose (submitted for publication)



Appendix A2 – Waning immune response after the third dose (preliminary unpublished data)



IgG Neutralizing Ab

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Fourth COVID-19 vaccine dose- mRNA1273

PI – Gili Regev-Yochay

Research Protocol

Background: The Omicron variant of concern (VOC) is currently rapidly spreading worldwide, with extremely high transmission rates, with an estimated R of >3 (Barnard et al., n.d.; Khoury et al., n.d.) . While the virulence of this VOC is yet unknown, even with a much lower virulence than the delta strain, with such high transmission, once again the pandemic will have severe effects on healthcare capacity. Thus, further effort to contain this VOC is required. Currently, the only successful key in containment of the SARS-CoV-2 pandemic was vaccination. Following the first two doses, extremely high vaccine effectiveness was reported from many real-world observational studies(Amit et al., 2021; Dagan et al., 2021; Haas et al., 2021). Several vaccines were reported with high vaccine efficacy and effectiveness, mostly, the mRNA vaccines, of which the BNT162b2 was rolled out in Israel. Yet, with time, waning of this effect was reported, both in immunogenicity as well as in vaccine effectiveness against infections and hospitalizations (Bar-On et al., 2021; Levin et al., 2021).

Following these reports, Israel initiated rollout of the third dose of BNT162b2 on July 29, initially to adults 60 years and older and within a few weeks expanded it to the whole population if at least 5 months have passed from the second dose. We have recently reported the early effects of the 3rd dose(Gilboa et al., 2021) , showing significant increase in IgG and Neutralizing antibodies, as well as increased T-cell activity in a small group of non-responders to first two doses. We have also submitted further data, showing the superiority of the third dose in aspects of immunogenicity, with significantly higher amounts of antibodies as well as improved quality as measured by higher avidity and neutralizing capacity (see yet unpublished data Appendix A1).

We now have preliminary, yet unpublished data, showing slow waning of the third dose immune response within 4 months after the third dose (Appendix A2). Simultaneously, Milo R. et al presented yet unpublished data on early waning of vaccine effectiveness of the third dose. While these data would not have been worrisome in the Delta VOC era, this may be different with the emergence of the Omicron VOC.

It was recently reported, by us and others that vaccine effectiveness against the Omicron VOC is decreased. This was shown by reduced neutralization efficiency of Omicron VOC compared to that of the Delta VOC or the wild type (Wu-1), by sera of vaccinated individuals. We have just reported (Nemet et al., n.d.) , accepted to NEJM) that sera from BNT162b 2-dose vaccinated individuals does not neutralize Omicron at all, while it neutralizes to a low level the Delta VOC and more so the Wu-

1. We compared this to 3-dose vaccinated, and also showed a significantly reduced neutralization of early 3-dose vaccinated as compared to the efficiency against Wu-1 or the delta VOC. Furthermore, data from Germany showed that neutralization of Omicron decreases within 3 months following the 3rd dose.

These data raise the question of when and will we need a 4th dose to cope with the emergence of Omicron. However, if we have reached the maximal effect of the current vaccine against Omicron, with a third dose, will a 4th dose have any added value? In this respect, several studies have demonstrated improved immunogenicity of 1 vaccine dose to SARS-CoV-2 recovered patients, yet a 2nd dose to these patients had very little added value. It is thus unclear whether a 4th dose of the current vaccine, which is not directed specifically at the Omicron VOC, can further improve our immunity and protection from this strain, and will it have any added value.

There is increasing data showing slight superiority of the Moderna mRNA1273 vaccine of the BNT162B2 vaccine(Khoury et al., n.d.-b). Boosting with mRNA1273 on previously BNT162b2 has not been reported. Yet, studies showing effectiveness of heterologous boosting (mostly of ChAdoX with BNT162b2) have been reported (Choi et al., 2021), including the large Cov-Boost (https://www.covboost.org.uk/). It is thus important to assess whether boosting with mRNA1273, of previously BNT162b2 fully vaccinated (3-doses) individuals, will be advantageous. Moreover, it is important to assess whether boosting with an Omicron modified vaccine will be advantageous over the original mRNA1273. While one may assume that it should be more efficient, since the immune system of previously vaccinated individuals has been primed towards the wild-type Spike protein, it is unclear whether there will be any advantage.

The Sheba HCW COVID Cohort was established on April 2020, when HCW were recruited to a large serology longitudinal follow up study. Since then, over 7000 HCW who were vaccinated by BNT162b2 are being followed monthly with blood sampling for immune responses, including mostly IgG, but also Neutralizing ab, avidity, microneutralization, IgA and cellular activation. Through this cohort we found the correlation between neutralizing ab levels and SARS-CoV-2 infections (Bergwerk et al., 2021) , the waning of immunity (Levin et al., 2021)and more. In our study, the median neutralizing ab titer of cases was 190, while it was significantly higher (530) among controls. Furthermore, (*Immune Correlates Analysis of the MRNA-1273 COVID-19 Vaccine Efficacy Clinical Trial*, n.d.) report that titer of 1000 or more are correlated with 96% vaccine efficacy and a titer of 100 is correlated with 91%, but this is for the era before Omicron. We and others have shown a decreased efficacy for Omicron of 8-fold to 24-fold compared to WT.

Here, we will study the potential immunogenicity of a 4th dose of COVID-19 vaccine, mRNA1273 (50ug), together with assessing safety and effectiveness in preventing infections.

Study Aims:

To assess the immunogenicity of a 4th dose, and its durability. This will be measured by following IgG, IgA, Pseudoneutralization assays, microneutralization, avidity, T-cell activity and B-cell repertoire and comparing them to a matched control group, who are participating in the Sheba COVID Cohort study. We will also assess safety and vaccine effectiveness by active surveillance of adverse events and by following incidence of SARS-CoV-2 infections.

Study design:

This is a prospective intervention study, to test the effect of a 4th dose mRNA1273 (Spikevax) 50 µcg , by comparing the immune response before and after the 4th dose, given to 150-200 volunteers, as well as comparing their responses with a control group of individuals vaccinated with 3 doses of BNT162b2 but without the 4th. All study participants would be health care workers from Sheba medical Center, who are participating in the Sheba COVID Cohort study and have a serology test from the previous 3 months. Participation in the study will be confidential and will not be disclosed to the worker's direct supervisor. Following the decision of the MOH to allow a 4th dose of COVID vaccine to HCW, we will recruit 150-200 volunteers, who received the 3rd dose at least 4 months previously, and have a known serology history (showing an immune response (even if just a low response) to the three previous doses, but with a recent relatively low IgG (below 700 BAU, or lower than 512 IU PNT50). The justification for choosing this cut-off is explained in the introduction. Volunteers will be tested before and after vaccination with a 4th dose of Spikevax , and followed for 180 days.

