IN THE HIGH COURT OF NEW ZEALAND
WELLINGTON REGISTRY

I TE KŌTI MATUA O AOTEAROA
TE WHANGANUI-A-TARA ROHE

CIV-2022-485-013

UNDER	the Judicial Review Procedure Act 2016
IN THE MATTER	of an application for judicial review of a decision made under the Medicines Act 1981
BETWEEN	**DCB**
	Applicants
AND	**THE MINISTER OF HEALTH**
	First Respondent
AND	**THE GROUP MANAGER OF THE NEW ZEALAND MEDICAL DEVICES SAFETY AUTHORITY (MEDSAFE)**
	Second Respondent
AND	**THE COVID-19 RESPONSE MINISTER**
	Third Respondent

REPLY AFFIDAVIT OF BYRAM BRIDLE IN SUPPORT OF APPLICATION FOR JUDICIAL REVIEW

Dated the 19th day of June 2022

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REPLY AFFIDAVIT OF BYRAM W. BRIDLE

I, **Byram W. Bridle**, Associate Professor, solemnly, sincerely, and truly declare and affirm:

1 I have previously prepared an affidavit in this matter dated 14 January 2022. I then later amended that affidavit to correct the exhibits and to make some minor corrections due to transposition issues - this I did in the affidavit affirmed 25 January 2022 (**First Affidavit**).

2 I subsequently prepared a further affidavit in this matter dated 1 May 2022 (**Further Affidavit**) to update the evidence and to provide the complete version of a figure titled *Disproportionate Number of Omicron Cases Amongst Vaccinated* that had been cut off in my First Affidavit.

3 I confirm that the evidence I gave in my First Affidavit and Further Affidavit are still valid.

4 Since my Further Affidavit, I received an affidavit from Mr. Christopher M. James, of Wellington, Group Manager of Medsafe, which included comments related to the evidence that I provided. This current affidavit represents my reply to Mr. James.

The Pfizer-BioNTech Product to Protect Against COVID-19 (also known as Comirnaty) Lacks the Characteristics of a Good Quality Vaccine

5 Paragraph 95 of Mr. James's affidavit states "I also do not agree with Dr Bridle's comment that a "good quality vaccine" only requires one or two doses for a person's lifetime. There are several widely used vaccines that require multiple doses/boosters (for example, diptheria, tetanus, pertussis), or require a seasonal dose as strains vary each year (such as influenza). The dosing schedule depends on a variety of factors including the infectious agent's pathology, mutagenicity (how frequently it mutates/evolves), and vaccine technology.".

6 Mr. James conflated several concepts, including the durability of vaccine-induced immunity with the mutation of viruses like SARS-CoV-2 and influenza viruses. These represent two very different and independent biological concepts. Further conflating this

is the fact that children under the age of five have immune systems that are quite immature.[1] This means they cannot respond well to vaccines. For colostrum-fed neonates there is another issue. In addition to having extremely immature immune systems, the antibodies transferred into their bodies from their mothers via breastfeeding also inhibit their ability to mount immune responses to vaccines. This is because the immune system is designed to mount only low-magnitude antibody responses if there is already a large bolus of antibodies in circulation. This reduces the risk of antibody concentrations going above a threshold that could cause excessive inflammation and/or autoimmune responses. For all these reasons vaccines must be administered multiple times in children under the age of five. However, this has nothing to do with the vaccines being of poor quality. It is due to the immaturity of the immune systems of the very young recipients, which is confounded by the presence of maternal antibodies in babies. In the context of Comirnaty, these issues are irrelevant because it has only been approved for children that are five years of age or older, when the immune system is at or near full maturity. This is the only scenario where the duration of immunity conferred by a vaccine (*i.e.*, how long it protects against a disease) can be properly evaluated.

7 Dr. James's comment about the vaccine against diptheria, tetanus, and pertussis can now be placed into a context that is relevant to the decision the court will be making about Comirnaty. Specifically, New Zealand's Immunization Schedule advises multiple doses between the ages of six weeks to four years of age, when the immune system is very immature. However, only two doses of this vaccine are recommended beyond the age of four, when the immune system has matured[2]. Specifically, this vaccine is recommended at eleven years of age in New Zealand, with the second dose in the context of a mature immune system recommended at sixty-five years of age, which is to account for the fact that immunological functions begin to decline in old age, which is another time when people respond poorly to vaccines. Note that this represents a fifty-four-year interval compared to the three- to six-month intervals being recommended by many countries around the world for booster doses of Comirnaty. This is at least a 100-fold difference in

[1]Georgountzou A, Papadopoulos NG. Postnatal Innate Immune Development: From Birth to Adulthood. Front Immunol. 2017 Aug 11;8:957. doi: 10.3389/fimmu.2017.00957. PMID: 28848557; PMCID: PMC5554489.
[2]https://www.health.govt.nz/our-work/preventative-health-wellness/immunisation/new-zealand-immunisation-schedule

the duration of immunity between Comirnaty and what would be defined as a good-quality vaccine. Comirnaty is only being considered for use in children five years of age or older. As such, it cannot come close to the performance of other vaccines that routinely get administered within this same age range.

8 Interestingly, the Health Regulatory Agency in Canada goes far beyond how I have defined a high-quality vaccine. Specifically, Health Canada states "An ideal vaccine is: safe with minimal adverse effects; effective in providing lifelong protection against disease after a single dose that can be administered at birth;…".[3] For the reasons already given, it is expecting a lot for a vaccine to provide life-long immunity after a single dose at birth. As such, my definition specifically applies to vaccines administered once the immune system has started to approach full maturity (*i.e.*, at about five years of age or so).

9 The Immunisation Advisory Centre in New Zealand agrees that "In the very young and very old the duration of immunity can be limited".[4] As such, these two demographics represent outliers when assessing the duration of immunity that a vaccine can induce.

10 Mr. James noted that vaccines against influenza viruses are administered annually. This has nothing to do with the vaccines being of low quality. Instead, annual flu vaccines tend to have relatively low effectiveness because influenza viruses mutate rapidly. Each year's iteration of the flu vaccine is based on the strains of the virus that were prevalent in the previous flu season. As such, we are always using vaccines that are outdated in the context of the new variants of influenza viruses that are in circulation. These vaccines are quite effective at protecting against the strains of influenza that were used to make them. However, after approximately one year, we are trying to use them against variants that are so different that they are somewhat akin to a new pathogen. Of note, naturally acquired immunity against influenza viruses, which represents the gold standard that we attempt to recapitulate with vaccines, has proven to last for more than ninety years.[5]

[3] https://www.canada.ca/en/public-health/services/publications/healthy-living/canadian-immunization-guide-part-1-key-immunization-information/page-14-basic-immunology-vaccinology.html

[4] https://www.immune.org.nz/vaccines/efficiency-effectiveness

[5] Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, Tumpey TM, Pappas C, Perrone LA, Martinez O, Stevens J, Wilson IA, Aguilar PV, Altschuler EL, Basler CF, Crowe JE Jr. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature. 2008 Sep 25;455(7212):532-6. doi: 10.1038/nature07231. Epub 2008 Aug 17. Erratum in: Nature. 2012 Oct 25;490(7421):570. PMID: 18716625; PMCID: PMC2848880.

11 Mr. James raises an important point about viruses, like influenza viruses, that are prone to mutation. Vaccines against them rapidly become irrelevant. This is why influenza vaccines must be re-designed on an annual basis, to try to target the new versions of the viruses. Comirnaty was designed to target the original Wuhan variant of SARS-CoV-2 that was identified two years and eight months ago. The currently dominant Omicron sub-variants are so different that Cormirnaty is now out of date and irrelevant. The previous Delta variant of SARS-CoV-2 could efficiently evade immune responses induced by Comirnaty, and the newer Omicron variant even more so.

Pfizer's Biodistribution Study Revealed Multiple Major Issues

12 Paragraph 103 of Mr. James's affidavit states "Dr Bridle's further affidavit focuses on this biodistribution study. Pfizer provided this study to Medsafe as part of its original application for the parent product. It was assessed, along with other non-clinical data, as part of Medsafe's non-clinical evaluation of the parent product. No major concerns were raised that necessitated a request for additional information or condition of provisional consent. This is consistent with the assessments conducted by other regulators, see for example the European Medicine Agency's assessment report at pages 45 — 48.".

13 It is unknown which version of the biodistribution study that Medsafe received. The version received by the Japanese health regulatory agency (Exhibit 2 PDF page 141-164 of my First Affidavit) only contained a portion of the data in the version that the United States Food and Drug Administration was recently compelled to release to the public by court order. This full English-language version that was attached as Schedule 3 of my Further Affidavit showed that many disconcerting findings were not disclosed in the Japanese version of the document. The data disclosed in the European Medicine Agency's assessment report was even more abbreviated than the Japanese document. One of the major revelations from the version of the biodistribution study released by court order by the United States Food and Drug Administration was that Pfizer had failed to disclose important findings such as the abject failure of the initial iteration of their study. The first iteration of the biodistribution study resulted in animals being killed by the 'vaccine', with

clear evidence of the vaccine being distributed throughout the body. The study director had to request direction following the failure of the study to demonstrate safety, at which point they were asked to repeat the study with a lower dose of the 'vaccine'. A more detailed discussion of this can be found in paragraph 25b of my Further Affidavit. The many other safety concerns raised by the biodistribution study can be found in paragraphs 14-72 of my Further Affidavit. The vast majority of these issues were not addressed by Mr. James, who stated in paragraph 104 of his affidavit "I do not attempt to respond to every point by Dr. Bridle about this study". The fact that Japanese health regulators received a highly abbreviated version of the study, and the European Medicines Agency has provided no evidence of having received fully disclosed biodistribution data from Pfizer is of substantial concern.

14 There are newly available data that also address Mr. James's apparent lack of concern about Pfizer's biodistribution study in rats. More recently, I discovered that Pfizer and the United States Food and Drug Administration failed to fully disclose relevant non-proprietary data from a second biodistribution study that was performed in mice. Specifically, Pfizer had released a version of their mouse-based biodistribution study in 2021 that was rapidly removed from most internet sources. It is attached to this affidavit as "**Exhibit 1**". The 'official' version that was more recently released by the United States Food and Drug Administration under a court order is attached to this affidavit as "**Exhibit 2**".

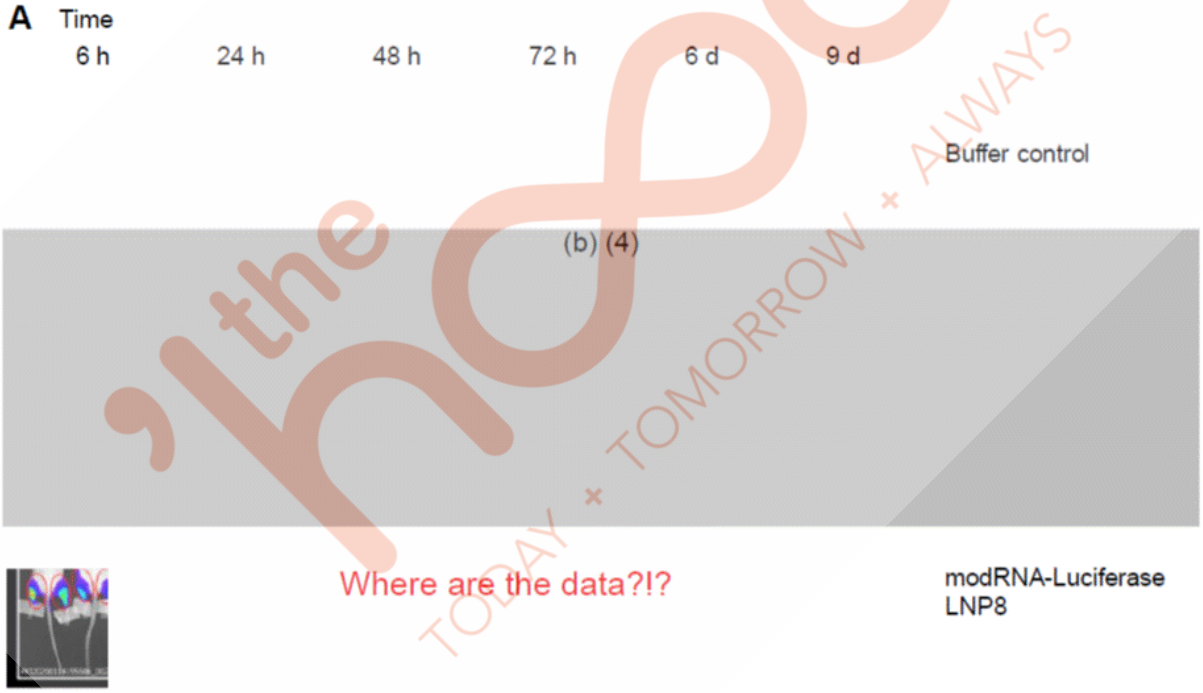
15 After placing exhibits 1 and 2 side-by-side I was able to further review the pharmacokinetic testing of a surrogate 'vaccine' that was akin to Pfizer-BioNTech's COVID-19 inoculation. In this case, the lipid nanoparticle (LNP) formulation was the same as the 'vaccine' being used in people. However, instead of carrying mRNA encoding the spike protein from the virus known as SARS-CoV-2, the LNPs carried the genetic blueprint for a protein called luciferase. Notably, the biodistribution of Comirnaty was never evaluated prior to its use in people.

16 Luciferase is the protein that allows fireflies to glow. When the mRNA gets into cells and those cells start manufacturing the luciferase, this can be externally imaged without harming the animal if the cells are relatively close to the skin and/or express lots of the

protein. This is a way to see where one would expect the spike protein to be expressed in the body and for how long after injection into a muscle.

17 As I was going through the newly released version of the document, I was surprised by how much redaction had been done. Most of the relevant non-proprietary data were blotted out. When compared to the originally released version of the document, I noticed that important data that had been redacted were for results that contradicted the key conclusions of the study.

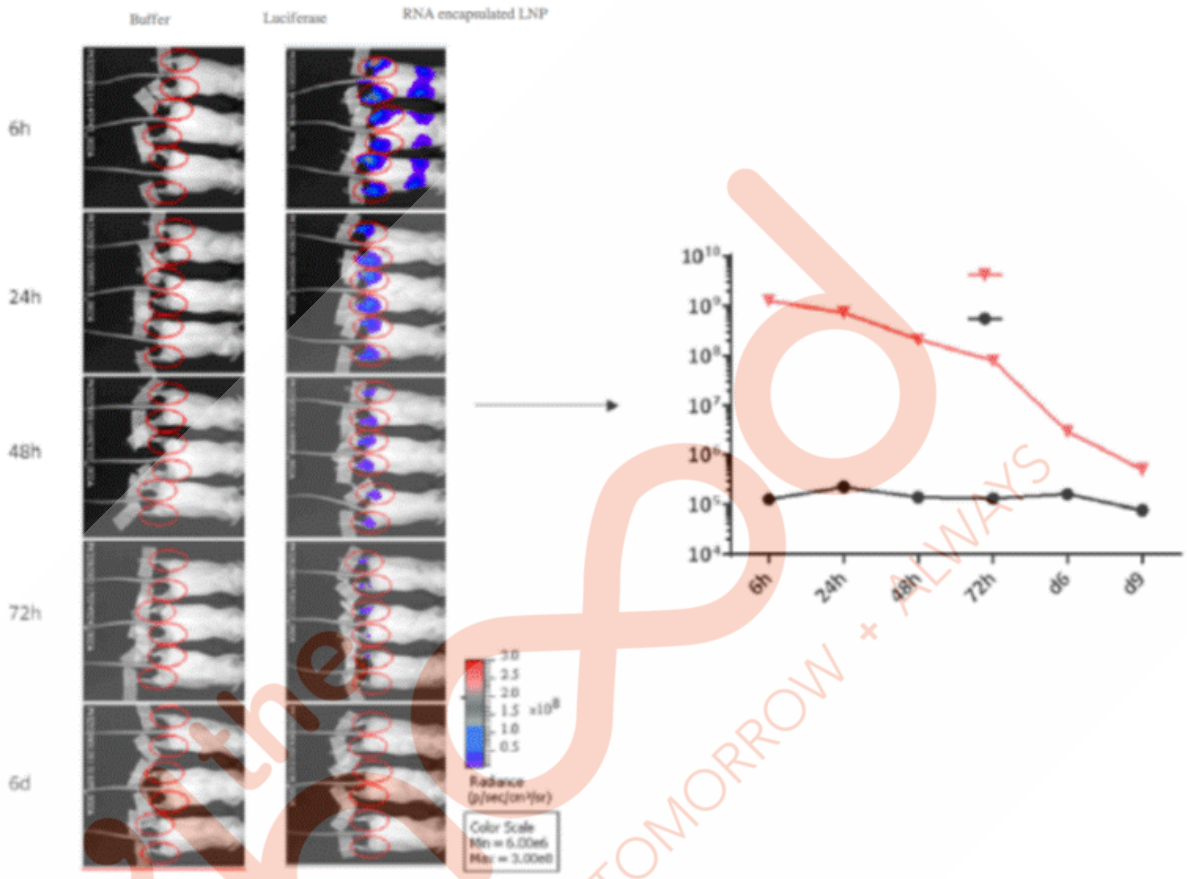
18 Figure 1A of Exhibit 2 attached to this Reply Affidavit was supposed to show the expression of luciferase over time in mice after administration of the surrogate 'vaccine'. Instead, this is what was published in the official report (the red text was my addition):



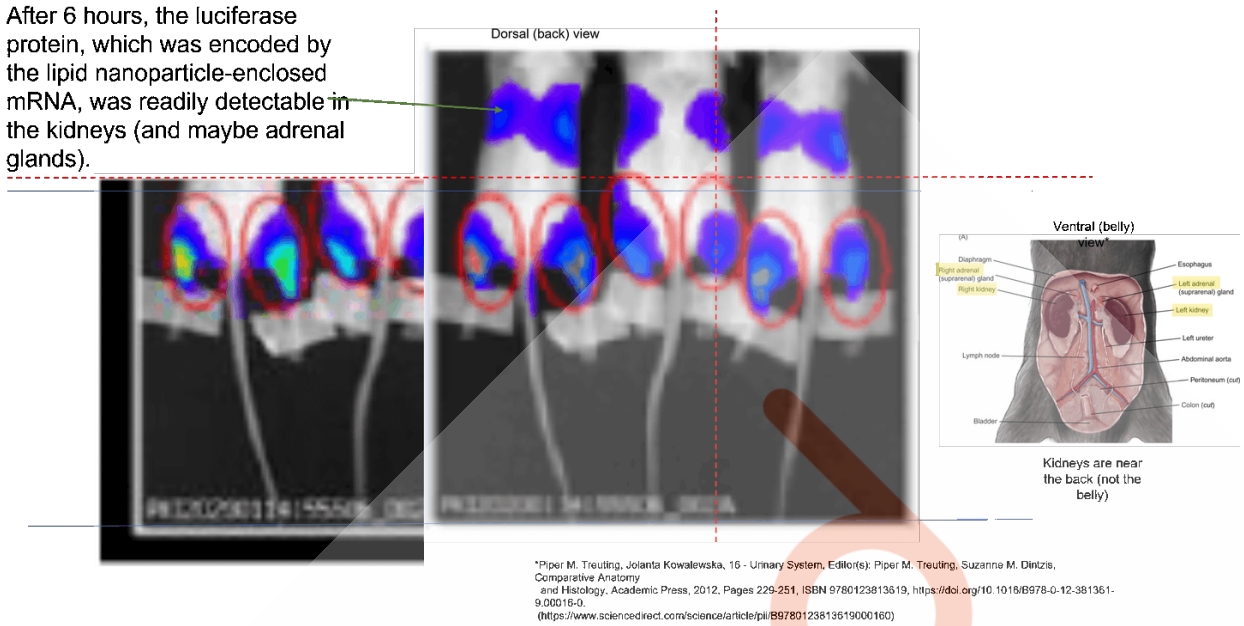
Note the single picture in the bottom left-hand corner. This shows the luciferase signal in two of three mice that had been injected. The 'vaccine' was injected into the calf muscle (the one at the back of your lower leg). It is standard practice to do intramuscular injections into the larger muscles of both hind limbs of mice. In people we usually do intramuscular injections into a single shoulder muscle.

