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Therapeutic Goods Administration

Nonclinical Evaluation Report

BNT162b2 [mRNA] COVID-19 vaccine (COMIRNATY™)

Submission No: PM-2020-05461-1-2

Sponsor: Pfizer Australia Pty Ltd

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TGA Health Safety
Regulation

NONCLINICAL EVALUATION REPORT

Submission type: New vaccine
Sponsor: Pfizer Australia Pty Ltd
Generic name: BNT162b2 [mRNA] COVID-19 vaccine
Trade name: COMIRNATY
Dose form and strength: Solution; 500 µg/mL (diluted to deliver 30 µg/0.3 mL per injection)
Vaccine Type: mRNA encapsulated in LNP

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SUMMARY

- Pfizer Australia Pty Ltd has applied for provisional registration of a new mRNA vaccine, BNT162b2 [mRNA] COVID-19 vaccine (COMIRNATY), in lipid nanoparticle (LNP) formulation [BNT162b2 (V9)] indicated for active immunisation to prevent COVID-19 disease caused by SARS-CoV-2 virus, in individuals 16 years of age and older. The proposed dosing regimen involved administering two doses of 30 µg of mRNA/0.3 mL per dose given 21 days apart intramuscularly (IM).
- The sponsor has generally conducted adequate studies on pharmacology and toxicity (GLP compliant repeat dose and developmental and reproductive toxicity studies) with BNT162b2 (V9). Limited pharmacokinetic studies were conducted with the LNP formulation and two novel lipid excipients (ALC-0159 and ALC-0315).
- BNT162b2 (V9) was found to be immunogenic in non-clinical studies in mice, rats and rhesus macaques. BNT162b2 (V9) induced humoral and cellular immune responses in mice and monkeys. However, antibodies and T cells in monkeys declined quickly after 5 weeks after the second dose of BNT162b2 (V9) raising long term immunity concerns.
- The vaccine protected monkeys from infection when challenged 55 days after the 2nd vaccine dose based on viral RNA load and radiographic lung lesions. The vaccine dose in monkeys (100 µg) was higher than the proposed clinical dose of 30 µg.
- Almost similar microscopic lung inflammation was observed in both challenged control and immunised animals after the peak of infection (Days 7/8). Rhesus macaques do not show clinical signs and generally develop only mild lung pathology from SARS-CoV-2 infection. There were no studies on protection of older animals from SARS-CoV-2 infection or duration of protection after immunisation. The animal studies were of short term; long term immunity was not assessed. The sponsor indicated that long term immunity would be addressed by human data.
- There are no distribution and degradation data on the S antigen-encoding mRNA. A whole body imaging study with a surrogate, luciferase expressing mRNA indicate that the vaccine LNP formulation is expected to deliver the mRNA effectively *in vivo*, the mRNA and translated antigen protein are mainly localised at the injection site, distributed in liver and likely draining lymph nodes, and nearly completely degraded in 9 days.
- The limited studies showed slow elimination of ALC-0315 and retention in liver, and complete elimination of ALC-0159 in 14 days, with the latter eliminated in faeces most likely by biliary excretion. Both lipids are also eliminated by amide or ester hydrolysis.
- BNT162b2 (V9) was tolerated in rats without evidence of systemic toxicity. Rats administered BNT162b2 (V9) (3 IM doses once weekly at 30 µg/dose, ~200 times the clinical dose on a µg/kg basis) showed injection site inflammation, clinical pathology (increases in white blood cells, basophils, eosinophils, neutrophils, large unstained cells, fibrinogen, and acute phase proteins), and tissue pathology (hypercellularity of draining lymph nodes, spleen and bone marrow), consistent with immune stimulation and inflammation responses as well as minimal vacuolation of portal hepatocytes without evidence of liver injury. All effects were either partially or fully reversible after 3 weeks of recovery. However, the dosing interval (one week) was not ideal given the immune response peaks 2-3 weeks after dosing, and the clinical dosing interval is 3 weeks. Given the availability of clinical data, another repeat dose study in animals is not considered necessary. The shortcoming of the repeat dose toxicity study design should not preclude approval of the vaccine.

- A combined reproductive and developmental study showed no adverse effects on female fertility, embryofetal development and post-natal development (up to weaning) in rats. Female rats administered IM four times (21 and 14 days prior mating and gestation days 9 and 20) with 30 µg/dose BNT162b2 showed transient decreases in maternal weight gain after each injection and injection site inflammation. Neutralising antibodies were induced in dams and detected in fetuses and pups.
- There are no repeat dose or reproductive toxicity studies specifically with the novel excipients. Findings in the studies with the LNP vaccine formulation do not appear to be due to the lipid excipients except for hepatocyte vacuolation, which was probably a manifest of hepatocyte uptake of lipids. Given that both the novel excipients are amino or amino/PEG lipids, potential lifetime exposure is expected to be low, and the availability of clinical data, the lack of repeat dose toxicity studies with the excipients in a second species is acceptable. No significant increase in cytokines was observed in the repeat dose study in rats with the V8 variant; however the number of animals studied for cytokines was small ($n=3$) and there was high inter-animal variation. One *in vitro* study using human PBMC gave inconclusive results. Therefore, the potential of the LNP or the vaccine formulation for complement activation or stimulation of cytokine release was not adequately assessed in nonclinical studies.
- Considering the chemical structure of the excipients and limited potential lifetime exposure, the excipients are not expected to pose genotoxicity or carcinogenicity potential.

CONCLUSIONS AND RECOMMENDATION

- Primary pharmacology studies indicate the vaccine elicits both neutralizing antibody and cellular immune responses to the spike (S) antigen in mice and monkeys, and conferred some protection of monkeys from infection.
- Antibodies and T cells in monkeys declined quickly over 5 weeks after the second dose of BNT162b2 (V9), raising concerns over long term immunity, which will be assessed by clinical studies according to the Sponsor.
- Repeat dose toxicity studies with the proposed vaccine and a variant, both in the LNP formulation, in rats raised no safety issues. Findings were consistent with immune stimulation and inflammation responses (injection site inflammation, increased body temperature, leucocytosis, increased large unstained cells, fibrinogen and acute phase proteins, and hypercellularity of lymphohaematopoietic tissues). Hepatocyte vacuolation (probably lipid vacuoles) was not associated with evidence of liver injury and was reversible.
- The toxicity of the LNP formulation and novel excipients ALC-0159 and ALC-0315 was assessed in one species as part of the repeat dose study with the vaccine. Neither the mRNA nor the lipid excipients of the LNP formulation are expected to have genotoxic potential. However, the potential of the LNP or the vaccine formulation for complement activation or stimulation of cytokine release was not adequately assessed in nonclinical studies. Further investigation (i.e., analysis of complement activation and cytokine stimulation) is recommended unless this particular concern is addressed by clinical data. The absence of a repeat dose toxicity study in a second species and genotoxicity studies with the novel excipients was adequately justified by the Sponsor.
- A combined reproductive and developmental study showed no adverse effects on female fertility, embryofetal development and post-natal development (up to weaning) in rats. Pregnancy category B1 is considered acceptable.

- Short term protection studies, lack of pharmacokinetic data for the S antigen-encoding mRNA (BNT162b2 V9), suboptimal dosing interval in the repeat dose study, lack of repeat dose toxicity studies in a second species and genotoxicity studies with the novel excipients, and lack of studies investigating potential for autoimmune diseases were noted. However, these deficiencies are either adequately justified by the Sponsor or addressable by clinical data.
- There are no nonclinical objections to the provisional registration of the vaccine. Long term immunity, vaccine induced autoimmune diseases were not studied in the nonclinical program and should be addressed by clinical data post provisional registration. Nonclinical studies on complement activation and stimulation of cytokine release are recommended unless these issues are addressed by clinical data.
- The draft Product Information should be amended as directed on pages 15-16.

ASSESSMENT

General comments

Pfizer Australia Pty Ltd has applied for provisional registration of a new biological entity, BNT162b2 [mRNA] COVID-19 vaccine (COMIRNATY), under section 23AA of the Act. This vaccine is indicated for active immunisation to prevent COVID-19 disease caused by SARS-CoV-2 virus, in individuals 16 years of age and older. BNT162b2 is an mRNA vaccine in lipid nanoparticle (LNP) formulation (LNP-BNT162b2), expressing the full-length SARS-CoV-2 spike glycoprotein (S). The proposed dosing regimen is two doses of 30 µg of mRNA/0.3 mL per dose given 21 days apart intramuscularly (IM).

Module 4 comprised of nonclinical studies with a number of variants of mRNAs encoding the S protein including BNT162b2 [V8 and the final commercial candidate V9] formulated in LNP formulations. Pivotal studies were conducted with the clinical formulation. It was indicated by the sponsor that the BNT162b2 V8 and V9 variants have identical amino acid sequence with slight differences in their codon optimisation sequences for better antigen expression.

The vaccine formulation contains two novel excipients, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (**ALC-0159**) and ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (**ALC-0315**).

Pharmacology

BNT162b2 mRNA (V9) encodes the full-length of the SARS-CoV-2 Spike-glycoprotein with the modification of two nucleosides at residues 986 and 987 (replaced by proline; expressed protein named as P2 S). BNT162b2 mRNA is encapsulated in LNP, which is expected to enable entry into host cells, expression of the S protein, and elicitation of both antibody and cellular immune responses. The S protein binds to the host cell receptor, angiotensin-converting enzyme 2 (ACE2) to gain entry into host cells (Chou et al. 2020).

The *in vitro* expression of S antigen was confirmed in mammalian cells (human embryonic kidney cells) using western blot, flow cytometry and immunofluorescence assays.

Immunogenicity was assessed in wild type mice and rhesus monkeys, and also in rats in the repeat dose toxicity studies and the developmental and reproductive study. A protection study was conducted in rhesus monkeys since ACE2 of rhesus macaques has high homology to human ACE2 and greater binding activity for SARS-CoV-2 than ACE2 of other laboratory animals (Wan et al. 2020). Antigen-specific enzyme linked immunosorbent assays (ELISA) and virus neutralisation assays using pseudovirion (mouse study and rat repeat dose toxicity study) or wild-type SARS-CoV-2 virus (monkey study and rat repeat dose and reproductive toxicity study) were used to characterise the humoral response. Evaluation of cellular responses included the examination of CD8⁺ and CD4⁺ T cell responses. A few limitations were noted in the submitted pharmacology studies. While mouse models in the pharmacology studies could be used to assess immunogenicity of the vaccine, wild type mice could not be used for assessing protective efficacy of the vaccine since the SARS-CoV-2 S protein does not effectively bind to ACE2 of wild type mice to initiate replication (Zhao et al. 2020). Thus, protective efficacy was assessed only in one animal species, the rhesus monkey.

S antigen expression

P2 S antigen expression was demonstrated in HEK293 cells (human embryonic kidney cells) *in vitro* transfected with BNT162b2 mRNA using a commercial transfection kit, BNT162b2 mRNA formulated in the LNP formulation or in a human cell line transfected with a modified pcDNA3.1 construct encoding P2S. S protein encoding mRNAs on modified RNA (modRNA) platforms demonstrated slightly higher transfection rates compared to self-amplifying mRNAs (saRNAs). However, S antigen expression was higher by the saRNAs compared to the modRNAs. This is expected considering the replicative nature of the saRNA platform.

The expressed S protein co-localised with an endoplasmic reticulum (ER) marker, suggesting the S protein is synthesised and processed within the ER for surface expression or secretion. The expressed P2 S had high binding affinity to human ACE2 peptidase domain and an anti-RBD human neutralising antibody ($K_D \sim 1.2$ nM) and also bound to antibodies from COVID-19 convalescent patients. CryoEM analysis of the purified S2 protein expressed from DNA confirmed the prefusion conformation of the P2 S similar to previously reported structures of P2 S (Cai et al. 2020; Henderson et al. 2020, Wrapp et al. 2020).

Immunogenicity

BNT162b2 (V9) was found to be immunogenic in mice and monkeys, inducing both humoral and cellular immunity. IgG antibodies in mice had more potent affinity for the receptor binding domain (RBD) (K_D 0.99 nM) of the S protein compared to S1 (K_D 12 nM) of the S protein, both with high on rate (k_{on}) and low off rate (k_{off}).

In mice, a single IM dose of BNT162b2 (V9) at 0.2, 1 or 5 μ g/animal induced anti-S1, anti-RBD and virus-neutralising antibodies, which were detectable from day 7 and titres increased with increasing dose and time over 28 days after immunisation (last sampling time). Antibodies induced in mice were both of IgG2 and IgG1 subtypes, with IgG2a slightly higher than IgG1 at the 1 and 5 μ g immunisation dose, but lower than IgG1 at 0.2 μ g, suggesting a T_H1 -biased immune response at high doses (≥ 1 μ g) and a T_H2 -biased response at low doses.

In monkeys, BNT162b2 (V9) induced similar anti-S1 IgG and SARS-CoV-2 neutralising antibodies at the IM dose of 30 or 100 μ g (5 and 17 μ g/kg, respectively, based on a body weight of 6 kg). A booster dose 3 weeks after the first dose markedly increased antibody titres. Antibodies gradually decreased after immunisation, and 4 weeks after the booster dose, the S1-binding and neutralising titres reduced by 3-7 fold. Serum antibodies in the immunised monkeys were not followed up beyond 4 weeks after the booster dose. The Sponsor stated that long term immunity was being assessed in ongoing Phase 1 studies in the US and Germany up to 2 years following vaccination, and these data would provide a more accurate measure of long-term immunity.

BNT162b2 (V9) also induced cellular immune responses in mice and monkeys. The vaccine elicited a T_H1 -dominant T cell response after a single dose of 1 or 5 μ g in mice and two doses (30 or 100 μ g) in monkeys. In mice, BNT162b2 immunisation at ≥ 1 μ g induced high levels of T1 responses as shown by $IFN\gamma^+$ CD4 and CD8 splenocytes, increased cytokine release of T1 type cytokines ($IFN\gamma$, IL-2, $TNF\alpha$) by splenocytes after S peptides-stimulation *ex vivo*, and very low levels of T_H2 cytokines (IL-4, IL-5, IL-13) except for unexplained high levels of IL-4, IL-5, IL-13 in one out of 8 animals.

BNT162b2 immunisation also induced proinflammatory cytokines such as GM-CSF, $TNF-\alpha$, IL-6 and IL-18, in addition to $IFN-\gamma$, in splenocytes. BNT162b2 increased CD8⁺, CD4⁺ and T_{FH} cells (but not T_H1) in draining lymph nodes. Similar to findings in mice, S-specific T_H1 -dominant $IFN\gamma/IL2/TNF\alpha$ + T-cell responses were detected in all immunised monkeys with a dose-dependent increase in S-specific CD4⁺ T cell responses despite a T_H2 -biased response in one out of 6 monkeys. As in mice, the vaccine also induced a CD8⁺ T cell response in monkeys. The Sponsor indicated that clinical data has also demonstrated a strong T_H1 -dominant CD4⁺ T cell and $IFN\gamma$ + CD8⁺ T cell response following two doses of 30 μ g BNT162b2.

BNT162b2 (V8) induced humoral and cellular immune responses in mice similar to BNT162b2 (V9) when administered intramuscularly. However, lower titres of functional neutralising antibodies were observed compared to BNT162b2 (V9) immunisation. Therefore, BNT162b2 (V9) was found to be more immunogenic in mice compared to BNT162b2 (V8), validating the suitability of BNT162b2 (V9) as the clinical candidate.

Both BNT162b2 variants (V9 and V8) elicited immune responses (neutralising antibodies) in rats as investigated in repeat dose toxicity studies.

BNT162b2 (V9) at 0.05 to 15 µg/mL did not significantly induce proinflammatory cytokines (TNF- α , IL-6, IFN- γ , IL-1 β) and chemokines (MIP-1 β and MCP-1) in human peripheral blood mononuclear cells (PBMCs) *in vitro*. However, the *in vitro* study used PBMCs from only 3 donors and results were highly variable between donors, and the findings were inconclusive.

Protection against infection

BNT162b2 (V9) provided protection against SARS-CoV-2 challenge in rhesus monkeys after two immunisation doses of 100 µg/dose. No viral RNA was detected in bronchoalveolar lavage (BAL), with reduced viral RNA detected in nasal swabs, and no radiographic (X-ray and CT) lesions in immunised, challenged animals. In contrast, viral replication and lung radiographic abnormalities were evident in un-immunised, challenged controls. There was no evidence of vaccine-elicited disease enhancement as detected from radiographic analysis. No clinical signs were observed in any challenge groups, which is consistent with other studies in SARS-CoV-2-infected non-human primates (Muñoz-Fontela et al. 2020). While monkeys are not a good animal model of severe COVID-19 disease in humans, they are susceptible to viral replication and develop relatively mild lung pathology from SARS-CoV-2 infection. Very mild inflammation of lung tissues was observed in both control and vaccinated groups 7 or 8 days after challenge, with no significant difference in inflammation score. Comparatively younger monkeys (2-4 years old) were used in this study, which might have contributed to the absence of clinical signs and very mild histological pathology of lungs after virus challenge (Yu et al. 2020, Blair et al. 2020, Johansen et al. 2020). Overall, the vaccine protected monkeys from SARS-CoV-2 infection based on viral RNA load and radiographic lung lesions.

The T_H1-biased response observed in mice and monkeys suggests low risk of antibody-dependent enhancement (ADE) and vaccine-associated enhanced respiratory disease (VAERD).

Although no other LNP-mRNA vaccine has been approved yet in Australia, the pharmacological findings above are consistent with findings from nonclinical studies with several mRNA vaccines under clinical trials using similar LNP platform technology (influenza, rabies and Zika virus infections) (Sahin et al. 2014, Pardi et al. 2018, Pardi et al. 2017).

Although the immune mechanism of prevention from SARS-Cov-2 infection is still not well understood, both antibody-mediated and T cell-mediated immunity are known to be important contributors for effective protection against SARS-CoV-2 (Tay et al. 2020). Furthermore, if neutralising antibody-mediated protection is incomplete, cytotoxic CD8⁺ T cells are crucial for viral clearance (Arunachalam et al. 2020). One study found that among people who had recovered from COVID-19, 100% had S protein-specific CD4⁺ T cells in the circulation and 70% had S protein-specific CD8⁺ T cells in the circulation (Grifoni et al. 2020). Preclinical studies show a protective role of T cells in host defence against SARS-CoV (Zhao & Perlman 2010).

The pharmacology studies indicated that BNT162b2 (V9) induces humoral and cellular immune responses in mice and monkeys. The vaccine protected monkeys from infection when challenged 55 days after the 2nd vaccine dose, but the vaccine dose (100 µg) in monkeys was higher than the proposed clinical dose of 30 µg (. There were no studies on protection of older animals from SARS-CoV-2 infection or duration of protection after immunisation. Long term immunity needs to be addressed from clinical studies. The Sponsor referred to an ongoing 2-year clinical trial in US and Germany, which is expected to address long-term immunity.

Safety pharmacology

Limited safety pharmacology parameters *e.g.* body temperature were investigated in the toxicity study, in accordance with the WHO guideline on nonclinical evaluation of vaccines (WHO 2005).

Pharmacokinetics

Pharmacokinetic studies are generally not required for a vaccine per relevant guidelines; however, they are recommended for novel excipients or adjuvants used in the vaccine formulation, and in some cases for the antigen. The LNP in BNT162b2 contains two novel excipients, pharmacokinetics of which were studied in animal species and *in vitro*. In addition, tissue distribution of luciferase expressed by luciferase-encoding mRNA as a surrogate of the vaccine mRNA in the LNP formulation was also studied.

mRNA/expressed protein distribution and degradation

The biodistribution of the mRNA and expressed antigen encoded by the mRNA component of BNT162b2 is expected to be dependent on the LNP distribution. To visualise the tissue distribution of the mRNA-LNP formulation, mRNA encoding luciferase was formulated in an LNP formulation (LNP8) similar to the BNT162b2 vaccine. Following an IM injection of the luciferase mRNA formulation in mice, luciferase was detected by whole body imaging mainly at the injection site, which declined to the background level after 9 days. Luciferase was also seen in liver, which disappeared in 48 hours. This is consistent with other kinetics studies with mRNA-LNPs (Pardi et al. 2015). Sensitivity of the imaging detection system was low. Distribution to other tissues, e.g. draining lymph nodes, is highly likely (Lindsay et al. 2019), but the level was probably below the limit of detection of the imaging system.

The vaccine mRNA is expected to be degraded by multiple pathways [e.g. deadenylation, endonuclease-mediated decay (Garneau et al. 2007)], while the antigen protein undergoes proteolysis as for endogenous proteins. There are no data on the kinetics of BNT162b2 mRNA degradation. In mice injected with the luciferase mRNA, the absence of expressed protein by 9 days after dosing indicates that mRNA has been degraded.

Novel excipients

A single-dose intravenous (IV) study in rats using an LNP encapsulating luciferase mRNA demonstrated that both novel lipid excipients, ALC-0159 and ALC-0315 in the LNP formulation rapidly distributed from plasma to liver, which was the only organ collected for analysis. The particle size, encapsulation efficiency and lipid composition (relative to mRNA concentration) in the LNP were similar to the LNP used in the vaccine. The elimination of both lipids were slow, with elimination $t_{1/2}$ (from plasma) of 73 h for ALC-0159 and 139 h for ALC-0315. Tissue retention in liver was also shorter for ALC-0159 than ALC-0315; the concentration of ALC-0315 in liver tissue remained detectable on day 14, while ALC-0159 was no longer quantifiable. Both excipients were excreted in faeces (~50% of dose for ALC-0159 and ~1% of dose for ALC-0315 in 14 days). No unchanged lipids were detected in urine.

The distribution of lipid nanoparticles encapsulating mRNA encoding luciferase was investigated by monitoring of a radiolabelled lipid-marker after a single IM injection to Wistar rats. Major uptake of the lipid marker, probably representing the lipid nanoparticles, was noted in the injection site and liver with low distribution in spleen, adrenal glands and ovaries (distribution was not investigated in draining lymph nodes). The total radioactivity recovery was very low at all time-points (range = 20 – 60%), possibly because the draining lymph nodes of the injection site were not collected and faeces, urine, carcass and cage-wash samples were not analysed. The Sponsor stated that the tissues collected for this study were a standard panel of tissues, which did not include the draining lymph nodes. Doses higher (50 and 100 µg mRNA/animal) than those proposed in humans (30 µg mRNA) were tested in rats (avg. BW ~ 225 g), which showed clinical signs of piloerection, hunched body, decreased activity and irregular respiration. This might indicate toxicity of the LNP formulation at high doses. The 50 µg mRNA/animal dose is ~ 370 × the proposed human dose on a µg/kg BW basis.

In vitro metabolic studies showed slow, minor hydrolysis of both novel lipids in all species (mouse, rat, monkey and human). ALC-0159 was converted to N,N-ditetradecylamine and most likely PEG

(not determined in the study) by slow amide hydrolysis, and ALC-0315 to 2-hexyldecanoic acid and hydroxyalkyl-azanediy l moieties by ester hydrolysis and bis-hydrolysis. In rats given a single IV dose of LNP encapsulating luciferase-encoding mRNA (similar to the LNP in the clinical formulation), no metabolites of ALC-0159 were detected, while 2-hexyldecanoic acid in plasma, [(4-hydroxybutyl)azanediy l]dihexanol in plasma, liver, faeces and urine, and glucuronide conjugate of [(4-hydroxybutyl)azanediy l]dihexanol in urine were detected as metabolites of ALC-0315. The metabolites were not quantified.

In summary, the limited pharmacokinetic studies indicate that the vaccine LNP formulation is expected to deliver the mRNA effectively *in vivo* and the antigen expressed mainly at the injection site, liver and probably in draining lymph nodes. The limited studies showed slow elimination of ALC-0315 and retention in liver, and complete elimination of ALC-0159 in 14 days, with the latter eliminated in faeces most likely by biliary excretion.

Toxicity

A 17-day GLP compliant repeat-dose toxicity study (Study 20GR142) was conducted in rats using the IM route with the clinical candidate vaccine BNT162b2 (V9). The rats received 3 doses of the vaccine 7 days apart at 30 µg/dose (~120 µg/kg body weight), ~200 times the clinical dose on a µg/kg basis (30 µg, equivalent to 0.6 µg/kg per dose for a 50 kg person).