As controls, a sub-cohort of similar HCW, who are recruited to the Sheba COVID Cohort study (IRB 8008-20) and are followed monthly with serology tests, and are not receiving a 4th dose of any type. The control group, all of whom signed an informed consent and allowed blood samples to be used for further immunologic studies, will be matched by age, gender, time from 3rd vaccine dose and IgG titers, and will be followed similarly, as by the original 8008-20 protocol.

On recruitment, volunteer will:

20. Receive a detailed explanation and sign the informed consent form (appendix ICF)
21. Fill an initial inclusion/ exclusion criteria questionnaire.
22. Fill a general comorbidity questionnaire (Appendix Q1), additionally they will be screened for COVID-19 symptoms such as fever, cough, anosmia
23. Have up to 40cc blood drawn for all serology and cellular immunity tests.
24. Perform a PCR for SARS-CoV-2 test
25. Receive the 4th dose of Spikevax (Moderna mRNA 1273) 50 µcg.

26. Will have a physician check-up and follow up for 15 minutes after receiving the dose.

Six additional visits will follow as described in the research timeline:

Research Timeline:

Visit number		1	2	3	4	5	6	7
	Day	0	7	14	21	60	90	180
Vaccine	Moderna-mRNA1273 50 µcg	X						
Blood sample	IgG	X	X	X	X	X	X	X
	PNT	X	X	X	X	X	X	X
	microneutralization	X	X	X	X	X	X	X
	IgA	X	X	X	X	X	X	X
	Tcell activity	X		X		X		X
	Bcell repertoire	X		X		X		X
Nasal/NP swab	SARS-CoV-2 PCR	X	X	X	X	X	X	X
Questionnaires	Background comorbidity	X						
	Adverse events			X		X		
MD checkup		X	**	**	**	**	**	**

*Visit day is allowed to be +/-2days of planned time.

** If any symptoms or AE will be reported, a physician checkup will be added to the visit.

Inclusion Criteria:

16. Has participated in the Sheba COVID Cohort serology study, and has history of IgG and Neutralizing ab in the recent year.
17. Age: Volunteer must be at least 18 years of age, at the time of signing the informed consent.
18. Sex: Male or Female. All female volunteers of reproductive age will be requested to use contraceptive measures for the two months following enrolment.
19. Received 3 doses of BNT162b2 with the 3rd dose at least 4 months previously.
20. Have a serology test within the previous 3 months of 700 BAU or less.
21. Responded to the previous vaccine doses, i.e. at least one IgG>100.
22. Medical Conditions: Volunteers with any medical condition are allowed, as long as they adhere to the criteria above.
23. Agreed to attend all visits and signed the informed consent

Exclusion Criteria:

11. Had previous SARS-CoV-2 infection (detected by either PCR, anti-S IgG before the 1st vaccine dose, anti-N IgG at any stage).

12. Had an allergic response to any of the previous BNT162b2 doses.
13. Has history of myopericarditis.
14. Report that they do not feel well or have a fever on the day of vaccination.
15. Pregnant on day of recruitment.

Criteria for premature cessation of the study:

- Increased rates of immediate adverse events, higher than those reported in previous studies (11 per million) https://www.cdc.gov/mmwr/volumes/70/wr/mm7002e1.htm)

Insurance: This is an investigator initiated trial and therefore is insured by Inbal.

Funding: This study is funded by the Sheba Medical Center, without external funding of any pharmaceutical company. All costs of the tests conducted by the virological unit will funded by the virological unit and all tests conducted in the laboratories of the Sheba medical center will be funded by them.

Control group: Will consist of a subgroup of the Sheba HCW COVID Cohort, who will match the volunteers for the 4th dose, by age, gender, time since 3rd dose and serology status (i.e. <700 IgG but at least one measure >100). This sub-cohort consists of >700 HCW that have already signed informed consent to participate in studies regarding immunogenicity of the BNT162b2 vaccine (IRB – 8008-20), including performing additional immunology tests on samples obtained. Any samples sent out are de-identified. All parameters and their trajectories will be compared between the Study participants and the Control group.

Vaccine storage and transport: Vaccines will be delivered by thermal shipping and will be stored in 2-8° C.

Statistical analysis: Sample size was determined to identify a 3-fold difference before and after the fourth dose, 190 participants are needed.

Matching of the control group, will follow the algorithm used in our study (Bergwerk et al, NEJM). The Cohort from which the controls are picked included >2000 HCW, with known serology result below 700.

Breakthrough infections will be defined as occurring after at least 8 days since 4th dose. Infection incidence will be compared between the intervention and the control group. Statistical analysis would be conducted in collaboration between the Gertner Institute for health policy and epidemiology and Sheba infection prevention and control unit using accepted methods for comparison between two groups in a case-control study and with SAS statistical program. All data regarding participants would be stored in secured computers and would be available only for this specific study

purpose. Any data transferred to a third party will be de-identified, the identification key will be kept by the PI.

Primary outcomes:

Serology tests as detailed below, including IgG, pseudoneutralization, microneutralization, avidity and IgA. These outcomes will be compared between pre- and post- 4th dose of Spikevax 50 µcg as well as with those outcomes in the control group.

Adverse event reporting of vaccinated individuals by an electronic survey that will be filled from visit 2. This will be compared to adverse event reporting of individuals previously reporting after the 3rd dose from the Sheba HCW COVID Cohort, matched by gender and age. Adverse event questionnaire – Appendix Q2.

Serious adverse events will be defined as any adverse event that resulted in death, hospitalization, permanent damage, required treatment in the emergency room or was life threatening.

Secondary outcomes:

T-cell activity and B-cell repertoire as described below.

SARS-CoV-2 incidence and specifically Omicron VOC incidence.