19 In stark contrast, the version of this figure from Exhibit 1 of this Reply Affidavit, which is denoted as figure 2 in that version of the report looks like this…

Figure 2 Luciferase RNA encapsulated LNP in vivo luminescence in BALB / C mice administered intramuscularly



The image at the top of the right column of images (the luciferase group at six hours post-injection) appeared to be almost identical to the only image in the 'official' report (*i.e.*, Exhibit 2 of this Reply Affidavit), but it was not cropped as extensively. This is very important because the only anatomical location that the 'vaccine' reportedly went to outside of the injection site was the liver. So, I did a head-to-head comparison between the two images…



The images appear to be identical, but with the luciferase signal reduced in the version on the left (which was from the 'official' report in Appendix 2 of this Reply Affidavit). After imaging, the software used to analyze the images can be used to manipulate the visual intensity of the signal. Reducing the signal intensity can be a strategy to visualize only high-intensity signals, with low-intensity positive signals becoming invisible. These mice are on their bellies, so the imaging is of their backs. I have twenty years of experience working with mice and rats. As such, I can definitively state that this signal pattern is suggestive of readily detectable expression of the mRNA-encoded protein in the kidneys, with possible involvement of the adrenal glands. This would be consistent with the rat biodistribution study that I previously commented on. These images are suggestive of cropping to exclude evidence of biodistribution to the kidneys and possibly the adrenal glands.

20 The rest of figure 1 from the 'official' report in Exhibit 2 of this Reply Affidavit looks like this…

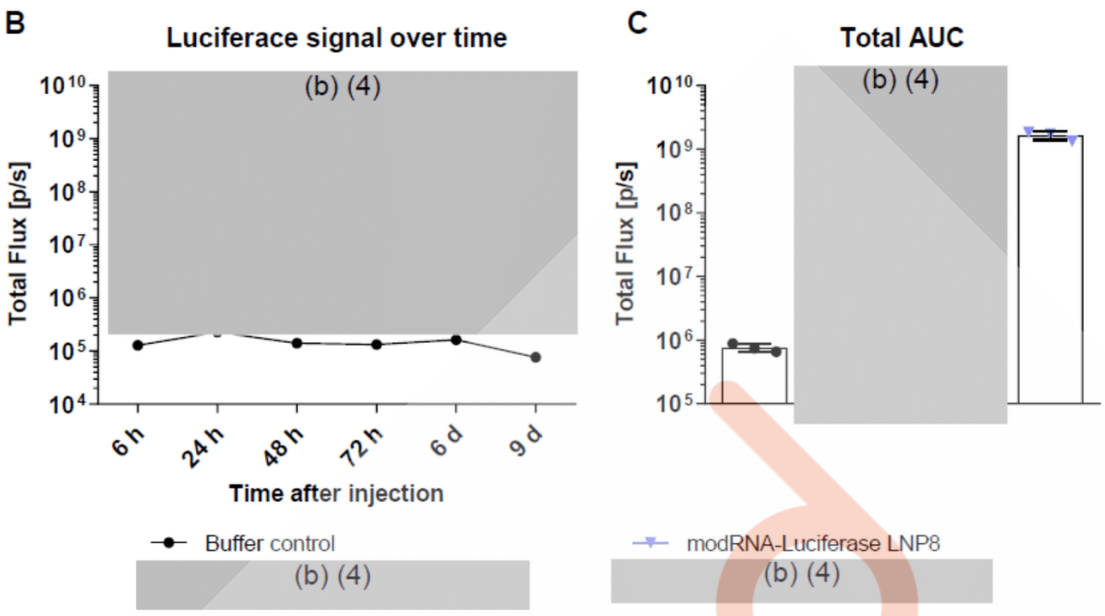
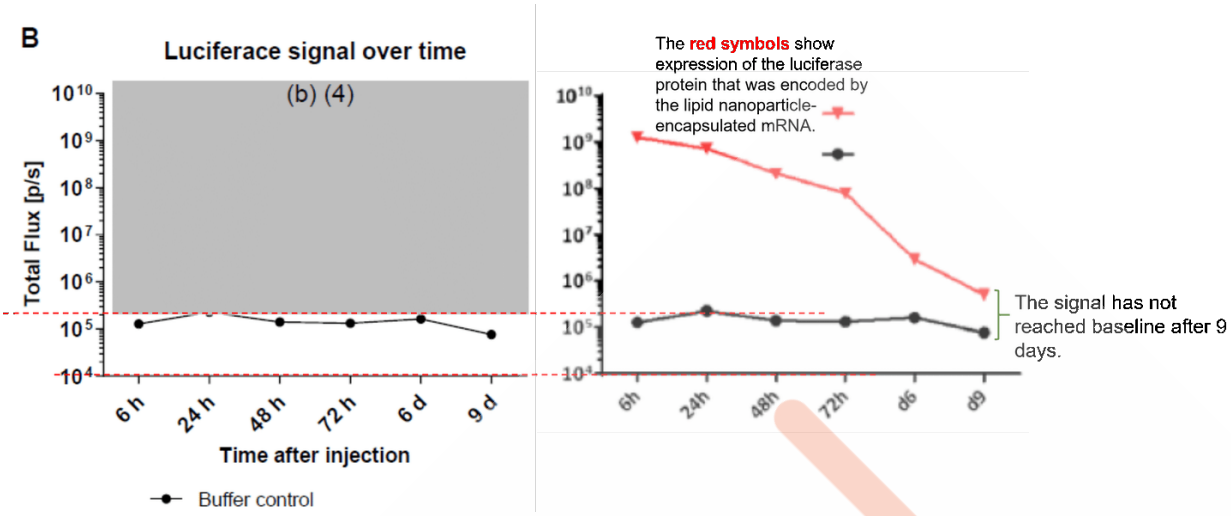


Figure 1: Bioluminescence measurement using the LNP-formulated modRNA encoding for luciferase
BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or DPBS only. A) At different time points after injection, the luciferase expression *in vivo* was measured by luciferin application. After 9 d, the reporter expression dropped to background levels. B) Quantification of luciferase signal over time and C) as total area under the curve (AUC, ±SD). p/s: photons per second

Note the conclusion that "reporter expression dropped to background levels". This suggests that the protein encoded by the mRNA 'vaccine' could no longer be detected in the mice after nine days. This 'official' version of the graph only shows the amount of signal for the mice that got injected with the carrier solution only (*i.e.*, it did not contain the 'vaccine'; so, these mice were what we call 'negative controls').

21 Here is this graph placed alongside the one from the version of the report in Exhibit 1 of this Reply Affidavit…



Placing the graphs side-by-side shows that the redaction hid the fact that the luciferase signal from the surrogate 'vaccine' did not reach baseline.

22 The only biodistribution data that were disclosed in the 'official' report in Exhibit 1 of this Reply Affidavit looked like this…

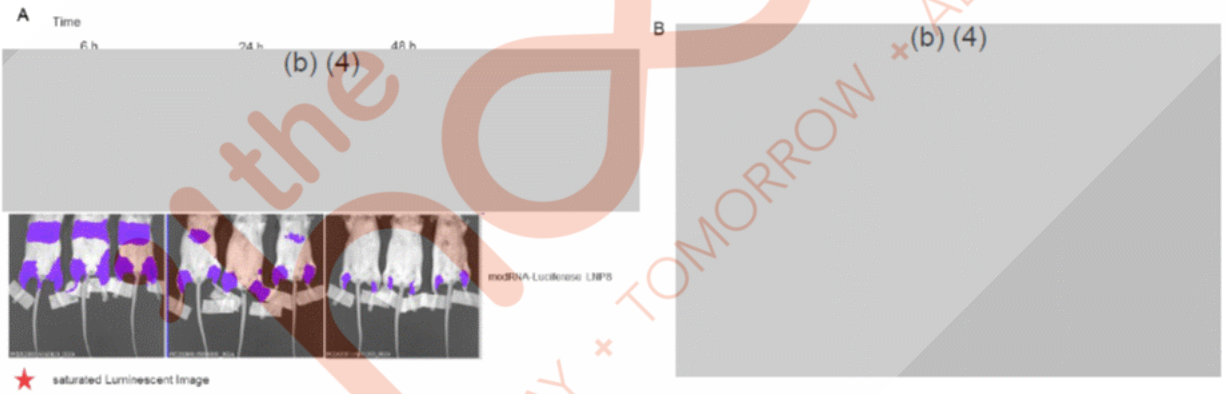


Figure 2: Bioluminescence measurement in the liver using the LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase. A) At 6 h, 24 h, and 48 h after injection, the luciferase expression *in vivo* was measured by luciferin application. B) Quantification of luciferase signal in the liver over time (mean ±SD). p/s: photons per second.

The official report only disclosed biodistribution to the liver. Note that for this imaging, the mice were belly up, since the liver is relatively close to the belly side of a mouse, and the signal spans the width of the belly, which aligns with the shape, size, and location of the liver (unlike the punctuated signals on the left and right of the backs of the mice in paragraph 19 above, which was suggestive of involvement of the kidneys). It would have

been nice to have images of the upper bodies of the mice to determine whether or not there were any signals in that region, especially the head.

23 Some people think that distribution of a mRNA vaccine to the liver is not a safety issue because it has been assumed that degradation and removal from the body occurs here. However, these data demonstrate substantial acute (short-term) expression throughout the liver of the protein encoded by the mRNA blueprint in the 'vaccine' (in this case, the blueprint encoded luciferase; the signal of which is shown as the blue/purple shaded areas in the image in paragraph 22 above).

24 For a mRNA vaccine encoding the spike protein of SARS-CoV-2, this would mean the liver cells would be covered with the spike protein. The first dose would induce antibody responses. This means that subsequent doses would force liver cells to express the spike protein in the presence of spike-specific antibodies. This would be expected to result in killing of self liver cells; a form of autoimmunity. This could potentially put some individuals at risk of developing autoimmune diseases such as autoimmune hepatitis. Of concern, autoimmune hepatitis is emerging as a safety signal following receipt of COVID-19 'vaccines'.[6-10]

[6]Garrido I, Lopes S, Simões MS, Liberal R, Lopes J, Carneiro F, Macedo G. Autoimmune hepatitis after COVID-19 vaccine - more than a coincidence. J Autoimmun. 2021 Dec;125:102741. doi: 10.1016/j.jaut.2021.102741. Epub 2021 Oct 26. PMID: 34717185; PMCID: PMC8547941.

[7]McShane C, Kiat C, Rigby J, Crosbie Ó. The mRNA COVID-19 vaccine - A rare trigger of autoimmune hepatitis? J Hepatol. 2021 Nov;75(5):1252-1254. doi: 10.1016/j.jhep.2021.06.044. Epub 2021 Jul 8. PMID: 34245804; PMCID: PMC8264276.

[8]Pinazo-Bandera JM, Hernández-Albújar A, García-Salguero AI, Arranz-Salas I, Andrade RJ, Robles-Díaz M. Acute hepatitis with autoimmune features after COVID-19 vaccine: coincidence or vaccine-induced phenomenon? Gastroenterol Rep (Oxf). 2022 Apr 27;10:goac014. doi: 10.1093/gastro/goac014. PMID: 35498817; PMCID: PMC9046091.

[9]Camacho-Domínguez L, Rodríguez Y, Polo F, Restrepo Gutierrez JC, Zapata E, Rojas M, Anaya JM. COVID-19 vaccine and autoimmunity. A new case of autoimmune hepatitis and review of the literature. J Transl Autoimmun. 2022;5:100140. doi: 10.1016/j.jtauto.2022.100140. Epub 2022 Jan 4. PMID: 35013724; PMCID: PMC8730708.

[10]Chen Y, Xu Z, Wang P, Li XM, Shuai ZW, Ye DQ, Pan HF. New-onset autoimmune phenomena post-COVID-19 vaccination. Immunology. 2022 Apr;165(4):386-401. doi: 10.1111/imm.13443. Epub 2022 Jan 7. PMID: 34957554.

Distribution of mRNA-Loaded Lipid Nanoparticles Throughout the Body is a Major Safety Concern

25 Paragraphs 104 and 104.1 of Mr. James's affidavit states "I do not attempt to respond to every point by Dr Bridle about this study, but note the following: One of Dr Bridle's main concerns seems to be that this study demonstrates that the LNP does not remain in the shoulder muscle but rather spreads through the body. However, it is not clear from Dr Bridle's affidavit why he considers that is cause for concern. Lipids (fats) are found in every membrane in the body and you would expect lipid nanoparticles to spread in the body. This study does not show that the mRNA component (the vaccine) follows the same distribution pattern. Dr Bridle also does not explain why it would be concerning for mRNA to spread in the body. Pfizer conducted a study in rats on the distribution of mRNA coding for luciferase, which was considered by Medsafe as part of the non-clinical evaluation of the parent product. The result of this study was that the mRNA remained mainly at the injection site, with some being found in the liver. No safety concerns were identified as a result of this study.".

26 Mr. James admitted that "you would expect lipid nanoparticles to spread in the body". I agree with this assessment of systemic distribution of the lipid nanoparticles that serve as the vehicle for carrying SARS-CoV-2 spike protein-encoding mRNAs. This contradicts public messaging, which suggested that a dose of Comirnaty would largely remain at the injection site, with some being taken by the cells of the immune system to the local draining lymph nodes where an immune response would be initiated. Indeed, this is how traditional vaccines function. It is concerning that public health officials did not reveal this atypical feature of Comirnaty.

27 Mr. James is correct that the biodistribution study reported by Pfizer only looked at the lipid nanoparticles, not the spike protein. This represents one of the many key weaknesses of the study; one that regulatory agencies should have identified, leading to insistence on a more comprehensive biodistribution study being performed. Specifically, Pfizer should have been required to study the biodistribution of the lipid nanoparticles, the mRNA that they contain, as well as the spike protein encoded by the mRNA. In this biodistribution

study, the biodistribution of the spike protein could not even be evaluated because only a surrogate 'vaccine' was used. Remarkably, the study was not even conducted with the same formulation of Comirnaty being used in people.

28 Mr. James pointed out that the study did not prove that the mRNA carried by the lipid nanoparticles had the same biodistribution as the latter. Although mRNA was not directly assessed in tissues, it is carried into tissues inside the lipid nanoparticles. Therefore, in the absence of data to the contrary, it is a safe assumption that mRNA would go to the same places as the lipid nanoparticles that are carrying the mRNA.

29 Mr. James was uncertain why systemic distribution of mRNAs encapsulated in lipid nanoparticles might be concerning. Spreading throughout the body of lipid nanoparticles that carry mRNAs encoding the spike protein of SARS-CoV-2 represents a major safety issue for multiple reasons. I covered the many reasons for this concern quite extensively on pages 43-47 of exhibit BRI-2 of my First affidavit and in paragraphs 62-67 of my Further Affidavit. To directly address Mr. James's uncertainty, I will reiterate just two of the many issues.

30 First, the lipid nanoparticles themselves are known to be proinflammatory. Inflammation can cause bystander damage to tissues. If this occurs in sensitive tissues such as the ovaries or testes, for example, this could potentially result in fertility issues. In fact, repeated dosing with lipid nanoparticles has been known for many years to be contraindicated due to toxicity issues. This has even been acknowledged by manufacturers of the current COVID-19 'vaccines'. In fact, this is one of the reasons why lipid nanoparticles were re-purposed for use as a vaccine technology. Their traditional use for systemic delivery of drugs and gene therapies required repeated dosing; something for which the toxicity issues could not be overcome, especially if doses were administered repeatedly over relatively short timeframes, such as three to four doses in less than one year. As such, it was hypothesized that they would be safer in the context of vaccines because most good quality vaccines only require one, or at most, two doses in people aged five or older to provide life-long immunity. As such, administering more than two doses of lipid nanoparticle-based

vaccines like Comirnaty is contraindicated unless long-term safety can be proven in the context of clinical trials specifically designed to assess repeated dosing beyond two boluses.

31 As pointed out by Mr. James, cells throughout the entire body are encapsulated by lipids (fats). Consequently, when the lipid nanoparticles get distributed throughout the body, they can fuse with the fat layer surrounding cells in the various tissues. This causes their mRNA cargo to be released inside the cells throughout the body, thereby allowing these cells to begin manufacturing the protein encoded by the mRNA. In the case of Comirnaty, the protein that would be made is the spike protein from SARS-CoV-2, which is specifically designed to be anchored on the surface of the cells. In addition, some of the protein will be digested inside the cells and pieces will be displayed on the surface in the context of specialized molecules called major histocompatibility complex molecules. These are designed to show pieces of proteins to facilitate killing of spike-expressing cells by T cells, which are a critical component of an immune response. This leads to a second major safety issue. Cells throughout the body, including those in the muscle at the injection site, would express the spike protein and become targets for autoimmune responses. Especially after the second dose. This is also likely why people with pre-existing naturally acquired immunity are at increased risk of experiencing adverse events after a single dose as discussed in my First Affidavit. The pre-existing spike-specific antibodies and T cells induced after recovery from natural infection would be able to immediately recognize and kill cells throughout the body as they begin to manufacture and then express spike proteins on their surface.

Lipid Nanoparticles Used in Comirnaty are Known to be Toxic, Especially if Administered Repeatedly

32 Paragraph 104.2 of Mr. James's affidavit states "Dr Bridle claims that LNPs can be toxic. Dr Altman also comments that Comirnaty uses a "completely new LNP delivery system". While the specific LNPs used in Comirnaty are novel, LNP delivery systems in general are not new. There are several approved medicines that use LNP delivery systems with good safety profiles (for example, Caelyx, Ambisome).".

33 The manufacturers of mRNA COVID-19 'vaccines' have admitted that the LNP delivery systems are toxic, and this is also a well-established fact in the peer-reviewed scientific literature. I discussed this in paragraph 30 above and in greater detail in paragraphs 62-67 of my Further Affidavit, with multiple references provided.

34 It is concerning that Mr. James would use the drugs Caelyx and Ambisome as examples to try to demonstrate the safety of the LNPs used as a component of Comirnaty. The most obvious issue is that Caelyx and Ambisome use completely different LNP formulations than Comirnaty. So, this is an irrelevant comparison; like comparing apples to oranges. There is a large array of different LNPs, with wide variation in their formulations and biological properties. Indeed, Mr. James stated that "the specific LNPs used in Comirnaty are novel". Of particular concern, cationic lipids, such as those introduced into cells throughout the body by Comirnaty, are known to be toxic.[11]

35 Second, Caelyx and Ambisome have very concerning safety profiles. They are only used to treat individuals with very serious diseases for which death is otherwise highly likely. Caelyx is used to treat lethal cancers. Ambisome is used to treat serious fungal infections in patients that are highly immunosuppressed or with infections around the brain.

36 The known side-effects of Caelyx include but are not limited to hives, difficulty breathing, swelling of your face, lips, tongue, or throat, fever, chills, lightheadedness, shortness of breath, itching, warm or tingly feeling, headache, pain or tightness in your chest or throat, back pain, fast heartbeats, blue-coloured skin, lips, or nails, blisters or ulcers in your mouth, red or swollen gums, trouble swallowing, pain, redness, numbness and peeling skin on your hands or feet, tiredness, mouth sores, skin sores, easy bruising, unusual bleeding, pale skin, cold hands and feet, weakness, tiredness, and swelling of your ankles or feet. Some of these side-effects are caused by the lipid nanoparticles themselves (*i.e.*, without the drug payload inside of them).[12]

[11] Cui S, Wang Y, Gong Y, Lin X, Zhao Y, Zhi D, Zhou Q, Zhang S. Correlation of the cytotoxic effects of cationic lipids with their headgroups. Toxicol Res (Camb). 2018 Mar 22;7(3):473-479. doi: 10.1039/c8tx00005k. PMID: 30090597; PMCID: PMC6062336.