Another GLP rat study (Study 38166) was conducted with different versions of BNT162 mRNA including BNT162b2 (V8), which is closely related to BNT162b2 (V9). Three doses of BNT162b2 (V8) was administered to rats at 100 µg/dose (equivalent to ~400 µg/kg per dose). Both studies used an adequate number of animals (10/sex/treatment group, plus 5/sex/group for observation of recovery).

The dosing interval was not optimal given that the immune response peaks 2-3 weeks after dosing, and the clinical dosing interval is 3 weeks. In addition, the novel lipid excipients have long elimination half-lives. Repeat dose toxicity studies with a dosing interval of 2 or 3 weeks would be more appropriate for investigating the potential toxicity of the vaccine. The Sponsor indicated that *“As platform data was available, a shortened administration paradigm was used in the repeat dose toxicity studies in order to assess the toxicity of the vaccine with a shorten study timeline allowing more rapid transition into clinical trials.”* Platform data were not provided to the TGA for review. Given the availability of clinical data, another repeat dose study in animals is not considered necessary. The shortcoming of the repeat dose toxicity study design should not preclude approval of the vaccine.

Major toxicities

Treatment related findings were increased body temperature, acute inflammation at the injected site with oedema and erythema, increased WBC, neutrophils, large unstained cells (LUC), eosinophils, basophils, and fibrinogen in the repeat dose study in rats with BNT162b2 (V9). The albumin/globulin ratio was decreased, and acute phase proteins, α2-macroglobulin and α1-acid glycoprotein increased. Transient lower reticulocytes (day 4 only) and slightly lower red cell mass (days 4 and 17) were noted. Spleen weights were increased, associated with enlarged spleen and lymph nodes (draining lymph nodes of the injection site). The findings were consistent with immune stimulation and inflammatory responses. Increased LUC has been reported for pixatimod, an immune stimulating agent (Hammond et al. 2018) or acute viral infections (Nixon et al. 1987).

Similar findings were observed in the study with BNT162 variants. In rats dosed with 100 µg BNT162b2 (V8), in addition to the findings for the V9 variant, local reactions increased with repeated dosing, body weight gain after the first 2 doses were lower than that of the control group and there was a small increase in serum GGT (2-3 fold). Serum GGT was also determined in the V9 study with no vaccine-related changes in GGT observed, but the GGT assay sensitivity in the V9 study (LOQ 3 IU/L) was lower than the assay in the V8 study (LOQ < 1 IU/L). No increases in serum ALT, AST, AP or bilirubin were observed in either study.

Macroscopic pathology and organ weight changes were also similar for the two variants and included increased size of draining (iliac) lymph nodes and increased size and weight of spleen. Treatment related microscopic findings were seen at the injection sites and in surrounding tissues (mixed cell inflammation, mostly neutrophils), draining lymph nodes (hypercellularity of germinal centre and increased plasma cells, mostly plasmablasts), bone marrow (hypercellularity of haematopoietic cells, primarily myeloid cells), spleen (increased haematopoiesis and germinal centre), and liver (vacuolation of hepatocytes in the portal region), consistent with immune responses and inflammatory reactions except for hepatocyte vacuolation (minimal in severity), probably lipid vacuoles.

Clinical pathology findings (except for serum globulin), and spleen (haematopoiesis), bone marrow and liver lesions were fully reversed after the 3-week recovery phase. Injection sites, spleen (increased germinal centre) and lymph node findings persisted during the 3-week recovery phase (both variants V9 and V8), and body temperature remained at an increased level at the end of the 3-week recovery phase (for the V8 variant).

An immune response (binding and pseudovirus neutralising antibodies for BNT162b2 [V8] and SARS-CoV-2 neutralizing antibodies for BNT162b2 [V9]) was elicited by the BNT162b2 (V8 and V9) antigen in rats.

Novel excipients

The toxicity of LNP formulation or the novel excipients alone was not specifically studied. In the repeat dose toxicity studies with the clinical candidate vaccine (BNT162b2 V9) and BNT162b2 V8, the doses of ALC-0159 and ALC-0315 ranged from 214 – 636 µg/kg and 1720 – 4700 µg/kg, respectively, equivalent to 1284 – 3816 µg/m² (ALC-0159) and 10,320 – 28,200 µg/m² (ALC-0315). The exposure in rats were up to ~100 times the clinical exposure based on dose per body surface area (53 and 430 µg/dose, respectively, for ALC-0159 and ALC-0315, equivalent to 35 and 285 µg/m², respectively, for a 50 kg person with a body surface area of 1.51 m²). It is uncertain whether any findings discussed above were related to these excipients. It is probable that hepatocyte vacuolation be due to uptake of these lipids by hepatocytes. Conflicting findings were reported in the literature on the induction of complement activation and cytokine release, an acute immunological response that can lead to anaphylactic-like shock, by LNPs or LNPs encapsulating mRNA or siRNA in animal studies (Sabnis et al. 2018, Sedic et al. 2018). The potential of the LNP or the vaccine formulation for complement activation or stimulation of cytokine release was not adequately assessed in nonclinical studies. One *in vitro* study using human PBMC gave inconclusive results. No significant increase in cytokines was observed in the repeat dose study in rats with the V8 variant; however the number of animals studied for cytokines was small ($n=3$) and there was high inter-animal variation with no empty LNP control.

Safety of the novel excipients was not assessed in a second species. No further data would be submitted as stated by the Sponsor. In response to the TGA enquiry regarding the toxicity assessment of the novel excipients in the LNP formulation, the Sponsor referred to the evaluation of the siRNA product Onpattro™ (patisiran)^{1,2} administered as a LNP formulation, which is approved in the US, Europe and Canada (not reviewed by TGA) for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis) by IV infusion every 3 weeks. The LNP in Onpattro™ is composed of DLin-MC3-DMA, PEG2000-C-DMG, DSPC, and cholesterol. No nonclinical safety concerns regarding excipients, impurities, or degradation products were identified in the FDA and EMA evaluations. The primary toxicity observed in both rats and monkeys was an elevation in liver enzymes, with hepatocyte vacuolation. Therefore, the Sponsor argued that ALC-0315 and ALC-0159 would have

¹ US Food and Drug Administration, Center for Drug Evaluation and Research. Onpattro (patisiran) NDA 210922 multidiscipline review, 19 Mar 2018.
https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210922Orig1s000MultiR.pdf Accessed: Jan 2021.

² <https://www.ema.europa.eu/en/documents/assessment-report/onpattro-epar-public-assessment-report.pdf>

similar toxicity profile to the lipids DLin-MC3-DMA and PEG2000-C DMG, respectively, as they were structurally and functionally similar (See Section 1.4). However, while the pegylated lipids (ALC-0159 and PEG2000-C DMG) are structurally similar, the structures of ALC-0315 and DLin-MC3-DMA are not similar. Nonetheless, given that both the novel excipients are amino or amino/PEG lipids and the potential lifetime exposure is expected to be low (see discussion below), the Sponsor's justification for not conducting repeat dose toxicity studies with the novel excipients in a second animal species is acceptable.

Genotoxicity

No genotoxicity studies were conducted for the vaccine. This is in line with relevant guidelines for vaccines. There were also no genotoxicity studies with the novel excipients. The sponsor stated that the novel lipid excipients are not expected to be genotoxic based on *in silico* analysis (Derek Nexus 6.1.0, Derek Knowledgebase 2020 version 1.0 and Sarah Nexus 3.1.0, Sarah Model 2020.1 Version 1.8) of the novel lipids and their primary metabolites (reports not provided).

The Sponsor justified the absence of genotoxicity studies with the novel lipid exposures based on the threshold of toxicological concern (TTC) concept.³ The human exposure to ALC-0159 and ALC-0315 as part of the LNP-BNT162b2 vaccine is 53.4 µg and 430 µg, respectively. Assuming the vaccine would be administered twice per year, the TTC for less-than-lifetime (LTL) exposure would be 19.16 mg/day, based on the TTC of 1.5 µg for a mutagenic substance over a lifetime of 70 years and linear extrapolation to an LTL TTC according to the ICH M7 guideline (1.5 µg x 365 days x 70 years/2 days = 19,162.5 µg). The calculated acceptable LTL exposure is 360 and 45 fold higher than the exposure to ALC-0159 and ALC-0315, respectively, from the vaccine.

The Sponsor also argued that ALC-0159 and ALC-0315 were structurally and functionally similar to the lipid excipients PEG-2000-C-DMG and DLin-MC3-DMA, respectively, used in the siRNA drug Patisiran.^{1,2} Both DLin-MC3-DMA and PEG-2000-C-DMG were found to be negative in a full genotoxicity test battery of patisiran in the LNP formulation.

Considering the above, the novel excipients in BNT162b2 are not expected to pose a genotoxic risk.

Carcinogenicity

Carcinogenicity studies were not conducted. This is acceptable based on its duration of use. The novel lipid excipients are not expected to be carcinogenic based on the low exposure, duration of exposure, absence of structure alerts for mutagenicity (see discussion above).

Reproductive toxicity

A GLP compliant, reproductive and developmental toxicity study was conducted in rats. The rats were dosed twice (21 and 14 days) before the start of mating and twice during gestation (gestation days 9 and 20). The dose administered to rats was 30 µg mRNA/dose, ~200 times the proposed clinical dose on a µg/kg basis. This study was adequately conducted in terms of dosing period and group size (44 female rats per treatment group) with 22 females/group for caesarean sectioning at the end of the gestation and remaining 22 females/group allowed to litter and raise pups until weaning prior to sacrifice and examination. Post-natal development of pups until weaning was also assessed.

The study showed no adverse effects on female fertility, embryofetal development and post-natal development (up to weaning) in rats when given four IM injections of the BNT162b2 (V9) vaccine. The female rats showed a transient decrease in body weight gain after each injection and swelling at

³ ICH guideline M7(R1) (2017): Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk.

the injection site(s) with or without limping and piloerection. The vaccine at the human dose (30 µg) induced an active immune response to SARS-CoV-2 in all rat dams from the vaccine group. The exposure of fetuses and pups to vaccine-specific antibodies was also demonstrated.

Pregnancy classification

The sponsor has proposed Pregnancy Category B1. The Pregnancy Category B1 is considered appropriate for this product as no embryofetal effects have been noted in the combined reproductive and development study in rats.

Local tolerance

As discussed above, evidence of inflammatory cell infiltrates at the injection site were observed post IM dosing. In both studies with BNT162b2 (V8 and V9), local inflammation persisted, though partially recovered, after the 3-week recovery phase after the last dose. In addition, local clinical edema and erythema at the injection site in rats was resolved prior to each subsequent administration of the vaccine.

Immunotoxicity

No dedicated immunotoxicity study was conducted. An *in vitro* study on stimulation of cytokine release in human PBMC cells provided inconclusive results. As expected, immune-stimulatory effects were observed in pharmacology and repeat dose toxicity studies. No vaccine-related systemic intolerance or mortality was observed in the studies.

No significant release of cytokines was observed in the repeat dose study with BNT162b2 variant V8, however, the number of animals studied for cytokines was small ($n=3$) and there was high inter-animal variation. IFN- γ was increased in animals immunised with BNT162b2. IFN- γ has been found to play a role in autoimmunity (Lees 2015; Pollard et al. 2013). Thus, autoimmune diseases are a potential risk of the vaccine. This is addressable by the ongoing 2-year clinical studies.

Paediatric use

BNT162b2 is not proposed for paediatric use and no specific studies in juvenile animals were submitted.

Comments on the Nonclinical Safety Specification of the Risk Management Plan

Key safety concerns arising from nonclinical data are adequately identified in the Safety Specification of the Risk Management Plan (Part II, Module SII) dated 29 November 2020. The developmental and reproductive toxicity study findings are not noted in the RMP.

PRODUCT INFORMATION

Round 1 Evaluation — Milestone 3

The following comments refer to the revised draft Product Information document (Version: pfpcovii10121) accompanying the sponsor's correspondence of 5 January 2021. Where changes are suggested, text proposed to be inserted is underlined and text to be deleted is shown struck-through.

4.5 Interactions with other medicines and other forms of interactions

There are no nonclinical interaction studies. The proposed statements should be assessed by the clinical evaluator.

4.6 Fertility, pregnancy and lactation

Effects on fertility

The statement proposed in Section 5.3 *Preclinical safety data – Reproductive toxicity* should be moved here with minor modification.

“In a combined fertility and developmental toxicity study, female rats were intramuscularly administered COMIRNATY prior to mating and during gestation (4 full human doses of 30 µg each, spanning between pre-mating day 21 and gestation day 20). SARS CoV-2 neutralising antibodies were present in maternal animals from prior to mating to the end of the study on postnatal day 21 as well as in fetuses and offspring. There were no vaccine related effects on female fertility and pregnancy rate. Animal studies do not indicate direct or indirect harmful effects with respect to reproductive toxicity (see Section 5.3 Preclinical safety data).”

Use in pregnancy

As discussed in the assessment, Pregnancy Category B1 is considered appropriate for this product as no embryofetal effects have been noted in a combined reproductive and development study in rats. Following changes are recommended.

“Pregnancy Category B21

There is limited experience with use of COMIRNATY in pregnant women. Animal studies do a combined fertility and developmental toxicity study in rats did not show vaccine-related harmful effects on embryofetal development, did not indicate direct or indirect harmful effects with respect to pregnancy, embryo/fetal development, r post-natal development (see Effects on fertility Section 5.3 Preclinical safety data). Administration of COMIRNATY in pregnancy should only be considered when the potential benefits outweigh any potential risks for the mother and fetus.”

Use in lactation

It is recommended that findings of the rat study be stated.

“It is unknown whether BNT162b2 is excreted in human milk. A combined fertility and developmental toxicity study in rats did not show harmful effects on offspring development before weaning (see Effects on fertility).”

5.1 Pharmacodynamic properties

Mechanism of action

Statements on the mechanism of action are supported by nonclinical data. Minor editorial changes are suggested.

“The nucleoside-modified messenger RNA in COMIRNATY is formulated in lipid nanoparticles, which enable delivery of the non-replicating RNA into host cells to direct transient expression of the SARS-CoV-2 spike (S) antigen. The mRNA codes for membrane-anchored, full-length S with two point mutations within the central helix. Mutation of these two amino acids to proline locks S in an antigenically preferred prefusion conformation. COMIRNATY elicits both neutralising antibody and cellular immune responses to the spike (S) antigen, which may contribute to protection against COVID-19.”

5.3 Preclinical safety data

Statements regarding general and reproductive toxicity should be removed from this section. The statement on genotoxicity and carcinogenicity is acceptable.

“Non-clinical data reveal no special hazard for humans based on conventional studies of repeat dose toxicity.”

General toxicity

Rats intramuscularly administered COMIRNATY (receiving 3 full human doses once weekly, generating relatively higher levels in rats due to body weight differences) demonstrated some injection site oedema and erythema and increases in white blood cells (including basophils and eosinophils) consistent with an inflammatory response, as well as vacuolation of portal hepatocytes without evidence of liver injury. All effects were reversible.

Genotoxicity/Carcinogenicity

Neither genotoxicity nor carcinogenicity studies were performed. The components of COMIRNATY (lipids and mRNA) are not expected to have genotoxic potential.

Reproductive toxicity

Reproductive and developmental toxicity were investigated in rats in a combined fertility and developmental toxicity study where female rats were intramuscularly administered COMIRNATY prior to mating and during gestation (receiving 4 full human doses that generate relatively higher levels in rat due to body weight differences, spanning between pre-mating day 21 and gestational day 20). SARS CoV-2 neutralising antibody responses were present in maternal animals from prior to mating to the end of the study on postnatal day 21 as well as in foetuses and offspring. There were no vaccine related effects on female fertility, pregnancy, or embryo-foetal or offspring development. No COMIRNATY data are available on vaccine placental transfer or excretion in milk.”

MAIN BODY OF REPORT

1. INTRODUCTION

1.1. BACKGROUND

Pfizer Australia Pty Ltd has applied to register a new recombinant vaccine, a prophylactic mRNA vaccine (code: BNT162b2) encapsulated in solid lipid nanoparticles (COMIRNATY). The vaccine is indicated for active immunisation to prevent COVID-19 disease caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus, in individuals 16 years of age and older. The vaccination schedule consists of two doses of 30 µg of BNT162b2 mRNA in 0.3 mL each dose, administered intramuscularly (IM) twice at 21 days intervals.

1.2. RELATED VACCINES

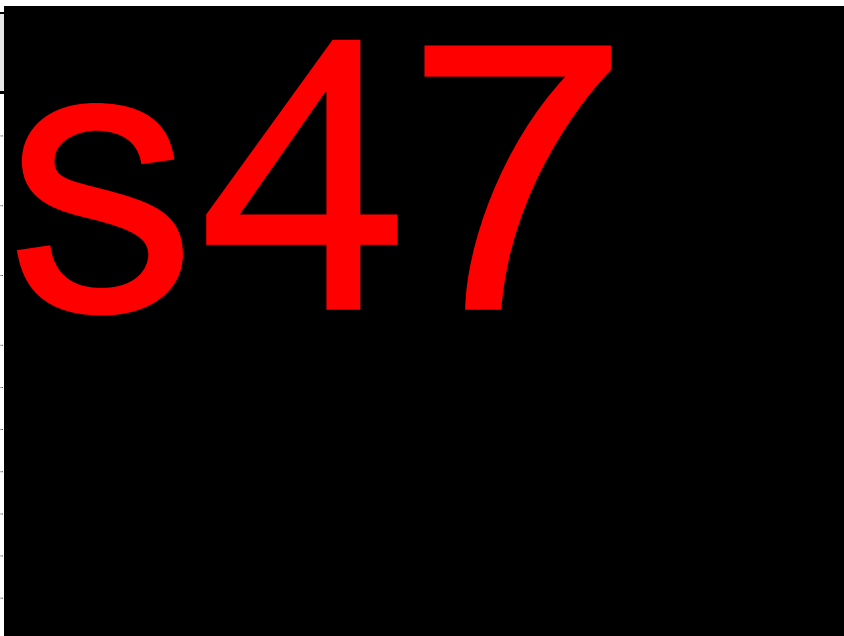
BNT162b2 COVID-19 vaccine is a nucleoside-modified mRNA based solid-lipid nanoparticle formulation to be registered for coronavirus disease. There is currently no mRNA vaccine registered in Australia.

1.3. PRODUCT FORMULATION

BNT162b2 vaccine is supplied as a preservative-free concentrated suspension formulation for intramuscular (IM) administration after dilution. The vaccine contains a nucleoside-modified messenger RNA, encoding the SARS-CoV-2 Spike-glycoprotein (S), produced by *in-vitro* transcription process and encapsulated into solid-lipid nanoparticles (LNP). The LNP is composed of four lipids: ALC-0315, ALC-0159, DSPC, and cholesterol. Quantities of mRNA, lipids and excipients are presented below in Table 1-1.

Table 1-1. Product formulation

Ingredient
BNT162b2 mRNA
[(4-hydroxybutyl)azanediyl]bis(hexane-6,1-diyl)bis(2-hexyldecanoate)*
2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide#
1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)
Cholesterol
Potassium chloride
Disodium hydrogen phosphate
Potassium dihydrogen phosphate
Sodium chloride
Sucrose
Water for injections



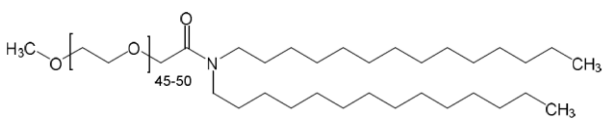
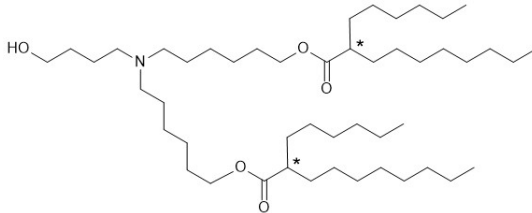
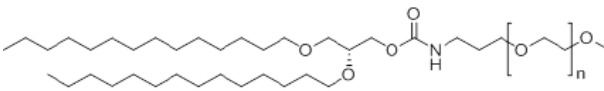
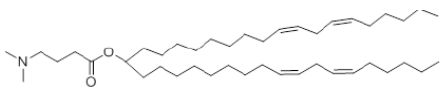
* company code = ALC-0315; # company code = ALC-0159

1.4. EXCIPIENTS

2-[(Polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) and [(4-hydroxybutyl)azanediyl]bis(hexane-6,1-diyl)bis(2-hexyldecanoate)(ALC-0315) are novel excipients and are not listed on the TGA's ingredient database. These excipients will be referred to as ALC-0159 and ALC-0315 in this report, respectively. As per the Sponsor's statement, ALC-0315 has been

optimised for RNA encapsulation and intracellular delivery, and ALC-0159 has been engineered to regulate LNP size (40 – 180 nm) and transfection potency. Batch data for multiple batches of proposed vaccine showed particle size (D50) in the range of 58 – 94 nm (Table 1-3). The lipids protect the mRNA from degradation, and facilitate cell uptake, hence they are a major determinant of potency (Jayaraman et al. 2012). The Sponsor indicated that these novel excipients, ALC-0159 and ALC-0315 are structurally and functionally similar to the lipid excipients PEG2000-C DMG and DLin-MC3-DMA, respectively, used in a siRNA-LNP drug product Onpattro™ (patisiran), which is approved in the US, Europe (by EMA) and Canada.

Details of these lipids are shown below.

Product	Novel lipid excipient (CAS no, Molecular formula and weight in Da)	Structure	Chemical name
BNT162b2 V9	ALC-0159 (CAS no. 1849616-42-7, (C ₂ H ₄ O) _n C ₃₁ H ₆₃ NO ₂ , n=45-50 and 2400-2600 Da)		2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide
	ALC-0315 (CAS no 2036272-55-4, C ₄₈ H ₉₅ NO ₅ and 766 Da)		((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate)
Onpattro™ (patisiran)	PEG2000-C-DMG [NA, C ₂ H ₄ O) _n C ₃₆ H ₇₃ NO ₅ , n=47 and 2650±300 Da]		(α-(3'-[1,2-di(myristyloxy)propanoxy]carbonylamino)propyl)-ω-methoxy, polyoxyethylene)
	DLin-MC3-DMA [NA, C ₄₃ H ₇₉ NO ₂ and 642.09 Da]		(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate

1.5. BATCHES USED IN MODULE 4 STUDIES

The development of COVID-19 vaccine included several variants of the BNT162 mRNA, which are tabulated in the following table (Table 1-2).

Table 1-2. Variants of BNT162 mRNA vaccine candidates

BNT162 vaccine candidate	RNA type	Encoded antigen
BNT162a1	Non-modified uridine mRNA	SARS-CoV-2 receptor binding domain, a secreted variant
BNT162b1	Nucleoside modified mRNA	SARS-CoV-2 receptor binding domain, a secreted variant
BNT162b2 (V8 and V9)	Nucleoside modified mRNA	Full length SARS-CoV-2 spike protein bearing mutations preserving neutralisation-sensitive sites
BNT162b3	Nucleoside modified mRNA	SARS-CoV-2 receptor binding domain, a membrane-bound variant

BNT162 vaccine candidate	RNA type	Encoded antigen
BNT162c1	Self-amplifying mRNA	SARS-CoV-2 receptor binding domain, a secreted variant
BNT162c2	Self-amplifying mRNA	Full length SARS-CoV-2 S protein bearing mutations preserving neutralisation-sensitive sites

The V9 variant was selected as the clinical candidate. V8 and V9 have identical amino acid sequences of the encoded antigens and differ only in their codon optimisation sequences, which were designed to improve antigen expression (V9) with higher content of cytosine ribonucleotides.

Main pharmacology studies and the pivotal repeat-dose study have been conducted with BNT162b2 (V9). Immunogenicity and toxicity studies were also conducted with the V8 variant. All mRNA variants tested in the nonclinical studies were formulated with the LNP.

Several manufacturing process changes for BNT162b2 mRNA, including production scale, were introduced during development. The proposed commercial scale manufacturing process includes use of linearised plasmid DNA template for mRNA production, whereas in early development phases PCR-amplification of DNA template was used. The characteristics of the BNT162b2 LNP batches used in the nonclinical studies are given in the following table (*Sponsor's response to TGA nonclinical questions*).