Detailed Serological and immunological assays:

18. **IgG antibody assay** – Using a commercially available test (SARS-CoV-2 IgG II Quant (Abbott, IL, USA)) we will measure the quantity of IgG antibodies against the SARS-CoV-2 receptor binding domain (RBD). This commercial test will be performed according to manufacturer's instructions
19. **IgA antibody assay-** To test if The forth vaccine dose generates an additive IgA response which may be important in neutralizing SARS-CoV-2 in addition to IgG we will measure IgA levels in serum. A 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4ºC with 50µl per well of 2µg/ml for detection of IgA antibodies. After blocking with 5% skimmed milk at 25ºC for 60 minutes, serum samples will be added to antigen coated wells. The plate will be incubated at 25ºC for 120 minutes, washed and a HRP-conjugated isotype specific antibody (anti-human IgA HRP conjugate (Abcam, MA, USA, product number: ab7383) (diluted 1:2000)) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will

be measured at 450nm using a micro-plate reader (Sunrise, Tecan). ELISA index value below 1.1 negative and equal or above 1.1, positive.

20. **Avidity –** to measure the quality of IgG antibodies we will use urea as a chaotropic reagent and test the strength of interaction between the IgG and the viral antigen (the RBD). Specifically, a 96 well microtiter Polysorb plate (Nunc, Thermo, Denmark) will be coated overnight at 4°C with 50µl per well of 1µg/ml of RBD antigen. After blocking with 5% skimmed milk at 25°C for 60 minutes, serum samples will be diluted 1:100, 1:400 and 1:1000 with 3% skimmed milk and added to antigen coated wells. The plate will be incubated at 25°C for 120 minutes, and following washing each sample,incubated either with the addition of 6M urea or PBS for 10 min. After washing, a goat anti-human IgG horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, PA, USA Code: 109-035-088) (diluted 1:15000) will be added to each well for 60 min. After washing, incubation of TMB Substrate Solution (Abcam) for 5 min and the addition of stop solution (2N HCl), the OD of each well will be measured at 450nm using a micro-plate reader (Sunrise, Tecan). Avidity index will be calculated as the ratio (in percentage) between sample OD with 6M urea and sample OD with PBS.
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    b. We will stimulate T cells by SARS-CoV-2 peptides to analyze the specific activation of CD4 and CD8 T cells in each cohort by flow cytometry using fluorescent probes.
    c. The frequency and specificity of SARS coV-2 specific memory B cells will be tested by flow cytometry using fluorescent probes corresponding to spike protein from different variants. Probe-binding B cells will be sorted by flow cytometry and single-cell immunoglobulin and whole transcriptome sequencing will be performed. From the sequencing reactions, constructs will

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Benefits of participating in the study:

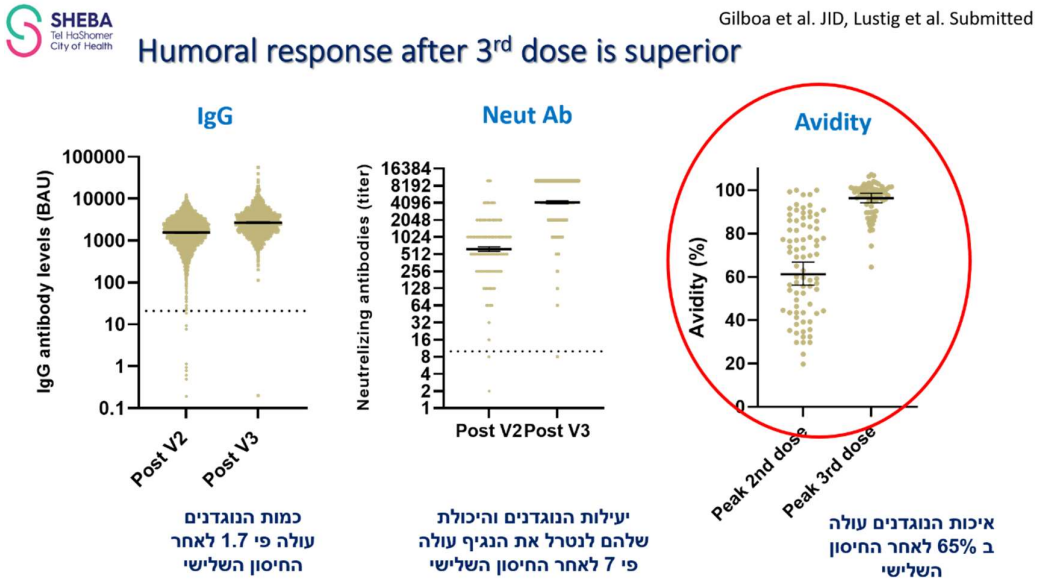
The potential benefit of improved protection from SARS-CoV-2. Currently, according the effectiveness of the previous doses, and in the absence of effective drug therapy, it is expected that an additional dose will raise the level of antibodies and thus raise the protection from SARS-CoV-2.

Risks in participating in the study:

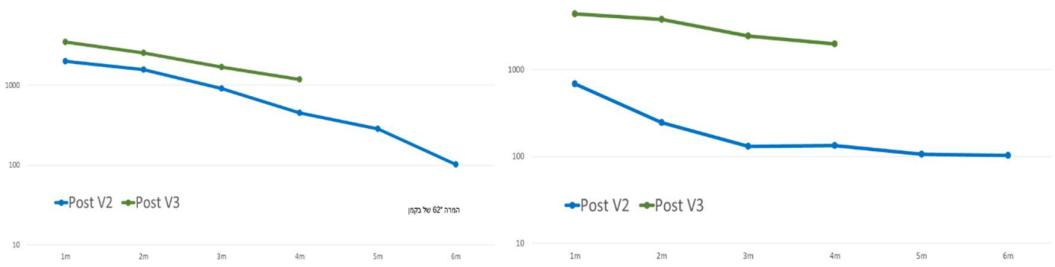
The known previous adverse events are expected to occur, these included frequent local reactions (mostly local pain), as well as systemic reactions including fatigue, fever, lymphadenopathy, myalgia, these have typically lasted up to 48 hours. Rarely, myocarditis has occurred (1:6,000-24,000, mostly in young men). It is yet unknown if a 4^{th} dose will have additional adverse reactions.

Some concerns may be raised regarding potential affinity maturation of B cells specific for ancestral variant epitopes encoded by the vaccine, skewing of the T cell response and depletion of memory T cells against other pathogens. The latter point with respect to T cells is not supported by convincing evidence in humans to suggest that this may be the case in the long term nor that it may be detrimental to human health. Many different vaccines are administered at regular intervals in humans and significant detrimental effects to the immune system's response to specific and other pathogens have not been clearly documented.