[12] https://www.rxlist.com/caelyx-drug.htm

37 The known side-effects of Ambisome include but are not limited to the following: fever, shaking, chills, flushing (warmth, redness, or tingly feeling), loss of appetite, dizziness, nausea, vomiting, stomach pain, diarrhea, headache, shortness of breath, fast breathing 1 to 2 hours after the infusion is started, sleep problems (insomnia), or skin rash, swelling or pain at injection site, muscle or joint pain, unusual tiredness, weakness, muscle cramping, changes in the amount of urine, painful urination, numbness or tingling of arms or legs, vision changes, hearing changes (*e.g.*, ringing in the ears), dark urine, severe stomach or abdominal pain, yellowing eyes or skin, swelling ankles or feet, fast/slow/irregular heartbeat, cold sweats, blue lips, easy bruising or bleeding, other signs of infection (*e.g.*, fever, persistent sore throat), mental/mood changes, seizures, black stools, or vomit that looks like coffee grounds. Some of these side-effects are caused by the lipid nanoparticles themselves.[13]

38 Risk-benefit analyses make the use of dangerous drugs like Caelyx and Ambisome appropriate only in situations where the disease is usually going to be fatal in the absence of treatment. This is in stark contrast to SARS-CoV-2, which does not cause COVID-19 in many children. And when an infection does progress to cause COVID-19, the disease represents a very low risk to children, especially in the context of the relatively avirulent Omicron variant. Further, Comirnaty is being used prophylactically in healthy people whereas drugs like Caelyx and Ambisome are used therapeutically in sick individuals. As such, the bar for safety needs to be set extremely high for children that have many quality life years ahead of them.

[13] https://www.rxlist.com/ambisome-side-effects-drug-center.htm

mRNA 'Vaccines' Accumulate in the Ovaries Where Safe Concentrations of Lipid Nanoparticles, mRNA, and SARS-CoV-2 Spike Proteins Have Never Been Established

39 Paragraph 104.3 of Mr. James's affidavit states "Dr Bridle raises a concern about distribution of LNPs to the ovaries. He says that "the study director concludes that the ovary is a main target organ for distribution of the LNPs" (at [54]). That is not correct: the study concludes that "total recovery [...] was greatest in the liver, with much lower total recovery in spleen, and very little recovery in adrenals glands and ovaries." The result of the study was that less than 0.1% of the injected dose of LNP was found in the ovaries."

40 I was correct in stating that the study director concluded that the ovaries were a main target organ for distribution of LNPs. On page 9 of the study report, which was attached to my Further Affidavit as Schedule 3, the study director stated that "Over 48 hours, [3H]-08-A01-C01 [*i.e.*, the LNPs] distributed mainly to liver, adrenal glands, spleen and ovaries, with maximum concentrations observed at 8-48 hours post-dose.". The quotation cited by Mr. James was with respect to the percentage of the initial dose that ended up in the ovaries. As I noted in paragraphs 43-47 of my Further Affidavit, it is misleading to refer to the percentage of the dose in the ovaries, which are extremely tiny organs. When it comes to toxicities, it is the concentration that a drug reaches in a tissue that matters. The percentage of the dose that arrived there is irrelevant. A low percentage of the dose accumulating in a tiny organ can amount to a concerningly high concentration, as was the case for the LNPs used in Comirnaty that accumulated in the ovaries.

41 Further, Mr. James has not accounted for the fact that there is no defined safe concentration of the novel LNPs in Comirnaty for any tissues.

42 Finally, in the context of the ovaries, Mr. James also did not account for the fact that it was just the LNPs assessed in the biodistribution study. For Comirnaty, these LNPs carry mRNA encoding the spike protein from SARS-CoV-2. Once these mRNAs get into cells they can make multiple copies of the spike protein. This represents a self-amplification of the effective dose that can vary from person to person depending on how metabolically active their cells are. In other words, different cells from different people have the potential

to manufacture different, but undefined quantities of the spike protein from the mRNAs delivered by the LNPs. Like the LNPs themselves, there is no defined safe concentration for spike proteins in the ovaries, or any other tissues for that matter. Worse, when ovarian cells begin expressing the spike protein on their surface, they can become targets for spike-specific antibodies and T cells, which could result in autoimmune reactions in the ovaries.

Pfizer's Development and Reproduction Study Assessed by Regulatory Agencies Had a Major Flaw That Was Overlooked

43 Paragraph 104.3 of Mr. James's affidavit states "I also note, as discussed above, that the development and reproduction study found no effects on fertility. To the extent that the presence of some LNP in the ovaries might have raised a potential fertility or reproduction concern, that was discounted by the DART study."

44 Pfizer's development and reproductive toxicity study was fatally flawed, and health regulators should have insisted that it be repeated in at least one animal model (and preferably more) that was more appropriate. The spike protein that Comirnaty gets a person's cells to manufacture is a highly bioactive molecule in the body, with many potential mechanisms of harm. In addition to presenting this evidence in my First Affidavit and Further Affidavit, the potential mechanisms of harm have been reviewed elsewhere.[14] Human cells express what is known as the high-affinity receptor for the SARS-CoV-2 spike protein. This means the spike protein can bind very strongly to human cells, which provides multiple mechanisms of action for potential harm.[14] In stark contrast, the rats that were used in Pfizer's reproductive toxicity study express what is known as the low-affinity receptor for the spike protein.[15] This means the spike protein cannot bind strongly to rat cells. This was confirmed by the observation that, unlike humans, rats are highly resistant to developing COVID-19 when they are infected by SARS-CoV-2.[15] This is

[14]Karrow NA, Shandilya UK, Pelech S, Wagter-Lesperance L, McLeod D, Bridle B, Mallard BA. Maternal COVID-19 Vaccination and Its Potential Impact on Fetal and Neonatal Development. Vaccines (Basel). 2021 Nov 18;9(11):1351. doi: 10.3390/vaccines9111351. PMID: 34835282; PMCID: PMC8617890.

[15]Pach S, Nguyen TN, Trimpert J, Kunec D, Osterrieder N, Wolber G. ACE2-Variants Indicate Potential SARS-CoV-2-Susceptibility in Animals: A Molecular Dynamics Study. Mol Inform. 2021 Sep;40(9):e2100031. doi: 10.1002/minf.202100031. Epub 2021 Aug 10. PMID: 34378348; PMCID: PMC8420607.

because the virus cannot grab onto the cells in the respiratory tract of rats very well. Any animal model that expresses the low affinity receptor for the spike protein, such as the rats used in Pfizer's study, would not be appropriate to elucidate potential toxicities. In other words, Pfizer's reproductive toxicity study utilized an animal model that is incapable of demonstrating potential toxicities related to the SARS-CoV-2 spike protein.[15] This biased the results towards demonstrating safety. More appropriate animal models would include mice that have been genetically engineered to express the same high-affinity receptor for the SARS-CoV-2 spike protein that humans have, or mink, which naturally express the high-affinity receptor and are susceptible to developing severe COVID-19.

45 There was a second fatal flaw with the reproductive toxicity study performed by Pfizer. For an unknown reason, they failed to treat the males with Comirnaty. Only the females were treated. As such, there was no assessment of potential reproductive toxicity in males.[16]

46 Also, the reproductive toxicity study was not conducted for Pfizer, but rather by Pfizer and BioNTech, which represents a serious conflict of interest.[15] Specifically, nine out of the ten authors, including the corresponding author, were employed by Pfizer or BioNTech, which are the companies that own Comirnaty.

Shedding of COVID-19 'Vaccine' Components is Not Only Plausible, but It Has also Been Proven

47 Paragraph 104.3 of Mr. James's affidavit states "Dr Bridle says he has a significant concern that urine and faecal samples were not analysed, because of the "world-wide debate about potential shedding of mRNA 'vaccine' components and/or the spike protein." "Vaccine shedding" is a theory that has gained traction amongst people who are fearful of COVID-19 vaccines. For vaccines based on a live attenuated (weakened) virus, it is possible for a vaccinated person to "shed" small amounts of the weakened virus. However, the

[16]Bowman CJ, Bouressam M, Campion SN, Cappon GD, Catlin NR, Cutler MW, Diekmann J, Rohde CM, Sellers RS, Lindemann C. Lack of effects on female fertility and prenatal and postnatal offspring development in rats with BNT162b2, a mRNA-based COVID-19 vaccine. Reprod Toxicol. 2021 Aug;103:28-35. doi: 10.1016/j.reprotox.2021.05.007. Epub 2021 May 28. PMID: 34058573; PMCID: PMC8163337.

Comirnaty vaccine does not contain any virus. There is no plausible mechanism by which shedding could occur for the Comirnaty vaccine. There is no legitimate debate to be had about "vaccine shedding".".

48 There is a plausible mechanism whereby shedding of components of the Cormirnaty 'vaccine' or the spike protein that it encodes could occur. The biodistribution study that was attached as Schedule 3 to my Further Affidavit demonstrated that low concentrations of the lipid nanoparticle component of Comirnaty accumulated in the lungs, skin, small and large intestines, salivary glands, and bladder. Seeding of spike-encoding mRNAs into cells in these tissues would allow them to manufacture amplified quantities of the spike protein. This means there would be the potential for spike-encoding mRNA and/or the spike protein to be present in aerosols exhaled from the lungs, sweat or oil secreted from the skin, excretion in faeces due to seeding of the intestines, and excretion in saliva and/or urine.

49 Further, there is published evidence of mRNA from COVID-19 'vaccines' being shed in breast milk of lactating women who were inoculated.[17] Although the mRNA was at low concentrations, a safe concentration of SARS-CoV-2 spike protein-encoding mRNAs in human breast milk has never been established. This is particularly important since low concentrations of mRNA can be amplified into higher concentrations of proteins since multiple proteins can be made from a single mRNA molecule. This is akin to a home builder being able to construct multiple houses from a single blueprint. Regardless, shedding of a mRNA vaccine component in breast milk provides proof of principle that contradicts the conclusion drawn by Mr. James. Distribution of mRNA from COVID-19 'vaccines' into breast milk is not surprising in light of Pfizer's biodistribution study that provides clear evidence of their lipid nanoparticles trafficking throughout the body via the blood; proteins in blood, including antibodies, tend to get concentrated in breast milk for delivery to suckling infants.

[17]Low JM, Gu Y, Ng MSF, Amin Z, Lee LY, Ng YPM, Shunmuganathan BD, Niu Y, Gupta R, Tambyah PA, MacAry PA, Wang LW, Zhong Y. Codominant IgG and IgA expression with minimal vaccine mRNA in milk of BNT162b2 vaccinees. NPJ Vaccines. 2021 Aug 19;6(1):105. doi: 10.1038/s41541-021-00370-z. PMID: 34413319; PMCID: PMC8376902.

50 The extent of shedding of mRNA 'vaccines' or their components from routes in the body other than breast milk remains unknown. Of concern is the fact that samples that could confirm or refute the concept of shedding of vaccine components were acquired in Pfizer's biodistribution study but were never analyzed. These samples are likely still archived. Health regulatory agencies should have insisted that these shedding samples be analyzed prior to any public rollout, especially when it was known, as stated by Mr. James in paragraph 104.1 of his affidavit, that mRNA-loaded lipid nanoparticles spread throughout the body. In fact, assuming the shedding samples are archived, this request could still be made. Alternatively, a simple, rapid unbiased third-party analysis of shedding samples (*e.g.*, saliva, urine, faeces, skin swabs) from any vaccinated people could easily be conducted to resolve this issue.

51 Both the plausible mechanism of action for shedding of vaccine components and the proof that this does occur suggest it is inappropriate to conclude "There is no legitimate debate to be had about "vaccine shedding". Even if this issue remained a "theory" as postulated by Mr. James (affidavit, paragraph 104.4), it would be inappropriate to dismiss it out of hand. Theories are foundational components of science that are routinely debated and formally tested in research studies. It is also inappropriate to insinuate that professionals with deep expertise in vaccinology who have identified these legitimate safety concerns are merely "fearful of COVID-19 vaccines" (paragraph 104.4 of Mr. James's affidavit). In fact, to maximize the safety of the public, a regulatory agency should welcome robust scientific discussions among experts about legitimate safety issues related to novel medical technologies. To dismiss serious concerns that are supported by the peer-reviewed scientific literature and that can be tested using rapid and cheap methods does not follow the precautionary principle, which is to assume that any new medical technology may be unsafe until definitively proven otherwise. This basic principle of medical science has existed to minimize the risk of compromising the safety of the public.

Declared Remotely by the Dependent, stated as being located in Guelph, Ontario, Canada, before me at the city/town of GUELPH, in the province of Ontario on this day of June 19, 2022, in Accordance with O.Reg 431/20, Administering Declaration Remotely.

Lee Cole P05826

Commissioner for Taking Affidavits

(or as may be)

Byram W. Bridle

Dependent Byram W. Bridle

TABLE OF CONTENTS

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Terms and abbreviations used in this section

Terms and abbreviations not omitted or defined	ALC-0159 Added to this drug.
	PEG lipid
ALC-0315.	Aminoolipids added to this drug
[3h] -the	RadioLabeled [Cholesteryl-1,2-3H (N)] -Cholestryl Hexadecyl Ether: Radioactive Signs [Cholester Lil -1, 2-3H (N)] Hexadecyl ether
DSPC	1,2-Distearoyl-Sn-Glycero-3-Phosphocholine: 1,2-Jistealoyl-Sn-Glycero-3-Phosphoco Rin
GLP	Good Laboratory Practice: Standard of implementation of non-clinical trials on drug safety
LNP	Lipid-nanoparticle: Lipid nanoparticles
modrna	Nucleoside-Modified mRNA: Modified nucleoside mRNA
mRNA	Messenger RNA: Messenger RNA
m/z	M / Z (M Over Z): Give the weight of ions by unified atomic mass unit (= Dalton) A dimensionless amount obtained by dividing the amount of the number of ions by the absolute value of the number of ions.
PEG	Polyethylene Glycol: Polyethylene glycol
PK	Pharmacokinetics: Pharmacokinetics
Rna	Ribonucleic Acid: ribonucleic acid
There	Supernatant fraction obtained from liver homogenate by centrifuging at 9000 g□□□□□□□□ To A supernatant dispatched with 9000 g centrifuged
WHO	World Health Organization: World Health Organization

1. Summary

BNT162B2 (BionTech Code Number: BNT162, PFIZER Code Number: PF-07302048) is a heavy acute call
Susing syndrome coronavirus 2 (SARS-COV-2) spike glycoprotein (S protein) total length
Code modified nucleoside MRNA (MODRNA) and for infectious diseases with SARS-COV-2
Development has been developed as the essence of mRNA vaccines.In formulation of BNT162B2, two
Functional lipid ALC-0315 (amino lipid) and ALC-0159 (PEG lipid) and two structural lipids
As By mixing with DSPC (1,2-Distearoyl-Sn-Glycero-3-Phosphocholine) and cholesterol
Lipid nanoparticles (LNP) which encapsulate BNT162B2 are formed (hereinafter, "BNT162B2 encapsulated LNP").

ALC-0315 contained in LNP and ALC-0315 and
In vivo and in vitro tests and BNT162B2 to evaluate ALC-0159 absorption (PK), metabolism and excretion
In-vivo distribution test using luciferase or radiolabeled lipid as an alternative reporter
Conducted.

Based on the development of vaccines for the prevention of infections, based on the need to evaluate systemic exposure
(WHO, 2005; Infectious disease prevention vaccine non-clinical trial guidelines) 1, 2, BNT162B2 Encapsulated LNP muscles
By admission PK test did not conduct.Also, the other he contained in this drug is two lipids (cholester
Roll and DSPC is a naturally occurring lipid, and is considered to be metabolism as well as endogenous lipids.
available.in addition, BNT162B2 is degraded by ribonuclease in captured cells and nucleic acid
Thank you, S-protein derived from BNT162B2 is expected to be subject to proteolysis.From the above,
It was thought that no need to evaluate metabolism and excretion of these components.

LNP enclosed RNA encoding luciferase as an alternative reporter of BNT162B2 (Lucife
Laze RNA is enclosed in LNP with the same lipid configuration as BNT162B2 encapsulated LNP: Since then, "Lucifer
Zeros In the PK test, which was administered intravenously to Wistar Han rats), plasma, urine, feces and
Collect liver samples over time and in each sample ALC-0315 and ALC-0159 concentrations were measured.That
fruit, ALC-0315 and ALC-0159 have been shown to be promptly distributed from blood to the liver.Also,
ALC-0315 and ALC-0159 excreted about 1% and about 50% of doses as unchanged
In urine, all were less than the detection limit.

In vivo distribution test, luciferase RNA encapsulated LNP was intramuscularly administered to BALB / C mice.That
As a result, the expression of luciferase was found at the site of administration, and the expression level was low in the liver.
Also recognized.Expression at the administration site of luciferase is after administration It is recognized from 6 hours, and after administration 9 days
Was disappeared.After administration of the liver expression It was observed for 6 hours and disappeared by 48 hours after administration.Also,
Luciferase RNA encapsulated LNP radiolabeled body is intramuscularly administered into rats to quantitatively in vivo distribution.
When evaluated, the radioactivity concentration was the highest at the site of administration.The liver is the highest outside the administration site
It was (maximum of dose 18%).

Metabolism of ALC-0315 and ALC-0159 CD-1 / ICR mouse, Wistar Han or Sprague Dawley rats,
Cynomolgus monkeys or human blood, liver microsomes, liver In vitro using S9 fractions and hepatocytes
evaluated.Also, the above-mentioned rat intravenous administration For plasma, urine, feces and liver samples collected in PK test
In IN VIVO metabolism was also examined.From these in vitro and in vivo tests, ALC-0315 and
ALC-0159 is an ester bond and an amide bond hydration, respectively, in any animal species of testing
It has been shown to be slowly metabolized by solution.

From the above non-clinical pharmacokinetic evaluation, the circulating blood was reached LNP was shown to be distributed in the liver.
Also, Metabolism and feces excretion is involved in the disappearance of ALC-0315 and ALC-0159, respectively.
It was suggested.

2. Analysis Method

Report number: PF-07302048_06 [redacted]_072424

ALC-0315 and ALC-0315, which is a LNP constituent lipid in rat intravenous administration PK test (M2.6.4.3) of GLP non-application
ALC-0159 Developed LC / MS method with appropriate performance to quantify concentrations.That is, 20 µl
Plasma, liver homogenate (liver A homogenate is prepared using sections collected from three places.
Suitable for pooling, dilute with blank matrix), urine and feces homogenate (as appropriate, Blanc
Cumatrix diluted) Samples Internal standards (Removed by acetonitrile containing PEG-2000)
After protein, centrifuge and the supernatant We subjected to LC-MS / MS measurement.

3. Absorption

Report number: PF-07302048_06 [redacted]_ 072424, Overview Table: 2.6.5.3

Luciferase RNA encapsulated LNP is male to consider the in-vibration condition of ALC-0315 and ALC-0159
Wistar Han rats are administered in a single intravenous administration at a dose of 1 mg RNA / kg, with time (before administration, 0.1, 0.25,
Sparse plasma and liver on 0.5, 1, 3, 6 and 24 hours and 2, 4, 8 and 14 days after administration.
Collected by sampling Three / time pointed).ALC-0315 and ALC-0159 in plasma and liver
Measure the concentration, PK parameters were calculated (Table 1).Blood ALC-0315 and ALC-0159
After giving Slightly distributed to the liver by 24 hours.Also, 24 hours plasma concentration after administration is in the highest plasma
Density It was less than 1% (Figure 1).Close-end phase disappearance half-life (T2) is in plasma and in liver
The same level, ALC-0315 was 6 to 8 days, and ALC-0159 was 2-3 days.From the results of this test, the liver is in blood
from It was suggested that it is one of the major organizations that take ALC-0315 and ALC-0159.