Table 1-3. LNP characterisation in nonclinical studies

Study no.	Batch LNP	Size (nm)	Encapsulation efficacy (%)	RNA (mg/mL)	Lipid (mg/mL)			
					ALC-0315	ALC-0159	DSPC	Cholesterol
R-20-0072	LNP8 FM-1074-D	s47	■	■	■	■	■	■
072424 (PF-07302048_06Jul2)	FM-1261A	s47	■	■	■	■	■	■
185350	NC-0552-1	s47	■	■	■	■	■	■
R-20-0085	BNT162b2 (V9) (RBP020.2 LNP)	s47	■	■	■	■	■	■
R-20-0112								
VR-VTR-10671								
20GR142								
20256434								
LPT 38166	BNT162b2(V8) (RBP020.1 LNP)	s47	■	■	■	■	■	■

NA: not available in the certificate of analysis

Both RBP020.1 LNP RNA and RBP020.2 LNP RNA were produced using the manufacturing process starting with (linear) PCR product.

1.6. OVERSEAS REGULATORY STATUS

A similar application has been made in the USA, EU, Canada, Switzerland and New Zealand (all in October 2020). USA, UK and Canada have approved the product for emergency use (December 2020). EU and Switzerland have also approved conditional marketing authorisations.

2. PRIMARY PHARMACOLOGY

SARS-CoV-2 possesses a spike (S) glycoprotein, which binds to the host cell membrane protein ACE2 to enter human cells. The virus-ACE2 binding results in the release of the viral genome in the host cells. The S-protein has two functional units, S1 and S2 (Figure 2-1). For infection, S requires proteolytic cleavage by two host proteases, a furin-like protease between the S1 and S2 subunits, and by the serine protease TMPRSS2 at a conserved site directly preceding the fusion peptide (S2') (Bestle et al. 2020). While the membrane-proximal S2 furin cleavage fragment is responsible for membrane fusion, the membrane-distal S1 fragment, with its receptor-binding domain (RBD), recognises the host receptor and binds to the target host cell. During cell entry, the S1 fragment dissociates, and the S2 fragment undergoes a fold-back rearrangement to the post-fusion conformation in a process that facilitates fusion of viral and host cell membranes.

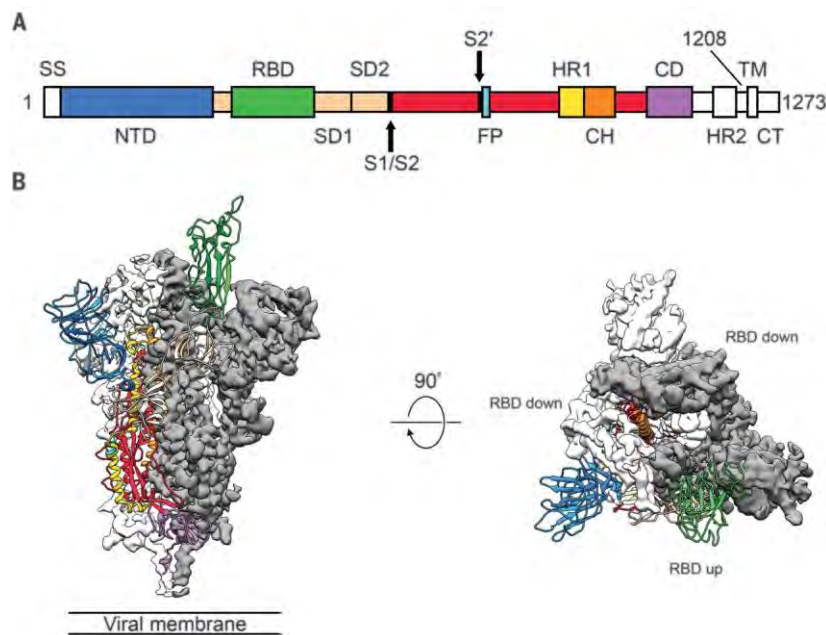


Figure 2-1. Structure of SARS-CoV-2 S protein in the prefusion conformation (Wrapp et al. 2020)

BNT162b2 mRNA encodes the full-length S protein with the modification of two nucleosides (residues 986 and 987, replaced by proline: K986P, V987P; P2 S). BNT162b2 is expected to induce antibodies targeting the S protein.

Several *in vitro* and *in vivo* studies in mice and non-human primates were performed to demonstrate S protein expression, immunogenicity and protective efficacy. Nonclinical pharmacology studies are shown in Table 2-1.

Table 2-1. Summary of nonclinical pharmacological studies (clinical candidate is in bold font)

Study objective	Test material (animal model)	Study number
Assessment of vaccine immunogenicity	BNT162b2 V9 (BALB/c mice)	R-20-0085
Assessment of vaccine immunogenicity	BNT162b2 V8 (BALB/c mice)	R-20-0054
Assessment of immunogenicity and protection against wild type SARS-CoV-2	BNT162b2 V9 (Lot # CoVVAC/270320) (Rhesus monkeys)	VR-VTR-10671
Characterising the immunophenotype in spleen and lymph node of mice treated with SARS-CoV-2 vaccine candidates	BNT162a1, BNT162b1, BNT162b2, BNT162c2 (BALB/c mice)	R-20-0112
<i>In vitro</i> expression of BNT162b2 drug Substance and drug Product	BNT162b2-RNA, BNT162b2 (ATM LNP) , Polymun batch RBP020.2 LNP with the lot: CoVVAC/270320 (HEK293T cells)	R-20-0211

Study objective	Test material (animal model)	Study number
<i>In vitro</i> expression of mRNA constructs encoding the SARS-CoV-2 Spike protein variants V8 and V9	BNT162b2 V8 and V9 (HEK293T cells)	R-20-0360
Structural and biophysical characterisation of SARS-CoV-2 spike glycoprotein (P2 S)	modified pcDNA3.1 construct encoding P2S antigen (tagged and untagged) (Expi293F cells)	VR-VTR-10741
<i>In vitro</i> study of cytokine/chemokine secretion	BNT162b2 V9 (human peripheral blood mononuclear cells)	R-20-0357

2.1. IMMUNOGENICITY IN MICE

Study No. R-20-0085: Immunogenicity of BNT162b2 (V9) in Mice

BALB/c mice ($n = 8 \text{ ♀} / \text{group}$)

Test item: BNT162b2 V9: RNA batch: RNA-RF200321-06

Polymun batch RBP020.2 LNP with the lot: CoVVAC/270320

Single IM dosing; 20 μL of vaccine containing 0.2 μg , 1 μg or 5 μg of BNT162b2 V9 in PBS+300 mM sucrose; Control: PBS+300 mM sucrose

Study details

Blood collected at day 7, 14, and 21 to analyse the antibody immune response by ELISA and pseudovirus-based neutralisation assay (pVNT)

Blood collected at day 28 for ELISA, pVNT analyses and affinity measurements of vaccine-induced antibodies towards recombinant SARS-CoV-2 S and RBD-domain *via* Surface Plasmon Resonance (SPR); Additional analysis of the T-cell response in splenocytes by ELISpot, luminex assay and intracellular cytokine staining (ICS)

Major findings

Detection of IgG antibodies

- Dose dependent \uparrow in S1 (both IgG1 and IgG2 subtypes) and RBD specific IgGs over time in all immunised groups. The IgG2a/IgG1 ratio was similar for both 1 μg and 5 μg doses (~ 1.3 and ~ 1.15 , respectively) whereas a higher IgG1 was obtained at 0.2 μg dose (IgG2a/IgG1 ratio ~ 0.6).
- No IgG was detected in either pre-immunisation groups or control groups.
- Serum antibody titres against S1 and RBD increased in a dose-dependent manner (statistically significant) over time in all immunised groups compared to the control (Figure 2-2, Figure 2-3).

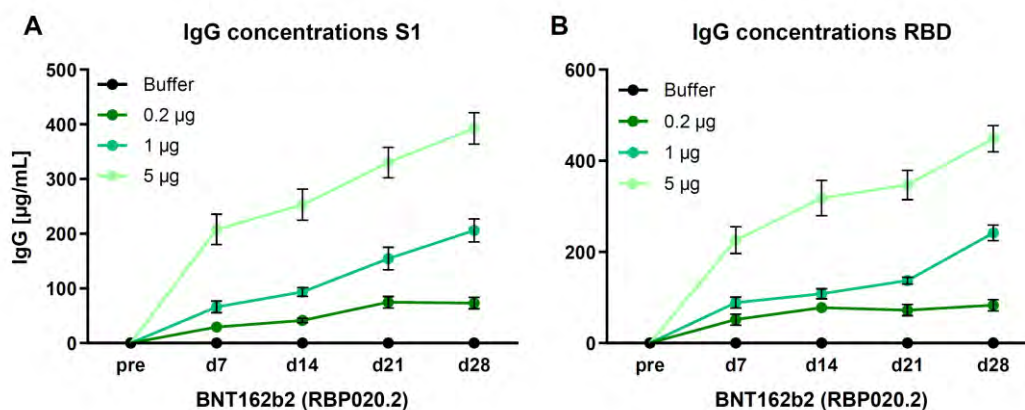


Figure 2-2. Kinetics of the antibody concentration against the viral antigen [Group mean antibody concentrations are shown (\pm SEM)]

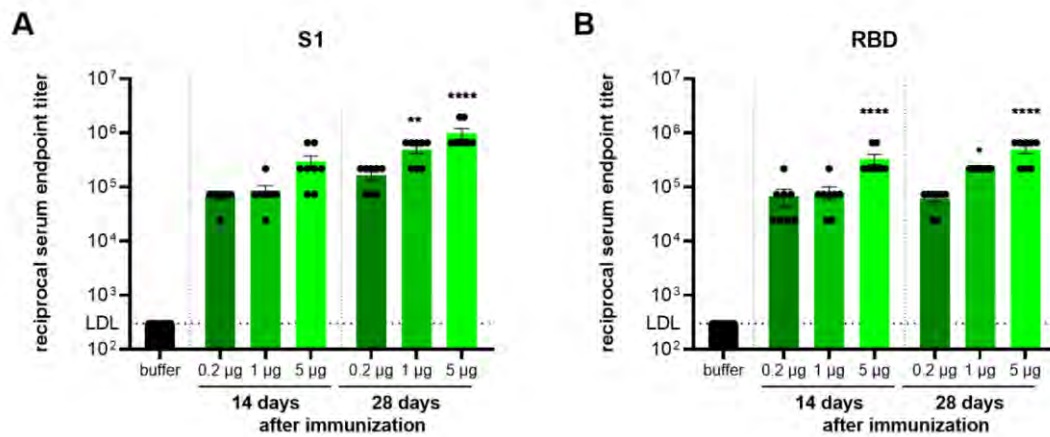


Figure 2-3. Serum Ab titres at day 14 and 28 after immunisation (S1, day 28: $p = 0.0082$ and < 0.0001 for 1 and 5 µg, respectively; RBD, day 28: $p = 0.011$ and < 0.0001 for 1 and 5 µg, respectively)

- BNT 162b2-induced IgG demonstrated affinity for S1 (K_D : 12 nM) and the RBD (K_D : 0.99 nM) both with high on-rate (geometric mean k_{on} : 3.33×10^4 /Ms for S1-specific affinity; 6.02×10^5 /Ms for RBD-specific affinity) and low off-rate (geometric mean k_{off} : 4.00×10^{-4} /s for S1-specific affinity; 5.97×10^{-4} /s for RBD-specific affinity).
- Functional neutralising antibodies developed in all animals in a dose dependent manner as analysed by pVNT (day 14-28), with higher titres on days 21 and 28 than on day 14.

Antibody titres on day 28	BNT162b2		
	0.2 µg	1 µg	5 µg
Anti S1 protein total IgG [µg/mL]	73.0 ± 10.4	205.9 ± 21.0	392.7 ± 28.9
Anti RBD protein total IgG [µg/mL]	83.1 ± 12.3	241.7 ± 17.2	448.6 ± 28.6
pVN ₅₀ titre [reciprocal dilution]	33.0 ± 9.8	192.0 ± 31.4	312.0 ± 35.1

Cellular immune response

- S protein or RBD-specific peptide pools (S protein: 315 overlapping peptides; RBD: 48 overlapping peptides) induced IFN-γ responses in T cells from both fresh and frozen splenocytes of immunised animals (higher response for the vaccine dose of 1 or 5 µg than the 0.2 µg dose).
- Both reactive CD8⁺ and CD4⁺ T-cell responses were observed in splenocytes from the groups treated with 5 µg RNA and stimulated with the S protein peptide pool (CD8⁺ > CD4⁺) compared to splenocytes stimulated with AH1-specific peptide pool (control) by ELISpot. No significant CD8⁺ and CD4⁺ T-cell responses were observed in cells stimulated with the RBD peptide pool.

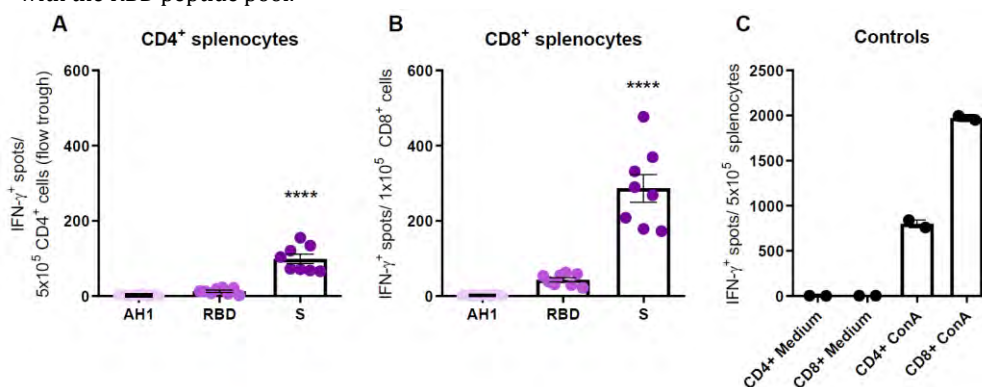


Figure 2-4. ELISpot analysis of splenocytes from 5 µg BNT162b2-immunised mice on day 28

- BNT162b2 immunisation induced an increased level of T_H1-specific and proinflammatory cytokines (IFN-γ, GM-CSF, TNF-α, IL-6, IL-18) in supernatants of stimulated splenocytes.
- 1 µg BNT162b2 induced more cytokines compared to other groups.
- S- peptide pool (0.1µg/mL/peptide) induced more cytokines compared to RBD- peptide pool (0.66 µg/mL/peptide). Very low levels of T_H2 cytokines (IL-4, IL-5, IL-13) were detected in S peptides stimulated cells (*not in cells stimulated by RBD peptides*), with high levels of IL-4, IL-5, IL-13 in only one animal (Figure 2-5).

- Reactive IFN- γ , TNF- α - and IL-2 secreting CD4⁺ and CD8⁺ T cells were detected after peptide stimulation by intracellular cytokine staining (ICS).

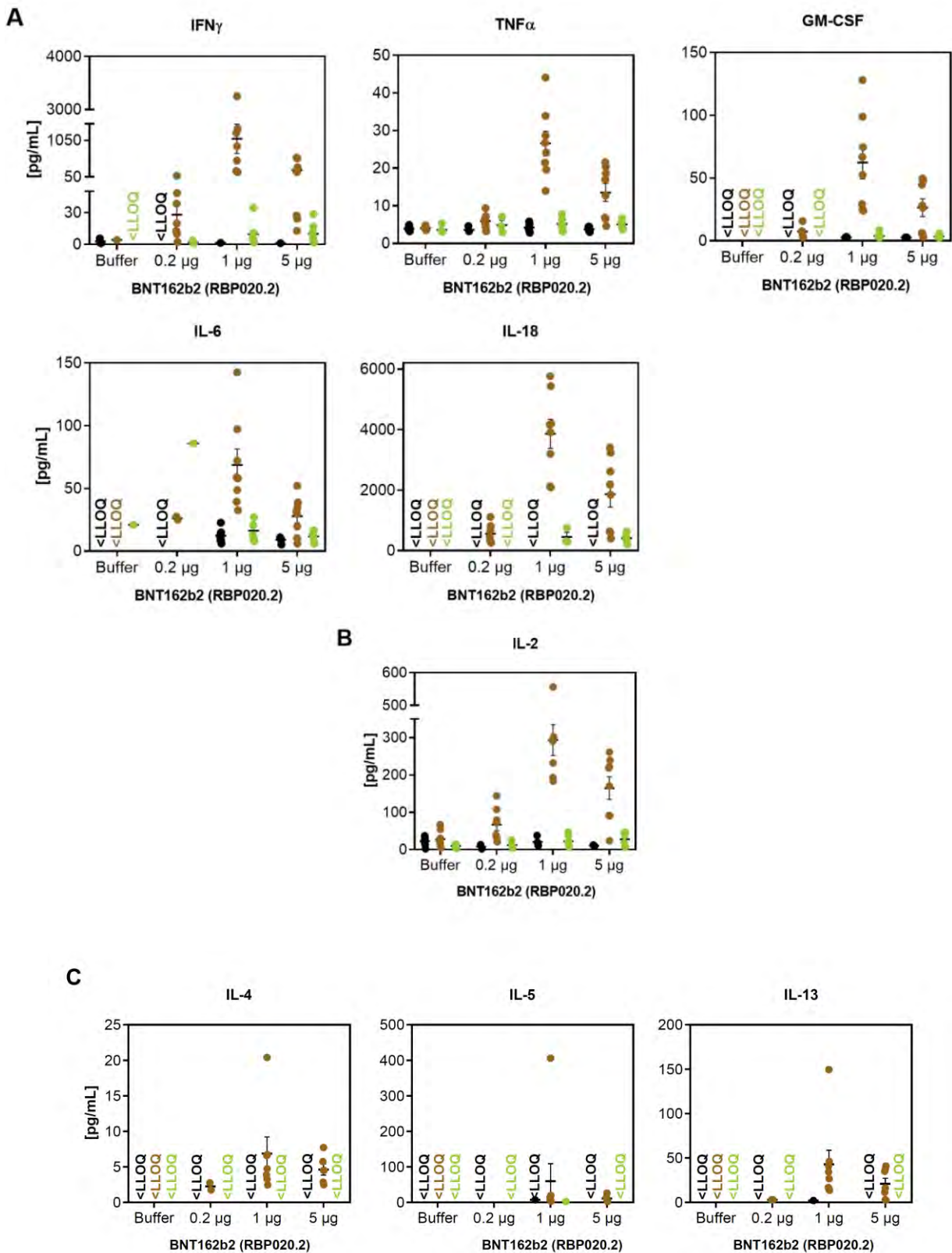


Figure 2-5. Cytokines in stimulated splenocytes 28 days after immunisation a. T_H1 specific cytokines; b. T_{eff} specific cytokines and c. T_H2 specific cytokines

Conclusion: BNT 162b2 induced anti-S and anti-RBD IgG and neutralising antibodies against the antigen, with high binding affinity to S1 and the RBD, as well as a T_H1-biased T cell response including a cytotoxic T-cell response.

Study No. R-20-0054: Immunogenicity of BNT162b2 (V8) in Mice

BALB/c mice ($n = 8 \text{ ♀ /group}$)

Test items: BNT162b2 (V8): RNA batch: RNA-KG200312-01; Polymun batch RBP020.1, LNP with the lot: CoVVAC/160320
Single IM dosing; 20 μL of vaccine containing 0.2 μg , 1 μg or 5 μg of **BNT162b2 V8** in PBS+300 mM sucrose
Control: PBS+300 mM sucrose

Study details

Blood collected at 7, 14, and 21 days after immunisation for a screening analysis and at day 28 for endpoint titration to analyse the antibody immune response by ELISA, and at 14, 21 and 28 days for the VSV/SARS-CoV-2-S pVNT

Blood collected at day 28 for affinity measurements (5 μg BNT162b2 dose cohort only) of vaccine-induced antibodies towards recombinant SARS-CoV-2 S and RBD-domain *via* SPR

Spleens collected for splenocytes isolation and analysis of T-cell responses using IFN- γ -specific ELISpot assays. Cytokine responses were assessed by luminex assays and ICS.

Major findingsDetection of IgG antibodies

- Dose dependent \uparrow in S1 (both IgG1 and IgG2 subtypes) and RBD specific IgGs over time in all immunised groups similar to findings in **Study No. R-20-0085** with the vaccine candidate BNT162b2 (V9). However, the concentration of both IgGs against S1 and RBD induced by BNT162b2 (V8) was lower than that induced by BNT162b2 (V9) (**Study No. R-20-0085**).
- The IgG2a/IgG1 ratio (Figure 2-6) was similar for all doses in both studies. Slightly higher IgG2a concentrations compared to IgG1 were observed in all doses of BNT162b2 (V8) whereas a higher IgG1 was noted at 0.2 μg dose of BNT162b2 (V9) (**Study R-20-0085**). No IgG was detected in either pre-immunisation groups or control groups.

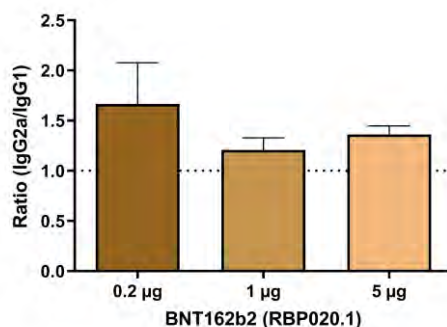


Figure 2-6. IgG2a/IgG1 subtype ratio on day 28

- Functional neutralising antibodies developed in animals treated with 1 and 5 μg of BNT162b2 (V8) in a dose dependent manner as analysed by pVNT, whereas much higher titres of antibodies developed in all doses of BNT162b2 (V9) (**Study R-20-0085**).
- BNT162b2 (V8) induced lower titre of functional neutralising antibodies (~ 3-5 fold) compared to BNT162b2 (V9).

Antibody titres on day 28	BNT162b2 V8		
	0.2 μg	1 μg	5 μg
Anti S1 protein total IgG [$\mu\text{g/mL}$]	74.0 \pm 21.3	296.2 \pm 37.2	558.4 \pm 53.
Anti RBD protein total IgG [$\mu\text{g/mL}$]	73.4 \pm 23.1	266.9 \pm 40.6	410.5 \pm 66.3
pVN ₅₀ titre [reciprocal dilution]	7.5 \pm 0.9	52.5 \pm 10.6	90.0 \pm 5.6

Cellular immune response

- S protein or RBD-specific peptide pools induced IFN- γ responses in T cells from splenocytes of immunised animals (higher response for the vaccine dose of 1 or 5 μg than the 0.2 μg dose).
- Both CD4⁺ and CD8⁺ cells displayed IFN- γ responses after stimulated with the S peptide pool. No significant CD8⁺ and CD4⁺ T-cell responses were observed in cells stimulated with the RBD peptide pool.
- 5 μg BNT162b2 immunisation induced an increased level of most T_H1-specific and proinflammatory cytokines (IFN- γ , GM-CSF, TNF- α , IL-6, IL-18) and IL-2 in supernatants of stimulated splenocytes. However, several values were below the lower level of quantification for statistical analysis (calculation of p values) in some treatment groups. The S peptide pool (0.1 $\mu\text{g/mL}$ peptide) induced more cytokines compared to the RBD peptide pool (0.165 $\mu\text{g/mL}$ peptide).
- The fraction of IFN- γ expressing CD3⁺CD4⁺ T cells was significantly higher for mice immunized with BNT162b2 compared to control ($p < 0.05$). Increased fractions of CD3⁺CD4⁺ T cells expressing the T_H2 cytokines IL-4 and TNF- α were observed in specific treatment groups (dose dependent \uparrow IL-4; \uparrow TNF- α in 0.2 μg treated group). No significant change was detected for IL-2.

- The fraction of IFN- γ -expressing CD3⁺CD8⁺ T cells was significantly higher for animals immunized with 5 μ g BNT162b2 compared to control ($p = 0.0055$). No significant change was detected for TNF- α and IL-2.
- The ICS results were considered inconclusive, as peptide-specific signals were in the range of medium background signals.

Conclusion: BNT162b2 (V8) induced anti-S and anti-RBD IgG and neutralising antibodies against the S1 antigen and the RBD at higher doses (1 and 5 μ g) as well as a T cell response.