Appendix A-1 – superiority of 3rd dose (submitted for publication)



Appendix A2 – Waning immune response after the third dose (preliminary unpublished data)



IgG Neutralizing Ab

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Final Protocol

Immunogenicity of a Fourth mRNA COVID-19 vaccine dose;

Homologous (4 doses of BNT162b2) (IRB-8980-21) or heterologous (mRNA1273 following 3 doses of BNT162b2) (IRB-9035-21)

Study protocol

Background: The Omicron variant of concern (VOC) is currently rapidly spreading worldwide, with extremely high transmission rates, with an estimated R of >3 (Barnard et al., n.d.; Khoury et al., n.d.) . While the virulence of this VOC is yet unknown, even with a much lower virulence than the delta strain, with such high transmission, once again the pandemic will have severe effects on healthcare capacity. Thus, further effort to contain this VOC is required. Currently, the only successful key in containment of the SARS-CoV-2 pandemic was vaccination. Following the first two doses, extremely high vaccine effectiveness was reported from many real-world observational studies(Amit et al., 2021; Dagan et al., 2021; Haas et al., 2021). Several vaccines were reported with high vaccine efficacy and effectiveness, mostly, the mRNA vaccines, of which the BNT162b2 was rolled out in Israel. Yet, with time, waning of this effect was reported, both in immunogenicity as well as in vaccine effectiveness against infections and hospitalizations (Bar-On et al., 2021; Levin et al., 2021)

Following these reports, Israel initiated rollout of the third dose of BNT162b2 on July 29, initially to adults 60 years and older and within a few weeks expanded it to the whole population if at least 5 months have passed from the second dose. We have recently reported the early effects of the 3rd dose(Gilboa et al., 2021) , showing significant increase in IgG and Neutralizing antibodies, as well as increased T-cell activity in a small group of non-responders to first two doses. We have also submitted further data, showing the superiority of the third dose in aspects of immunogenicity, with significantly higher amounts of antibodies as well as improved quality as measured by higher avidity and neutralizing capacity (see yet unpublished data Appendix A1).

We now have preliminary, yet unpublished data, showing slow waning of the third dose immune response within 4 months after the third dose (Appendix A2). Simultaneously, Milo R. et al presented yet unpublished data on early waning of vaccine effectiveness of the third dose. While these data would not have been worrisome in the Delta VOC era, this may be different with the emergence of the Omicron VOC.

It was recently reported, by us and others that vaccine effectiveness against the Omicron VOC is decreased. This was shown by reduced neutralization efficiency of Omicron VOC compared to that of the Delta VOC or the wild type (Wu-1), by sera of vaccinated individuals. We have just reported (Nemet et al., n.d.) , accepted to NEJM) that sera from BNT162b 2-dose vaccinated individuals does not neutralize

Omicron at all, while it neutralizes to a low level the Delta VOC and more so the Wu-1. We compared this to 3-dose vaccinated, and also showed a significantly reduced neutralization of early 3-dose vaccinated as compared to the efficiency against Wu-1 or the delta VOC. Furthermore, data from Germany showed that neutralization of Omicron decreases within 3 months following the 3rd dose.

These data raise the question of when and will we need a 4th dose to cope with the emergence of Omicron. However, if we have reached the maximal effect of the current vaccine against Omicron, with a third dose, will a 4th dose have any added value? In this respect, several studies have demonstrated improved immunogenicity of 1 vaccine dose to SARS-CoV-2 recovered patients, yet a 2nd dose to these patients had very little added value. It is thus unclear whether a 4th dose of the current vaccine, which is not directed specifically at the Omicron VOC, can further improve our immunity and protection from this strain, and will it have any added value.

The Sheba HCW COVID Cohort was established on April 2020, when HCW (including employees, students, volunteers and retired personnel), were recruited to a large serology longitudinal follow up study. Participation in the study is confidential and is not disclosed to the worker's direct supervisor. Since then, nearly 7000 HCW who were vaccinated by BNT162b2 are being followed monthly with blood sampling for immune responses, including mostly IgG, but also Neutralizing ab, avidity, microneutralization, IgA and cellular activation. Through this cohort we found the correlation between neutralizing ab levels and SARS-CoV-2 infections (Bergwerk et al., 2021) , the waning of immunity (Levin et al., 2021)and more. In our study, the median neutralizing ab titer of cases was 190, while it was significantly higher (530) among controls. Furthermore, (*Immune Correlates Analysis of the MRNA-1273 COVID-19 Vaccine Efficacy Clinical Trial*, n.d.) report that titer of 1000 or more are correlated with 96% vaccine efficacy and a titer of 100 is correlated with 91%, but this is for the era before Omicron. We and others have shown a decreased efficacy for Omicron of 8-fold to 24-fold compared to WT.

Here, we will study the potential immunogenicity of a 4th dose, together with assessing safety and efficacy in preventing infections.

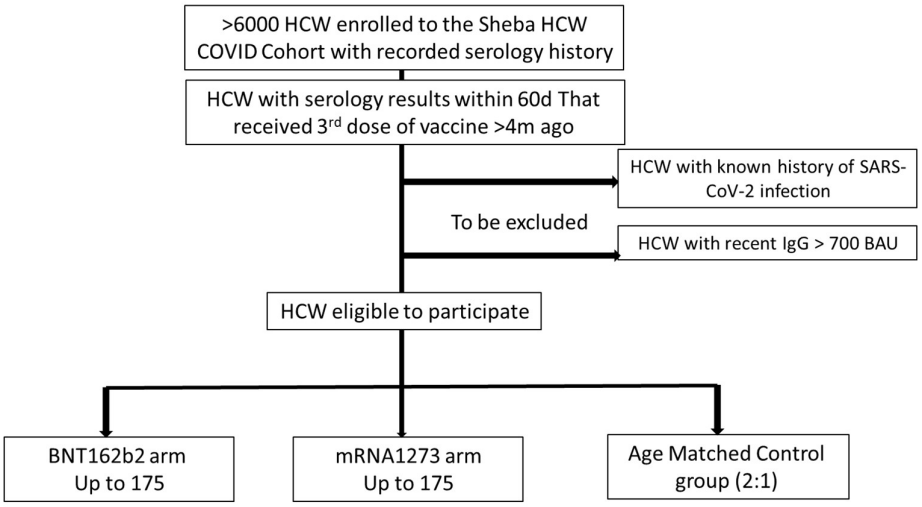
METHODS:

Study Design

This is an open-labled, controlled intervention trial. Two intervention arms are planned and participants will be designated to each arm on a time dependent manner. Eligible participants are those enrolled to the Sheba COVID-19 Cohort study (IRB 8008-20), who have been followed for serology testing, have an available test from the recent 60 days with a result of IgG of 700BAU or below (i.e., under 40 percentile of the full cohort on Dec 2021) and have received the third BNT162b2 dose at least 4 months earlier (see below full inclusion/exclusion criteria). Initially

volunteers will be designated to the BNT162b2 arm, and once approval of the second arm will be received, volunteers will be designated to the second – mRNA1273 arm. Age matched controls, who are eligible to be enrolled to the intervention arm will be selected for the efficacy analysis.