Conducted in this study On the examination results of Urinary and feces concentration of ALC-0315 and ALC-0159
It is Section M2.6.4.6.

Table 1 luciferase RNA encapsulated LNP in Wistar Han rats at a dose of 1 mg RNA / kg

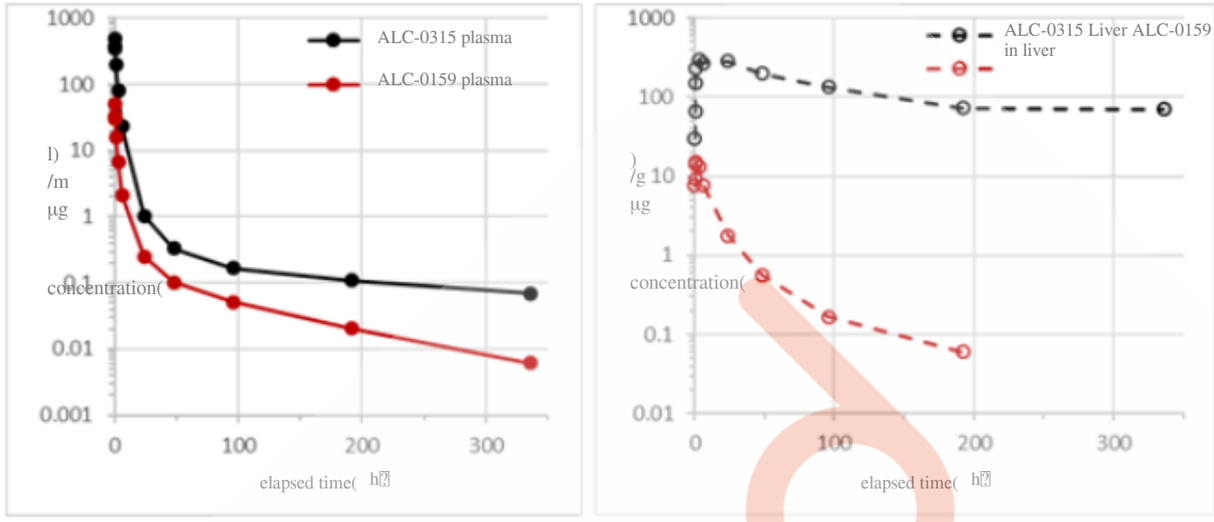
When given Pharmacokinetics of ALC-0315 and ALC-0159

Analyte	Analyze dose (mg/kg)	sex/Nt½[h]		AUCinf (µg•h/mL)	AUClast (µg•h/mL)	To the liver Distribution ratio (%)a
ALC-0315.	15.3	Male 3b	139	1030	1020	60
ALC-0159.	1.96	Male 3b	72.7	99.2	98.6	20

a. Calculated as the highest liver distribution amount (µg) / [dose (µg)].b. Each time point.Sparse sampling.

Figure 1 luciferase RNA encapsulated LNP in Wistar Han rats at a dose of 1 mg RNA / kg

When given Plasma and liver concentrations of ALC-0315 and ALC-0159



4. Distribution

Report number: R-[redacted]72, 185350, Overview Table: 2.6.5.5a, 2.6.5.5b

female Administer luciferase RNA encapsulated LNP to BALB / C mice (3 animals) and luciferase emission

As an alternative marker The vivo distribution of BNT162B2 was examined.That is, luciferase RNA encapsulation

LNP was administered intramuscularly at a dose of 1 µg RNA (total 2 µg RNA) in the left and right hindlimbs of mice.Then

Cypherase emission detection Luciferin, which is a light emitting substrate 5 minutes ago, is administered intraperitoneally, isoflurane hemp

Downward 6 and 24 hours after administration using Xenogen IVIS Spectrum in vivo, 6 and 24 hours and 2,

By measuring it on 3, 6 and 9 days, it is recommended with time with the same individual of luciferase protein

I was evaluated.As a result, expression at the site of administration of luciferase is administered Recognized from 6 hours,

After giving It disappeared on the 9th.Liver expression was also from 6 hours after administration, and disappeared by 48 hours after administration

I was.Distribution to the liver is a luciferase where topically administered Some of the RNA encapsulated LNP reaches circulating blood and liver

It was considered to indicate that it was incorporated in the needs. As detailed in M2.6.4.3, rats are

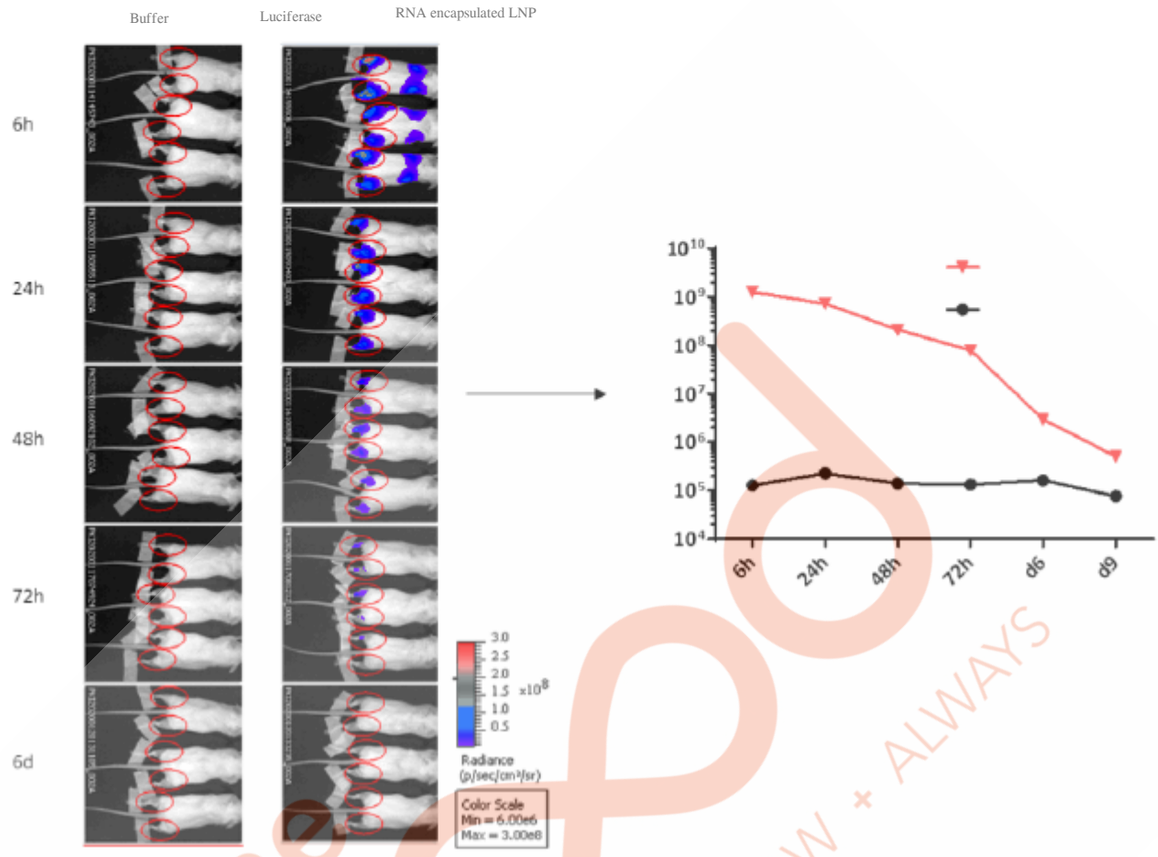
Laze When RNA encapsulated LNP is administered intravenously, the liver is the main of ALC-0315 and ALC-0159

It is suggested that it is a distributed organ, this is the finding of the test results that were intramuscularly administered to mice

The mixture was.In addition, a toxic finding finding of liver disorder is recognized in rat repeated dose toxicity test

Absent(M2.6.6.3).

Figure 2 Luciferase RNA encapsulated LNP in vivo luminescence in BALB / C mice administered intramuscularly



male and female Wistar Han rats, LNP labeled with [3H] -colesteryl hexadecyl ether ([3H] -CHE)

Luciferase using The RNA encapsulated LNP is intramuscularly administered at a dose of 50 μg RNA and 15 minutes after administration

Atmosphere Help plasma and tissues from 3 males and 3 males at each time of 1, 2, 4, 8, 24 and 48 hours

By measuring the radioactivity concentration by liquid scintillation counting method Review the vivo distribution of LNP

It was reported.Both male and female, the radioactivity concentration was the highest dosing site at any measurement.

After administration of radioactivity concentration in plasma The highest value was shown for 1 to 4 hours.In addition, liver, spleen, adrenal and

Distribution to the ovary was observed, and after administration that the radioactivity was the highest in these tissues 8 to 48

It was time.Total radiation recovery rate for doses other than the site of administration is the highest in the liver (maximum 18%)

spleen(1.0% or less), adrenal (less than 0.11%) and ovary (0.095% or less) significantly lower than the liver

won.In addition, the average concentration and tissue distribution pattern of radioactivity were roughly similar to male and female.

It is believed that the in vivo expression distribution of the antigen encoded by BNT162B2 depends on the LNP distribution.For this test

Luciferase Is the lipid configuration of RNA encapsulated LNP be identical to the application formulation of BNT162B2

The results of this test It is believed that the distribution of BNT162B2 encapsulated LNP is shown.

5. Metabolism

Report number: 01049-0[redacted]49-01049-020,0[redacted]49-021,01049-[redacted]2, [redacted] [redacted] [redacted]
PF-07302048_05 [redacted]_043725, Overview Table: 2.6.5.10a, 2.6.5.10b, 2.6.5.10c, 2.6.5.10d

CD-1 / ICR mouse, Wistar Han or Sprague Dawley rats, cynomolgus monkeys and humans

Chrome, liver In vitro metabolic stability of ALC-0315 and ALC-0159 using S9 fractions and hepatocytes

The sex was evaluated. ALC-0315 or ALC-0159 for each animal species Microsomer or liver S9 fraction (120)

Intercarding incubation) or hepatocytes (Add to 240 minutes incubation)

The proportion of unconstructed unaccurations after bath was measured.resulting in, ALC-0315 and ALC-0159

It is metabolically stable in animal species and test systems, and the ultimate percentage of unaccurate is More than 82%.

further Metabolic pathways of ALC-0315 and ALC-0159 were evaluated in vitro and in vivo.this

In the test, CD-1 mouse, Wistar Han rats, cynomolgus monkey and human blood, liver S9 fraction

And using hepatocytes IN Vitro metabolism was evaluated.In addition, plasma, urine, feces collected in rat PK test

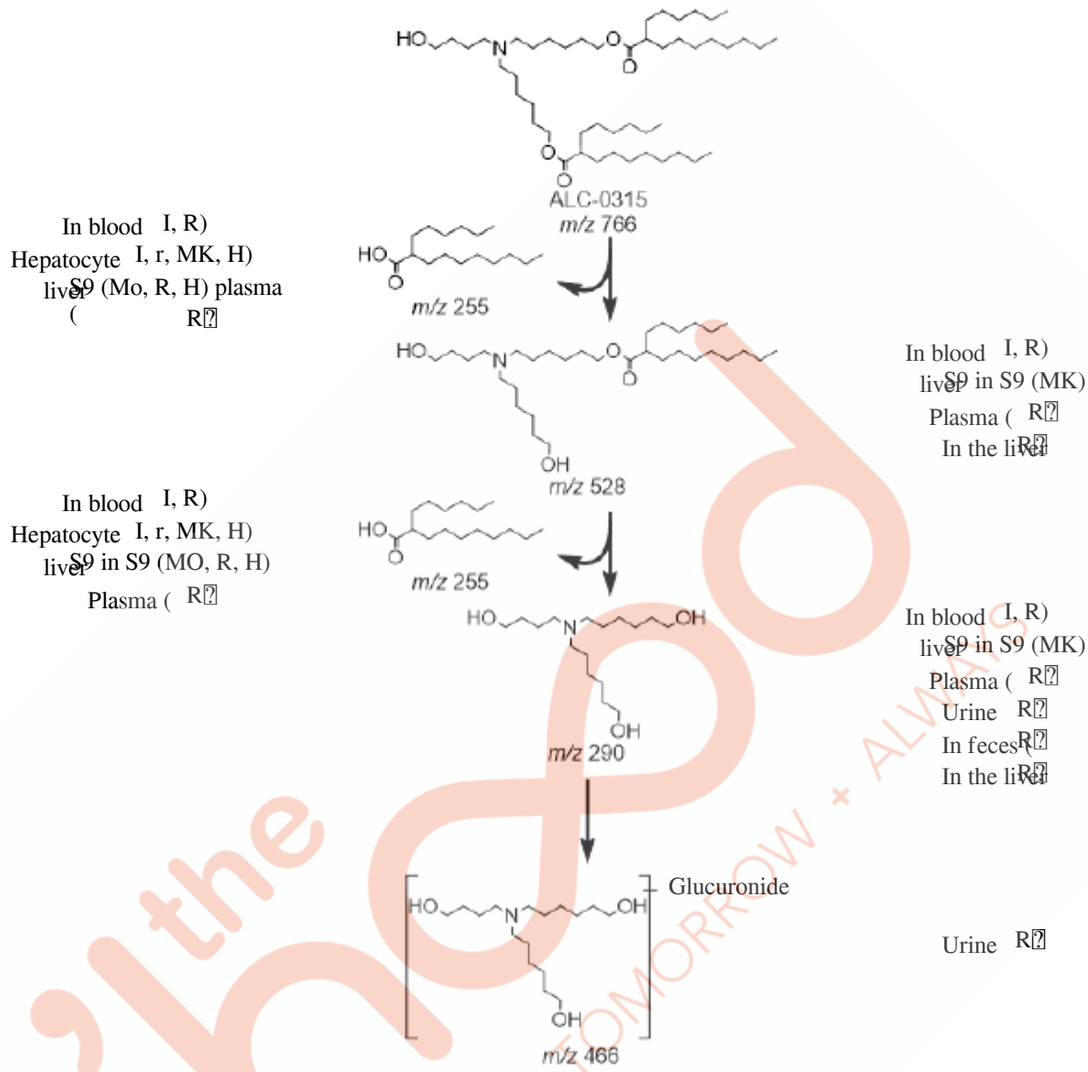
And liver samples, IN VIVO metabolism was evaluated (M2.6.4.3).From the test results, ALC-0315

When Metabolism of ALC-0159 is all slowly slow, and hydrolysis of ester bonds and amide bonds, respectively

It became clear that it is metabolized by. Metabolism by hydrolysis shown in Figure 3 and Figure 4

Was found in all animal species evaluated.

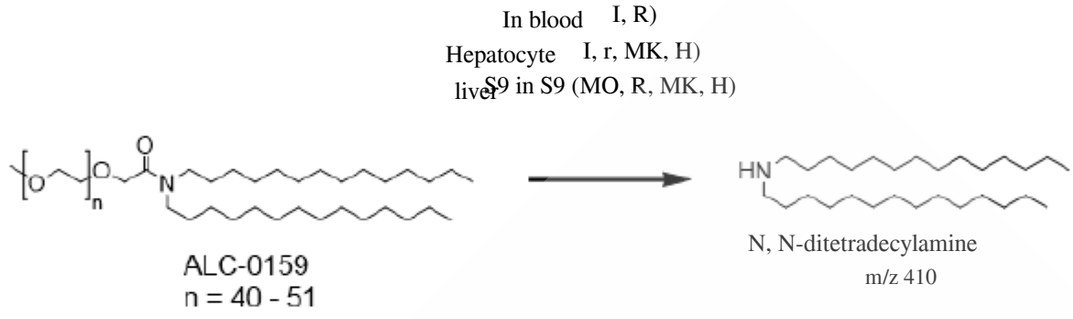
Figure 3 Estimated in vivo metabolic pathway of ALC-0315 in various animal species



H: Human, MK: Monkey, MO: Mouse, R: Rat

ALC-0315 is metabolized by receiving ester hydrolysis twice in succession.This two hydrolysis
By first, monoester metabolites (M / Z 528), then a dual-dose esterification metabolite (M / z 290) is formed
It is done.This double-dose esterification metabolite is further metabolized and glucuronic acid conjugate (M / Z 466)
However, this glucuronic acid conjugate is rats PK test was only detected in urine.In addition, two hydrolysis
Any acidic product of It was also confirmed that 6-hexyl decanoic acid (m / z 255).

Figure 4 Estimated in vivo metabolism pathway of ALC-0159 in various animal species



H: Human, MK: Monkey, MO: Mouse, R: Rat

ALC-0159 produces N, N-ditetradecylamine (M / Z 410) by hydrolysis of amide bonds

The pathway was the main metabolic pathway.This metabolite is blood and mice rats of mouse rats.

Sal-human hepatocytes and liver It was detected in the S9 fraction.Metabolites of ALC-0159 from in vivo samples

It was not confirmed.

6. Excretion

Luciferase PK test with intravenous administered intravenously to rats at a dose of 1 mg RNA / kg of RNA encapsulated LNP

(M2.6.4.3, ALC-0315 and ALC-0159 in urine and feces collected over time were measured.

None of the unchangeable bodies of ALC-0315 and ALC-0159 were not detected in urine.On the other hand, in the feces

ALC-0315 and ALC-0159 unchanged substances are detected, and the percentage per dose is about 1% and

about It was 50%.Also, as shown in Figure 3, the metabolites of ALC-0315 were detected in urine.

7. Pharmacokinetic drug interaction

The pharmacokinetic drug interaction test of this vaccine has not been conducted.

8. Other pharmacokinetic tests

Other pharmacokinetic tests of this vaccine have not been conducted.

9. Consideration and conclusion

Rats In the PK test, the concentration of ALC-0315 in plasma and liver is the highest concentration for 2 weeks after administration.

Every Decreased to 1/7000 and about 1/2-sq, and the ALC-0159 concentration is about 8000 minutes, respectively.

And about It decreased to one of 250 minutes.T-13 is the same in plasma and liver, ALC-0315, he is 6 to 8 days,

ALC-0159 was 2-3 days.Plasma T-13 values are distributed in tissues as LNP, each lipid.

It is then considered to indicate that it has been redistributed in plasma during the disappearance process.

Although the unchangeable body of ALC-0315 was hardly detected in any of urine and feces, rat PK test

Monomeric metabolites and dual esterification metabolites from feces and plasma samples collected 6-Hexy

Radecanoic acid detected glucuronic acid conjugate of dual-dose-esterified metabolites from urine.This metabolism

Process Although it is considered as the main loss mechanism of ALC-0315, quantitative data to verify this hypothesis is obtained

Absent.on the other hand, ALC-0159 was excreted in feces as an unchangeable body of dose.In vitro metabolic experiment

In the hydrolysis of the amide bond, it was slowly metabolized.

Because the in-vivo expression distribution of the antigen encoded by BNT162B2 is considered to depend on the LNP distribution,

BALB / C mice are intramuscularly administered luciferase RNA encapsulated LNP and alternative reporter protein

In-vivo distribution was examined.As a result, expression of luciferase is found at the site of administration,

The expression level was also observed in the liver but was also observed.Expression at the site of administration of luciferase was observed from 6 hours after administration and disappeared on 9 days after administration.The expression in the liver is observed from 6 hours after administration.

After giving It disappeared by 48 hours.Distribution to the liver is a circular luciferase RNA encapsulated LNP

It was considered to indicate that it was reached and taken up in the liver.Also, Lucifer in rats

Zeros When the radiolabel of RNA encapsulated LNP was administered intramuscularly, the radioactivity concentration is the highest value at the dosing site.

Indicated.Other than the site of administration, the liver was the highest and then detected in the spleen, adrenal and ovaries,

Total radioactivity recovery for dosages in these tissues was significantly lower than the liver.This result is

In-mouse biological distribution tests were encoded by luciferase expression in liver.In addition,

No toxic findings were observed showing liver injury in rat repeated dose toxicity tests (M2.6.6.3).