Study No. R-20-0112: Characterising the immunophenotype in spleen and lymph node of mice treated with SARS-COV-2 vaccine candidates

BioNTech SE, 55131 Mainz, Germany

Test items

ID	RNA platform	Antigen
BNT162a1	Non-modified RNA (uRNA)	RBD of S1S2 protein (V5)
BNT162b1	Modified RNA	RBD of S1S2 protein (V5)
BNT162b2	Modified RNA	S1S2 full length protein (V9)
BNT162c2	Self-amplifying RNA (saRNA)	S1S2 full length protein (V9)

The objective of this study

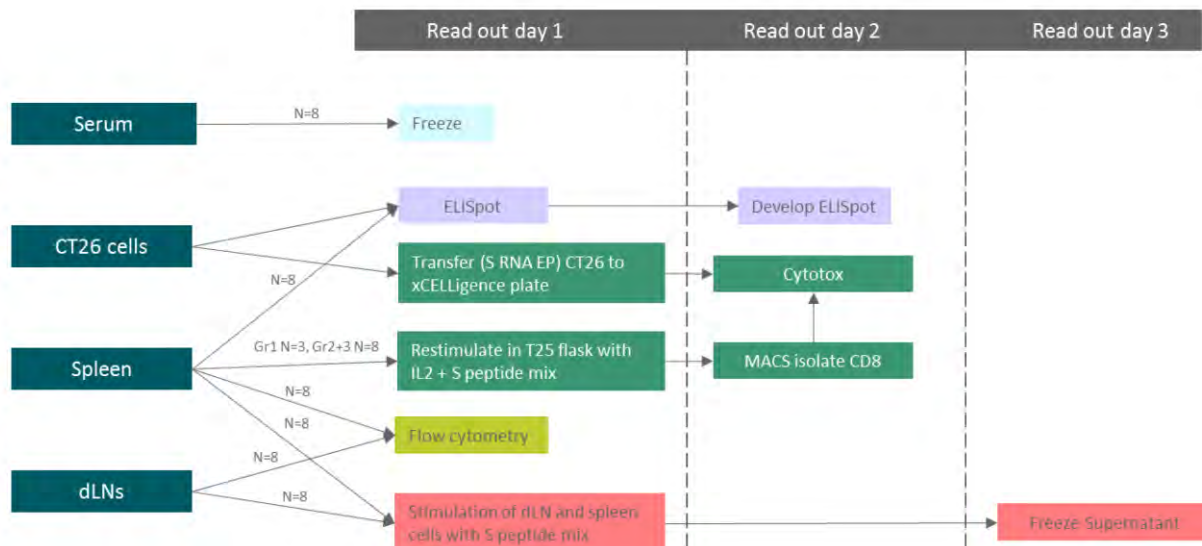
- Characterisation of T- and B-cell responses in the spleen, lymph nodes and blood.
- Determination of the ability of CD8⁺ T cells to kill cells presenting the vaccine-encoded antigen.
- Determination of neutralising antibody responses (analysis not performed)

Study details

BALB/c mice ($n = 8 \text{ ♀ /group}$)

Single IM dosing; 20 μ L of vaccine containing 0.2 μ g, 1 μ g or 5 μ g of BNT162b2 in PBS+300 mM sucrose was administered on day 0; Control: 0.9% NaCl

Blood collected at day 7. Serum and tissues were analysed at day 12 and day 27. The following diagram shows an overview of the subsequent analytical methods including sample allocation to the respective assays.



Analyses

- Serum was analysed to determine SARS-CoV-2 specific IgG responses.
- Splenocytes were tested for recognition of an S protein-specific peptide mix or S RNA-electroporated CT26 cells by secretion of IFN- γ (IFN- γ ELISpot assay).
- A fraction of splenocytes was stimulated overnight with an S protein-specific peptide mix and recombinant IL-2, and isolated CD8⁺ T cells were challenged on the next day for killing of S RNA-electroporated CT26 colon carcinoma cells (xCELLigence cytotoxicity assay).
- Splenocytes and draining lymph nodes (dLN; popliteal, iliac and inguinal, pooled) cells were analysed for T- (CD4⁺ T_{H1}, T_{H2}, T_{FH}, CD8⁺ T cells) and B-cell phenotype (GC, plasma, memory B cells).
- dLN and spleen cells were stimulated for 48 h with an S protein-specific peptide mix to analyse T-cell secreted cytokines (ProcartaPlex cytokine multiplex assay).

Major findings

S protein specific T-cell responses (IFN- γ secretion) were detected in groups receiving BNT162b2, BNT162b1 and BNT162c2 after stimulation with either S peptide or S RNA (mean spot counts > 1000 for b2 and c2 and 750 for b1) and to a lesser extent in BNT162a1 treated groups (mean spot counts 36-100).

T and B cell analysis in blood

Lymphocyte frequencies

- **BNT162b2** treatment increased CD8⁺ (34%) and decreased CD4⁺ T cells.
- BNT162b1, **BNT162b2** and BNT162c2 treatment induced an increase and activation of T_{FH} cells among CD4⁺ T cells (1.34%, 0.53% and 0.48% BNT162c2, **BNT162b2** and BNT162b1, respectively). ↓ B cells in all groups.

T cell activation

- BNT162b1 and **BNT162b2** treatment resulted in T cell activation (↑ CD44, CD38, PD-1 by CD8⁺ T cells and ICOS by both CD8⁺ and CD4⁺) and antigen specific secretion of cytokines by splenocytes. ↑ ICOS⁺ T_{FH} cells in all groups but most significantly for BNT162b1, **BNT162b2** and BNT162c2.

T and B cell analysis in dLNs

- ↑ CD8⁺ T cells in BNT162b2 groups, ↑ CD4⁺ T cells and T_{FH} in BNT162b1 and BNT162b2 groups. ↑ T_{H1} T in BNT162b1 and BNT162b2 groups (Figure 2-7).
- ↑ B cells in BNT162b1 and **BNT162b2** groups with higher numbers of antibody producing plasma B cells and germinal center B cells (Figure 2-8).
- Germinal center B cells demonstrated a class switch to IgG1 (BNT162a1, BNT162b1 and **BNT162b2**) or IgG2a (BNT162b1 and BNT162b2) in BNT162a1, BNT162b1 and **BNT162b2** groups only.

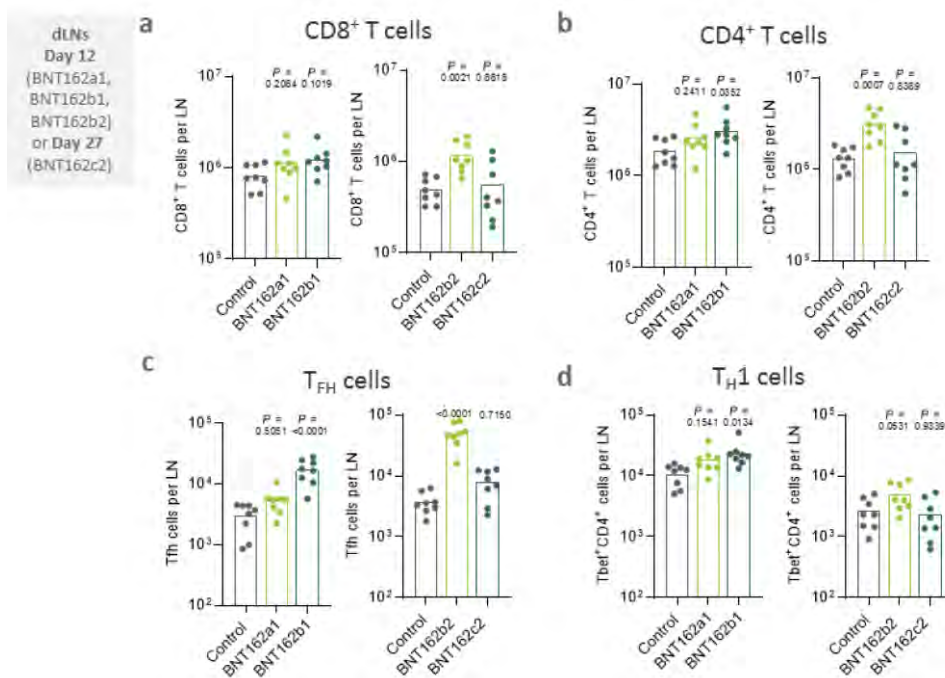


Figure 2-7. T cell response in the dLNs

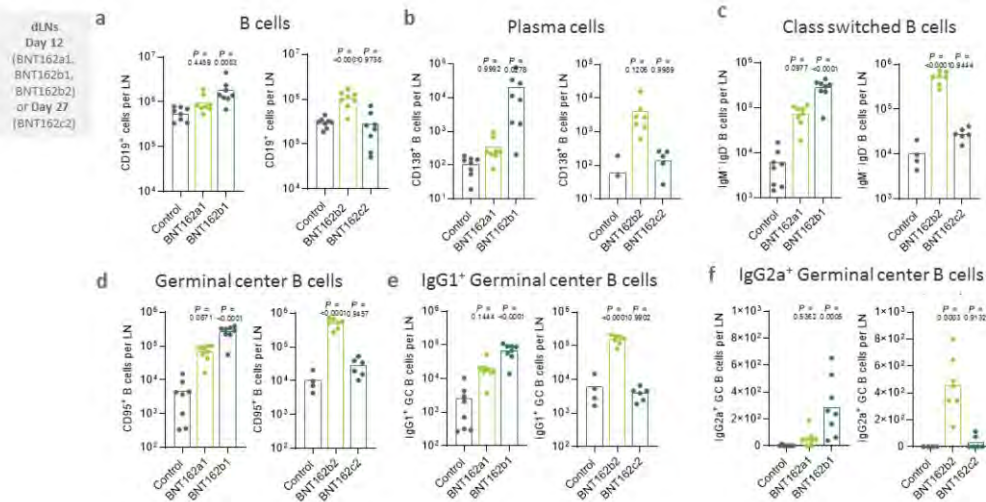


Figure 2-8. B cell response in the dLNs

T and B cell analysis in spleen (Figure 2-9 and Figure 2-10)

- ↑ T_{FH} cells, germinal center B cells and class switched B cells in BNT162b1 or **BNT162b2** groups similar to the findings in the spleen but less pronounced compared to blood and dLNs.
- Significant antigen-specific release of the T_{H1} cytokines IFN-γ and IL-2 was observed in the BNT162b1, **BNT162b2** and BNT162c2 groups (flow cytometry analysis and cytokine multiplex assay). Significant release of IL-2 and TNF in CD8⁺ T cells from BNT162b1 and **BNT162b2** groups.
- Significant release of T_{H1} cytokines IFN-γ and IL-2 from CD4⁺ T cells from BNT162b1 group, but not the T_{H2} cytokine IL-4. Although numbers were generally low and the variation between treated groups high, significant antigens specific secretion of IFN-γ among T_{FH} cells was detected in the **BNT162b2** group.
- A significant IL-2 response and a weak IFN-γ release were observed in splenocytes from BNT162a1 treated mice (3 of 8 mice, cytokine multiplex assay).
- Highest responses for both cytokines surpassing the upper limit of quantification for IFN-γ were found in the **BNT162b2** and BNT162c2 groups encoding the full-length S protein. Comparably weak or no secretion of the T_{H2} cytokines IL-4 and IL-5 was measured. Low but significant release of IL-4 and IL-5 was shown for **BNT162b2** and BNT162c2. IL-4 but not IL-5 was detected in BNT162b1 vaccinated mice (cytokine multiplex assay).
- High amounts of pro inflammatory IL-18 were released in the **BNT162b2** and BNT162c2 groups, and to lesser extent in the BNT162b1 and BNT162a1 groups.
- Proinflammatory cytokines (GM-CSF and IL-6) were elevated in the BNT162b1, **BNT162b2** and BNT162c2 groups.

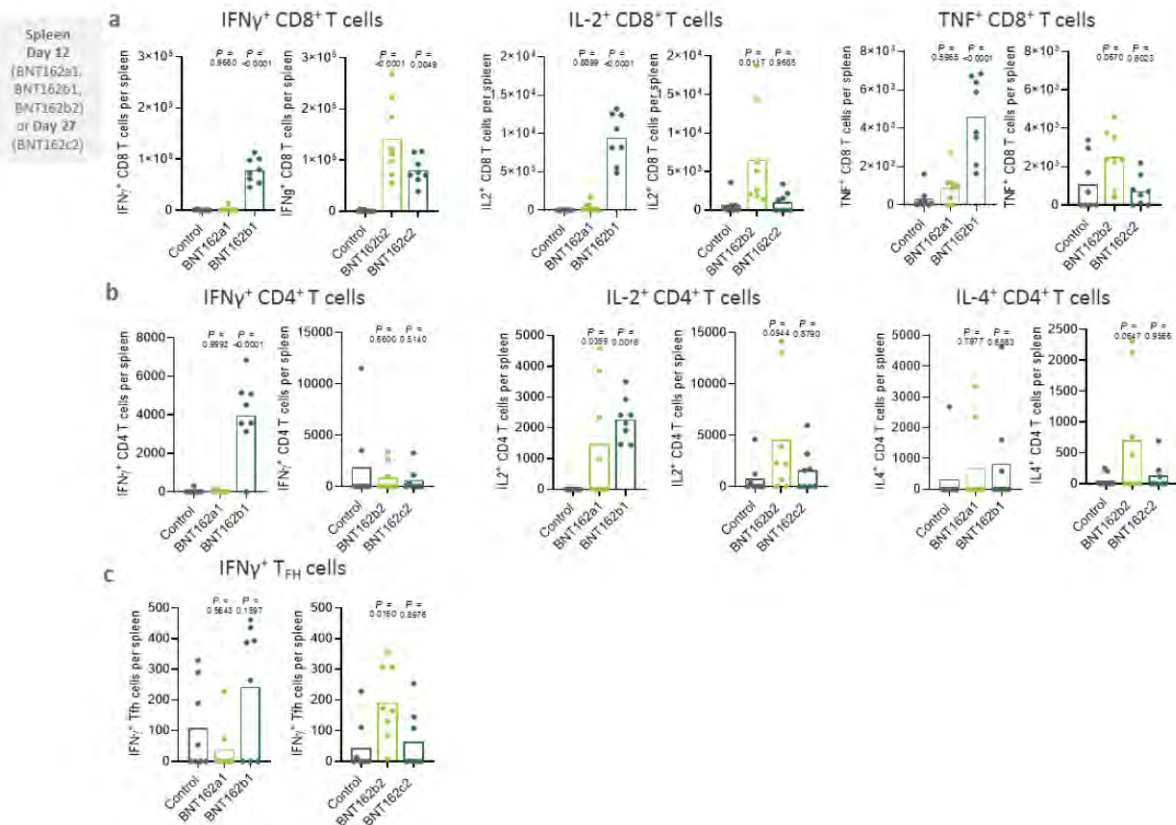


Figure 2-9. T cell response in spleens upon S peptide restimulation

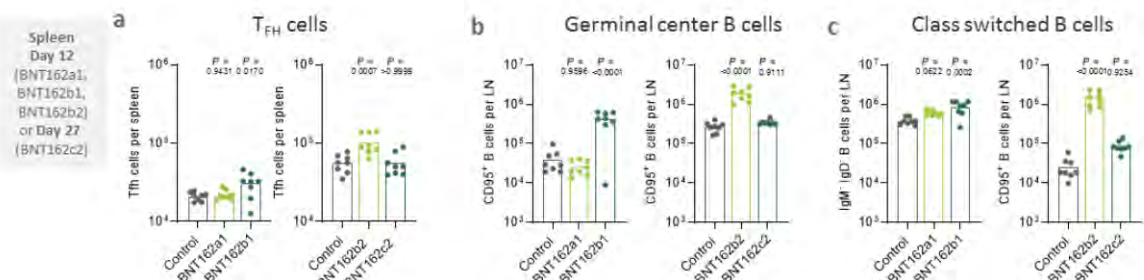


Figure 2-10. B cell response in the spleen upon S peptide restimulation (Note: labeling of the y-axis in figure b and c reflects the results observed in spleen as per the Sponsor's response to nonclinical questions)

Cytotoxicity towards S protein

- No relevant CT26 cell lysis was observed in the BNT162a1 group.
- For the BNT162b1 vaccinated group, a tendency for cell killing was observed in four out of eight mice given compared to control.
- More pronounced tumor cell lysis in eight out of eight mice was observed for splenocytes of mice vaccinated with **BNT162b2** or BNT162c2, which encode the full-length S protein. Overall, the detected effects were rather weak and warrant further optimisation of the assay. No quantitative and statistical analysis of this dataset was performed.

Conclusion: SARS-CoV-2 vaccine candidate **BNT162b2** was able to induce both T and B cell responses.

2.2. IMMUNOGENICITY AND PROTECTION STUDY IN MONKEYS

Study No. VR-VTR-10671: BNT162b2 (V9) Immunogenicity and Evaluation of Protection against SARS-CoV-2 Challenge in Rhesus Macaques

Pfizer Vaccine Research
 Pearl River, NY 10965

Rhesus monkeys ($n = 6 \sigma$ /group), 2-4 years old

Treatment phase

IM dosing on day 0 and 21, 0.5 mL of vaccine containing 30 μ g or 100 μ g of BNT162b2 V9 LNP

Control: saline

Blood samples were collected at pre-dose, and weekly after dosing to day 56. S-specific T-cell responses were analysed by ELISpot and ICS.

Challenge phase

Challenge virus: The working virus stock was generated from two passages of the USA-WA1/2020 isolate (a 4th passage seed stock purchased from BEI Resources; NR-52281) in Vero 76 cells.

The groups immunised with two IM doses of 100 μ g BNT162b2 (V9) (and 3 control monkeys) were challenged 55 days after the second immunisation with 1.05×10^6 plaque forming units of SARS-CoV-2 (strain USA-WA1/2020), split equally between the intranasal (IN) and intratracheal (IT) routes.

Three naive control monkeys were mock-challenged with cell culture medium (Sentinel). Nasal and oropharyngeal (OP) swabs were collected and bronchoalveolar lavage (BAL) was performed, and the samples were tested for the presence of SARS-CoV-2 RNA by RT-qPCR using the CDC-developed 2019-nCoV_N1 assay. Chest X-rays and CT scans were performed prior to challenge and at day 3 and 10 post challenge.

Histopathological assessments were performed at days 7 or 8 following infectious SARS-CoV-2 challenge in the BNT162b2-immunised animals ($n=6$) and saline-immunised control animals ($n=3$). Tissues collected and microscopically evaluated included lung, kidney, liver, spleen, skin, large and small intestine, heart [with coronary arteries], bone marrow, nasal septum, tongue, trachea, mediastinal lymph node, and mucocutaneous junctions.

Lung lesions were evaluated using a semi-quantitative scoring system with inclusion of cell types and/or distribution. An inflammation area score, based on the estimated area of the lung section with inflammation, was used to grade each lung lobe: 0=normal; 1=<10%; 2=11-30%; 3=30-60%; 4= 60-80%; 5=>80%. For each animal, the inflammation area score for each lung lobe was averaged to generate a single inflammation area score for that animal. That score was used to evaluate the severity of respiratory disease after SARS-CoV-2 challenge.

Immunogenicity

- S1 specific IgGs were detected by day 14 after the first dose, the IgGs markedly increased after dose 2 in a dose dependent manner. Negligible S1 specific IgG was detected in the control group.
- The geometric mean concentration (GMCs) of S1-binding IgG (30,339 U/mL and 34,668 U/mL at 30 μ g and 100 μ g doses, respectively) 7 days after the 2nd immunisation dose were significantly higher than the S1-binding IgG GMC (631 U/mL) of human convalescent serum (HCS) (Figure 2-11). IgG relatively rapidly decreased to ~4000 and ~6000 U/mL on day 56 (5 weeks after the 2nd immunisation dose).

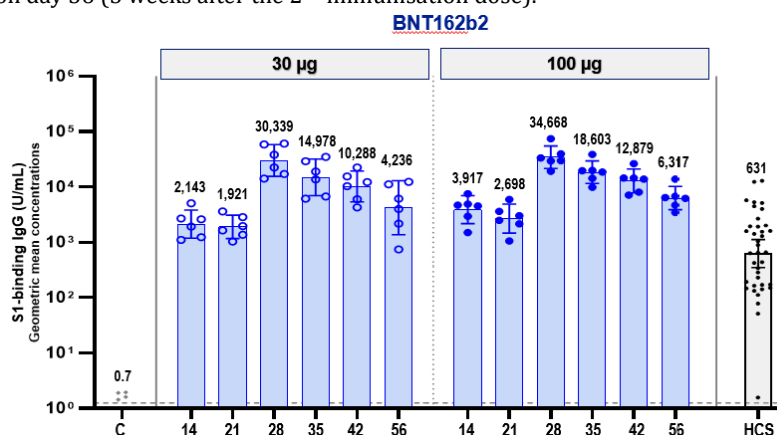


Figure 2-11. S1-binding IgG concentrations (GMCs) elicited by immunisation with BNT162b2 (V9). C - saline-immunisation control; HCS - human convalescent serum panel

- BNT162b2 (V9) administration stimulated 50% serum neutralising titres (VNT₅₀) as detected in monkey sera by day 14 after the first dose (measured by geometric mean titre or GMT). GMTs peaked 1 week after the second dose (GMT 962, 14 days after dose 2 of 30 μ g and GMT 1689 after dose 2 of 100 μ g) and lasted to day 56 with GMTs significantly higher than neutralisation GMTs of the HCS (GMT 94). By 5 weeks after the second dose, neutralising titres decreased by 3-5 fold from the peak (Figure 2-12).