Figure 1: Flow Chart of eligibility and study population selection



Inclusion Criteria:

24. Age: Volunteer must be at least 18 years of age, at the time of signing the informed conset.
25. Sex: Male or Female.
26. Received 3 doses of BNT162b2 with the 3rd dose at least 4 months previously.
27. Have a serology test within the previous 3 months of 700 BAU or less.
28. Responded to the previous vaccine doses, i.e. at least one IgG>100.
29. Medical Conditions: Volunteers with any medical condition are allowed, as long as they adhere to the criteria above.
30. Agreed to attend all visits and signed the informed consent

Exclusion Criteria:

16. Had previous SARS-CoV-2 infection (detected by either PCR, anti-S IgG before the 1st vaccine dose, anti-N IgG at any stage).
17. Had an allergic response to any of the previous BNT162b2 doses.
18. Had history of myopericarditis.
19. Reported that they do not feel well or have a fever on the day of vaccination.
20. Pregnant on day of recruitment.

Criteria for premature cessation of the study:

- Increased rates of immediate adverse events, higher than those reported in previous studies (11 per million) https://www.cdc.gov/mmwr/volumes/70/wr/mm7002e1.htm)

On recruitment, volunteer:

27. Receive a detailed explanation and signed the informed consent form
28. Filled an initial inclusion/ exclusion criteria questionnaire.
29. Filled a general comorbidity questionnaire, additionally they were screened for COVID-19 symptoms such as fever, cough, anosmia
30. Had 40cc blood drawn for all serology and cellular immunity tests.
31. Performed a PCR for SARS-CoV-2 test
32. Receive the 4th dose of BNT162b2 30µg or mRNA1273 50 µg
33. Had a physician checkup and followup for 15 minutes after receiving the dose.

Four additional visits followed as described in the research timeline:

Research Timeline:

Visit number		1	2	3	4
	Day	0	7	14	21
Vaccine	BNT162b2 30 µg or mRNA1273 50 µg	X			
Blood sample	IgG	X	X	X	X
	PNT	X	X	X	X
	microneutralization	X	X	X	X
	IgA	X	X	X	X
	Tcell activity	X		X	
	Bcell repertoir	X		X	
Nasal/NP swab	SARS-CoV-2 PCR	X	X	X	X
Questionnaires	Background comorbidity	X			
	Adverse events			X	

Primary outcomes:

Serology tests as detailed below, including IgG, pseudoneutralization, microneutralization and avidity. These outcomes were compared between pre- and post- 4th dose as well as with those outcomes in the second intervention arm and in the control group.

Adverse event reporting of vaccinated individuals by an electronic survey that will be on day 5 and will be repeatedly sent from visit 2. Serious adverse events will be followed by active surveillance, on each visit, and by direct contact of the research team if a visit has been missed. Serious AE are defined as any adverse event that resulting in death, hospitalization, permanent damage, requirs treatment in the emergency room or life threatening.

Secondary outcomes:

T-cell activity as in detailed methods appendix.

SARS-CoV-2 cumulative incidence and vaccine efficacy will be assessed, by active surveillance of weekly SARS-CoV-2 PCR testing regardless of any symptom. Additionally, symptoms will be assessed on all visits and participants will be encouraged to perform home antigen testing if any symptom occurs in between PCR testing, or upon exposure to SARS-CoV-2 detected individuals.

All data regarding participants would be stored in secured computers and would be available only for this specific study purpose. Any data transferred to a third party will be de-identified, the identification key will be kept by the PI.

Statistical Analysis Plan:

Sample size: To identify a 2-fold difference, in GMT of IgG between the two intervention groups, with alpha of 0.05 and beta of 0.8, 65 participants in each group are needed. To identify a 3-fold difference before and after the fourth dose for each group, 190 participants are needed. To detect a 20% difference in rate of adverse events between the two intervention arms, we will need 108 participants in each group.

To calculate cumulative incidence subjects will be included in follow up starting from day 8 after receiving the fourth dose. For Control participants, the start day will be on the day the matched intervention arm participant entered the study. Follow up will continue until the end of the study or until becoming positive. Analysis will be performed using two methods: Poisson regression with vaccine groups as the main covariates and calendar day and age-group as confounder covariates; and Cox regression, with calendar days for baseline hazard, left truncation for persons in the vaccine groups who are vaccinated later than January 3rd, including vaccine groups as the main covariates and age-group as confounding covariates. In secondary analyses, the effect of vaccine in two separate periods will be evaluated: from 8-14 days following vaccination and from 15-29 days following vaccination.

Benefits of participating in the study:

The potential benefit of improved protection from SARS-CoV-2. Currently, according the effectiveness of the previous doses, and in the absence of effective drug therapy, it is

expected that an additional dose will raise the level of antibodies and thus raise the protection from SARS-CoV-2.

Risks in participating in the study:

The known previous adverse events are expected to occur, these included frequent local reactions (mostly local pain), as well as systemic reactions including fatigue, fever, lymphadenopathy, myalgia, these have typically lasted up to 48 hours. Rarely, myocarditis has occurred (1:6,000-24,000, mostly in young men). It is yet unknown if a 4th dose will have additional adverse reactions.

Some concerns may be raised regarding potential affinity maturation of B cells specific for ancestral variant epitopes encoded by the vaccine, skewing of the T cell response and depletion of memory T cells against other pathogens. The latter point with respect to T cells is not supported by convincing evidence in humans to suggest that this may be the case in the long term nor that it may be detrimental to human health. Many different vaccines are administered at regular intervals in humans and significant detrimental effects to the immune system's response to specific and other pathogens have not been clearly documented.

General comorbidity computer based questionnaire: see Appendix A.

Adverse event questionnaire: See Appendix B.