From the above non-clinical pharmacokinetic evaluation, the circulating blood was reached LNP was shown to be distributed in the liver.

Also, Metabolism and feces excretion is involved in the disappearance of ALC-0315 and ALC-0159, respectively.

It was suggested.

10. Charts

The chart is shown in the text and outline table.

references

1 World Health Organization. Annex 1. Guidelines on the nonclinical evaluation of vaccines. In: WHO Technical Report Series No. 927, Geneva, Switzerland. World Health Organization; 2005:31-63.

2 Non-clinical trial guidelines for infection prevention vaccine (Medicine dike examination 0527 1, May 27, 2010)

2.6.5.1. PHARMACOKINETICS OVERVIEW

Test Article: BNT162b2

Type of Study	Test System	Test item	Method of Administration	Testing Facility	Report Number
Single Dose Pharmacokinetics					
Single Dose Pharmacokinetics and Excretion in Urine and Feces of ALC-0159 and ALC-0315	Rat (Wistar Han)	modRNA encoding luciferase formulated in LNP comparable to BNT162b2	IV bolus	Pfizer yet	PF-07302048_06 [redacted]_072424
Distribution					
In Vivo Distribution	Mice BALB/c	modRNA encoding luciferase formulated in LNP comparable to BNT162b2	IM Injection	[redacted]b	R- -0072
In Vivo Distribution	Rat (Wistar Han)	modRNA encoding luciferase formulated in LNP comparable to BNT162b2 with trace amounts of [3H]-CHE as non- diffusible label	IM Injection	[redacted]c	185350
Metabolism In Vitro and In Vivo Metabolism					
In Vitro Metabolic Stability of ALC-0315 in Liver Microsomes	Mouse (CD-1/ICR), rat (Sprague Dawley and Wistar Han), monkey (Cynomolgus), and human liver microsomes	ALC-0315.	In vitro	[redacted]d	01049-008
In Vitro Metabolic Stability of ALC-0315 in Liver S9	Mouse (CD-1/ICR), rat (Sprague Dawley), monkey (Cynomolgus), and human S9 liver fractions	ALC-0315.	In vitro	[redacted]d	01049-009

2.6.5.1. PHARMACOKINETICS OVERVIEW

Test Article: BNT162b2

Type of Study	Test System	Test item	Method of Administration	Testing Facility	Report Number
In Vitro Metabolic Stability of ALC-0315 in Hepatocytes	Mouse (CD-1/ICR), rat (Sprague Dawley and Wistar Han), monkey (Cynomolgus), and human hepatocytes	ALC-0315.	In vitro	[redacted]	01049-[redacted]
In Vitro Metabolic Stability of ALC-0159 in Liver Microsomes	Mouse (CD-1/ICR), rat (Sprague Dawley and Wistar Han), monkey (Cynomolgus), and human liver microsomes	ALC-0159.	In vitro	[redacted]	01049-[redacted]
In Vitro Metabolic Stability of ALC-0159 in Liver S9	Mouse (CD-1/ICR), rat (Sprague Dawley), monkey (Cynomolgus), and human S9 fractions	ALC-0159.	In vitro	[redacted]	01049-[redacted]
In Vitro Metabolic Stability of ALC-0159 in Hepatocytes	Mouse (CD-1/ICR), rat (Sprague Dawley and Wistar Han), monkey (Cynomolgus), and human hepatocytes	ALC-0159.	In vitro	[redacted]	01049-[redacted]
Biotransformation of ALC-0159 and ALC-0315 In Vitro and In Vivo in Rats	In vitro: CD-1 mouse, Wistar Han rat, cynomolgus monkey, and human blood, liver S9 fractions and hepatocytes In vivo: male Wistar Han rats	ALC-0315 and ALC-0159	In vitro or IV (in vivo in rats)	Pfizer thin	PF-07302048_05 [redacted]_043725

2.6.5.1. PHARMACOKINETICS OVERVIEW Test Article: BNT162b2

Type of Study	Test System	Test item	Method of Administration	Testing Facility	Report Number

ALC-0159 = 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide), a proprietary polyethylene glycol-lipid included as an excipient in the LNP formulation used in BNT162b2; ALC-0315 = (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate), a proprietary aminolipid included as an excipient in the LNP formulation used in BNT162b2; IM = Intramuscular; IV = Intravenous; LNP = lipid nanoparticles; S9 = Supernatant fraction obtained from liver homogenate by centrifuging at 9000
g. a. La Jolla, California.
b. , Germany.
c. [redacted]
, U[redacted]
Ch[redacted]ton, Connecticut.

2.6.5.3. PHARMACOKINETICS:

Test Article: modRNA encoding luciferase in LNP Report Number: PF-07302048_06 _072424 [redacted]

PHARMACOKINETICS AFTER A SINGLE DOSE

Species (Strain)	Rat (Wistar Han)	
Sex/Number of Animals	Male/ 3 animals per timepointa	
Feeding Condition	Fasted	
Method of Administration	IV	
Dose modRNA (mg/kg)	1	
How to LC-0159 (MG / KG)	1.96	
How do you have LC-0315 (MG / KG)	15.3	
Sample Matrix	Plasma, liver, urine and feces	
Sampling Time Points (h post dose):	Predose, 0.1, 0.25, 0.5, 1, 3, 6, 24, 48, 96, 192, 336	
Analyte	ALC-0315.	ALC-0159.
PK Parameters:	Meanb	Meanb
AUCinf (µg•h/mL)c	1030	99.2
Aaclast (µg • h / ml)	1020	98.6
Initial t½ (h)d	1.62	1.74
Terminal elimination t½ (h)e	139	72.7
Estimated fraction of dose distributed to liver (%)f	59.5	20.3
Dose in Urine (%)	Ncg	Ncg
Dose in Feces (%)h	1.05	47.2

ALC-0159 = 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide), a proprietary polyethylene glycol-lipid included as an excipient in the LNP formulation used in BNT162b2; ALC-0315 = (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate), a proprietary aminolipid included as an excipient in the LNP formulation used in BNT162b2; AUCinf = Area under the plasma drug concentration-time curve from 0 to infinite time; AUClast = Area under the plasma drug concentration-time curve from 0 to the last quantifiable time point; BLQ = Below the limit of quantitation; LNP = Lipid nanoparticle; modRNA = Nucleoside modified messenger RNA; PK = Pharmacokinetics; t½ = Half-life.
a. Non-serial sampling, 36 animals total.
b. Only mean PK parameters are reported due to non-serial sampling.
c. Calculated using the terminal log-linear phase (determined using 48, 96, 192, and 336 h for regression calculation).
d. ln(2)/initial elimination rate constant (determined using 1, 3, and 6 h for regression calculation).
e. ln(2)/terminal elimination rate constant (determined using 48, 96, 192, and 336 h for regression calculation).
f. Calculated as follows: highest mean amount in the liver (µg)/total mean dose (µg) of ALC-0315 or ALC-0159. g. Not calculated due to BLQ data. h. Fecal excretion, calculated as: (mean µg of analyte in feces/ mean µg of analyte administered) × 100

2.6.5.5A. PHARMACOKINETICS: ORGAN DISTRIBUTION

Test Article: modRNA encoding luciferase in LNP Report Number: R- -0072 [redacted]

Species (Strain):	Mice (BALB/c)
Sex/Number of Animals:	Female/3 per group
Feeding Condition:	Fed adlibitum
Vehicle/Formulation:	Phosphate-buffered saline
Method of Administration:	Intramuscular injection
Dose (mg/kg):	1 µg/hind leg in gastrocnemius muscle (2 µg total)
Number of Doses:	1
Detection:	Bioluminescence measurement
Sampling Time (hour):	6, 24, 48, 72 hours; 6 and 9 days post-injection

Time point	Total Mean Bioluminescence signal (photons/second)		Mean Bioluminescence signal in the liver (photons/second)
	Buffer control	modRNALuciferase in LNP	modRNALuciferase in LNP
6 hours	1.28 x 105	1.26 × 10 9	4.94 × 107
24 hours	2.28 x 105	7.31 × 10 8	2.4 × 10 6
48 hours	1.40 × 10 5	2.10 × 10 8	Below detectiona
72 hours	1.33 × 10 5	7.87 × 107	Below detectiona
6 days	1.62 × 105	2.92 × 10 6	Below detectiona
9 days	7.66 × 10 4	5.09 x 105	Below detectiona

LNP = Lipid nanoparticle; modRNA = Nucleoside modified messenger RNA.
a. At or below the background level of the buffer control.

2.6.5.5B. PHARMACOKINETICS: ORGAN DISTRIBUTION CONTINUED

Test Article: [3H]-Labelled LNP-mRNA formulation containing ALC-0315 and ALC-0159

Report Number: 185350

Species (Strain):	Rat (Wistar Han)
Sex/Number of Animals:	Male and female/3 animals/sex/timepoint (21 animals/sex total for the 50 µg dose)
Feeding Condition:	Fed adlibitum
Method of Administration:	Intramuscular injection
Please:	50 µg [3H]-08-A01-C0 (lot # NC-0552-1)
Number of Doses:	1
Detection:	Radioactivity quantitation using liquid scintillation counting
Sampling Time (hour):	0.25, 1, 2, 4, 8, 24, and 48 hours post-injection

Sample	Mean total lipid concentration (µg lipid equivalent/g (or mL) (males and females combined)							% of administered dose (males and females combined)						
	0.25 h	1 h	2 h	4 h	8 h	24 h	48 h	0.25 h	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	0.271	1.48	2.72	2.89	6.80	13.8	18.2	0.001	0.007	0.010	0.015	0.035	0.066	0.106
Bladder	0.041	0.130	0.146	0.167	0.148	0.247	0.365	0.000	0.001	0.001	0.001	0.001	0.002	0.002
Bone (femur)	0.091	0.195	0.266	0.276	0.340	0.342	0.687	-	-	-	-	-	-	-
Bone marrow (femur)	0.479	0.960	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	0.007	0.013	0.020	0.016	0.011	0.010	0.009
Eyes	0.010	0.035	0.052	0.067	0.059	0.091	0.112	0.000	0.001	0.001	0.002	0.002	0.002	0.003
Heart	0.282	1.03	1.40	0.987	0.790	0.451	0.546	0.018	0.056	0.084	0.060	0.042	0.027	0.030
Injection site	128	394	311	338	213	195	165	19.9	52.6	31.6	28.4	21.9	29.1	24.6
Kidneys	0.391	1.16	2.05	0.924	0.590	0.426	0.425	0.050	0.124	0.211	0.109	0.075	0.054	0.057
Large intestine	0.013	0.048	0.093	0.287	0.649	1.10	1.34	0.008	0.025	0.065	0.192	0.405	0.692	0.762
Liver	0.737	4.63	11.0	16.5	26.5	19.2	24.3	0.602	2.87	7.33	11.9	18.1	15.4	16.2
Lung	0.492	1.21	1.83	1.50	1.15	1.04	1.09	0.052	0.101	0.178	0.169	0.122	0.101	0.101

2.6.5.5B. PHARMACOKINETICS: ORGAN DISTRIBUTION CONTINUED

Test Article: [3H]-Labelled LNP-mRNA formulation containing ALC-0315 and ALC-0159 Report Number: 185350

Sample	Total Lipid concentration (µg lipid equivalent/g [or mL]) (males and females combined)							% of Administered Dose (males and females combined)						
	0.25 h	1 h	2 h	4 h	8 h	24 h	48 h	0.25 h	1 h	2 h	4 h	8 h	24 h	48 h
Lymph (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	1.37	-	-	-	-	-	-	-
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	12.3	0.001	0.009	0.008	0.016	0.025	0.037	0.095
Pancreas	0.081	0.207	0.414	0.380	0.294	0.358	0.599	0.003	0.007	0.014	0.015	0.015	0.011	0.019
Pituitary gland	0.339	0.645	0.868	0.854	0.405	0.478	0.694	0.000	0.001	0.001	0.001	0.000	0.000	0.001
Prostate (males)	0.061	0.091	0.128	0.157	0.150	0.183	0.170	0.001	0.001	0.002	0.003	0.003	0.004	0.003
Salivary glands	0.084	0.193	0.255	0.220	0.135	0.170	0.264	0.003	0.007	0.008	0.008	0.005	0.006	0.009
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	1.28	1.30	1.47	0.024	0.130	0.319	0.543	0.776	0.906	0.835
Spinal cord	0.043	0.097	0.169	0.250	0.106	0.085	0.112	0.001	0.002	0.002	0.003	0.001	0.001	0.001
Spleen	0.334	2.47	7.73	10.3	22.1	20.1	23.4	0.013	0.093	0.325	0.385	0.982	0.821	1.03
Stomach	0.017	0.065	0.115	0.144	0.268	0.152	0.215	0.006	0.019	0.034	0.030	0.040	0.037	0.039
Tests (Males)	0.031	0.042	0.079	0.129	0.146	0.304	0.320	0.007	0.010	0.017	0.030	0.034	0.074	0.074
Thymus	0.088	0.243	0.340	0.335	0.196	0.207	0.331	0.004	0.007	0.010	0.012	0.008	0.007	0.008
Thyroid	0.155	0.536	0.842	0.851	0.544	0.578	1.00	0.000	0.001	0.001	0.001	0.001	0.001	0.001
Uterus (females)	0.043	0.203	0.305	0.140	0.287	0.289	0.456	0.002	0.011	0.015	0.008	0.016	0.018	0.022
Whole blood	1.97	4.37	5.40	3.05	1.31	0.909	0.420	-	-	-	-	-	-	-
Plasma	3.97	8.13	8.90	6.50	2.36	1.78	0.805	-	-	-	-	-	-	-
Blood: plasma ratio	0.815	0.515	0.550	0.510	0.555	0.530	0.540	-	-	-	-	-	-	-

2.6.5.5B. PHARMACOKINETICS: ORGAN DISTRIBUTION CONTINUED

Test Article: [3H]-Labelled LNP-mRNA formulation containing ALC-0315 and ALC-0159 Report Number: 185350

-- = Not applicable, partial tissue taken; [3H]-08-A01-C0 = An aqueous dispersion of LNPs, including ALC-0315, ALC-0159, distearoylphosphatidylcholine, cholesterol, mRNA encoding luciferase and trace amounts of radiolabeled [Cholesteryl-1,2-3H(N)]-Cholesteryl Hexadecyl Ether, a nonexchangeable, non-metabolizable lipid marker used to monitor the disposition of the LNPs; ALC-0159 = 2-[(polyethylene glycol)-2000]-N,N--ditetradecylacetamide), a proprietary polyethylene glycol-lipid included as an excipient in the LNP formulation used in BNT162b2; ALC-0315 = (4--hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate), a proprietary aminolipid included as an excipient in the LNP formulation used in BNT162b2; LNP = Lipid nanoparticle; mRNA = messenger RNA.
a. The mean male and female blood:plasma values were first calculated separately and this value represents the mean of the two values.

2.6.5.9. PHARMACOKINETICS: METABOLISM IN VIVO, RAT

Test Article: modRNA encoding luciferase in LNP Report Number: PF-07302048_05 _043725 [redacted]

Species (Strain):	Rat (Wistar Han)
Sex/ Number of animals	Male/ 36 animals total for plasma and liver, 3 animals for urine and feces
Method of Administration:	Intravenous
Dose (mg/kg):	1
Test System:	Plasma, Urine, Feces, Liver
Analysis Method:	Ultrahigh performance liquid chromatography/ mass spectrometry

Biotransformation	m/z	Metabolites of ALC-0315 Detected			
		Plasma	Urine	Feces	Liver
N-dealkylation, oxidation	102.0561a	ND	ND	ND	ND
N-Dealkylation, oxidation	104.0706 b	ND	ND	ND	ND
N-dealkylation, oxidation	130.0874	ND	ND	ND	ND
N-Dealkylation, oxidation	132.1019b	ND	ND	ND	ND
N-dealkylation, hydrolysis, oxidation	145.0506a	ND	ND	ND	ND
Hydrolysis (acid)	Brother .2330	+	ND	ND	ND
Hydrolysis, hydroxylation	271. Investing	ND	ND	ND	ND
Bis-Hydrolysis (Amine)	290.2690 b	+	+	+	+
Hydrolysis, glucuronidation	431.2650a	ND	ND	ND	ND
Bis-hydrolysis (amines), glucuronidation	464.2865a	ND	ND	ND	ND
Bis-hydrolysis (amines), glucuronidation	466.3011b	ND	+	ND	ND
Hydrolysis (amine)	528.4986 b	+	ND	ND	+
Hydrolysis (amine), Glucuronidation	704.5307 b	ND	ND	ND	ND
Otachi and Ashi D	778.6930a	ND	ND	ND	ND
Otachi and Ashi D	780.7076 b	ND	ND	ND	ND
Hydroxylation	Achieve.	ND	ND	ND	ND
Sulfation	844.6706	ND	ND	ND	ND
Sulfation	846.6851b	ND	ND	ND	ND
Glucuronidation	940.7458	ND	ND	ND	ND
Glucuronidation	942.7604 b	ND	ND	ND	ND

Note: Both theoretical and observed metabolites are included.
m/z = mass to charge ratio; ND = Not detected; + = minor metabolite as assessed by ultraviolet detection.
a. Negative ion mode.
b. Positive ion mode.

2.6.5.10A. PHARMACOKINETICS: METABOLISM IN VITRO

Test article: alc-0315
Report Numbers: 01049- 008
01049-00[redacted]
01049-01[redacted]

Type of Study:	Stability of ALC-0315 In Vitro		
Study System:	Liver Microsomes + NADPH	S9 Fraction + NADPH, UDPGA, and alamethicin	Hepatocytes
ALC-0315 Concentration:	1 µM	1 µM	1 µM
Duration of Incubation (min):	120 min	120 min	240 min
Analysis Method:	Ultra-high performance liquid chromatography-tandem mass spectrometry		

Incubation time (min)	Percent ALC-0315 remaining														
	Liver Microsomes					Liver Said Frazy				Hepatocytes					
	Mouse (CD-1/ICR)	Rat (SD)	Rat (WH)	Monkey (Cyno)	Human	Mouse (CD-1 / ICR)	Rat (SD)	Monkey (Cyno)	Human	Mouse (CD-1 / ICR)	Rat (SD)	Rat (WH)	Monkey (Cyno)	Human	
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
15	98.77	94.39	96.34	97.96	100.24	97.69	98.85	99.57	95.99	-	-	-	-	-	
30	97.78	96.26	97.32	96.18	99.76	97.22	99.62	96.96	97.32	101.15	97.75	102.70	96.36	100.72	
60	100.49	99.73	98.54	100.00	101.45	98.61	99.62	99.13	94.98	100.77	98.50	102.32	98.21	101.44	
90	97.78	98.66	94.15	97.96	100.48	98.15	98.85	98.70	98.33	101.92	99.25	103.09	100.01	100.36	
120	96.54	95.99	93.66	97.71	98.31	96.76	98.46	99.57	99.33	98.85	97.38	99.61	96.36	100.72	
180	-	-	-	-	-	-	-	-	-	101.15	98.88	103.47	95.64	98.92	
240	-	-	-	-	-	-	-	-	-	99.62	101.12	100.00	93.82	99.64	
t½ (min)	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 240	> 240	> 240	> 240	> 240	

-- = Data not available; ALC-0315 = (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate), a proprietary aminolipid included as an excipient in the lipid nanoparticle formulation used in BNT162b2; Cyno = Cynomolgus; NADPH = Reduced form of nicotinamide adenine dinucleotide phosphate; NC = not calculated; SD = Sprague Dawley; t½ = half-life; WH = Wistar-Han; UDPGA= uridine-diphosphate-glucuronic acid trisodium salt.