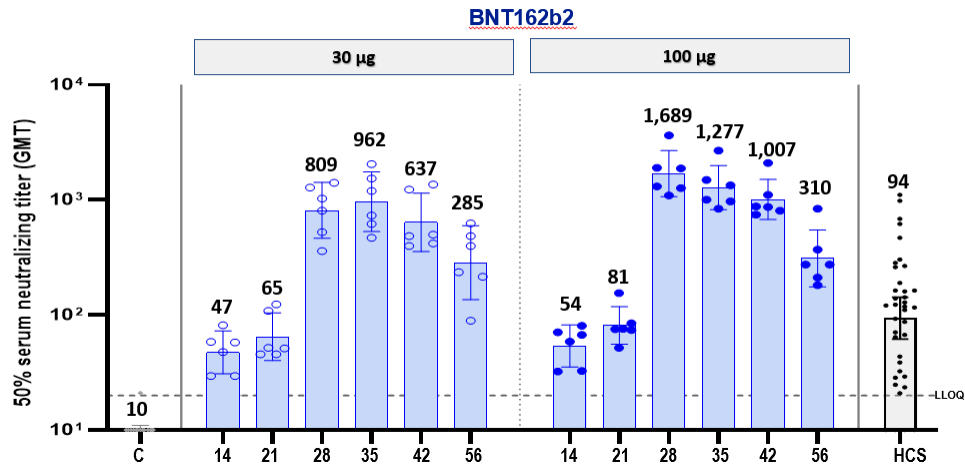


Figure 2-12. 50% serum neutralising titres (GMTs) elicited by immunisation with BNT162b2 (V9). C – saline-immunisation control; HCS – human convalescent serum panel

- Strong IFN- γ and low IL-4 responses were detected in BNT162b2-immunised animals (ELISpot analysis) on day 28 and 42 (Figure 2-13).
- BNT162b2 administration induced a high frequency of S-specific CD4⁺ T cells producing IFN- γ , IL-2, or TNF- α but a low frequency of CD4⁺ cells that produce IL-4, indicating a T_H1-biased response except for one monkey, which has a slightly higher T_H2 response than T_H1. S-specific IFN- γ producing CD8⁺ T cell responses were also observed in immunised animals (flow cytometry ICS analysis). The vaccine also increased IFN- γ CD8⁺ T cells (Figure 2-14).
- The T-cell responses were similar between the two dose levels. S protein specific T cell responses decreased from day 28 to day 42 by <2 fold (not determined on day 56), although the decrease was relatively small or there was no significant change for CD8⁺ T cells in the high dose group (100 µg)

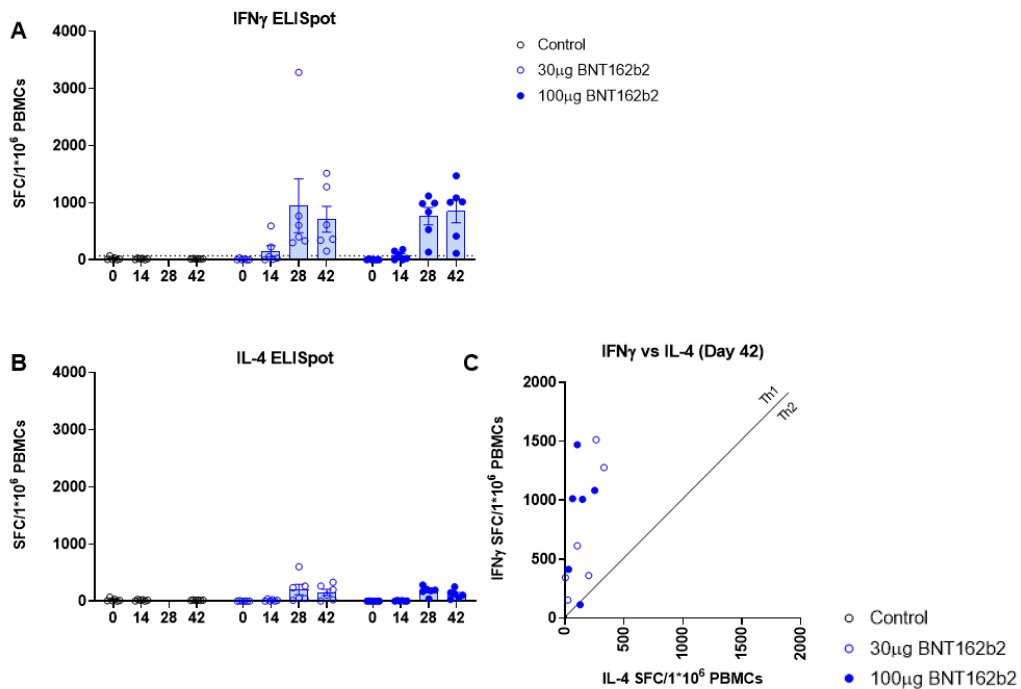


Figure 2-13. IFN- γ and IL-4 ELISpot results in BNT162b2- and Control-immunized animals

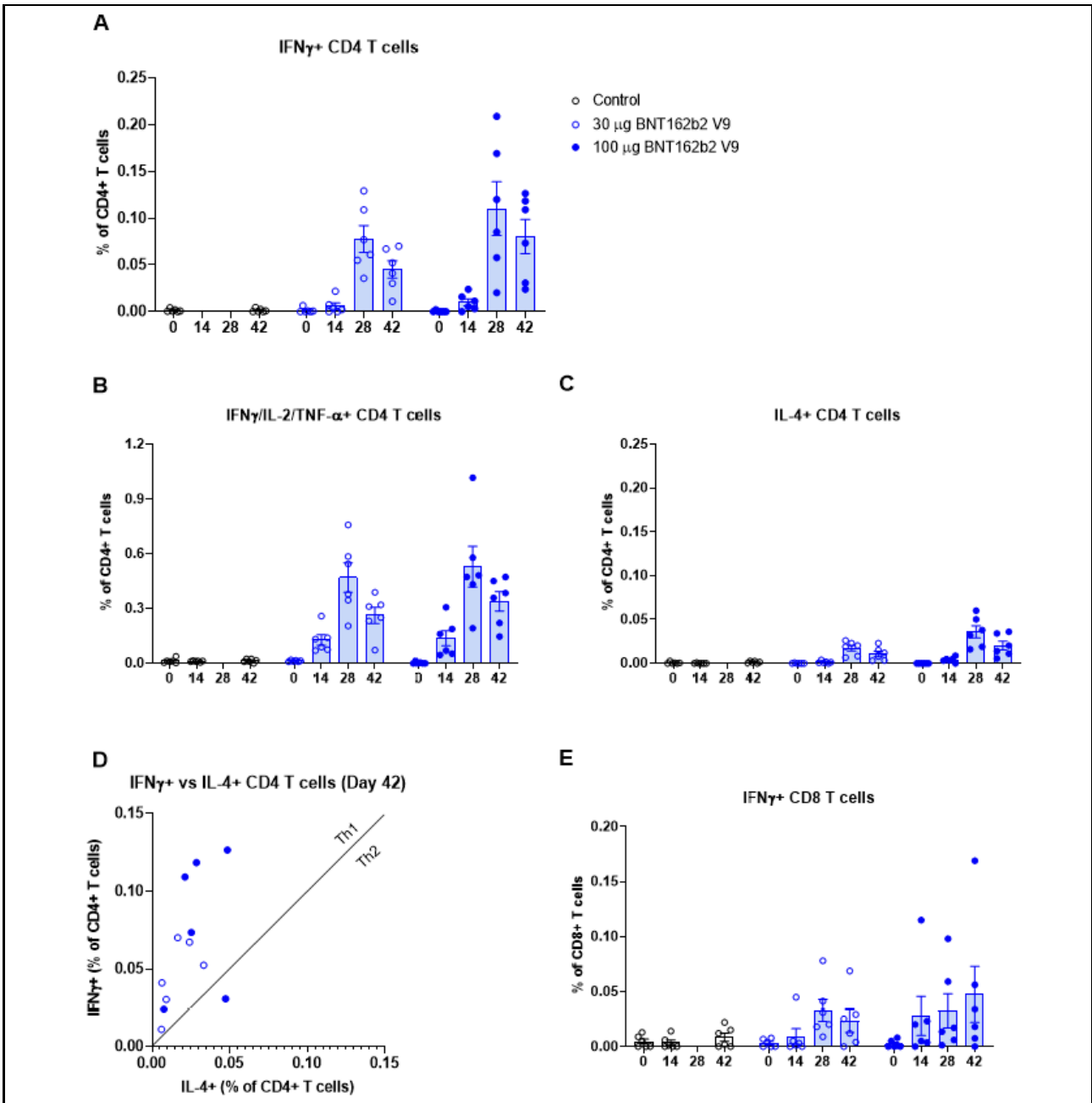


Figure 2-14. S-specific CD4 and CD8 T-cell responses in BNT162b2- and control-immunised animals (ICS assay)

Protection

- At the time of challenge, SARS-CoV-2 neutralising titres ranged from 260 to 1,004 in the BNT162b2 (V9)-immunised animals. Neutralising titres were undetectable in animals from the control-immunised and sentinel groups.
- Viral RNA was detected in BAL fluid from 2/3 control-immunised animals on day 3 and from 1/2 on day 6 post challenge whereas no viral RNA was detected in BAL fluid from the BNT162b2 (V9)-immunised and SARS-CoV-2 challenged macaques (statistically significant) (Figure 2-15).
- Viral RNA was detected in nasal swabs obtained from control-immunised animals on days 1 (viral RNA: $\sim 10^4 \log_{10}$ copies), 3 ($\sim 10^4 \log_{10}$ copies), and 6 ($\sim 10^3 \log_{10}$ copies) post challenge. High viral RNA copies were detected in nasal swabs obtained from BNT162b2 (V9)-immunised animals on Day 1 (viral RNA: day 1 $\sim 10^5 \log_{10}$), but RNA copies were below the limit of detection from day 3 (less than $\sim 10^2 \log_{10}$ copies).
- Low levels of RNA were observed from OP swabs in immunised monkeys on days 1 and 3, compared to the control-immunised monkeys.

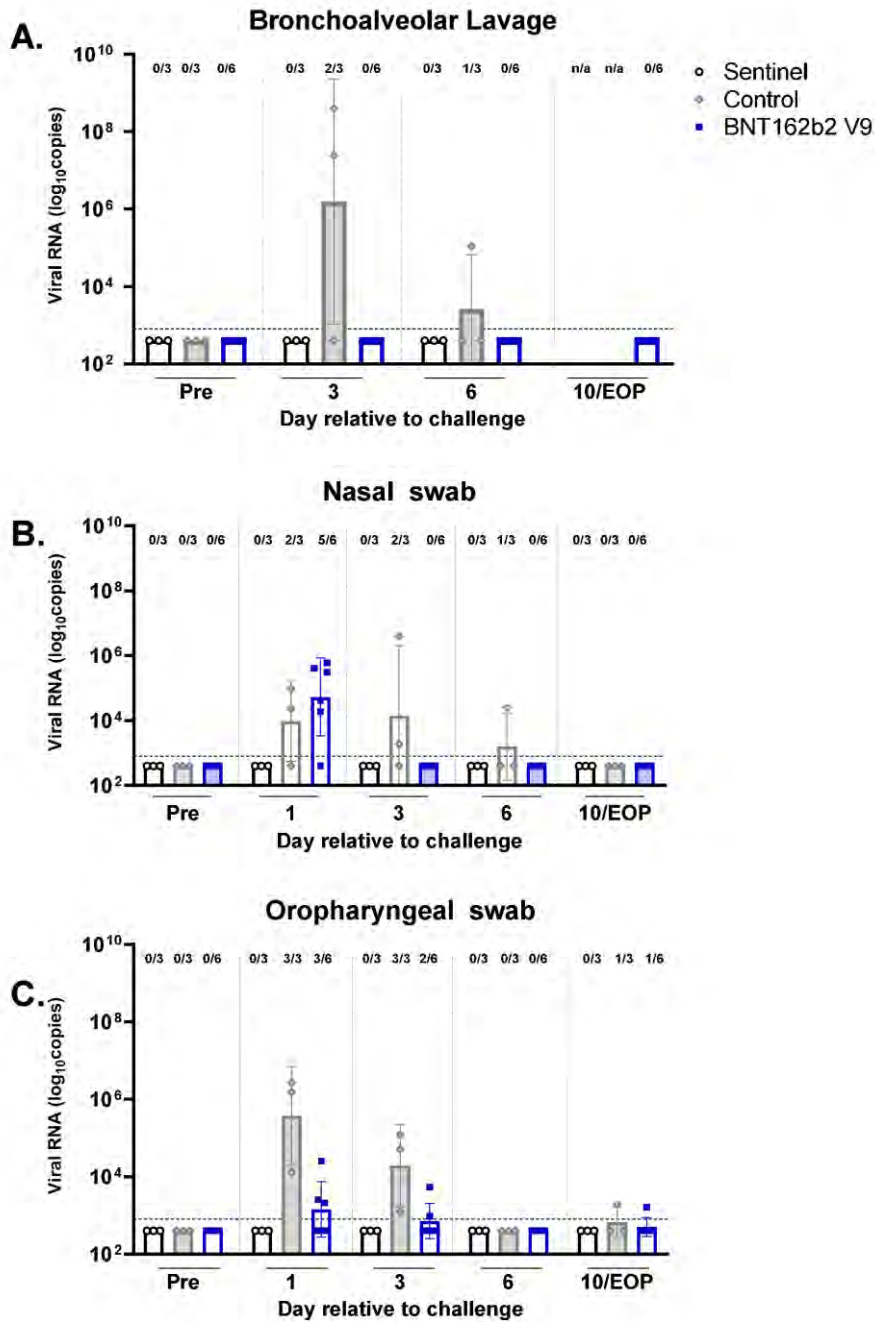


Figure 2-15. Viral RNA in BAL fluid and nasal and oropharyngeal swabs of animals after infectious SARS-CoV-2 challenge

- No significant clinical signs of illness were observed in any group of animals including control despite the presence of viral RNA in BAL fluid from challenged control animals.
- Radiographic evidence (X-ray) of pulmonary abnormality was observed in challenged controls but not in challenged BNT162b2-V9 immunised animals nor in unchallenged sentinels. A very slight increase in the CT score was observed in challenged immunised animals on day 10 compared to day 3 relative to challenge; however, both X-ray and CT scores were lower compared to control challenged animals (Figure 2-16)
- No radiographic evidence of vaccine-elicited enhanced disease was observed (images were not provided).

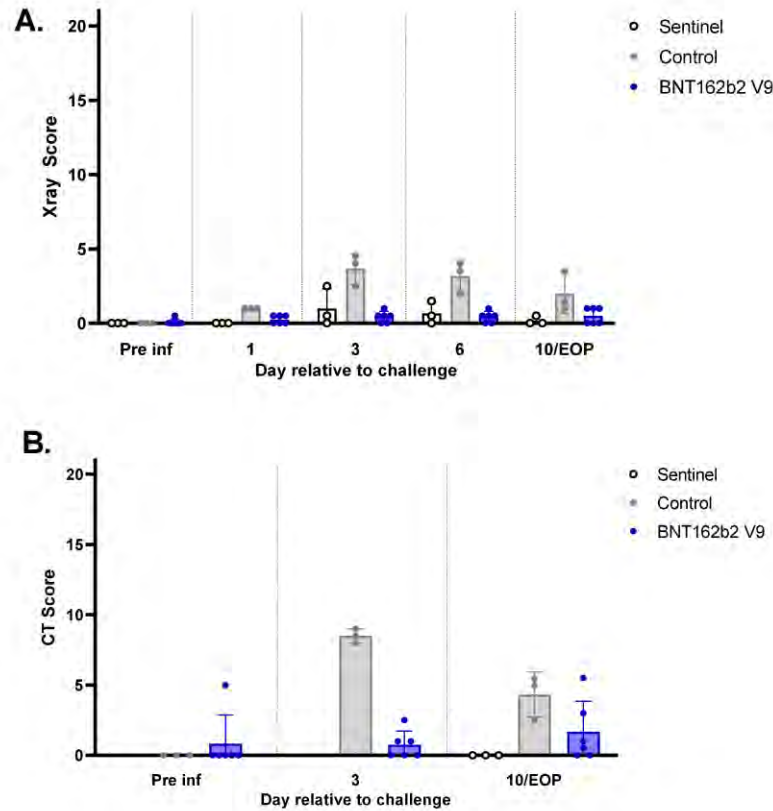


Figure 2-16. Radiograph and CT scores of lungs after challenge [Radiograph (A) and CT (B) scores were assigned on a scale of 1-20. The height of the bars indicates the mean score].

- No significant gross pathology findings were noted in any organ.
- Slight lung inflammation (< 10%) was observed in both saline and BNT162b2-V9 immunised animals with slightly lower inflammation score in immunised animals.
- Inflammatory cell infiltrates including macrophages, neutrophils, lymphocytes, plasma cells, and some eosinophils in both control and immunised animals.
- No evidence of enhanced respiratory disease was observed.
- No other significant microscopic effect was observed in other tissues analysed.

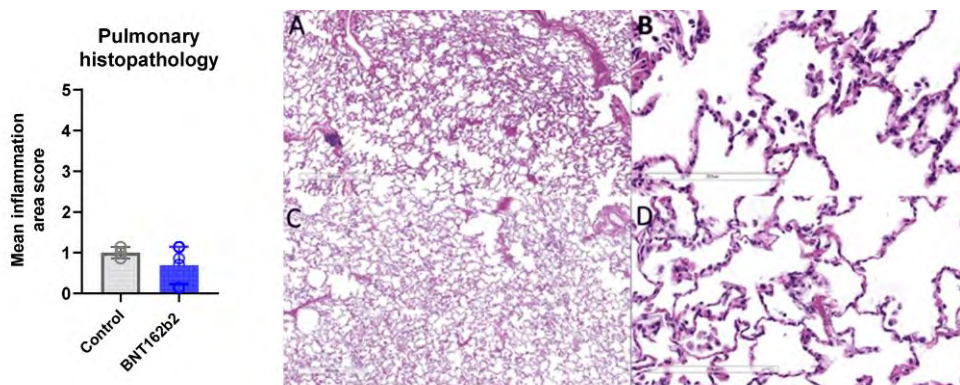


Figure 2-17. Lung inflammation area score after IN/IT SARS-CoV-2 challenge (left panel: bars indicate the geometric mean area scores (lung inflammation) within each group on day 7 or 8 after IN/IT SARS-CoV-2 challenge. Saline-immunized and challenged animals (Control; n=3) are shown in grey and BNT162b2-vaccinated and challenged animals (BNT162b2; n=6) are shown in blue; Right panel: photomicrographs of lung sections from control animals and lungs from BNT162b2-immunized and challenged animals (A, C and B, D: 2.5 × and 20 x objective, respectively).

Conclusion

- BNT162b2 (V9) was found to be immunogenic in monkeys inducing T_H1 biased T cell responses.
- BNT162b2 (V9) provided protection of the 2-4 years old rhesus macaque monkeys from infectious SARS-CoV-2 challenge with reduced detectable viral RNA in the respiratory system compared to the saline control.

- Slight lung inflammation was noted in both challenged control and immunised animals with slightly lower inflammation score in immunised animals compared to control.

2.3. EXPRESSION OF ANTIGENS

Different *in vitro* methods were performed to analyse S protein expression (i.e, SARS-CoV-2 P2 S), which are described in the following table.

Study no. R20-0211: Analysis of the expression of SARS-CoV-2 P2 S antigen																
<p>Test items: BNT162b2-RNA (DS) and BNT162b2-LNP (DP)</p> <p>Control: GFP encoding modified RNA (positive); non-transfected cells (negative)</p> <p>Study details</p> <p>HEK293T cells transfected with either BNT162b2-RNA (DS) or BNT162b2-LNP (DP) were incubated for 18 h before harvesting for analyses. A commercial transfection kit (RiboJuice mRNA transfection kit) was used in the transfection of cells with BNT162b2-RNA. Cells transfected with GFP were examined microscopically for successful transfection</p>																
<p>Assessment of transfection frequencies</p> <p>Western blot of cell lysate was performed with transfected HEK293 cells to confirm BNT162b2-RNA (DS) expression and FACS analysis to assess transfection frequencies and viability of cells transfected with BNT162b2-RNA (DS) or BNT162b2-LNP (DP).</p> <p>Major findings</p> <ul style="list-style-type: none"> • Western blot analysis confirmed the expression of the BNT162b2 antigen in HEK293T cells. • Both BNT162b2-RNA (DS) and BNT162b2-LNP (DP) demonstrated high transfection frequencies with BNT162b2-LNP (DP) exhibiting slightly higher transfection frequency compared to BNT162b2-RNA (DS). No significant differences in cell viability were observed among all cell groups (Figure 2-18). 																
<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <p>Transfection frequency</p> <table border="1"> <caption>Transfection frequency data</caption> <thead> <tr> <th>Group</th> <th>anti-S1-AB positive cells [%]</th> </tr> </thead> <tbody> <tr> <td>non transfected</td> <td>~5</td> </tr> <tr> <td>DS BNT162b2-RNA 1 µg</td> <td>~85</td> </tr> <tr> <td>DP BNT162b2 1 µg</td> <td>~95</td> </tr> </tbody> </table> </div> <div style="text-align: center;"> <p>Viability</p> <table border="1"> <caption>Viability data</caption> <thead> <tr> <th>Group</th> <th>viable cells [%]</th> </tr> </thead> <tbody> <tr> <td>non transfected</td> <td>~95</td> </tr> <tr> <td>DS BNT162b2-RNA 1 µg</td> <td>~95</td> </tr> <tr> <td>DP BNT162b2 1 µg</td> <td>~90</td> </tr> </tbody> </table> </div> </div>	Group	anti-S1-AB positive cells [%]	non transfected	~5	DS BNT162b2-RNA 1 µg	~85	DP BNT162b2 1 µg	~95	Group	viable cells [%]	non transfected	~95	DS BNT162b2-RNA 1 µg	~95	DP BNT162b2 1 µg	~90
Group	anti-S1-AB positive cells [%]															
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non transfected	~95															
DS BNT162b2-RNA 1 µg	~95															
DP BNT162b2 1 µg	~90															
<p style="text-align: center;">Figure 2-18. FACS analysis of transfection frequency and cell viability</p>																
<p>Assessment of cellular localisation</p> <p>Immunofluorescence staining of HEK-293 cells transfected with BNT162b2-RNA (DS) was used to investigate whether the construct was processed within the endoplasmic reticulum (ER).</p> <p>Major findings</p> <ul style="list-style-type: none"> • Co-localisation of the S-protein antigen with an ER marker was detected, suggesting the S protein is processed within the ER (Figure 2-19). • The non-transfected cells did not express the S1 protein. 																

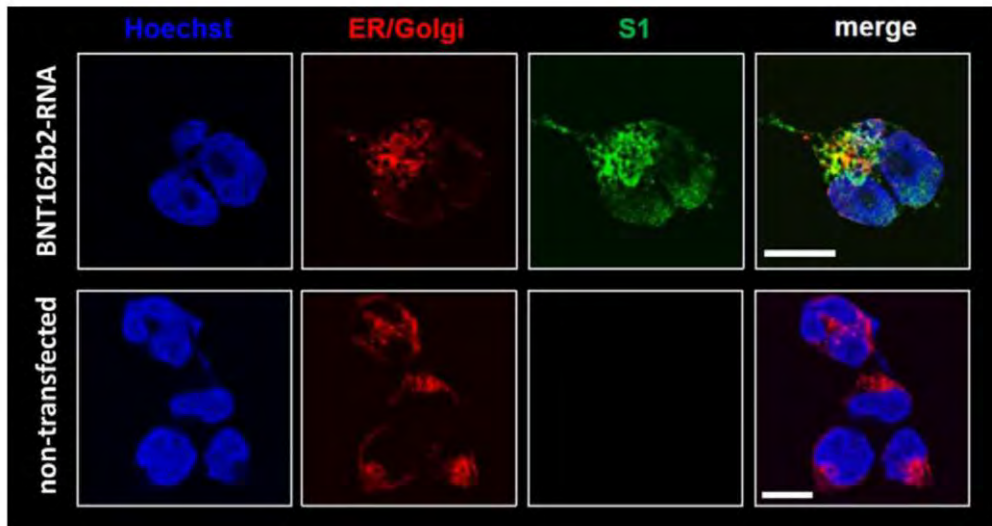


Figure 2-19. Immunofluorescence staining of transfected cells

Study no. R-20-0360: Analysis of the expression of antigens from the mRNA constructs encoding S protein

Test items: different mRNA constructs encoding S antigen including V8 (RNA batch: RNA-KG200312-01) and V9 constructs (RNA batch: RNA-RF200321-06) in different RNA platforms (modRNA = nucleoside-modified mRNA, saRNA = self-amplifying RNA, uRNA = non-modified uridine-containing mRNA)
Control: GFP encoding modified RNA (positive); non-transfected cells (negative)

Study details

s47
[Redacted text]

Major findings

- modRNAs (V9 and V8) demonstrated high transfection frequencies compared to saRNAs.
- saRNA constructs showed higher expression rates than the other two RNA platforms (modRNAs and uRNA).
- No significant differences in cell viability were observed among all cell groups.

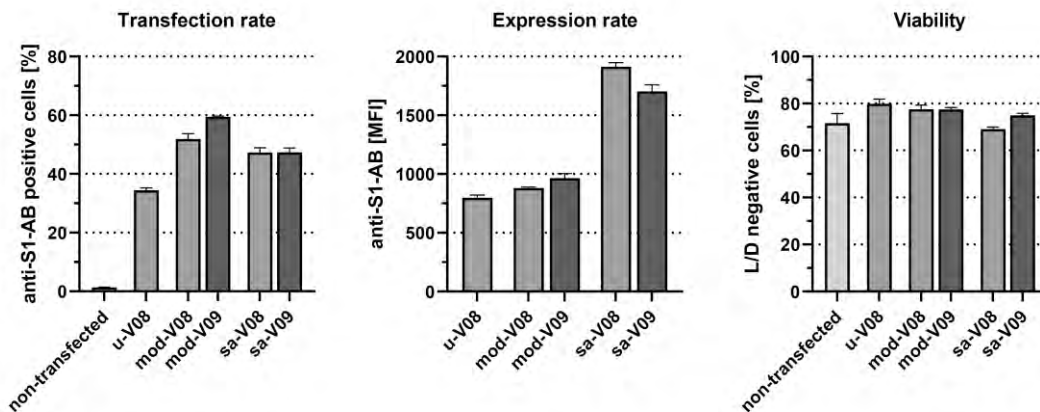


Figure 2-20. FACS analysis of transfection, antigen expression and cell viability

2.4. STRUCTURAL CHARACTERISATION OF S ANTIGEN (P2 S)

Study no. VR-VTR-10741: Structural and biophysical characterisation of SARS-CoV-2 spike glycoprotein (P2 S)

s47

[Redacted text]

Major findings

- Surface expression of P2 S in Expi293F cells was confirmed by staining with antibodies against the RBD, S1, and S2 regions of the full-length S protein (binding RBD > S2 > S1).
- Human ACE2 peptidase domain, therapeutic antibody CR3022 and two neutralising antibodies isolated from a COVID-19 convalescent patient, B38 and H4, bound to surface expressed P2 S (Figure 2-21).