Summary of Protocol Changes:

Protocol	Version	Version	Section	Change
8980-21	1	2	All	English translated
8980-21	2	4	Footer	Date: December 15, to December 26, 2021
			Background	Added detail on ab as correlates of protection
			Study Aim	Clarified the use of the matched control group
			Study Design	Clarified and further detail on the Sheba COVID HCW Cohort, from which the control group will be matched
			1st visit activities	Additions: Inclusion/exclusion criteria questionnaire Screening for COVID-10 symptoms Physician checkup
			Inclusion Criteria	Clarification on Medical conditions: Volunteers with any medical condition are allowed as long as they adhere to other inclusion criteria.
			Exclusion Criteria	Additions: Report they do not feel well or have fever on the day of vaccination. Pregnant on day of recruitment
			Criteria for premature cessation of study	Section was added
			Insurance	Section was added
			Control group	Detail about their previous enrollment, IRB approval was added
			Vaccine storage and transport	Section was added
			Statistical analysis	Section was added
			Detailed Serological assays	IgA antibody assay subsection was added
			Risks in participating in the study	Detail added regarding potential risk of affinity maturation and skewing of T cell response, and depletion of memory Tcells.
9035-21	1	2	Footer	Date: December 30, 2021 to Jan 3, 2022

			Background	Addition of the fact that the MOH has approved a 4th dose of COVID vaccine to HCW.
Final	8980-21	9035-21	Title	Changed to : Immunogenicity of a fourth mRNA COVID-19 vaccine dose; Homologous or heterologous
			Organization of sections	Change in order, (1) study design, for clarification a flow chart of eligibility and study population was added. (2) inclusion/exclusion criteria (3) research timeline (4) Outcomes (5) statistical analysis plan (6) Benefits and Risks in participating.
			Study Design	An open-label, two arm intervention. Participant intervention designation is time dependent.
			Statistical Analysis plan	Includes more detail on assessment of cumulative incidence of infections and vaccine efficacy

Statistical Analysis Plan (SAP)

Immunogenicity of a Fourth mRNA COVID-19 vaccine dose;

Homologous (4 doses of BNT162b2) (IRB-8980-21) or heterologous (mRNA1273 following 3 doses of BNT162b2) (IRB-9035-21)

Introduction

This statistical analysis plan (SAP) provides detailed methodology for summary and statistical analysis of the data collected in studies 8980-21 and 9035-21.

Study Aims: The major objective of this study is to describe the immune responses following a 4th dose of either BNT162b2 or mRNA1273, following 3 doses of BNT162b2, when the third dose was given at least 4 months earlier.

Immune responses to be assessed:

1. Anti-SARS-CoV-2 RBD IgG
2. Anti-SARS-CoV-2 IgA
3. Avidity of IgG antibodies
4. Sars-CoV-2 pseudovirus neutralization
5. SARS-CoV-2 micro-neutralization of different VOCs available and wild type
6. T-cell activation by elispot
7. T-cell activation by flow cytometry
8. B cell repertoire

Of the above, (1), (4) and (5) are primary outcomes and other immune responses are secondary and exploratory outcomes.

The second major objective is to assess safety of a 4th dose. The following solicited local and systemic adverse events will be reported and the proportion of participants reporting each event among the 4th dose recipients will be calculated with its 95%CL.

1. Local reactions, including pain, redness, swelling or itching in the injection site.
2. Systemic reaction, including lymph node swelling (local or generalized), fever > 37.5, fatigue, myalgia, headache, facial nerve palsy, paresthesia, allergic reaction.

Unsolicited AE will also be collected on each visit until visit 5.

Secondary aim: to assess incidence and characteristics of breakthrough infections in 4th dose recipients and compare them to infections in the control group, and between recipients of either vaccine.

Study Design: This is an open-label, non-randomized trial, to assess the immune response of a 4th dose of either BNT162b2 or mRNA1273. We plan to enroll 150-200 participants to each arm. A control group, which will not recieive a 4th dose, but has similar baseline characteristics (from the same eligibile sub-cohort) will be selected for comparisons. For each vaccine recipient 2 matched controls will be selected. Several measures of immunogenicity will be measured and reported:

1. Immune responses before and after the fourth dose will be compared.
2. Immune responses at peak, following 4th dose will be compared to that after 3rd and 2nd doses.
3. Immune responses at peak following the 4th dose, will be compared to that of the control group, which did not receive a 4th dose.
4. Immune responses at peak and the rate of waning of the two vaccines will be compared.

Randomization: This a non-randomized, open-label trial. Designation to an intervention arm will be time dependent. Enrollment to the first intervention arm (BNT162b2) will take place during two consecutive days. Once approval of protocol 9035-21 will be given, two days of enrollment to the second arm will take place.

Blinding: This study is open-labeled, unblended.

Control group: From the sub cohort of all eligible individuals , an age matched (+/-5 years age difference allowed), will be selected in a 1:2 ratio to each participant in each of the intervention arms. A single control will be allowed to serve as a matched control of both intervention groups. Perfect age and gender will be preferred. Control group will be encouraged through text messages, e-mails and telephone calls to obtain a once weekly RT-PCR test for COVID-19. They will be instructed to report any positive COVID-19 test obtained regardless of whether it was taken as part of the study. Additionally they will receive a computer based questionnaire to assess their compliance with COVID-19 testing.

Study Objectives and Endpoints:

Objective	Estimate	Endpoint (outcome measure)
Primary		
To assess immune response to a 4th COVID-19 mRNA vaccine	Geometric mean titer (GMT) at each time point	SARS-CoV-2 RBD-IgG Pseudoneutralization, microneutralizaion of Delta, Omicron and WT strains
	Geometric mean fold rise	Before and after 4th dose
	Geometric mean ratios	Peak of 2nd vs. 3rd vs. 4th dose Intervention group vs. control group Comparison of the 2 intervention groups
To assess safety of a 4th COVID-19 mRNA vaccine	Number of reported AE and the proportion of reported events in each group	Immediate reactions (allergic, within 30 min of vaccine administration)
	Number of reported AE and the proportion of reported events in each group	Solicited local reactions from day 5 to day 21 (pain, rash, redness, itching, swelling)
	Number of reported AE and the proportion of	Solicited systemic AE (lymph node swelling, fever > 37.5,

	reported events in each group	fatigue, myalgia, headache, facial nerve palsy, paresthesia, late allergic reaction
Secondary		
To assess immune response to a 4th COVID-19 mRNA vaccine	Geometric mean titer (GMT) at each time point	IgA, T cell activity, avidity, microneutralization of various VOC
To assess SARS-CoV-2 breakthrough infection incidence and characterize breakthrough infections	Breakthrough infection rate in each group (intervention and control)	PCR positivity Rapid Ag test positivity N-gene Ct value COVID-19 symptoms ER or hospitalization

Endpoints:

Primary Endpoints:

Primary Immunogenicity Endpoints:

1) Anti SARS-CoV-2 RBD IgG titers in BAU at each time point. GMT of the groups will be calculated with their 95% confidence interval.
2) Pseudoneutralization titers at each time point for each group will similarly be calculated.
3) Microneutralization titers of Delta, Omicron and WT strains before 4th dose and on each following visit.