2.6.5.10B. PHARMACOKINETICS: METABOLISM IN VITRO
CONTINUED

Test article: alc-0159
Report Numbers: 01049- 020 01049-021
01049-02[redacted]

Type of Study:	Stability of ALC-0159 In Vitro		
Study System:	Liver Microsomes + NADPH	S9 Fraction + NADPH, UDPGA, and alamethicin	Hepatocytes
ALC-0159 Concentration:	1 µM	1 µM	1 µM
Duration of Incubation (min):	120 min	120 min	240 min
Analysis Method:	Ultra-high performance liquid chromatography-tandem mass spectrometry		

Incubation time (min)	Percent ALC-0159 remaining													
	Liver Microsomes					Liver Said Frazy				Hepatocytes				
	Mouse (CD-1/ICR)	Rat (SD)	Rat (WH)	Monkey (Cyno)	Human	Mouse (CD-1 / ICR)	Rat (SD)	Monkey (Cyno)	Human	Mouse (CD-1 / ICR)	Rat (SD)	Rat (WH)	Monkey (Cyno)	Human
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
15	82.27	101.24	112.11	100.83	99.59	98.93	84.38	91.30	106.73	-	-	-	-	-
30	86.40	93.78	102.69	85.12	92.28	91.10	90.87	97.96	107.60	100.85	93.37	113.04	90.23	106.34
60	85.54	98.34	105.38	86.36	95.53	102.85	97.97	105.56	104.97	94.92	91.81	105.07	92.93	101.58
90	85.41	95.44	100.90	94.63	97.97	90.75	93.51	108.33	109.36	94.28	90.25	112.80	94.59	92.67
120	95.87	97.10	108.97	93.39	93.09	106.76	92.70	105.74	119.59	87.08	89.47	104.11	97.51	96.04
180	-	-	-	-	-	-	-	-	-	94.92	93.96	102.90	89.81	93.66
240	-	-	-	-	-	-	-	-	-	102.75	94.93	98.79	92.93	102.57
t½ (min)	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 120	> 240	> 240	> 240	> 240	> 240

-- = Data not available; ALC-0159 = 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide), a proprietary polyethylene glycol-lipid included as an excipient in the lipid nanoparticle formulation used in BNT162b2; Cyno = Cynomolgus; NADPH = Reduced form of nicotinamide adenine dinucleotide phosphate; NC = not calculated; SD = Sprague Dawley; WH = Wistar-Han; UDPGA= uridine-diphosphate-glucuronic acid trisodium salt.

2.6.5.10C. PHARMACOKINETICS: METABOLISM IN VITRO CONTINUED

Test article: alc-0315

Report Number: OF-07302048_05 [redacted]_043725

Type of study		Metabolism of ALC-0315 In Vitro											
Study system		Blood				Hepatocytes				Liver Said Frazy			
ALC-0315 concentration		10 µM				10 µM				10 µM			
Duration of incubation		24 h				4 h				24 h			
Analysis Method:		Ultrahigh performance liquid chromatography/ mass spectrometry											
Biotransformation	m/z	Blood				Hepatocytes				Liver Said Frazy			
		Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human
N-dealkylation, oxidation	102.0561a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-Dealkylation, oxidation	104.0706 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-dealkylation, oxidation	130.0874	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-Dealkylation, oxidation	132.1019b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-dealkylation, hydrolysis, oxidation	145.0506a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis (acid)	Brother .2330	+	+	ND	ND	+	+	+	+	+	+	ND	+
Hydrolysis, hydroxylation	271. Investing	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bis-Hydrolysis (Amine)	290.2690 b	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Hydrolysis, glucuronidation	431.2650a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bis-hydrolysis (amines), glucuronidation	464.2865a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bis-hydrolysis (amines), glucuronidation	466.3011b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis (amine)	528.4986 b	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Hydrolysis (amine), glucuronidation	704.5307 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Otachi and Ashi D	778.6930a	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Otachi and Ashi D	780.7076 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxylation	Achieve.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulfation	844.6706	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulfation	846.6851b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucuronidation	940.7458	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucuronidation	942.7604 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: Both theoretical and observed metabolites are included.
m/z = mass to charge ratio; ND = Not detected; + = metabolite present.
a. Negative ion mode.
b. Positive ion mode.

2.6.5.10D. PHARMACOKINETICS: METABOLISM IN VITRO CONTINUED

Test article: alc-0159

Report Number: OF-07302048_05 [redacted]_043725

Type of study		Metabolism of ALC-0159 In Vitro											
Study system		Blood				Hepatocytes				Liver Said Frazy			
ALC-0159 concentration		10 µM				10 µM				10 µM			
Duration of incubation		24 h				4 h				24 h			
Analysis Method:		Ultrahigh performance liquid chromatography/ mass spectrometry											
Biotransformation	m/z	Blood				Hepatocytes				Liver Said Frazy			
		Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human
Oh, it's THY ACON, LKY	107.0703 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Oh, it's THY ACON, LKY	151.0965b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Oh, it's THY ACON, LKY	195.1227 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis, N-Dealkylation	214. Stere	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-Dealkylation, oxidation	227.2017	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis (amine)	410.4720b	+	+	ND	ND	+	+	+	+	+	+	+	+
N, Lky	531.5849 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
N-Dealkylation	580. Step	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Oh, THY AICO, OY	629. Greatness	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxylation	633.6931 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ω-Hydroxylation, Oxidation	637.1880b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis (acid)	708.7721 b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: Both theoretical and observed metabolites are included.
m/z = mass to charge ratio; ND = Not detected; + = metabolite present.
a. Negative ion mode.
b. Positive ion mode.

Exhibit Note

This is the attachment marked "Exhibit 2" referred to in the Affidavit/Declaration of Byram W. Bridle' Sworn/Confirmed/Declared at Guelph, Ontario, Canada, this 19th day of June 2022, before me:

Signature: PO5896

BioNTech SE
An der Goldgrube 12
55131 Mainz, Germany
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R&D STUDY REPORT No. R-20-0072

EXPRESSION OF LUCIFERASE-ENCODING MODRNA AFTER I.M. APPLICATION OF GMP-READY ACUITAS LIPID NANOPARTICLE FORMULATION

Version 03
Date: 27 Nov 2020

Reported by (b) (6)

Test item: modRNA encoding luciferase
Key words: COVID-19, modRNA, biodistribution, mouse, bioluminescence assay

This R&D report consists of 36 pages.

TABLE OF CONTENTS

LIST OF FIGURES

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LIST OF ABBREVIATIONS

ALC-0159	Proprietary PEG-lipid included as an excipient in the LNP formulation from Acuitas
ALC-0315	Proprietary amino-lipid included as an excipient in the LNP formulation from Acuitas
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AUC	Area under the curve
BALB/c	Mouse strain used in this study
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BNT162	BioNTech's vaccine program against COVID-19
ConA	Concanavalin A
COVID-19	Coronavirus disease 2019
DPBS	Dulbecco's phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
FELASA	Federation of European Laboratory Animal Science Associations
GMP	Good manufacturing practice
HRP	Horseradish peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
i.m.	Intramuscularly
IP-10	Interferon-gamma induced protein 10
IVIS	*In vivo* imaging system
LNP	Lipid nanoparticle
Luc	Luciferase (from firefly *Pyractomena lucifera*)
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein 1β
modRNA	Nucleoside-modified mRNA
NBT	Nitro blue tetrazolium
OD	Optical density
p/s	Photons per second
SD	Standard deviation
SOP	Standard operating procedure
S protein	Spike protein
saRNA	Self-amplifying mRNA
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TNF	Tumor necrosis factor

RESPONSIBILITIES

Person responsible for the study:	(b) (6) (b) (6) BioNTech (b) (6) (b) (6)	26NOV2020 Date
Author:		
	(b) (6) BioNTech SE	Date
Reviewer:	(b) (6) (b) (6) (b) (6) BioNTech (b) (6)	26NOV2020 Date
QA representative:	(b) (6) (b) (6) BioNTech SE	27NOV 2020 Date

Meaning of the signatures:

Person responsible for the study: I am responsible for the content of the R&D report and confirm that it represents an accurate record of the results. This study was performed according to the SOPs and methods as well as the rules and regulations described in the report.

Author: I am the author of this document.

Reviewer: I reviewed the R&D report and confirm that this document complies with the scientific and technical standards and requirements.

QA representative: I confirm that this document complies with the relevant quality assurance requirements.

Approval of the author via e-mail according to CC-20-0081 (see attachment).
e-g (b) (6) 27NOV2020

1 SUMMARY

BioNTech is developing RNA-based vaccines designed to protect against the novel coronavirus disease that emerged in 2019 (COVID-19). The BNT162 project involves testing three RNA platforms, which are under development at BioNTech with the surface or spike protein (S protein) of the novel coronavirus (SARS-CoV-2) as the viral antigen. These RNAs will be formulated with a GMP-compatible LNP formulation provided by Acuitas.

In this study, the GMP-ready formulation containing the amino-lipid ALC-0315 and the PEG-lipid ALC-0159 (in this report referred to as LNP 8 which is the identical composition as used in BNT162) was tested in comparison with a (b) (4) (b) (4) by Acuitas, (b) (4) and an in-house formulation, (b) (4) to characterize the biodistribution of luciferase expressed by LNP-formulated nucleoside-modified mRNA (modRNA). Activation of the innate immune system, formation of antibodies against luciferase, and T-cell activation were also assessed.

Four groups of three BALB/c mice were injected intramuscularly (i.m.) with a total dose of 2 µg/animal of (b) (4) LNP8- or (b) (4) -formulated modRNA encoding luciferase or with buffer (DPBS) as control. At 6 h, 24 h, 48 h, 72 h, 6 d, and 9 d after injection, the *in vivo* luciferase expression was measured by luciferin application. Serum samples were taken 1 day before and 6 h after immunization as well as on day 9 for quantification of the activation of the innate immune system in a Luminex-based multiplex assay and antigen-binding antibody analysis in an IgG-specific ELISA. Splenocytes were isolated on 9 d to assess the T-cell response by IFN-γ ELISpot Assay. Doses of RNA in the other biodistribution study were 50 and 100 µg mRNA/animal.

An approximately 20-fold higher luciferase expression at the injections site was observed for modRNA-Luciferase (b) (4) and the GMP-ready modRNA-Luciferase LNP8 when compared to modRNA formulated with (b) (4) The difference between the area under the curve for (b) (4) and LNP8-formulated modRNA compared to buffer control as well as to (b) (4) formulated modRNA was statistically significant. In addition, luciferase expressed from the (b) (4) -formulated modRNA showed limited drainage to the liver compared to LNP8-formulated modRNA.

A multiplex assay showed that the innate immune system was temporally activated by (b) (4) and LNP8-formulated modRNA. The activation was more pronounced for (b) (4) formulated modRNA than for modRNA formulated with LNP8, indicating a formulation-related effect rather than a payload or expression level effect.

Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9. However, a strong antigen-specific IFN-γ T-cell response was measured by ELISpot assay on day 9 for (b) (4) and LNP8-formulated modRNAs, with statistically significant differences between these test groups, the buffer control, and the (b) (4) group.

In conclusion, despite different biodistribution characteristics, both Acuitas LNPs allowed a high antigen expression level thereby inducing a strong T- but not B-cell response on day 9 post immunization.

(b) (6)	26 NOV 2020
Responsible Person: (b) (6) BioNTech (b) (6) (b) (6)	Date

2 GENERAL INFORMATION

2.1 Sponsor and Test Facilities

Sponsor

BioNTech RNA Pharmaceuticals GmbH
An der Goldgrube 12
55131 Mainz
Germany

Test Facility

BioNTech SE
An der Goldgrube 12
55131 Mainz
Germany

2.2 Participating Personnel

Responsible person: (as defined in SOP-100-024)	(b) (6) (b) (6) BioNTech (b) (6)
Author:	(b) (6) BioNTech SE
Experimenter: Immunization, blood sampling, ELISA, ELISpot	(b) (6) BioNTech (b) (6)
Experimenter: Luminex-based multiplex assay	(b) (6) BioNTech (b) (6)

2.3 Study Dates

Start of experiments: 14 JAN 2020

Completion of experiments: 23 JAN 2020

2.4 Guidelines and Regulations

All experiments are executed in accordance with the existing standard operating procedures and described processes from BioNTech SE. Applicable documents are listed below.

- Animal test application approval number: G18-12-007
- SOP-030-071 Abtöten von Mäusen
- SOP-030-072 Fixiergriff und Ohrmarkierung bei Mäusen
- SOP-030-073 Betäubung bei Mäusen
- SOP-030-074 Blutentnahme bei Mäusen
- SOP-030-078 Isolierung muriner Splenozyten
- SOP-030-079 Intramuskuläre Applikation bei Mäusen
- SOP-030-110 IFN-γ ELISpot murin
- SOP-090-013 Biological safety in laboratories

2.5 Changes and Deviations

Not applicable. There is no formal R&D plan available.

2.6 Documentation and Archive

Study reports are stored and archived according to SOP-100-003 Archiving of Paper-Based Documents.

Raw data and evaluated data are saved at:

- P:\BioNTechRNA\RN_R0030_AIRVAC\24_Preclinic\01_Vakzine Testing in vivo Luc\IM#88 GMP ready LNP Acuitas modRNA
- Lab books:
    - No. 1455 (complete study plan including results)
    - No. 1835 (IVIS images and quantification, Luciferase ELISA, ELISpot)
    - No. 1593 page 71-84 (Luminex-based multiplex assay)

3 INTRODUCTION

3.1 Background

In December 2019, an outbreak of pneumonia of unknown cause in Wuhan, Hubei province in China started. The disease spread rapidly and in January 2020, the agent was identified. By 1 April 2020, infection with the novel coronavirus (SARS-CoV-2) was confirmed in approximately 820,000 people with more than 40,000 casualties[1]. A vaccine is urgently needed and BioNTech decided to develop a rapid vaccine project (BNT162) with the surface or spike protein (S protein) of the virus as the viral antigen.

The development of *in vitro* transcribed RNA as an active platform for the use in infectious disease vaccines is based on the extensive knowledge of the company in RNA technology, which has been gained over the last decade. The core innovation is based on *in vivo* delivery of a pharmacologically optimized, antigen-coding RNA vaccine to induce robust neutralizing Abs and accompanying/concomitant T-cell responses to achieve protective immunization with minimal vaccine doses (Vogel et al. 2017, Moyo et al. 2018, Pardi et al. 2017).

At BioNTech, three different RNA platforms formulated with lipid nanoparticles (LNPs) are under development, namely non-modified uridine-containing mRNA (uRNA), nucleoside-modified mRNA (modRNA) and self-amplifying RNA (saRNA). In the present study, an LNP-formulated modRNA encoding luciferase was used representatively to investigate the *in vivo* biodistribution and the immune response of the vaccine candidates.

LNP formulations from a third party provider (Acuitas) were tested in comparison to the in-house formulation (b) (4) Acuitas (b) (4) (b) (4) (b) (4) Acuitas also provided an LNP formulation that is cGMP-ready, namely LNP8, which contains two proprietary lipids (ALC-0159 and ALC-0315) and has the identical composition as the LNP formulation used in the BNT162 program.

3.2 Objectives

The objective of this study was to investigate the biodistribution of luciferase expressed by the LNP-formulated modRNA using bioluminescence measurements in BALB/c mice, as well as innate immune system activation, formation of antibodies against luciferase and T-cell activation.

[1] Coronavirus disease (COVID-2019) situation report 72, World Health Organization; /www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports

3.3 Study Design

1/50th and 1/100th the dose of the other study in rats. Rats were ~220 g. Mice ~20 g or 1/11th the weight. Therefore, this dose is effectively ~4.5x lower.

Four groups of three BALB/c mice were injected intramuscularly (i.m.) in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or with buffer as control on day 0. At 6 h, 24 h, 48 h, 72 h, 6 d, and 9 d after injection, the *in vivo* luciferase expression was measured by luciferin application.

In addition, serum samples were collected on day -1 and 6 h and 9 d post immunization and cytokine/chemokine level determination (multiple) and on day 1 and 9 for luciferase-specific ELISA. On day 9, spleens were resected for immunological analysis using IFN-γ ELISpot assays.

Dose was unclear in text. Dose was ~2.25 lower in this expt. (2 ug/mouse)

Table 1: Study design

Group	Treatment	Dose [µg/ mouse]	Formulation	Treatment schedule	End of experiment	Sample collection
1	Buffer control	N/A	N/A	Day 0	Day 9	Serum ELISA on days -1 and 9, serum for Multiplex assay on day -1, 6 h, and 9 d; splenocytes for ELISpot on day 9
2	modRNA-Luciferase (b) (4)	2 µg (1 µg/leg)	(b) (4)	Day 0	Day 9	
3	modRNA-Luciferase (b) (4)	2 µg (1 µg/leg)	Acuitas proprietary	Day 0	Day 9	
4	modRNA-Luciferase LNP8 (GMP-ready)	2 µg (1 µg/leg)	ALC-0315:ALC-0159:DSPC:Chol	Day 0	Day 9	

4 MATERIALS AND METHODS

4.1 Test Item

- LNP-formulated modRNA encoding luciferase diluted to 0.05 mg/mL to obtain a dose of 1 µg in 20 µL application volume. For CoAs see Appendix 2: Certificates of Analysis of RNA and LNPs.
- Acuitas LNPs:
  - (b) (4)
  - LNP8 modRNA Luc, RNA-EH190611-01c, batch FM-1074-D, 90% encapsulation, 1.0 mg/mL encapsulated RNA, diameter 71 nm, polydispersity 0.053, storage temperature -80°C.
- BioNTech LNP:
  - (b) (4)

Table 2: Lipid component formulations

Formulation	Lipids			
	Functional lipid 1	Functional lipid 2	Structural lipid 1	Structural lipid 2
(b) (4)	Acuitas proprietary	Acuitas proprietary	Acuitas proprietary	Acuitas proprietary
LNP8	ALC-0315	ALC-0159	DSPC	Cholesterol
(b) (4)	(b) (4)	(b) (4)	(b) (4)	(b) (4)

4.2 Control Item

- DPBS

4.3 Test System

- *Mus musculus*: 12 female BALB/c mice at an age of 9 weeks at study start with a body weight of approximately 25 g

Now can calculate an even more accurate dose equivalency (compared to rat study) b/c the body weights are indicated here (25 g, not 20 g). So, rats were ~225 g so mice were ~9x smaller. Thus, the equivalent rat dose would have been ~5.6 ug or 11.1 ug / animal. Therefore the dose used here is ~2.8x lower.

4.4 Materials

Table 3: Materials

Product name	Application/specification	Article no.	Working dilution	Provider
Dulbecco's phosphate-buffered saline (DPBS)	Buffer control	14190-094	1×	Thermo Fisher Scientific
Syringes 0.3 mL 30 G	Insulin syringes for i.m. application	4144150	N/A	BD
Syringes 0.5 mL 29 G	Insulin syringes for i.p. application	324824	N/A	BD
Luciferin	Substrate for *in vivo* luciferase imaging	122799-10	150 mg/kg	Perkin-Elmer
Microvette 500 Z-gel tubes	Blood sampling	2021-01-31	N/A	Sarstedt
Xenogen IVIS® Spectrum	*In vivo* BLI imager	-	N/A	Caliper Life Sciences
MaxiSorp plate	ELISA	439454	N/A	Thermo Fisher Scientific
QuantiLum recombinant luciferase	Positive control	E1701	100 ng/µL	Promega
Casein blocking buffer	10×	B6429	1×	Sigma-Aldrich
TMB ONE ECO-TEK	Chromogenic substrate for horseradish peroxidase	4380H	N/A	BIOTREND
Sodium bicarbonate	7.5% $NaHCO_3$	S8761-100 ml	N/A	Sigma-Aldrich
HCl	Hydrochloric acid solution volumetric, 0.1 M HCl	2104-50 ml	N/A	Sigma-Aldrich
RPMI1640 medium	Cell culture medium	61870	N/A	Gibco
Biotek Epoch	ELISA plate reader	-	N/A	Biotek
Mouse IFN-γ ELISpotPLUS kit	Kit for enumeration of cells secreting mouse IFN-γ	3321-4APT-2	N/A	Mabtech
ImmunoSpot® S5 Versa Analyzer	ELISpot plate reader	-	N/A	Cellular Technology Ltd.
RPMI1640 medium	Cell culture medium	61870	N/A	Gibco
Multiplex	PROCARTAPLEX 10 PLEX	PPX-10 - MXU63C9	N/A	Life Technologies GmbH
Bio-Plex 200	Multiplex reader	-	N/A	Bio-Rad
Anti-firefly luciferase antibody (mAb21),	Assay control ELISA	ab64564	1:1,000	Abcam
Mouse IgG isotype	Assay control ELISA	0107-08	1:100 as starting dilution	Southern Biotech
Goat anti-mouse IgG HRP	Secondary antibody ELISA	115-035-071	1:15,000 (if stored in 50% glycerol 1:7,500)	Jackson

Table 4: Software

Product name	Application	Provider
Living image	*In vivo* BLI quantification	Perkin-Elmer
Prism	Analysis	GraphPad Software Inc.
Excel	Animal monitoring, raw data ELISA	Microsoft Corp.
Bio-Plex Manager (6.1)	Bio-Plex reader	Bio-Rad
Gen5	Absorbance reader	Biotek
ImmunoCapture V6.3	ELISpot analysis	Cellular Technology Ltd.