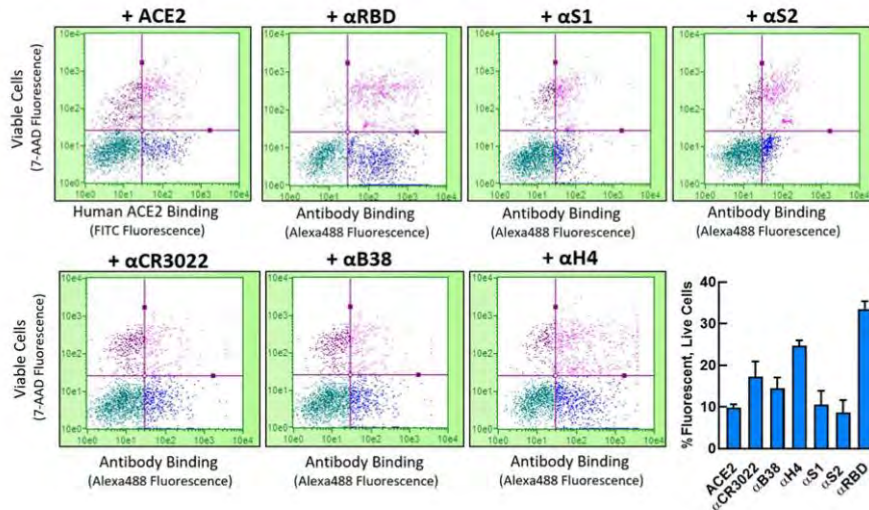
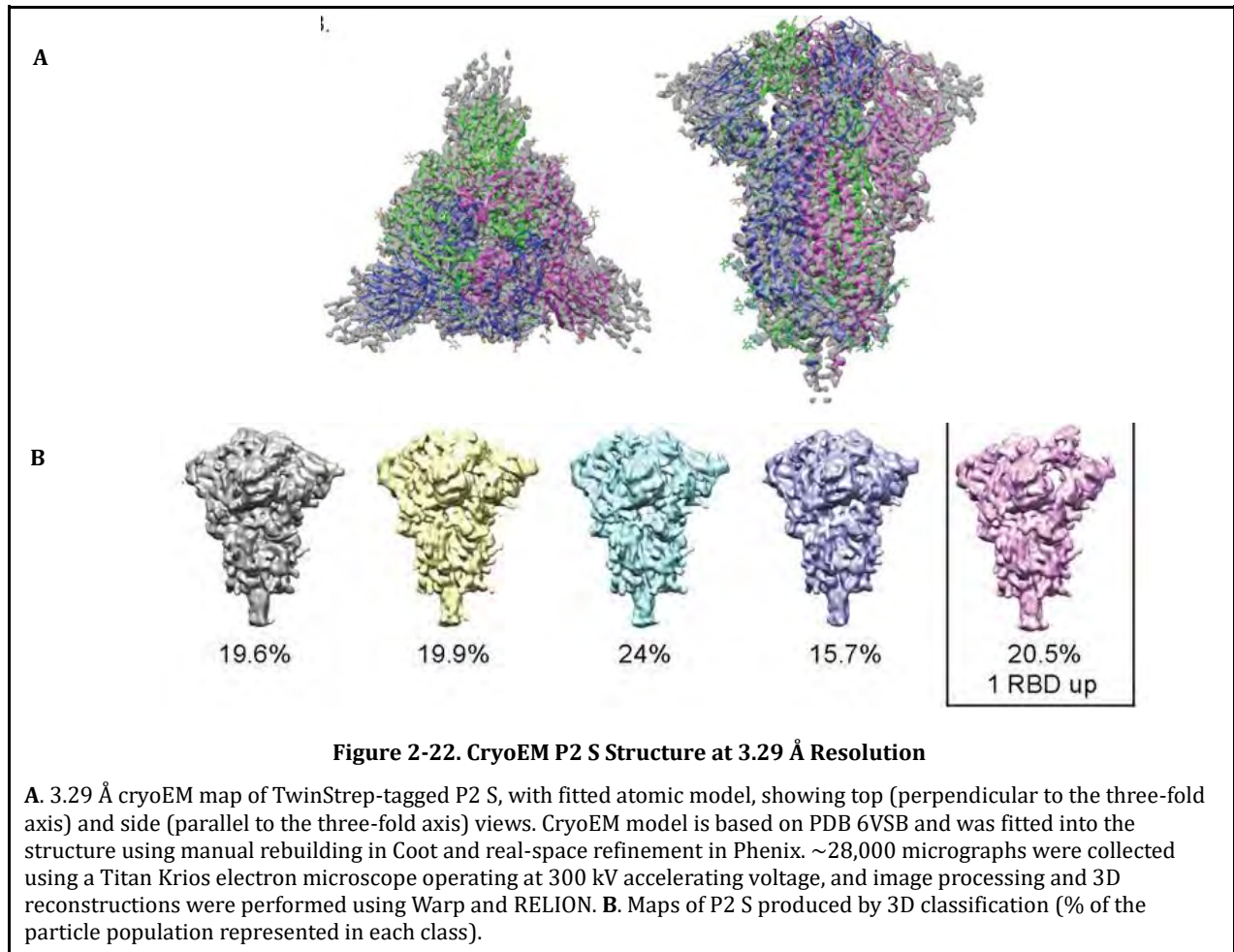


Figure 2-21. Binding to Cell Surface-Expressed Recombinant P2 S

- The trimeric P2 S bound to the human ACE2-PD, and an anti-RBD human neutralising antibody B38 with high affinity ($K_D = \sim 1.2$ nM).
- 2D classification of particles from cryoEM data revealed a particle population resembling the prefusion conformation of S protein. Processing and refinement of this dataset yielded a high-quality 3D map (nominal resolution of 3.29 Å) (Figure 2-22), into which a previously published atomic model (PDB ID: 6VSB) was fitted and rebuilt. The rebuilt model (Figure 2-22 A) shows good agreement with reported structures of prefusion full-length wild type S (Cai et al. 2020) and its ectodomain with P2 mutations (Wrapp et al. 2020).
- 3D classification of the dataset (Figure 2-22 B) showed a class of particles that was in the one RBD ‘up’ (accessible for receptor binding), two RBD ‘down’ (closed) conformation and represented 20.5% of the trimeric molecules. The remainder were in the all RBD ‘down’ conformation. The RBD in the ‘up’ conformation was less well resolved than other parts of the structure, suggesting conformational flexibility and a dynamic equilibrium between RBD ‘up’ and RBD ‘down’ states as also suggested by others (Cai et al. 2020; Henderson et al. 2020).



2.5. CYTOKINE/CHEMOKINE SECRETION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

Study No. R-20-0357	
Test system: Human peripheral blood mononuclear cells (PBMCs) (<i>n</i> = 3 healthy donors)	
Test items: BNT162b2 (V9): Polymun batch mRNA RBP020.2-LNP; lot: CoVVAC/270320; RNA batch: RNA-RF200321-06; Control: PBS	
Doses of BNT162b2: 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.5 and 3 µg LNP-mRNA per well, corresponding to 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8 and 15 µg/mL of BNT162b2	
S47	[Redacted]
[Redacted]	[Redacted]
[Redacted]	[Redacted]
[Redacted]	[Redacted]
[Redacted]	[Redacted]
Major findings	
<ul style="list-style-type: none"> All PBMCs showed basal to low levels of TNF-α, IL-6, IFN-γ, IL-1β across the doses, with no consistent and/or dose-dependent increases (Figure 2-23). All cytokines increased (~2-6 fold) at BNT162b2 dose of 15 µg/mL and IFN-γ at ≥ 8 µg/mL in the samples from donor 1. 	

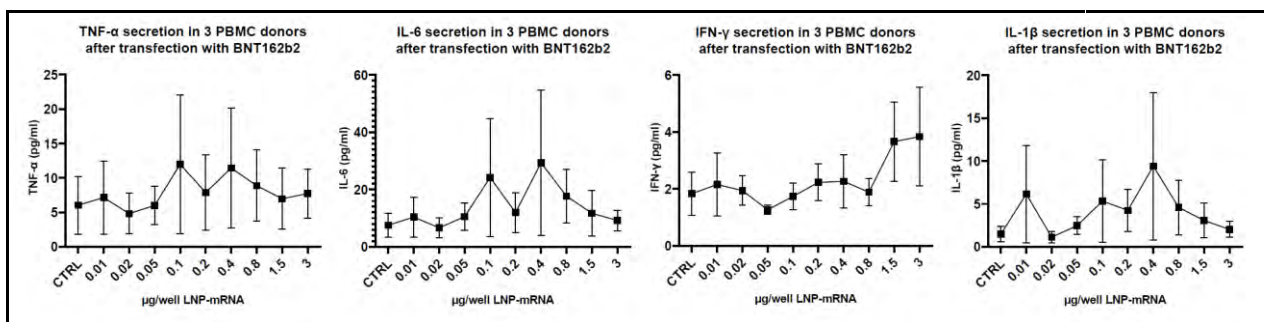


Figure 2-23. Cytokine secretion after transfection of PBMCs with BNT162b2 (mean ± SEM)

- PBMCs from all three donors showed basal to low levels of IP-10, MIP-1β and MCP-1 at doses up to 1 μg/mL of BNT162b2.
- A dose dependent increase in IP-10 secretion was observed at ≥ 1 μg/mL in donor 1 resulting in an increase in the average value for all the samples (higher standard deviations at higher doses). Inconsistent changes in the secretion of MIP-1β and MCP-1 were observed (Figure 2-24).

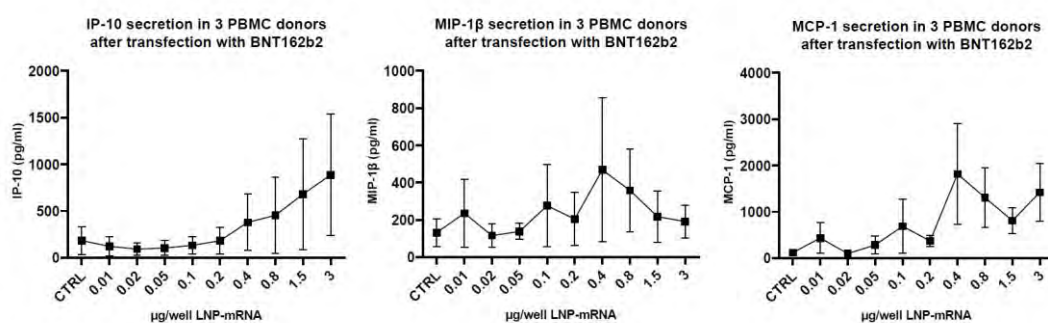


Figure 2-24. Chemokine secretion after transfection of PBMCs with BNT162b2 (mean ± SEM)

- ~ 100% cell viability was observed in PBMCs from two donors. A small, dose dependent decrease in cell viability (by up to 12% at 15 μg/mL) was observed in PBMCs from donor 2.

Conclusion

The transfection of human PBMCs with BNT162b2 resulted in low levels of proinflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-1β) secretion. Of the chemokines (IP-10, MIP-1β, MCP-1), only IP-10 secretion tended to be increased at ≥ 2 μg/mL BNT162b2. The results were highly variable across the samples due to low number of samples and inconclusive.

3. SAFETY PHARMACOLOGY

No safety pharmacology studies were conducted. Body temperatures and daily clinical observations were made in the repeat-dose toxicity studies.

4. PHARMACOKINETICS

4.1. PLASMA KINETICS

No absorption studies were conducted for BNT162b2, which is acceptable as per the WHO and EMA relevant guidelines (WHO, 2005; EMA, 1998). The LNP formulation of the vaccine contains two new excipients ALC-0159 and ALC-0315, for which pharmacokinetic studies were conducted (**Study 072424**).

The plasma kinetics and elimination of ALC-0159 and ALC-0315 were examined after a single intravenous (IV) dose of LNPs containing luciferase-encoding mRNA (1 mg/kg) in male Wistar Han

rats. The LNP composition was similar to the LNP in the BNT162b2 vaccine (Table 1-3). The ALC-0159 and ALC-0315 doses were 1.96 and 15.3 mg/kg, respectively.

Analyses

- 20 μ L aliquots of plasma, liver homogenate, urine, and faeces homogenate samples from the rats were centrifuged to obtain supernatant, which were analysed using LC-MS/MS. Blood and liver were collected predose and at 0.1, 0.25, 0.5, 1, 3, 6, 24, 48, 96, 192 and 336 h after dose ($n=3$ /time point). Faeces and urine were collected daily for 2 weeks.
- BLQ concentrations were not used in the PK calculations. The linear dynamic ranges of the standard curves for ALC-0159 and ALC-0315, were 4.88 to 2500 ng/mL for plasma, 19.53 to 10000 ng/g for liver, 4.88 to 2500 ng/mL for urine, and 6.592 to 3375 ng/g for faeces.
- The pharmacokinetic parameters were determined from pooled animal data using noncompartmental analysis in Watson LIMS 7.5.

Major findings

- Plasma concentrations of ALC-0159 and ALC-0315 decreased rapidly (initial $t_{1/2}$ of 1.74 and 1.62 h, respectively). ALC-0159 and ALC-0315 were slowly cleared from plasma, with terminal elimination $t_{1/2}$ of 72.7h and 139h, respectively. AUC_{last} was 98.6 μ g.h/mL for ALC-0159 and 1020 μ g.h/mL for ALC-0315, and AUC_{inf} 99.2 and 1030 μ g.h/mL, respectively (Figure 4-1).
- ALC-0159 and ALC-0315 concentrations in plasma dropped 8000 and 7000 fold, and in liver > 250 and 4 fold, respectively, 2 weeks after dosing.
- The liver appears to be the major site of uptake of both lipids from the blood (~ 20% of dose for ALC-0159 and ~60% of dose for ALC-0315 (based on the highest mean amount at 1 h and 24 h, respectively). Faecal excretion of unchanged lipids was ~47% and ~1% of dose for ALC-0159 and ALC-0315, respectively (Figure 4-2). Both lipids were below LOQ in urine.

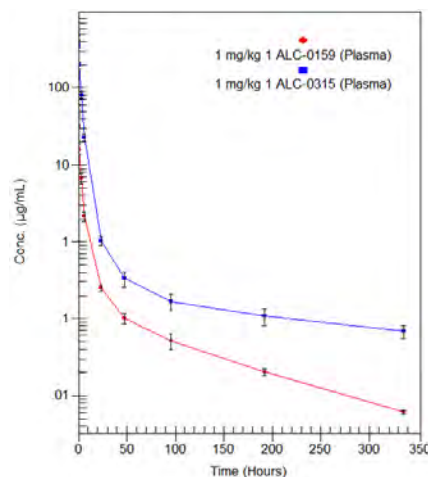


Figure 4-1. Mean plasma concentrations of ALC-0315 and ALC-0159

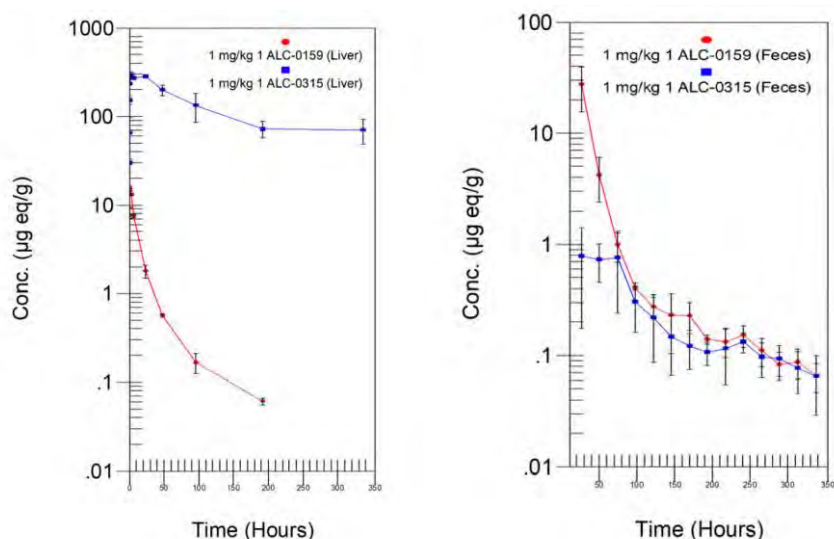


Figure 4-2. Mean liver and faecal concentrations of ALC-0315 and ALC-0159

4.2. DISTRIBUTION

The distribution of LNP-BNT162b2 (V9) mRNA or expressed S protein was not studied.

4.2.1. Study R-20-0072

The expression of luciferase encoding mRNAs encapsulated in different LNP formulations after IM administration to BALB/c mice was investigated.

Table 4-1. Study design

Treatment group	mRNA dose (µg)	Formulation
Buffer control (DPBS)	N/A	N/A
mRNA Luciferase LNP12	2 µg	DODMA:Chol: DOPE:PEGcerC16 (40:48:10:2); RNA-EH190611-01c, FSU-I#029, 79% encapsulation, 0.9053 mg/ml encapsulated RNA, <u>diameter 84 nm</u> , polydispersity 0.202, storage temperature +4°C.
mRNA Luciferase LNP5	2 µg	Acuitas proprietary; RNA-EH190611-01c, batch FM-1055-D, 79% encapsulation, 0.924 mg/ml encapsulated RNA, <u>diameter 108 nm</u> , polydispersity 0.091, storage temperature -80°C.
mRNA Luciferase LNP8	2 µg	"GMP-ready"; RNA-EH190611-01c, batch FM-1074-D, 90% encapsulation, 1.0 mg/ml encapsulated RNA, <u>diameter 71 nm</u> , polydispersity 0.053, storage temperature -80°C. The clinical candidate S antigen encoding mRNA is formulated with this formulation.
Study details		
<ul style="list-style-type: none"> LNP-formulated mRNA encoding luciferase was diluted to 0.05 mg/ml to obtain a dose of 1 µg in 20 µL application volume. The LNP formulations and control were administered to four groups of three female BALB/c mice (1 µg IM in each leg). At 6 h, 24 h, 48 h, 72 h, 6 d and 9 d after injection, <i>in vivo</i> imaging of luciferase expression was conducted using a Xenogen IVIS® Spectrum device, following the injection of luciferin (IP, 150 mg/kg) 5 min prior to imaging. Serum samples were collected on day 1 and 6 h and 9 d post immunisation for cytokine/chemokine level determination (multiplex) and on days -1 and 9 for luciferase-specific ELISA. On day 9, spleens were resected for immunological analysis using IFN-γ ELISpot. 		

Major findings

Expression of luciferase encoding mRNA

- Bioluminescence measurements indicated that all formulations resulted in mRNA expression with highest signal detected at 6 h at the injection site, which declined slowly over time until day 9 (Figure 4-3).
- Group mean luciferase expression from RNA formulated with LNP8 at the injection site at 6 h was $\sim 1 \times 10^9$ p/s. Luciferase expression slowly declined to $\sim 7 \times 10^7$ p/s at 72 h, and $\sim 3\text{--}5 \times 10^5$ p/s on day 9).

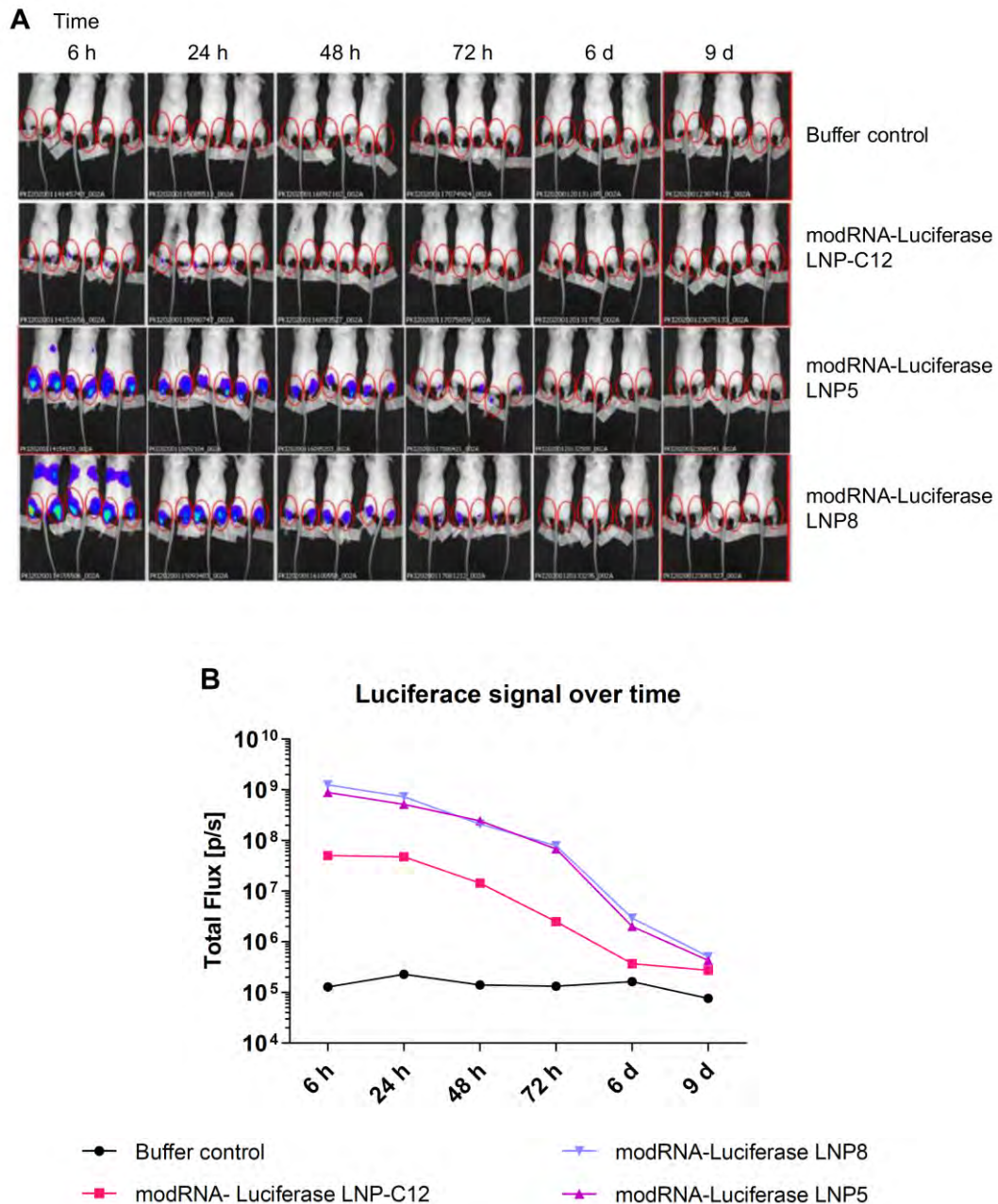


Figure 4-3. Bioluminescence measurement using the LNP-formulated mRNA encoding for luciferase

- Luciferase expressed by the LNP8-mRNA drained to the liver as visualised by luciferase expression at 6 h in the liver region (Figure 4-4).

- Group mean luciferase expression from LNP8-mRNA in the liver at 6 h was $\sim 4.94 \times 10^7$ p/s (Figure 4-4). No signal was detected in liver at 48 h post immunisation.

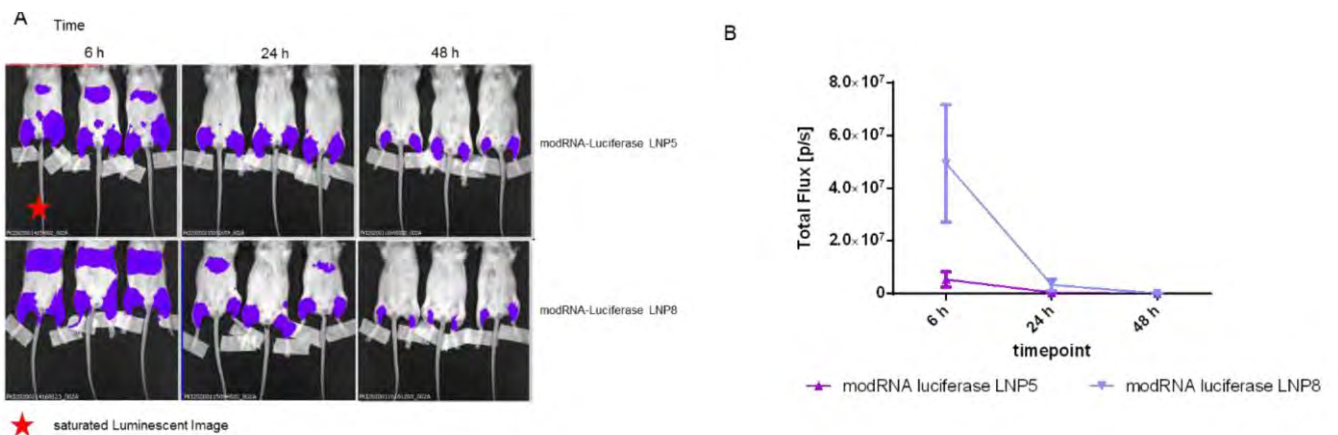


Figure 4-4. Bioluminescence measurement in the liver using luciferase encoding mRNA-LNP

Activation of the innate immune system

- Serum samples were tested for MCP-1, MIP-1 β , TNF- α , IFN- α , IFN- γ , IL-2, IL-6, IL-10, IL1- β and IP-10.
- No cytokines/chemokines were detected in the pre-dose serum.
- Immunisation with mRNA formulated with LNP8 induced MCP-1, IL-6 and IP-10 in all 3 animals and MIP-1 β in one animal at 6 h post immunisation. The cytokine levels were markedly lower than those induced by LNP5, which also induced IFN- α and IFN- γ . All chemokine/cytokine levels dropped to background levels at day 9.