Primary Safety Endpoints are as follows:

1) Immediate Reactions – participants will be followed for 30 min after recipient of the vaccine dose. Any immediate reaction will be reported. Primary endpoints that will be reported: The number of immediate reactions in each group, and proportion of immediate reactions. Comparisons between the groups and between age groups will be performed.
2) Local reactions (Pain, redness, swelling, rash or itching) will be collected via an electronic questionnaire from day 5 to day 21. The following measures will be assessed:
    a. Presence or absence
    b. Duration (in days)
    c. Severity (from mild-1, to severe-10)
3) Systemic AE (Lymph node enlargement, Fever>37.5, fatigue, myalgia, headache, facial nerve palsy, paresthesia, late allergic reaction) will be similarly collected via the electronic questionnaire from day 5 to 21. The following measures will be assessed:
    a. Presence or absence
    b. Duration(in days)

c. Severity (from mild-1, to severe-10), severity will be divided to three categories- 1-3- mild, 4-6 moderate, 7-10 severe

4) Serious AE will be collected through the 180 days of the study. Will be categorized according the MedDRA terms. The safety endpoints "SAEs from the vaccination (received in this study) through 6 months after the vaccination" will be summarized by system organ class and preferred term. Additionally, SAEs will be listed.

Derived variables for presence of each and any local or systemic adverse reaction within 21 days of each vaccination

Variable	Yes (1)	No (0)
Presence of each local reaction on any questionnaire	Participant reports the reaction as "yes" on any of the questionnaires (within 21 days of vaccination)	Participant reports the reaction as "no" on all of the questionnaires (within 21 days of vaccination)
Presence of any local reaction on any questionnaire	Participant reports any local reaction as "yes" on any of the questionnaires (within 21 days of vaccination)	Participant reports all local reactions as "no" on all of the questionnaires (within 21 days of vaccination)
Presence of each systemic AE on any questionnaire	Participant reports the reaction as "yes" on any of the questionnaires (within 21 days of vaccination)	Participant reports the reaction as "no" on all of the questionnaires (within 21 days of vaccination)
Presence of any systemic AE on any questionnaire	Participant reports any systemic AE as "yes" on any of the questionnaires (within 21 days of vaccination)	Participant reports all systemic AE as "no" on all of the questionnaires (within 21 days of vaccination)

Secondary endpoints:

Secondary Immunogenicity Endpoints:

1) Anti SARS-CoV-2 IgA at each time point. GMT of the groups will be calculated with their 95% confidence interval.
2) T cell activity, measured as Tcell activity/10^6 cells. Will be assessed on visits 1, 3, 5 and 7.

Secondary SARS-CoV-2 infection Endpoints

3) SARS-CoV-2 PCR will be performed by nasopharyngeal-oropharyngeal swabs in each visit, and while the pandemic surge is high (>10k newly detected cases/d) active weekly surveillance will continue, for both intervention and control arms.
4) Symptomatic COVID – any SARS-CoV-2 symptom will be requested to be reported. Upon reporting of such a symptom, rapid Ag test and PCR will be performed.

Exploratory endpoints:

Bcell repertoire – initially, on visits 1, 3, 5 and 7, whole blood will be drawn PBMCs separated, and frozen for later evaluation of the B cell repertoire.

Baseline variables:

Measurements or samples collected prior to the study vaccination in this study period are considered the baseline data for the assessments.

Demographics, Medical and vaccination History – have been collected upon initial enrollment to the HCW serology study. Yet, the general comorbidity computer-based questionnaire will be filled upon enrollment to this study once again, to reassure full data availablility. These data include date of birth, sex (male or female) , height and weight, comorbidities including hypertention, dyslipidemia, autoimmune disease, diabetes, heart disease, lung disease, coagulation disorder, immunosuppression (including organ transplant recipient, currently undergoing biologic therapy/chemotherapy, treated with corticosteroids, underwent a splenectomy, or diagnosed with HIV), liver disease and kidney disease.

Physical exam by physician / nurse, on vaccination day, including BP, pulse, will be measured following vaccine administration, and if any immediate reaction will develop.

Sample Size and Power

To identify a 2-fold difference, in GMT of IgG between the two intervention groups, with alpha of 0.05 and beta of 0.8, 65 participants in each group are needed. To identify a 3-fold difference before and after the fourth dose for each group, 190 participants are needed. To detect a 20% difference in rate of adverse events between the two intervention arms, we will need 108 participants in each group.

Safety Monitoring Committee reports:

Following each visit a Safety monitoring report will be sent to the committee for evaluation.

Immunogenicity Review

Due to the urgency to inform public health decisions. Following each timepoint, within 2 weeks, as the primary outcomes will be analysed and reported as needed. Data will be disseminated to public health officials as needed and presented to inform the scientific community.

General Analyses

The following descriptive statistics will be used to summarize continuous variables: number of non missing values, mean, standard deviation, median, range.

For binary variables: descriptive statistics for categorical variables (proportions) will be presented in percentage, and the 95%CI, when applicable.

For antibody titers the geometric means will be calculated as the mean of the assay results after making the logarithmic transformation and then exponentiating the mean to express results on the original scale. Two sided 95% CI will be obtained by taking log transformation of assay results, calculating the 95% CI with reference to students t-distribution and then exponentiating the confidence limits.

Geometric mean fold rises –(GMFR) are defined as ratios of the results after vaccination to the results before vaccination. GMFR are limited to participants with nonmissing values at both time points. They are calculated as the mean of the difference of logarithmically transformed assay result and exponentiating the mean. The associated 2-sided 95% Cis will be obtained by constructing Cis using students t-distribution and then exponentiating the confidence limits.

Geometric mean ratios – will be calculated as the mean of the difference of logarithmically transformed assay results and exponentiating the mean. Two-sided Cis will be obtained.