Table 5: Peptide pool for stimulation of splenocytes

Peptide pool MHC-I	
Name	**Sequence**
Firefly luciferase-1	GFQSMYTFV
Firefly luciferase-2	VPFHHGFGM
Firefly luciferase-3	VALPHRTAC

Consensus sequences from Limberis et al. 2009

4.5 Methods

4.5.1 Animal Care

4.5.1.1 General Information

BALB/c mice were delivered at the age of at least six weeks. Delivered mice were used for experiments after approximately one week of acclimatization. All experiments and protocols were approved by the local authorities (local welfare committee), conducted according to the FELASA recommendations and in compliance with the German animal welfare act and Directive 2010/63/EU. Only animals with an unobjectionable health status were selected for testing procedures.

All animals were registered upon arrival in the lab animal colony management system PyRAT (Scionics Computer Innovation GmbH, Dresden, Germany) and tracked until death. Each cage was labeled with a cage card indicating the mouse strain, gender, date of birth, and number of animals per cage. At the start of an experiment additional information was added such as the project and license number, the start of the experiment and details on interventions. Where necessary for identification, animals were arbitrarily numbered with earmarks.

4.5.1.2 Housing Condition and Husbandry

Mice were housed at BioNTech SE's animal facility under barrier and SPF conditions (An der Goldgrube 12, 55131 Mainz) in individually ventilated cages (Sealsafe GM500 IVC Green Line, TECNIPLAST, Hohenpeißenberg, Germany; 500 cm²) with a maximum of five animals per cage. The temperature and relative humidity in the cages and animal unit was kept at 20-24°C and 45-55%, respectively, and the air change (AC) rate in the cages at 75 AC/hour. The cages with dust-free bedding made of

debarked chopped aspen wood (Abedd LAB & VET Service GmbH, Vienna, Austria, product code: LTE E-001) and additional nesting material were changed weekly. Autoclaved sniff M-Z food (sniff Spezialdiäten GmbH, Soest, Germany; product code: V1124) and autoclaved water (tap water) were provided *ad libitum* and changed at least once weekly. All materials were autoclaved prior to use.

4.5.2 Animal Monitoring

Routine animal monitoring wa carried out daily and included in pection for dead animals and control of food and water supplies. Each animal's health was closely assessed at least once weekly. The general physical condition was assessed with regard to the following parameters:

- Body weight change
- Macroscopic assessment of activity level/ behavior
- Macroscopic assessment of general discomfort: drop in body temperature determined by touch and by visual inspection of ears and paws. Ears and paws appear pink in a healthy mouse, white in a mouse with discomfort indicated by reduced blood circulation
- Macroscopic assessment of fur condition and appearance of eyes, inspection of body cavities/ fluids
- Macroscopic assessment of irregularities in breathing ability
- Indication of pain
- Macroscopic assessment for signs of automutilation and or fighting

4.5.3 Endpoint of Experiment / Termination Criteria

Animals were euthanized in accordance with §4 of the German animal welfare act and the recommendation of GV-SOLAS by cervical dislocation or by exposure to carbon dioxide. Additionally, termination criteria applied according to the specification within the respective animal test approval as listed below. Body weight losses exceeding 20%, or a high severity level in any of the parameters found in Section 4.5.2 were on their own sufficient reason for immediate euthanasia.

4.5.4 Injection of Test and Control Items

Animals were anesthetized by inhalation of 2.5% isoflurane in oxygen and the injection site (hind leg) was shaved. Buffer or dissolved test item was applied i.m. into the *musculus gastrocnemius* at a volume of 20 µL. All mice received 1 µg in each leg. After injection and a short recovery phase from anesthesia, the animals were observed for any signs of discomfort due to the injection procedure.

4.5.5 Bioluminescence Measurements

The mice were monitored over a period of 9 days using *in vivo* imaging system (IVIS) measurements. Briefly, the Xenogen IVIS® Spectrum device was used for *in vivo* imaging according to the manufacturer's instruction. Approximately 6 h after LNP administration and 5 min prior to imaging, animals were injected for the first time intraperitoneally (i.p.) with luciferin (150 mg/kg, dosing volume: 300 µL, 29 G needle). Mice were anesthetized (2.5% isoflurane/O_2) and placed in the imager, first with the dorsal side exposed and then with the ventral side exposed, and luciferase activity was measured. Images were taken with exposure time and sensitivity set to 1 s, 10 s, and 1 min and bin 2, bin 4, or bin 8, respectively. The dorsal and ventral images were analyzed by visual inspection after aligning of sensitivities of each picture and used for illustration of findings. The images were analyzed using Living Image *in vivo* imaging 3.0 software, where the regions to be quantified (radiance) were drawn manually and calculated automatically (region of interest, ROI), to follow kinetics of the total fluxes (p/s) over time in a GraphPad file.

4.5.6 Blood Sampling via the *Vena Facialis*

Blood was sampled via the *vena facialis* according to SOP-030-074. In short, without prior anesthesia, mice were held tightly and using a lancet, the *v. facialis* was punctured in a precise and short movement. Blood was collected into Microvette 500 Z-gel tubes, subsequently the restraining grip was loosened. Blood samples were centrifuged at 10,000 ×g (room temperature) for 5 min and serum transferred to a pre-labeled 1.5 mL reagent tube before storage at -20°C.

4.5.7 Luminex-based Multiplex Assay (ProcartaPlex Multiplex Immunoassay)

The assay was performed according to manufacturer's protocol. Briefly, magnetic beads were added to the provided 96-well flat bottom plate and the beads were washed (wash buffer 1×) with the help of a hand-held magnetic plate washer. The antigen standard was reconstituted in universal assay buffer (1×), pooled in one tube, the volume adjusted to a final volume of 250 µL, serial diluted (4-fold serial dilution steps), and 50 µL was added to the designated wells. Serum samples were diluted 1:1 with the universal assay buffer and 50 µL added to the wells. The standard was measured in duplicates and the serum samples in triplicates. The plate was incubated on a plate shaker at 500 rpm) for 2 h covered with a black lid. After three wash steps, 25 µL of the ready-to-use detection antibody was added, incubated for 30 min on the shaker and washed three times. Streptavidin-PE (50 µL) were added and the plate incubated for 30 min on the shaker and washed three times. The beads were resuspended in 120 µL reading buffer, the plate was sealed, and data were acquired in the Bio-Plex 200 Luminex system.

4.5.8 Luciferase-specific ELISA

Luciferase-specific IgGs in serum samples obtained on study days 1 and 9 were detected using ELISA. Recombinant luciferase (100 ng/100 µL) protein was utilized to coat MaxiSorp plates at 4°C overnight. Upon washing and blocking using casein-based blocking buffer, serum samples were screened for luciferase-specific antibodies by incubation on plates for 1 h at 37°C. An anti-firefly luciferase antibody (mAb21) as well as a mouse IgG isotype were included as assay controls. Subsequently, plates were incubated with horseradish peroxidase (HRP)-labeled secondary anti-mouse IgG antibody for another 45 min at 37°C before 3, 3', 5, 5'-tetramethylbenzidine (TMB) ONE substrate was applied. Colorimetric detection was monitored and optical density read at 450 nm calculated to a wavelength reference of 620 nm (ΔOD 450–620 nm).

4.5.9 ELISpot Analysis

4.5.9.1 T-cell epitope prediction

The respective peptides for stimulation of splenocytes (Table 5) were used as published by Limberis et al. 2009, where the authors mapped the dominant and minor T-cell epitopes in BALB/c mice (GFQSMYTFV and VPFHHGFGM, VALPHRTAC, respectively) for monitoring cellular responses *in vivo*. No modifications have been added to the published peptides before peptide synthesis by JPT technologies GmbH.

4.5.9.2 Sample Collection and Processing

Spleens were removed on day 9 after euthanizing the mice, and single-cell suspensions were prepared (SOP-030-078). In brief, the removed organs were pressed through a 70 µm cell mesh using the plunger of a syringe to release the cells from the organ into a tube. After washing with PBS the cell pellet was incubated with erythrocyte lysis buffer, washed in PBS, and passed again through a 70 µm cell mesh. Resulting cells were resuspended in medium and counted.

4.5.9.3 IFN-γ ELISpot Assay

The IFN-γ ELISpot assay was used to measure IFN-γ release after *in vitro* stimulation of T cells as an indicator for the induction of antigen-specific T cells. ELISpot analysis was performed using the Mabtech Mouse IFN-γ ELISpot^{PLUS} kit. Isolated splenocytes were seeded to pre-coated ELISpot plates at 5 × 10^5 cells/well in 200 µL medium and stimulated with antigen-specific peptide pools composed of single peptides and a final concentration of 2 µg/mL per peptide, predicted as described in Section 4.5.9.1 overnight in a humidified incubator at 37°C. As peptide controls, splenocytes were incubated with 6 µg/mL of an irrelevant AH1 peptide derived from the endogenous retroviral gene product envelope glycoprotein 70 (gp70; AH1: amino acids 6 to 14). Splenocytes were incubated with medium alone as a negative control or with 2 µg/mL Concanavalin A (ConA) as an internal positive control, confirming the functionality of

the assay. Spots were visualized with a biotin-conjugated anti-IFN-γ antibody followed by incubation with streptavidin-alkaline phosphatase (ALP) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. Spot numbers were counted and analyzed using the ImmunoSpot® S5 Versa ELISpot Analyzer, the ImmunoCaptureTM image acquisition software, and the ImmunoSpot® Analysis software version 5. The quality control (QC) function of ImmunoSpot analysis software was used to limit false positive spot counts. All tests were performed in triplicate and spot counts were summarized as median values for each triplicate.

4.5.10 Statistical Analysis

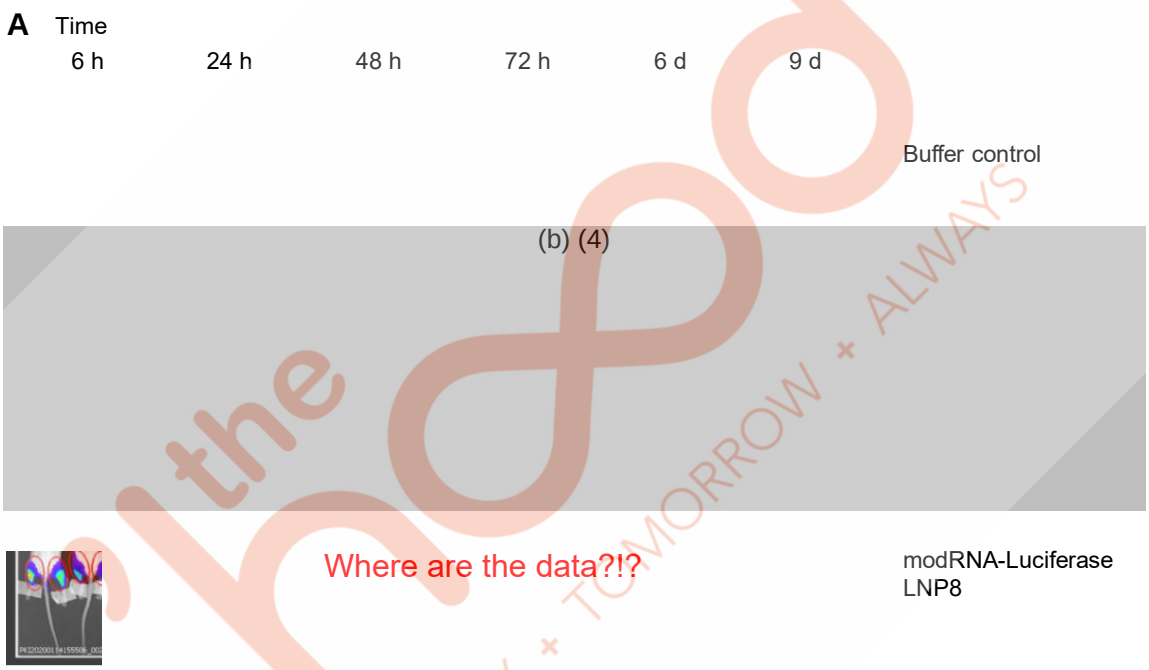
GraphPad Prism 8 software (La Jolla, USA) was used for statistical analysis and figure generation. All groups were compared by a one-way ANOVA with Tukey's multiple comparison post-test on each measurement day (area under the curve for bioluminescence assay, ELISA, and ELISpot analysis).

5 RESULTS

5.1 Bioluminescence Measurements

The biodistribution of luciferase expressed by the LNP-formulated modRNA after i.m. injection was assessed by bioluminescence measurements. Mice received a total dose of 2 µg (b) (4) LNP8, or (b) (4) -formulated modRNA, the control group received 20 µL DPBS only.

Mice were monitored over nine days and Luciferase signal was recorded and quantified (Figure 1).



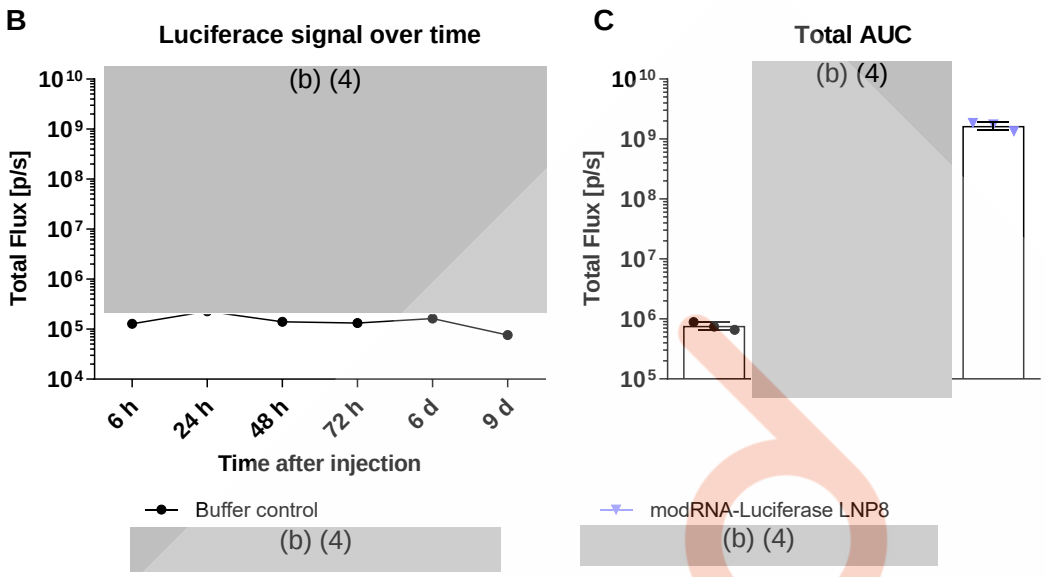


Figure 1: Bioluminescence measurement using the LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or DPBS only. A) At different time points after injection, the luciferase expression *in vivo* was measured by luciferin application. After 9 d, the reporter expression dropped to background levels. B) Quantification of luciferase signal over time and C) as total area under the curve (AUC, ±SD). p/s: photons per second

All formulations resulted in a modRNA-typical expression over time (Figure 1). Highest signal was detected at the first time points after immunization at the injection site and the signal declined slowly over time until day 9 (Figure 1B). Luciferase expressed by the modRNA formulated with Acuitas LNP8 drained to the liver as visualized by luciferase expression at 6 h in the liver region (b) (4) (b) (4) Acuitas (b) (4) formulations (Figure 1A and Figure 2). Group mean luciferase expression from RNA formulated with Acuitas (b) (4) LNP8 in the muscle at 6 h was approximately 1×10^9 p/s, (b) (4) (b) (4) Hence, Acuitas-formulated modRNA started at about 20-fold higher signal levels, stayed more than 20-fold higher until 72 h (~7×10^7 p/s for (b) (4) LNP8 vs (b) (4)) and declined then to a low level (b) (4) (b) (4) on day 9 (~$3–5 \times 10^5$ p/s).

Area under the curve calculation allowed comparing overall expression levels over the course of the experiment (Figure 1C). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison post-test comparing all groups with each other. Total luciferase expression from modRNA formulated with (b) (4) (b) (4) while total luciferase expression from modRNA formulated with (b) (4) LNP8 was approximately (b) (4) 1.5×10^9 p/s, respectively. The difference between the area under the curve for (b) (4) LNP8-formulated modRNA compared to buffer control (b) (4) was

statistically significant ($p < 0.0001$). (b) (4)
(b) (4)

5.2 Liver Expression (b) (4) LNP8

As mentioned in Section 5.1, luciferase expressed by the modRNA formulated with Acuitas LNP8 drained to the liver as visualized by luciferase expression at 6 h in the liver region (b) (4) Acuita (b) (4) (Figure 2A). Here the luciferase signal of modRNA formulated with (b) (4) LNP8 was quantified for better comparison. Group mean luciferase expression from RNA formulated with Acuitas (b) (4) in the liver at 6 h was (b) (4) while luciferase expression of RNA formulated with LNP8 was at about 4.94×10^7 p/s. Hence, luciferase expression from (b) (4) Acuitas (b) (4) (b) (4) compared to LNP8 (Figure 2B). The liver luciferase expression from RNA formulated with Acuitas LNP8 dropped to 2.4×10^6 p/s at 24 h, while the luciferase signal from RNA formulated with Acuitas (b) (4) No liver signal was detected at 48 h post immunization. Statistical analysis was not performed.

Figure 2: Bioluminescence measurement in the liver using the LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase. A) At 6 h, 24 h, and 48 h after injection, the luciferase expression *in vivo* was measured by luciferin application. B) Quantification of luciferase signal in the liver over time (mean ±SD). p/s: photons per second.

5.3 Luminex-based Multiplex Assay

Activation of the innate immune system was assessed in a Luminex-based multiplex assay (Procarta immunoassays). Serum samples (day -1 (pre), 6 h, and day 9) were tested for levels of the following chemokines and cytokines: MCP-1, MIP-1β, TNF-α, IFN-α, IFN-γ, IL-2, IL-6, IL-10, IL-1β, IP-10 (Figure 3). No cytokines/chemokines were detected in the pre-serum. (b) (4)
(b) (4)
(b) (4) Immunization with LNP8 induced slightly increased levels of MCP-1, IL-6, and IP-10 at 6 h post immunization (b) (4)

(b) (4) All chemokine/cytokine levels dropped to background levels at day 9. Statistical analysis was not performed for this assay.