Luciferase specific IgG expression

- No luciferase specific IgG was detected in any groups.

T cell responses

- Stimulation of splenocytes with luciferase peptide pools *ex vivo* induced IFN- γ responses in T cells of animals immunised with all LNP mRNA candidates.
- Group mean spot counts after stimulation of splenocytes with luciferase peptide pools were 53, 138, 676 and **519** spots per 5×10^5 cells for the buffer control, LNPC12, LNP5 and **LNP8** mRNA groups, respectively, as detected by IFN- γ ELISpot assay. The reactivity of splenocytes of the treatment groups to stimulation by an unrelated peptide, AH1 was very low (6-8 spots per 5×10^5 cells), and thus activation of T-cells with luciferase peptides is specific in the treatment groups (Figure 4-5).

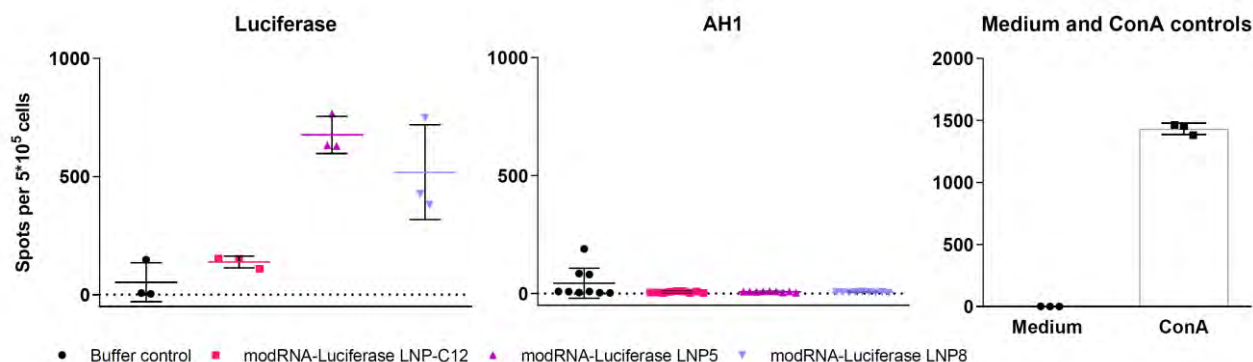


Figure 4-5. ELISpot analysis using splenocytes on day 9 after immunisation

Conclusion

- Expression of luciferase was largely limited to the injection site, with some distribution to liver.
- Luciferase signal reduced to background level at injection site and in liver in 216 hours and 48 hours, respectively.
- LNP8 induced an antigen-specific T cell response, but not B-cell response (no anti-luciferase antibodies).

4.2.2. Study 185350

The distribution of lipid nanoparticles (containing ALC-0315 and ALC-0159) encapsulating mRNA encoding luciferase, was investigated by monitoring of a radiolabelled (^3H -) lipid-marker after IM administration to Wistar rats.

Study details

- Lipid nanoparticle formulation [LNP size, lipid composition (relative to mRNA concentration), and encapsulation efficiency similar to LNP in BNT162b2 vaccine] along with trace amounts of radiolabelled lipid marker cholesteryl-1,2- ^3H (N)]-cholesteryl hexadecyl ether (Figure 4-6), was administered intramuscularly to 42 Wistar Han rats (21/sex; 8-11 week age) at a target dose of 50 μg mRNA/animal (1.29 mg total lipid/animal).

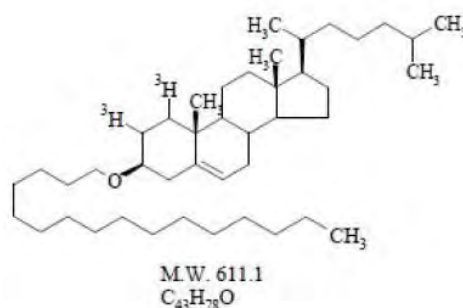


Figure 4-6. Structure of lipid marker cholesteryl-1,2- ^3H (N)]-cholesteryl hexadecyl ether (company code: [^3H]-CHE)

- An additional animal group of 21 male Wistar Han rats were injected intramuscularly at target dose of 2.57 mg total lipid/animal (100 μg mRNA/animal).
- Total radioactivity was measured by liquid scintillation counting of blood, plasma and tissue samples, collected at 0.25, 1, 2, 4, 8, 24 and 48 hours post-dose ($n=3$ /sex/timepoint).

- Tissue collection was extensive and typical. Urine, faeces and cage wash samples were also collected but not analysed for total radioactivity (possible future analysis of specific lipids by LC-MS/MS).

Major findings

Clinical observations

- *100 µg mRNA/rat dose group (♂ only):*
 - One animal was humanely killed after 30 h post-dose with necropsy showing prominent lobular architecture in the liver (no histology pictures provided). This animal was noted with decreased activity, brown staining on muzzle and irregular respiration at 24 h post-dose followed by additional observations of piloerection, hunched body and hypersensitivity to noise stimulus at 30 h post-dose.
 - Two additional animals were hunched and piloerect at 30 h post-dose.
 - All animals in this group lost bodyweight by an average of 7% with food hoppers untouched (raw data not provided).
- *50 µg mRNA/rat dose group (♂ + ♀):*
 - No significant adverse clinical observations in male animals.
 - One ♀ showed decreased activity and irregular respiration at 30 h post-dose. This ♀ was additionally hunched and piloerect at 48 h post-dose.

Tissue distribution

- The concentration of radioactive lipid marker reached the peak level in plasma (8.9 µg lipid eqv/mL) between 1 – 4 h post-dose and distribution mainly into liver, adrenal glands, spleen and ovaries over 48 h (Table 4-2). The concentration of radioactivity remained highest in injection site at all time-points.
- Concentrations were higher in plasma than in blood, with mean blood: plasma ratios of 0.5 – 0.6.

Table 4-2. Mean concentration of radioactivity (sexes combined) in tissue and blood following a single IM dose of 50 µg mRNA/rat

Sample	Total Lipid Concentration (µg lipid equiv/g (or mL))						
	0.25 min	1 h	2 h	4 h	8 h	24 h	48 h
Adipose tissue	0.057	0.100	0.126	0.128	0.093	0.084	0.181
Adrenal glands	0.27	1.48	2.72	2.89	6.80	13.77	18.21
Bladder	0.041	0.130	0.146	0.167	0.148	0.247	0.365
Bone (femur)	0.091	0.195	0.266	0.276	0.340	0.342	0.687
Bone marrow (femur)	0.48	0.96	1.24	1.24	1.84	2.49	3.77
Brain	0.045	0.100	0.138	0.115	0.073	0.069	0.068
Eyes	0.010	0.035	0.052	0.067	0.059	0.091	0.112
Heart	0.28	1.03	1.40	0.99	0.79	0.45	0.55
Injection site	128.3	393.8	311.2	338.0	212.8	194.9	164.9
Kidneys	0.39	1.16	2.05	0.92	0.59	0.43	0.42
Large intestine	0.013	0.048	0.09	0.29	0.65	1.10	1.34
Liver	0.74	4.62	10.97	16.55	26.54	19.24	24.29
Lung	0.49	1.21	1.83	1.50	1.15	1.04	1.09
Lymph node (mandibular)	0.064	0.189	0.290	0.408	0.534	0.554	0.727
Lymph node (mesenteric)	0.050	0.146	0.530	0.489	0.689	0.985	1.366
Muscle	0.021	0.061	0.084	0.103	0.096	0.095	0.192
Ovaries (females)	0.104	1.34	1.64	2.34	3.09	5.24	12.26
Pancreas	0.081	0.207	0.414	0.380	0.294	0.358	0.599
Pituitary gland	0.339	0.645	0.868	0.854	0.405	0.478	0.694
Prostate (males)	0.061	0.091	0.128	0.157	0.150	0.183	0.170
Salivary glands	0.084	0.193	0.255	0.220	0.135	0.170	0.264
Skin	0.013	0.208	0.159	0.145	0.119	0.157	0.253
Small intestine	0.030	0.221	0.476	0.879	1.279	1.302	1.472
Spinal cord	0.043	0.097	0.169	0.250	0.106	0.085	0.112
Spleen	0.33	2.47	7.73	10.30	22.09	20.08	23.35
Stomach	0.017	0.065	0.115	0.144	0.268	0.152	0.215
Testes (males)	0.031	0.042	0.079	0.129	0.146	0.304	0.320
Thymus	0.088	0.243	0.340	0.335	0.196	0.207	0.331
Thyroid	0.155	0.536	0.842	0.851	0.544	0.578	1.000
Uterus (females)	0.043	0.203	0.305	0.140	0.287	0.289	0.456
Whole blood	1.97	4.37	5.40	3.05	1.31	0.91	0.42
Plasma	3.96	8.13	8.90	6.50	2.36	1.78	0.81
Blood:plasma ratio	0.815	0.515	0.550	0.510	0.555	0.530	0.540

- Mean total radioactivity was greatest at the injection site followed by the liver with much lower total recovery in spleen, adrenal glands and ovaries (Table 4-2). The total radioactivity recovery was less than 100% at all time-points (range = 20 – 60%) probably due to difficulty in collecting entirety of injection site samples and the presence of radioactivity in the carcass, faeces and urine, which were not analysed.
- The tissue distribution pattern was similar in 100 µg mRNA/animal dose group as noted above for 50 µg mRNA/animal dose, with highest distribution into liver, adrenal glands and spleen.
- Draining lymph nodes to the site of injection should have been collected and analysed for radioactivity, given the increased size of draining lymph nodes seen in other nonclinical studies after dosing.

Conclusions

- Slow but significant distribution of lipid nanoparticles from the site of injection with major uptake into liver.
- Minor distribution in spleen, adrenal glands and ovaries over 48 h.
- Mean blood:plasma ratios of 0.5-0.6 indicating nanoparticles mainly present in plasma fraction of blood with peak concentrations in plasma at approx. 2 h post-dose.

4.3. METABOLISM

In vitro and *in vivo* studies were conducted to evaluate metabolism of novel lipids ALC-0159 and ALC-0315. The metabolism studies used LC/MS-MS bioanalytical methods for the detection of novel lipids and their metabolites. The major metabolic pathways for ALC-0159 and ALC-0315 in various species, as proposed by the sponsor, are shown in Figure 4-7 and Figure 4-8, respectively.

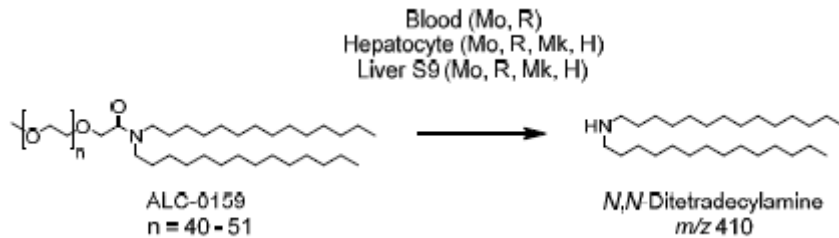


Figure 4-7. Proposed metabolic pathway for ALC-0159

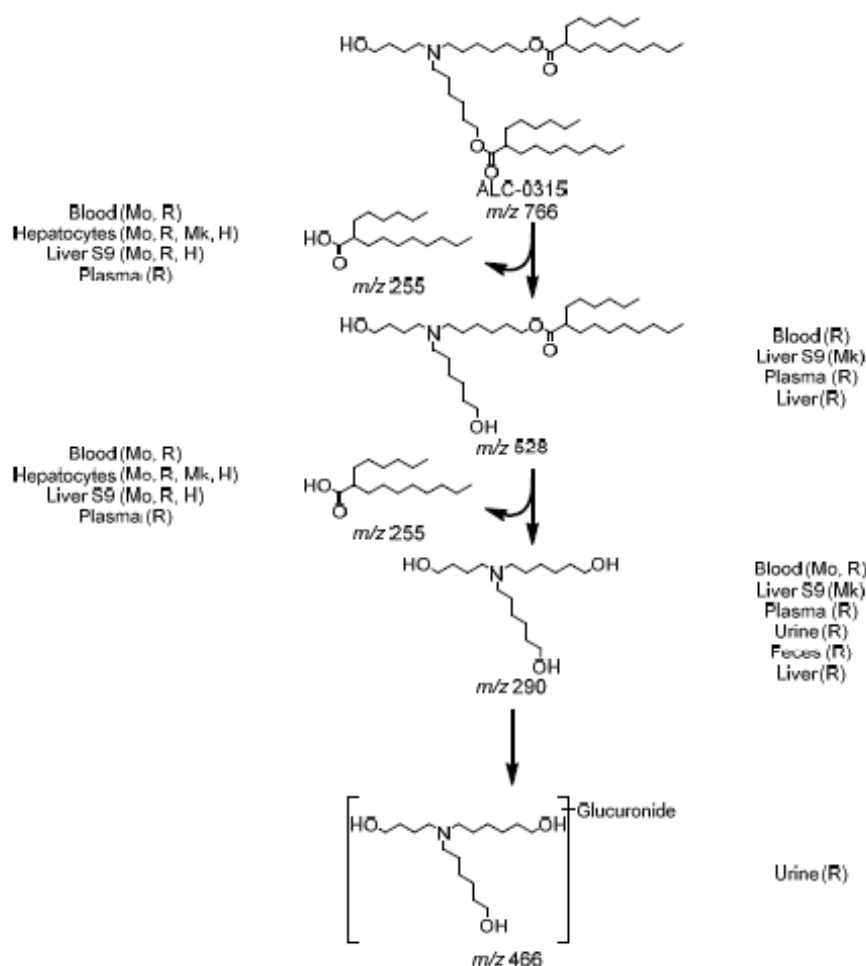


Figure 4-8. Proposed metabolic pathway for ALC-0315

In vitro studies indicated minor metabolism of ALC-0159 and ALC-0315 in liver, with most of the lipids found unchanged at the end of the 2 or 4 h incubation period (Table 4-3 and Table 4-4).

Table 4-3. Percent metabolism of ALC-0159 (1 µM in all assays) *in-vitro*

Study No./Test system	Species	Percent remaining unchanged ALC-0159								Half-life $t_{1/2}$ (min)	Cl_{int} (mL/min/kg)
		0 min	15 min	30 min	60 min	90 min	120 min	180 min	240 min		
01049-20020 Liver microsomal protein (0.5 mg/mL)	Mouse	100	82.3	86.4	85.5	85.4	95.9	-	-	>120	<45.5
	SD Rat	100	101.2	93.8	98.3	95.4	97.1	-	-	>120	<20.7
	Wistar Han Rat	100	112.1	102.7	105.4	100.9	109.0	-	-	>120	<20.7
	Monkey	100	100.8	85.1	86.4	94.6	93.4	-	-	>120	<16.9
	Human	100	99.6	92.3	95.5	98.0	93.1	-	-	>120	<14.5
01049-20021 Liver S9 protein (0.5 mg/mL)	Mouse	100	98.9	91.1	102.8	90.7	106.8	-	-	>120	-
	SD Rat	100	84.4	90.9	98.0	93.5	92.7	-	-	>120	-
	Monkey	100	91.3	98.0	105.6	108.3	105.7	-	-	>120	-
	Human	100	106.7	107.6	105.0	109.4	119.6	-	-	>120	-
01049-20022 Hepatocyte suspension (8x10 ⁴ cell)	Mouse	100	-	100.8	94.9	94.3	87.1	94.9	102.8	>240	<34.1
	SD Rat	100	-	93.4	91.8	90.3	89.5	94.0	94.9	>240	<13.5
	Wistar Han Rat	100	-	113.0	105.1	112.8	104.1	102.9	98.8	>240	<13.5
	Monkey	100	-	90.2	92.9	94.6	97.5	89.8	92.9	>240	<11.3
	Human	100	-	106.3	101.6	92.7	96.0	93.7	102.6	>240	<7.4

- Not determined

Table 4-4. Percent metabolism of ALC-0315 (1 µM in all assays) *in-vitro*

Study No./Test system	Species	Percent remaining unchanged ALC-0315								Half-life ($t_{1/2}$) min	Cl_{int} (mL/min/kg)
		0 min	15 min	30 min	60 min	90 min	120 min	180 min	240 min		
01049-20008 Liver microsomal protein (0.5 mg/mL)	Mouse	100	98.8	97.8	100.5	97.8	96.5	-	-	>120	<45.5
	SD Rat	100	94.4	96.3	99.7	98.7	96.0	-	-	>120	<20.7
	Wistar Han Rat	100	96.3	97.3	98.5	94.1	93.7	-	-	>120	<20.7
	Monkey	100	98.0	96.2	100	98.0	97.7	-	-	>120	<16.9
	Human	100	100.2	99.8	101.5	100.5	98.3	-	-	>120	<14.5
01049-20009 Liver S9 protein (0.5 mg/mL)	Mouse	100	97.7	97.2	98.6	98.1	96.8	-	-	>120	-
	SD Rat	100	98.8	99.6	99.6	98.8	98.5	-	-	>120	-
	Monkey	100	99.6	97.0	99.1	98.7	99.6	-	-	>120	-
	Human	100	96.0	97.3	95.0	98.3	99.3	-	-	>120	-
01049-20010 Hepatocyte suspension (8x10 ⁴ cells)	Mouse	100	-	101.1	100.8	101.9	98.8	101.1	99.6	>240	<34.1
	SD Rat	100	-	97.7	98.5	99.2	97.4	98.9	101.1	>240	<13.5
	Wistar Han Rat	100	-	102.7	102.3	103.1	99.6	103.5	100	>240	<13.5
	Monkey	100	-	96.4	97.8	100	96.4	95.6	93.8	>240	<11.3
	Human	100	-	100.7	101.4	100.4	100.7	98.9	99.6	>240	<7.4

- Not determined

Potential metabolites of ALC-0159 and ALC-0315 were analysed *in vitro* in blood, hepatocytes and liver S9 fraction and *in vivo* in rat plasma, urine, faeces and liver after a single IV dose of LNP containing luciferase mRNA (Report **043725**). No unique *in-vivo* metabolites of ALC-0159 were observed in the rat pharmacokinetic study, but N,N-ditetradecylamine formed by slow amide hydrolysis was identified in hepatocytes and liver S9 fractions from mouse, rat, monkey and human.

The deesterified metabolite of ALC-0315 (formed by ester hydrolysis) was observed *in vitro* in mouse and rat blood; monkey liver S9 fraction; and *in vivo* in rat plasma, urine, faeces and liver. Glucuronide conjugate of this deesterified metabolite was observed in rat urine (Table 4-5).

Table 4-5. Metabolic profile of ALC-0315

	<i>In vitro</i> : Blood	<i>In vitro</i> : hepatocytes (10 µM; 4h incubation)	<i>In vitro</i> : liver S9 fractions	<i>In vivo</i> : 14day rat pharmacokinetic

m/z: Metabolic pathway*	(10 µM; 24h incubation)								(10 µM; 24h incubation)				study (15.3mg/kg ALC-0315 IV; 5 time-points over 0.1-336h)			
	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human	Plasma	Urine	Faeces	Liver
104.07: N-Dealkylation, oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
132.10: N-Dealkylation, oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145.05: N-Dealkylation, hydrolysis, oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
255.23: Hydrolysis (acid)	+	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-
290.27: Bis-hydrolysis (amine)	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+
466.30: Bis-hydrolysis (amine), glucuronidation	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
528.50: Hydrolysis (amine)	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	+

* Not all potential metabolites are listed in this table. Other potential metabolites, not listed in this table, were not detected in any of the samples. + = metabolite identified; - = Not detected

5. REPEAT-DOSE TOXICITY

Two rat studies examining the toxicity of repeated doses of different versions of the mRNA vaccine [BNT162a1, BNT162b1, BNT162c1, BNT162b3c, BNT162b2 (V8) and BNT162b2 (V9)] were submitted. One of the studies evaluated the clinical vaccine formulation. The repeat dose studies were conducted using the clinical route of administration and local tolerance was assessed. The highest dose (in absolute terms) used in the proposed clinical trial was evaluated in the studies.

Table 5-1. Overview of repeat-dose toxicity studies

Species & strain	Study	Duration	Route	Dose (µg/animal); frequency	Date	GLP
Rat (Wister Han)	38166	17 days	IM	0, 10, 30, 100; QWx3	17 September 2020	Yes
	20GR142	17 days	IM	0, 30; QWx3	23 November 2020	Yes

5.1. RAT**5.1.1. 17 days study: The vaccine candidate BNT162b2 (V9)**

Study: 20GR142 Laboratory: Pfizer Worldwide Research & Development, Drug Safety Research & Development, Eastern Point Road, Groton, USA Date: First dose: 06 July 2020 GLP: Yes Lot No.: COVVAC/270320 (BNT162b2 [V9]), BCV/040620 (BNT162b3c)	Strain: Wistar Han, Age: ~9 weeks old Test article: BNT162b2 (V9): 0.5 mg/mL RNA encoding the full SARS-CoV-2 Spike (S) P2 variant protein BNT162b3c: 0.5 mg/mL RNA encoding membrane-anchored, trimerised variant of the RBD of the SARS-CoV-2 S protein Route and frequency: IM, weekly on days 1, 8 and 15 Dose: 30 µg RNA, 60 µL/administration site/dose Vehicle: 0.9% sterile saline Strength: 0.5 mg/mL Group size: 10/sex (main) + 5/sex (recovery) Duration: 17 Days (± 3 weeks recovery phase)
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Mortality: none*Clinical signs:* none treatment related*Local tolerance:*

mRNA	Day	Dose (µg)	Post dose (h)	Oedema		Erythema	
				♂	♀	♂	♀
Control	1-15	0	-	-	-	-	-
BNT162b2 (V9)	1	30	24	++	++	+	+
			144	-	++	-	+
	15		24	++	++	-	+
			72	+	++	-	+
BNT162b3c	1	30	24				
			144				
	15		24				
			72				

Note: - no irritation, + slight irritation, ++ moderate irritation, +++ high irritation

Body weight gain: none treatment related*Food consumption:* no treatment-related effects*Ophthalmology:* no treatment-related effects

Body temperature: administrations of BNT162b2 or BNT162b3c on test days 1, 8, and 15 led to slightly, but statistically significantly increased body temperatures compared to the control animals after each dose by ~1°C in males and 0.3-0.9°C in females (Day 15 values: control ♂ 37.34°C, ♀ 38.02°C; BNT162b2 ♂ 38.37°C (P<0.001), ♀ 38.15°C; BNT162b3c ♂ 38.43°C (P<0.001), ♀ 38.35°C)

Haematology (day 17):

mRNA	Control		BNT162b2		BNT162b3c	
	♂	♀	♂	♀	♂	♀
Sex						
WBC (10 ³ /µL)	3.84	2.16	8.83†	5.70†		
Monocytes (10 ³ /µL)	0.071	0.056	0.234†	0.154†		
Neutrophils (10 ³ /µL)	0.674	0.409	4.449†	2.469†		
Large Unstained Cells (10 ³ /µL)	0.026	0.010	0.209†	0.132†		
Fibrinogen (mg/dL)	253.1	217.2	596.7†	596.7†		
Eosinophil (10 ³ /µL)	0.056	0.029	0.141†	0.092†		
Basophil (10 ³ /µL)	0.003	0.001	0.017†	0.008†		

† p <0.01

In addition, transiently lower ($0.44 \times -0.27 \times$ on day 4 for both sexes) and higher ($1.2-1.31 \times$ on day 17, ♀ only) reticulocyte counts, and minor lower red blood cell mass (red blood cell count, haemoglobin and haematocrit on day 17) were noted in both BNT162b2 (V9) and BNT162b3c treated groups *cf.* controls. Higher mean cell haemoglobin concentration (Day 4, ♂), lower mean cell haemoglobin [MCH] and higher red cell distribution width (Day 17; both sexes) were also observed in treated groups *cf.* control.