Methods to manage missing data

Participants will receive 4 adverse events electronic questionnaires, participants who will not reply to any of the questionnaires will be contacted by telephone to answer a phone questionnaire, participants who will not reply to any of the questionnaires will be considered as missing data.

Missing serology results will not be imputed.

No additional imputation will be applied to other missing data.

Graphical summaries of the data will be presented using graphpad, including bar plots, scatter plots or line plots.

Addition to Statistical Analysis Plan (SAP)

Immunogenicity of a Fourth mRNA COVID-19 vaccine dose;

Homologous (4 doses of BNT162b2) (IRB-8980-21) or heterologous (mRNA1273 following 3 doses of BNT162b2) (IRB-9035-21)

Jan 6, 2022

The study SAP was not changed. Yet, calculation of vaccine effectiveness were not part of the initial protocol since the expected number of events of COVID-19 infections was estimated to be too low to calculate the VE with sufficient power. Due to the extremely high rate of newly detected cases in Israel (18,000 new cases on Jan 5, 2022, and rapidly increasing effective reproduction number-R- that was 1.84 on Jan 2, 2022), the number of events of COVID-19 infections in all three groups- BNT162b2, mRNA1273 and controls, was much higher than initially expected. Thus, estimation of VE was added to the statistical plan, as follows:

Vaccinated subjects entered follow up 7 days after vaccine day unless they become positive before that. They are followed until being positive or end of follow up (30th of Jan 2022). The Moderna group were vaccinated about a week after BNT. The control group started follow up on 27th of Dec 2021 but since the vaccinated follow-up starts 7 days after vaccine day we started their follow up only from the 3rd of Jan 2022. The Control group was followed until being vaccinated or end of follow up. As a result the BNT and Control groups have identical period of follow up whereas the Moderna started follow up a week later. We split the control group for a control for BNT and a control to Moderna according to their matching (some are control for both). The cumulative incidence of each treatment group and its control are given below.

	group	day	cuminc	lowerCI	upperCI
1	Control	23	0.2148884	0.1519313	0.2731719
2	Control	28	0.2525738	0.1849619	0.3145769
3	BNT	23	0.1699346	0.1082381	0.2273627
4	BNT	28	0.1830065	0.1193810	0.2420351

	group	day	cuminc	lowerCI	upperCI
1	Control	17	0.2558150	0.1800064	0.3246150
2	Control	19	0.2558150	0.1800064	0.3246150
3	Moderna	17	0.2068966	0.1296463	0.2772903

4 Moderna 19 0.2068966 0.1296463 0.2772903

We applied a cox regression and used the calendar days for baseline hazard and vaccinated subjects were entered at their start of follow up by left truncation. The estimated coefficients are:

n= 692, number of events= 128

	coef	exp(coef)	se(coef)	z	Pr(>|z|)	
groupBNT	-0.3586	0.6986	0.2252	-1.593	0.111264	
groupmodern	-0.1138	0.8925	0.2424	-0.469	0.638862	
agec40-59	-0.3365	0.7143	0.2281	-1.475	0.140090	
agec60+	-0.8848	0.4128	0.2629	-3.366	0.000764	***

In a poisson regression I get (removing daily date effects and intercept):

	Estimate	Std. Error	z value	Pr(>|z|)
groupBNT	-0.3574	0.2252	-1.5874	0.1124
groupmodern	-0.1139	0.2424	-0.4698	0.6385
agec40-59	-0.3336	0.2280	-1.4629	0.1435
agec60+	-0.8786	0.2629	-3.3423	0.0008

Efficacy and 95% CI

	efficacy	lower_CI	upper_CI	pvalue
groupBNT	0.3005	-0.0875	0.5501	0.1124
groupmodern	0.1077	-0.4352	0.4452	0.6385
agec40-59	0.2837	-0.1200	0.5418	0.1435
agec60+	0.5846	0.3047	0.7519	0.0008

Additionally, as a sensitivity analysis, we examined change in efficacy in treatment groups from day 15

The poisson regression model (removing daily date effects and intercept):

	Estimate	Std. Error	z value	Pr(>|z|)
vacBNT	0.1068	0.5231	0.2043	0.8381
vacBNT_15+	-0.4416	0.2468	-1.7897	0.0735
vacmodern	-0.7208	0.4422	-1.6301	0.1031
vacmodern_15+	0.1787	0.2887	0.6190	0.5359
agec40-59	-0.3293	0.2280	-1.4440	0.1487
agec60+	-0.8714	0.2629	-3.3152	0.0009

Efficacy and 95% CI

	efficacy	lower_CI	upper_CI	pvalue
vacBNT	-0.1128	-2.1019	0.6008	0.8381

vacBNT_15+ 0.3570 -0.0429 0.6036 0.0735
vacmodern 0.5136 -0.1570 0.7956 0.1031
vacmodern_15+ -0.1957 -1.1054 0.3210 0.5359
agec40-59 0.2805 -0.1249 0.5398 0.1487
agec60+ 0.5816 0.2997 0.7501 0.0009

The differences between before and after 15 days was tested in 2 ways:

Using likelihood ratio test between the 2 models gives a nonsignificant improvement in the fit

.

Model 1: npos ~ group + agec + datec
Model 2: npos ~ vac + agec + datec
Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1 199 197.08
2 197 192.89 2 4.1874 0.1232

Testing the difference in the coefficients before and after 15 days for each treatment group I g et:

difference STD.Err p-value
BNT 0.5485 0.5752 0.3403
Moderna -0.8995 0.5156 0.0811

Both tests did not show a significant difference.

A sub-analysis of only symptomatic cases was also performed

The Poisson model estimates:

Coefficients:

Estimate Std. Error z value Pr(>|z|)
(Intercept) -4.28076 0.53421 -8.013 1.12e-15 ***
groupBNT -0.56402 0.25292 -2.230 0.02575 *
groupmodern -0.37637 0.27844 -1.352 0.17646
agec40-59 -0.29961 0.24641 -1.216 0.22402
agec60+ -0.91834 0.28976 -3.169 0.00153 **

Efficacy

efficacy lower_CI upper_CI pvalue
groupBNT 0.4311 0.0660 0.6535 0.0257
groupmodern 0.3137 -0.1845 0.6023 0.1765
agec40-59 0.2589 -0.2012 0.5428 0.2240
agec60+ 0.6008 0.2956 0.7738 0.0015

On January 2nd 2022 Israeli ministry of health decided to allow all health care workers to receive a fourth dose of the BNT162b2 vaccine. Matched controls who received the fourth dose of the vaccine were censored since the vaccination date.