Serum chemistry (day 17):

mRNA	Control		BNT162b2		BNT162b3c
	♂	♀	♂	♀	
Sex	♂	♀	♂	♀	S47
Albumin	3.50	3.60	3.43	3.07 †	
Globulin	1.89	1.84	2.08*	1.91	
Album/globulin	1.88	1.96	1.65†	1.61†	
Total protein	5.39	5.44	5.51	4.98 †	

*p <0.05; † p <0.01, significantly different from control based on trend or pairwise comparison

Acute phase proteins (day 17):

mRNA	Control		BNT162b2		BNT162b3c
	♂	♀	♂	♀	
Sex	♂	♀	♂	♀	S47
α 2-Macroglobulin (µg/mL)	14	33.1	991†	521†	
α 1-Acid Glycoprotein (µg/mL)	47.7	96	1836†	1492†	

† p <0.01

Of the clinical pathology findings, only globulin in the BNT162b2 group remained higher than the control group (2.26 *cf.* 2.10 g/dL, P<0.05) after the recovery phase.

Urinalysis: none treatment related

Organ weights (bodyweight relative): Spleen: ↑ by 1.4-1.6 fold and 1.5 fold in BNT162b2 and BNT162b3c groups, respectively, compared to control (statistically significant).

Gross Pathology: oedema and erythema were observed in 4-7 animals and 3-7 animals in BNT162b2 and BNT162b3c groups, respectively, compared to 1 in control (n=10); enlarged draining lymph node in 2 and 4 animals in BNT162b2 and BNT162b3c groups, respectively, compared to nil in control (n=10) and S47.

Histopathology:

	Control		BNT162b2		BNT162b3c
	Male	Female	Male	Female	
Injection site (number examined)#	10 (5)	10 (5)	10 (5)	10 (5)	S47
Inflammation	4	5	10	10	
Minimal	4	5	- (5)	- (5)	
Mild	-	-	7	7	
Moderate	-	-	3	3	
Oedema	-	-	9	10	
Mild	-	-	8	9	
Moderate	-	-	1	1	
Lymph node, draining (number examined)#†	10 (4)	10 (5)	9 (5)	10 (5)	
Increased cellularity, plasma cell	-	-	7 (4)	9 (4)	
Minimal	-	-	1 (4)	1 (4)	
Mild	-	-	4	1	
Moderate	-	-	2	7	
Increased cellularity, germinal centre	2	2 (1)	6 (4)	5 (3)	
Minimal	1	1 (1)	2 (2)	3 (1)	
Mild	1	1	4 (1)	2 (2)	
Macrophage infiltration	-	-	- (3)	- (3)	
Minimal	-	-	- (2)	- (1)	
Mild	-	-	- (1)	- (2)	
Lymph node, inguinal (number examined) #	9 (5)	10 (5)	10 (5)	10 (5)	
Increased cellularity, plasma cell	-	-	1	2	
Minimal	-	-	1	2	

Increased cellularity, germinal centre	1 (2)	1 (2)	5 (3)	6 (1)
Minimal	- (2)	1 (2)	1 (3)	3 (1)
Mild	1	-	4	3
Macrophage infiltration	-	-	-	-
Minimal	-	-	-	-
Liver (number examined)	10	10	10	10
Hepatocyte vacuolation, periportal	-	-	5	10
Minimal	-	-	5	10
Spleen (number examined)	10 (5)	10 (5)	10 (5)	10 (5)
Increased cellularity, haematopoietic cell	-	-	10	9
Minimal	-	-	10	9
Increased cellularity, germinal centre	-	-	5 (1)	6 (2)
Minimal	-	-	5 (1)	6 (2)
Bone marrow, sternum (number examined)	10	10	10	10
Increased cellularity, haematopoietic cell	-	-	10	10
Minimal	-	-	10	10

s47

Recovery data in brackets; † lymph node not specified in the study report but the sponsor confirmed that it was iliac lymph node; Lesion grading: minimal = grade 1, mild = grade 2, moderate = grade 3

- Similar histological findings for the two mRNA variants
- Inflammation at the injection site (mixed cell inflammation, mostly neutrophils and oedema; inflammatory cells frequently infiltrated and expanded epimysium, perimysium, and endomysium and separated and surrounded myofibers and/or blood vessels in skeletal muscle, and occasionally extended into SC tissues and into extra-capsular tissue of the joint)
- ↑ cellularity of germinal centres (minimal to mild) of draining and inguinal lymph nodes and spleen
- ↑ plasma cells (minimal to moderate; mostly immature as plasmablasts) in cortical, medullary and subcapsular sinuses of lymph nodes, with greater incidence and severity of in the draining lymph node (minimal to moderate) than inguinal lymph node (minimal); no findings in mesenteric lymph node
- ↑ haematopoiesis (minimal; primarily myeloid precursors) in the bone marrow and spleen red pulp
- Vacuolation of hepatocytes (minimal; small clear round membrane bound structure in cytoplasm) in the portal region

After the 3-week recovery phase, oedema at the injection site, hepatocellular vacuolation, increased haematopoiesis of bone marrow and spleen were no longer present. Other findings partially recovered (lower incidence and/or severity).

- Injection site: inflammation (mostly lymphocytes and plasma cells with few neutrophils)
- Spleen: Increased cellularity of germinal centres (minimal)
- Lymph nodes (draining and inguinal) : increased cellularity of germinal centres (minimal to mild) and plasma cells (minimal; mature plasma cells replaced plasmablasts), infiltration of macrophages (minimal to mild); s47

Conclusion: Most findings were related to immune responses to the vaccine and fully or partially reversed after the 3-week recovery phase. Hepatocellular vacuolation was probably due to the uptake of LNP or dissociated lipids (the tissue section was not stained for lipids). The vaccine induced NAb in rats. Cytokine release was not assessed.

Immunogenicity assessment: NAb was determined by a microneutralisation assay based on cytopathic effects of SARS-CoV-2 virus (2019-nCov/Italy-INMI1) in Vero E6 cells. No NAb was detected in the control animals or pre-dose samples of treated animals. All treated animals developed NAb; geometric mean NAb titres are shown in the table below.

Test day	BNT162b2		BNT162b3c	
	♂	♀	♂	♀
Day 17	1114	2501	s47	
Day 38 (recovery day 21)	5120	5120		

5.1.2. 17 days study: Variants of BNT162 mRNA

<p>Study: 38166 Laboratory: Laboratory of Pharmacology and Toxicology (LPT), GmbH & Co. KG, Hamburg, Germany Date: 17 September 2020 (First dose: 17 March 2020) GLP: Yes Lot No.: CoVVAC/090320 (BNT162a1), CoVVAC/100320 (BNT162b1), CoVVAC/130320 (BNT162c1), CoVVAC/160320 (BNT162b2 [V8])</p>	<p>Strain: Wister Han rats Age: ~7-9 weeks old Test articles:</p> <ul style="list-style-type: none"> • BNT162a1 (uRNA-LNP, RBD of the S protein) • BNT162b1 (mRNA-LNP, RBD of the S protein) • BNT162b2 (V8; mRNA-LNP, mutated full-length S protein) • BNT162c1 (self-amplifying mRNA-LNP, RBD of the S protein) <p>Route and frequency: IM, QW ×3 (days 1, 8, 15) (except for BNT162c1 on day 1 and 8) Dose: BNT162a1:10 and 30 µg/animal; BNT162b1: 30 and 100 µg/animal; BNT162c1: 30 µg/animal and BNT162b2: 100 µg/animal, 20 to 100 µL/administration site/dose Vehicle: Phosphate buffered saline, 300 mM sucrose/Solution Strength: 0.5 mg/mL Group size: 10/sex (main) + 5/sex (recovery) Duration: 17 days (±3 weeks recovery) (The animals administered with BNT162c1 were sacrificed on test day 10 after two doses and after recovery on test day 31)</p>
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Only the findings from the control animals and the animals treated with BNT162b2 (V8) are summarised below. Findings for other variants except for BNT162c1 were generally similar to those for BNT162b2.

Mortality: none

Clinical signs: none treatment related

Local tolerance:

mRNA	Day	Dose (µg) [#]	Post dose (h)	Oedema		Erythema		I/H Induration/Hardening	
				♂	♀	♂	♀	♂	♀
Control	1-15	0	-	-	-	-	-	-	-
BNT162b2 (V8)	1	100	24	+	+	+	-	-	-
			144	-	-	-	-		
	15		24	++++	++++	-	-		
			528/480 [^]	-	-	-	-		

Note: - no effect, + very slight irritation; ++ up to slight irritation, +++ up to moderate irritation, ++++ up to severe irritation; [^] erythema

Body weight gain:

mRNA	Dose (µg)	Body weight difference from day 1 (%)			
		Day 9		Day 16	
		♂	♀	♂	♀
Control	0	21.3	11.7	37.1	16.7
BNT162b2	100	-4.1	1.2	4.8	6.4

Note: the body weight was reduced up to 11.3% in male animals compared to the control group on test days 9 and 16 (statistically significant at $p \leq 0.01$ on test days 9 and 16) whereas an ~ 6.8% reduction of body weight was noted in females compared to the control group from day 2 to 16 (statistically significant at $p \leq 0.01$ or $p \leq 0.05$ on test days 9 and 16). At the end of the recovery period, there was no significant difference in body weight between the control and BNT162b2 treated groups.

Food consumption: no treatment-related effects

Ophthalmology: no treatment-related effects

Body temperature: administrations of BNT162b2 (100 µg) on test days 1, 8, and 15 (3 administrations) led to slightly increased body temperatures at 4 h p.a. and/or 24 h p.a. compared to the control animals (statistically significant).

During the recovery period, the body temperature remained at a slightly higher level compared to the control group in all treated groups.

Haematology: (all values compared to control, statistically significant changes)

BNT162b2 100 µg on day 17

- ↑ WBC cells: 118.7% ♂ and 111% ♀
- ↑ neutrophils: 605.8% ♂ and 679.8% ♀
- ↑ eosinophils: 419.3% ♂ and 509.6% ♀
- ↑ basophils: 146.7% ♂ and 105.3% ♀
- ↑ large unstained cells (LUC): 685.2% ♂ and 594.8% ♀
- ↑ Fibrinogen: 205.2% ♂ and 160.2% ♀
- ↓ haemoglobin: 9.1% ♂ and 12.7% ♀
- ↓ haematocrit (HCT): 11.9% ♂ and 13.5% ♀
- ↓ platelets: 29.2% ♂ and 34.1% ♀
- ↓ red blood cells: 9.8% ♀ only

In addition, ↓ absolute (72.1% ♂ and 48.2% ♀) and relative (74.3% ♂ and 47.7% ♀) reticulocytes were observed on day 4 only.

Serum chemistry (day 17, % relative differences to the control group):

mRNA	BNT162b2	
	♂	♀
Sex		
Albumin	-5.9*	-11*
Globulin	+23.1*	+17.7*
GGT [^]	+198.1*	+317.4*

* Statistically significant; ^ Control group: 1.62 IU/L increased, the range of individual data noted for the GGT activity in several tests groups slightly exceeded the range of historical data [LPT historical data mean (range of individual data): 2.28 (0.4-5.1) ♂, 2.43 (0.2-4.8) ♀]

Acute phase proteins (fold increase compared to control, statistically significant, p<0.01)

Acute phase protein	mRNA	BNT162b2	
	Sex	♂	♀
α1-acid glycoprotein	Day 4	6.9	5.6
	Day 17	20.7	15.9
α2-macroglobulin	Day 4	54.3	75.3
	Day 17	217	121

Cytokines (n=3): IFN-γ, TNF-α, IL-1β, IL-6, and IL-10 at 6 h after dosing were increased in all groups including the control group compared to predose values. There was no clear difference between the control and treated groups (noting small sample size and variations between animals).

Urinalysis: none treatment related

Organ weights (bodyweight relative): ↑ spleen (by 1.2-1.6 × in all groups compared to control)

Gross Pathology: indurated injection sites in > 6 animals/sex/groups (n=10) compared to nil in control; enlarged iliac lymph node and enlarged spleen in tested groups compared to nil in control

Histopathology: test item-related microscopic findings at the end of dosing included

- Inflammation at the injection site and surrounding tissues (all treated groups)
- ↑ cellularity of germinal centres (minimal to moderate) and increased plasma cells (minimal to moderate) in the draining (iliac) lymph node (all treated groups)
- ↑ haematopoiesis (minimal) in the bone marrow and spleen (all treated groups)
- Vacuolation of hepatocytes (minimal to mild) in the portal region (all treated groups).

All microscopic findings in injection sites and lymph node persisted during the recovery phase with evidence of partial recovery (decreased severity score compared to dosing phase; Day 31 for group 6; Day 38 for all other groups). Spleen, bone marrow and Liver effects were fully recovered at the end of the 3-week recovery phase.

All clinical pathology findings (including cytokines, acute phase proteins) were reversible.

Immunogenicity assessment

The immunogenicity results with BNT162b2 (V8), the closely related variant of BNT162b2 (V9) are provided below. Serum samples were collected from the treated main study animals at day 17 as well as from the treated recovery cohorts at day 38.

Test systems

- Recombinant His-tagged SARS-CoV-2-S spike protein S1 domain coated in high-protein binding 96-well plate
- Recombinant His-tagged SARS-CoV-2-S spike protein RBD sub-domain coated in high-protein binding 96-well plate
- HEK293T/17 (ATCC® CRL-11268™; for VSV/SARS-CoV-2-S pseudovirus generation)
- VERO 76 (ATCC® CRL-1587™; for VSV/SARS-CoV-2-S pseudovirus titration and pseudovirus neutralisation testing)

Major findings

- Immunisation with **BNT162b2** elicited IgG against the S1 and the RBD of SARS-CoV-2 S, which increased over time after the last dose. The group mean IgG concentrations against S1 and RBD are given in the following table.

		Mean IgG concentration [mg/mL]
17 days after first immunisation	Against S1	1.76 ± 0.16
	Against RBD	2.33 ± 0.19
38 days after first immunisation	Against S1	3.46 ± 0.52
	Against RBD	4.90 ± 0.87

- BNT162b2 (V8) administration elicited neutralising antibodies against pseudovirus infection. Neutralising antibody titres in vaccinated animals increased over time after the last dose. Serum titres resulting in 50% pseudovirus neutralisation exceeded the upper limit of quantification (ULOQ) of a reciprocal titre of 1536 in more than 8 out of ten animals on Day 38, and therefore a neutralisation titre of 90% was evaluated (Figure 5-1).

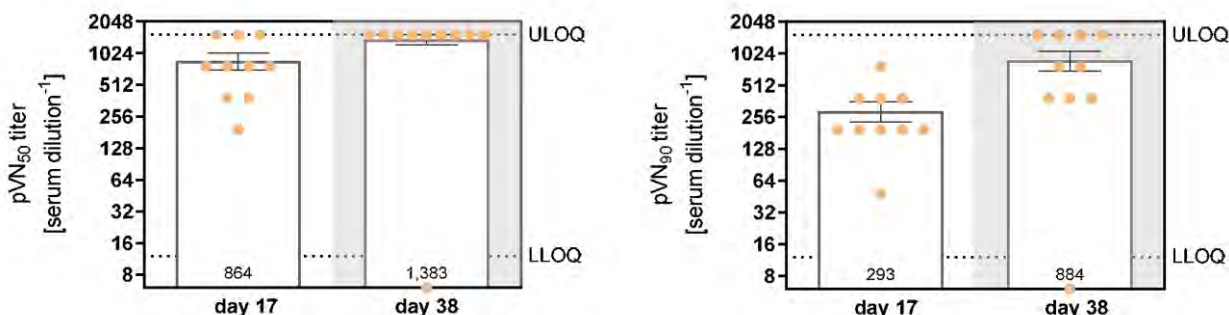


Figure 5-1. Pseudovirus Neutralisation Activity in Rats after BNT162b2 V8 Immunisation

6. REPRODUCTIVE AND DEVELOPMENTAL STUDIES

A single reproductive and developmental study has been conducted, assessing potential effects of three COVID-19 vaccine candidates (BNT162b1, BNT162b2 and BNT162b3 in lipid nanoparticles) on fertility and pre-/post-natal development in female Wistar rats (Table 6.1). The proposed clinical dose (30 µg/dose) was evaluated in the reproductive and developmental study.

Table 6.1. Overview of reproductive toxicity study

Species & strain	Study type	Dose and Route	Frequency	Study	GLP
Rat (Wistar)	Fertility & embryofetal development	0, 30 µg/dose mRNA in 0.06mL; IM	4 doses to ♀ rats: 21 & 14 days before mating and GD9 & GD20	20256434 [RN9391R58]	Yes

6.1. FERTILITY AND EMBRYOFETAL DEVELOPMENT

<p>Study: 20256434 [RN9391R58] Laboratory: Charles River Laboratories, Saint-Germain-Nuelles, France Date: 22 December 2020 (first dose: 27 July 2020) GLP: Yes Lot No.: <i>BNT162b1:</i> Batch No.: RBP020.3 LNP; Lot No.: CoVVAC/100320 <i>BNT162b2:</i> Batch No.: RBP020.2 LNP Lot No.: CoVVAC/270320 <i>BNT162b3:</i> Batch No.: RBP020.8 LNP Lot No.: BCV/040620</p>	<p>Strain: Wistar[Han] rats Age: ~ 11 weeks Test articles: BNT162b1; BNT162b2; BNT162b3 Group size = 44 ♀/group (22 ♀/group for caesarean and another 22 ♀/group for littering) Dose: 0, 30 µg/dose IM; 21 & 14 days before mating and GD9 & GD20. Duration: Treatment from 21 days prior to mating – GD20; termination on GD21 (caesarean subgroup) & LD21 (littering subgroup) Postnatal development assessed in 4 pups/sex/litter; termination on PND21 (extra pups culled on PND4).</p>
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Maternal mortalities: None treatment related in BNT162b2 treated groups.

Maternal body weight gain: ↓ ~ 12-14% during pre-mating period in BNT162b2 treated group *cf.* control; complete recovery at the end of gestation period. Reduced mean food consumption was noted after first dose administered; the effects were reversible at the end of dosing regimen.

Maternal clinical signs: Swelling (~ 100% treated rats in pre-mating period & ~ 10-50% treated rats in gestation period *cf.* control), in some rats, associated with limping and/or piloerection.

Oestrus cycling: no treatment related effects on mean length and regularity of the oestrous cycles (BNT162b2 *cf.* control).

Mating: No significant effect on mating index (97.7-100% for all groups) and pre-coital interval (2.7-3.0 days for all groups).

Fertility: No significant effect on fertility index (95-100%) and pregnancy rate (93-100% for all groups).

Parturition: One ♀ in the BNT162b3 group was euthanised on LD1 showing hunched posture, pale, marked piloerection, bleeding at the vulva, distended/purple abdomen. One ♀ each in the BNT162b1 and BNT162b3 groups were euthanised due to all stillborn pups or total litter death. No macroscopic findings were noted in maternal necropsy.

Gestation length: Mean duration of gestation was ~22 days in all groups. Gestation index was 95-100% for all groups.

Litter values:

- No significant effect on number of corpora lutea, number of implantations, post-implantation loss, live litter size, sex ratio and mean fetal weight.
- Increased pre-implantation loss in BNT162b2 group *cf.* the concurrent control group, but within historical control data range.

Finding	Group				Historical control data range (WI[Han])
	Control	BNT162b1	BNT162b2	BNT162b3	
Pre-implantation loss: (mean %)	4.1	4.8	9.8*	8.0	2.6 – 13.8 ^a

* P<0.05 (Dunnett non-parametric 2 sided test); a: Historical data based on 27 studies including 568 pregnant rats (GD20: 562), year not specified.

Fetal variations/malformations:

- Incidences of increased fetal abnormalities in treated groups *cf.* concurrent controls but within historical control data range for Wistar rats. The incidence of short supernumerary lumbar ribs was comparable between the treated and control groups. The incidence of supernumerary lumbar ribs was higher in the treated group *cf.* concurrent control. The historical control data from CRL, France (Lyon) include two entries: one for supernumerary lumbar ribs with a range of 0-5.3% for fetus and 0-22.7% for litter, and a second entry for thoracolumbar-full with range of 0-9.7% for fetus and 0-50.0% for litter. The sponsor, based on clarification provided by the study authors, stated that “supernumerary lumbar ribs” and “thoracolumbar, full” are two terms (in different versions of a preclinical software) used for the same finding (D21-2058752). Therefore, historical control data range used for supernumerary lumbar ribs in the table below is that stated for thoracolumbar-full in the study report (page 1102). Thus, the higher incidence of supernumerary lumbar ribs relative to the concurrent control group is not considered to be treatment-related.

Abnormality	Fetal incidence; [litter incidence]				Historical control data range (%) - WI(Han)
	Control	BNT162b1	BNT162b2	BNT162b3	
Right-sided aortic arch (<i>malformation</i>)	0/133 [0/21]	0/135 [0/20]	1/132 [1/21]	s47	0-1.0 ^c [0-5.0]
Small mouth & jaw agnathia (<i>malformation</i>)	0/277 [0/21]	0/282 [0/20]	1/276 [1/21]	s47	GD20: 0-0.4 ^b [0-4.8]
Absent lung lobe (<i>anomaly</i>)	0/133 [0/21]	0/135 [0/20]	1/132 [1/21]	s47	0-0.71 ^d [0-4.17]
Gastroschisis	0/277	0/282	1/276	s47	0-0.5 ^c

(malformation)	[0/21]	[0/20]	[1/21]	[0-5.0]
Transposed umbilical artery (variation)	7/133 [6/21]	12/135 [9/20]	13/132 [8/21]	0-10 ^c [0-44]
Short, misshapen & fused mandibles (malformation)	0/144 [0/21]	0/147 [0/20]	1/144 [1/21]	0-0.95 ^a [0-4.8]
Incomplete hyoid ossification (anomaly)	0/144 [0/21]	1/147 [1/20]	1/144 [1/21]	0-2 ^a [0-5]
Wavy ribs (anomaly)	0/144 [0/21]	1/147 [1/20]	1/144 [1/21]	0-3.4 ^a [0-12.5]
Incomplete ossification of thoracic centrum (anomaly)	1/144 [1/21]	0/147 [0/20]	3/144 [3/21]	0-9.6 ^a [0-37.5]
Incomplete ossification of cervical arch (anomaly)	0/144 [0/21]	0/147 [0/20]	2/144 [2/21]	0-5.8 ^a [0-19.0]
Lumbar, number = 7 (anomaly)	1/144 [1/21]	0/147 [0/20]	3/144 [2/21]	0-3.2 ^a [0-13.6]
Supernumerary lumbar ribs (anomaly)	3/144 [3/21]	5/147 [3/20]	12/144 (8.3%) [6/21, 28.6%]	0-9.7 ^a [0-50.0]

a: Historical data from 17 studies, 275 litters & 1721 fetuses at CRL Lyon (year: not specified); b: Historical data from 3 studies, 70 litters & 812 fetuses at CRL Horsham (year: not specified); c: Historical data from 7 studies, 148 litters & 1618 fetuses at CRL Horsham (year: not specified); d: Historical data from 16 studies, 350 litters & 1931 fetuses at CRL Montreal (year: 2006-14).

Pup development:

- No significant difference between BNT162b2 and control groups for weaning index, pinna unfolding, eye opening, pupillary reflex, auditory reflex and pup body weight gain.
- No significant clinical observations in BNT162b2 group except few (1-3) pups in this group were pale and cold to touch for first three days of litter date.
- No significant abnormalities in BNT162b2 group pups at post-lactation necropsy (PND21). One pup showed liver hernia in this group at PND4 cull.

Maternal necropsy:

- Firm, pale and enlarged injection site was noted in many (4-9/43) animals of BNT162b2 group. Solid, dark heterogeneous mass adherent to liver tissue in one animal.

Toxicokinetics: No toxicokinetic data

Serum antibody titres (microneutralisation assay using WI-MNSARS-CoV-2 virus based on cytopathic effect): BNT162b2 elicited immune response in females during gestation and in pups during postnatal development.

SARS-CoV-2 neutralising antibody mean titres

Interval/Occasion	Control	BNT162b1	BNT162b2	BNT162b3
Pre-dosing	5.0	5.0	5.3	
Prior to mating	5.0	525.6	3886.4	
GD21 (Dams)	5.0	987.0	3445.5	
LD21	5.0	3377.9	3620.4	
GD21 (Fetuses)	5.0	293.4	640.0	
PND21 (Pups)	5.0	4017.1	4561.4	

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