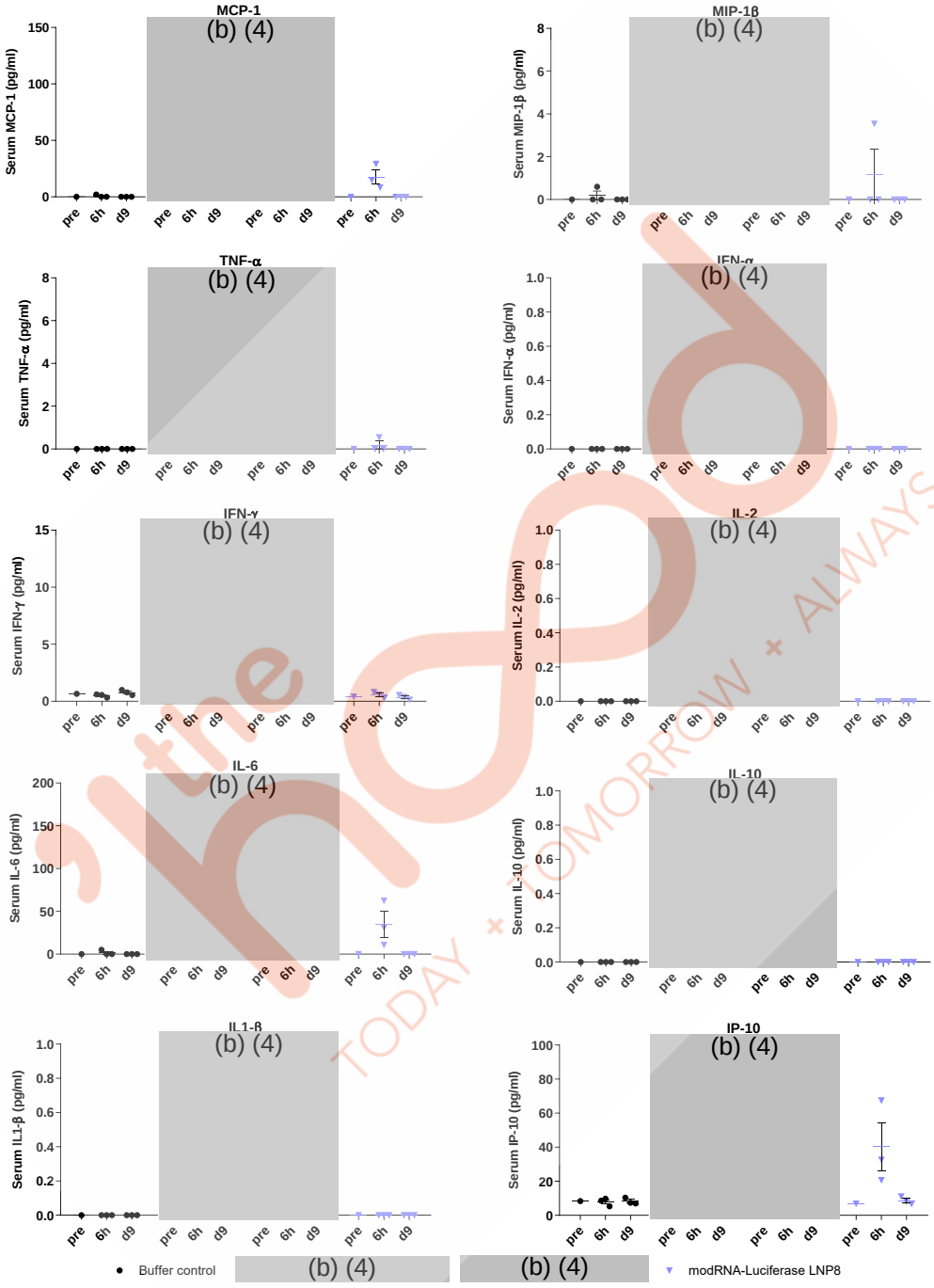


Figure 3: Activation of the innate immune system by LNP-formulated modRNA encoding for luciferase

BALB/c mice were injected i.m. in the right and left hind leg with each 1 µg of LNP-formulated modRNA encoding luciferase or DPBS only. Serum samples (day -1 (pre), 6 h, and 9 d) were assessed for presence of indicated chemokines/cytokines in a Luminex-based multiplex immunoassay.

5.4 Luciferase-specific ELISA

Luciferase-specific IgGs in serum samples obtained on study days -1 and 9 were investigated by ELISA.

Before immunization, no luciferase-specific IgGs were detected (day -1, Figure 4). Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9 post immunization.

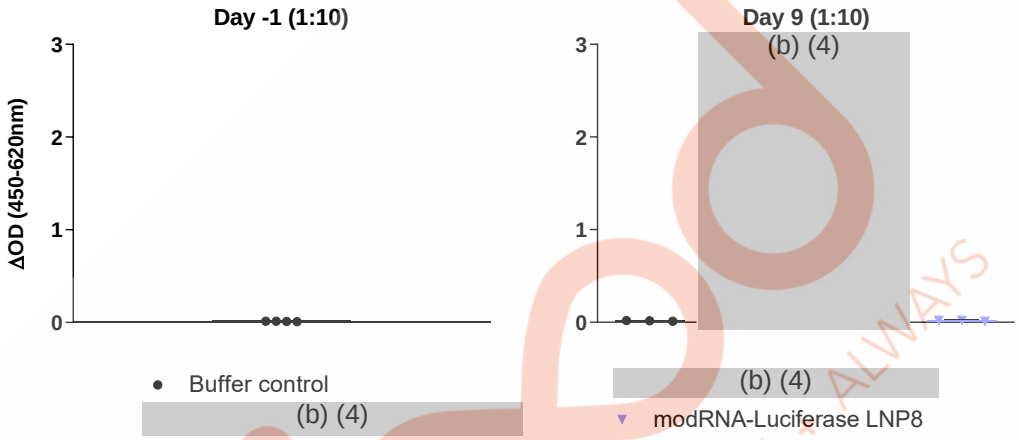


Figure 4: Luciferase-specific IgG ELISA on days -1 and 9

BALB/c mice were immunized with 1 µg/leg luciferase-encoding modRNA on day 0. Serum samples were collected on days -1 and 9 and the total amount of antigen-specific immunoglobulin G (IgG) was measured via ELISA. The serum 1:10 diluted. Individual ΔOD values are shown by dots; group mean values are indicated by horizontal bars (±SD).

5.5 IFN-γ ELISpot Assay

Mice were euthanized on day 9 and splenocytes were isolated to assess T-cell responses by ELISpot analysis. Splenocytes were stimulated with luciferase-specific peptide pools (Table 5) and IFN-γ secretion was detected. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparison post-test comparing all groups with each other. Control measurements were performed using an irrelevant peptide pool, medium only, or Concanavalin A.

Stimulation of splenocytes with MHC I-specific peptide pools induced IFN-γ responses in T cells of animals immunized with all modRNA LNP candidates (Figure 5). Group mean values of 53 spots per 5 × 10^5 cells were counted for animals injected with buffer control after stimulation with MHC I-specific luciferase peptide pools. The high spot count in can be attributed to reactivity of T cells of one mouse in group 1. Splenocytes of this mouse react also to the stimulation with AH1, the negative control. The group mean values are also 44 spots be 5 × 10^5 cells for the AH1 control, thus the response

of the control group to the luciferase-specific peptide pool can be considered unspecific. Group mean spot counts after stimulation with MHC I-specific peptide pools were (b) (4) (b) (4) 519 spots per 5 × 10^5 cells for the group treated with LNP8-formulated modRNA. The reactivity of splenocytes of the treatment groups to the negative control was very low (6-8 spots per 5 × 10^5 cells), thus activation of T-cells with luciferase peptides is highly specific in the treatment groups.

(b) (4)

(b) (4) The differences between the groups treated (b) (4) LNP8-formulated modRNAs compared to buffer control were statistically significant ((b) (4) p = 0.0051 for modRNA-luciferase LNP8). The (b) (4) and LNP8 groups displayed significantly higher MHC I-specific IFN-γ secretion (b) (4) (b) (4) p = 0.0163 for modRNA-luciferase LNP8). (b) (4) (b) (4)

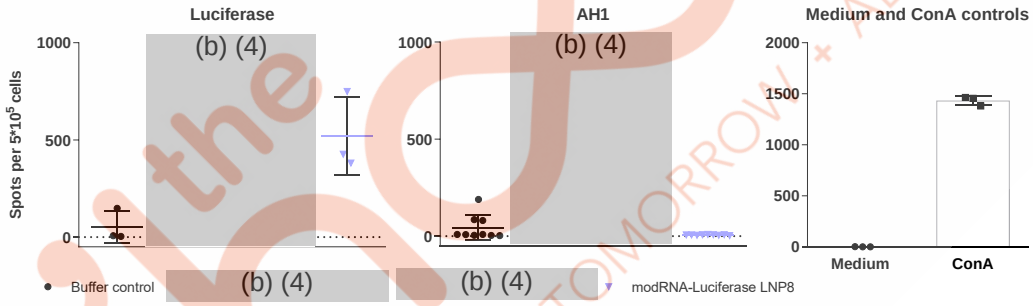


Figure 5: ELISpot analysis using splenocytes on day 9

ELISpot assay was performed using splenocytes isolated on day 9 after prime immunization. Splenocytes were stimulated with MHC I-specific luciferase peptide pools and IFN-γ secretion was measured to assess T-cell responses. Individual spot counts are shown by dots; group mean values are indicated by horizontal bars (±SD).

6 CONCLUSION

After injection of LNP-formulated modRNAs, an approximately 20-fold higher luciferase expression at the injections site was observed in a bioluminescence assay for (b) (4) the GMP-ready modRNA-Luciferase LNP8 (b) (4) (b) (4) The difference between the area under the curve for (b) (4) LNP8-formulated modRNA compared to buffer control (b) (4) was statistically significant. (b) (4) (b) (4)

A multiplex assay showed that the innate immune system was temporally activated by (b) (4) LNP8-formulated modRNA. (b) (4) (b) (4)

Treatment with modRNA with all tested LNP formulations did not induce the formation of luciferase-specific IgGs on day 9 as measured by ELISA.

However, a strong antigen-specific IFN-γ T-cell response measured by ELISpot assay on day 9 for (b) (4) LNP8-formulated modRNAs, with statistically significant differences between these test groups and the buffer control (b) (4)

7 DOCUMENT HISTORY

Reasons for changes compared to previous version:

Minor editorial changes, such as the correction of typing errors, are not listed.

Sections	Version 01	Version 02	Reason for change
1	-	More detailed information about LNP8 added	Clarification that LNP8 contains ALC-0159 and ALC-0315
3.1			
6			
4.1		Table added	Details of LNP lipid component formulations

Sections	Version 02	Version 03	Reason for change
4.1	-	Test item information updated	Diameter of LNP8 modRNA was corrected to 71 nm

8 REFERENCES

Limberis MP, Bell CL, Wilson JM. Identification of the murine firefly luciferase-specific CD8 T-cell epitopes. Gene Ther. 2009;16(3):441-7.

Moyo N, Vogel AB, Buus S, Erbar S, Wee EG, Sahin U et al. Efficient Induction of T Cells against Conserved HIV-1 Regions by Mosaic Vaccines Delivered as Self-Amplifying mRNA. Molecular therapy. Methods & clinical development. 2018; 12, 32-46.

Pardi N, Hogan MJ, Pelc RS, Muramasu H, Andersen H, DeMaso CR et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. Nature. 2017; 543 (7644), 248-251.

Vogel AB, Lambert L, Kinnear E, Busse D, Erbar S, Reuter KC et al. Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. Molecular therapy: the journal of the American Society of Gene Therapy. 2017; 26 (2), 446-455.

9 APPENDIX

Appendix 1: Animal Observations

		-5 days p.a. 09.01.2020		1 days p.a. 15.01.2020			3 days p.a. 17.01.2020			6 days p.a. 20.01.2020		
treatment	Lab ID	weight in g	health	weight in g	% change weight	health	weight in g	% change weight	health	weight in g	% change weight	health
Buffer control	1-1	21.4	No observations	21.7	101.0	No observations	21.5	100.4	No observations	21.7	101.3	No observations
	1-2	22.5	No observations	22.8	101.2	No observations	22.2	98.6	No observations	22.7	100.8	No observations
	1-3	23.0	No observations	23.3	101.4	No observations	23.5	102.3	No observations	23.4	101.5	No observations
modRNA-Luciferase (b) (4)	2-1	20.7	No observations	21.0	101.4	No observations	20.9	100.7	No observations	20.8	100.3	No observations
	2-2	21.5	No observations	22.5	104.6	No observations	22.3	103.8	No observations	23.2	107.9	No observations
	2-3	21.2	No observations	21.4	100.8	No observations	21.3	100.4	No observations	21.3	100.5	No observations
Acuitas LNPs modRNA luciferase	3-1	21.3	No observations	21.5	100.8	No observations	21.4	100.5	No observations	21.0	98.4	No observations
	3-2	21.2	No observations	21.2	99.9	No observations	21.0	99.1	No observations	21.0	99.2	No observations
	3-3	20.9	No observations	21.1	101.0	No observations	22.3	107.0	No observations	21.7	104.1	No observations
modRNA-Luciferase LNP8 (GMP-ready)	4-1	22.2	No observations	23.2	104.7	No observations	22.9	103.2	No observations	23.5	106.1	No observations
	4-2	21.6	No observations	22.4	103.8	No observations	22.5	104.3	No observations	21.9	101.6	No observations
	4-3	20.5	No observations	20.8	101.3	No observations	20.9	101.7	No observations	20.9	101.9	No observations

Appendix 2: Certificates of Analysis

Confidential

ACUITAS THERAPEUTICS

R&D Formulation Characterization Summary:

Batch ID	LNP ID	mRNA ID	Encaps (%)	Encaps mRNA (mg/mL)	Yield (mg)	mRNA/Lipid Ratio (mg/umol)	Particle Diameter (nm)	Poly-dispersity
[redacted]								
FM-1074 -D	LNP 8	mod Luc RNA-EH190611-01c	90%	1	0.36	0.025	71	0.053

Notes

Formulated 09-Dec-19

Diluent: 300 mM sucrose in PBS

Recommended storage at approximately -80 °C. Avoid repeated freeze/thaw cycles.

(b) (6)

(b) (6) Research Associate

11-Dec-19

Date

Page 1 of 1

ACD-008.01

Confidential

ACUITAS THERAPEUTICS

R&D Formulation Characterization Summary:

Batch ID	LNP Variant	mRNA ID	Encaps (%)	Encaps mRNA (mg/mL)	Yield (mg)	mRNA/Lipid Ratio (mg/umol)	Particle Diameter (nm)	Poly-dispersity
[redacted]								

(b) (4)

Notes

(b) (4)

(b) (6)

(b) (6) Research Associate

26-Nov-19
Date

Page 1 of 1

ACD-008.01

1 | Analytical Datasheet

Test items – Formulation: Analytical Data Sheet

Formulation experiment number: FSU-I#029
Responsible: MeSc, JaKs
Experimenter: MnSt, JaKs
Date of preparation: 06Jan20

Physicochemical characterisation:

Sample batch number	Formulation				pH	Osmolality (mOsmol/kg)	Size		Zeta (mV)	Encapsulation efficiency (%)	RNA conc. (mg/mL)	LNP are conform ?
	Lipid Mix	Batch	RNA	Batch			Z-Av (nm)	PDI				
[redacted]												

(b) (4)

(b) (4)

RNA Certificate of Analysis

(Version 17)

BIONTECH
RNA Pharmaceuticals

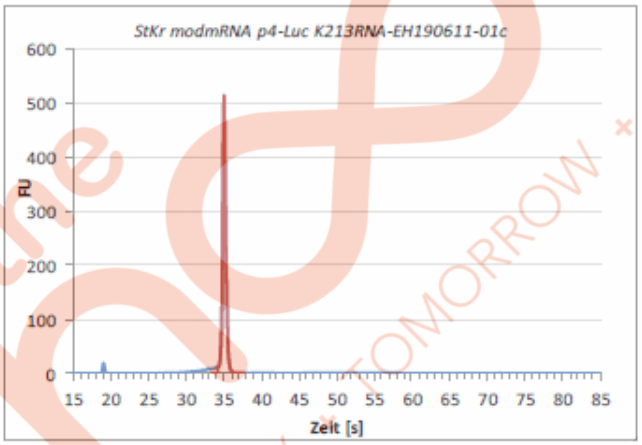
Customer	Date of Order	RNA-ID	Project Number
(b) (6)	27.05.2019	RNA-EH190611-01c	RN9095R00
Cap	**Construct**		
(b) (4)	modmRNA p4-Luc		
Purification	**Storage Buffer**		
dsRNA removal	H2O		
Modification	**Concentration [µg/µl]**	**Aliquot volume [µl]**	**Total amount [µg]**
m1Y	5,75	10600	60950

Quality Control:

Instrument:	Agilent 2100 Bioanalyzer
Run:	mRNA Nano_2019-06-14_001

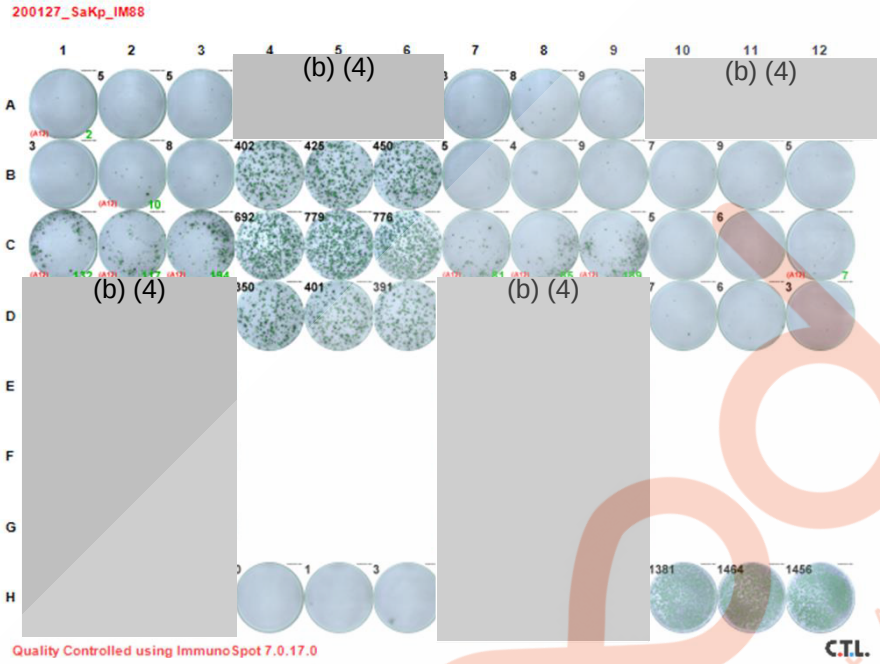
Results:

Peak height (FU):	514,963
Integral (%):	90



Appendix 3: Raw Data IFN-γ ELISpot

Plate 1: Left, luciferase peptides; right, AH1 ELISpot (irrelevant peptide)



Luc AH1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Group 1, animal 1			Group 3, animal 3			Group 1, animal 1			Group 3, animal 3		
B	Group 1, animal 2			Group 4, animal 1			Group 1, animal 2			Group 4, animal 1		
C	Group 1, animal 3			Group 4, animal 2			Group 1, animal 3			Group 4, animal 2		
D	Group 2, animal 1			Group 4, animal 3			Group 2, animal 1			Group 4, animal 3		
E	Group 2, animal 2						Group 2, animal 2					
F	Group 2, animal 3						Group 2, animal 3					
G	Group 3, animal 1						Group 3, animal 1					
H	Group 3, animal 2			Medium			Group 3, animal 2			**ConA**		

Group 1: Buffer control; Group 2: (b) (4) (2x1 µg); Group 3: (b) (4) (2x1 µg); Group 4: modRNA-luciferase LNP8 (GMP-ready) (2x1 µg)

Appendix 4: Statistical Analysis

Bioluminescence assay

Group mean values, bioluminescence assay, luciferase signal over time

Time point	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
	N = 3			N = 3
6 h	128046,667			1,2589e+009
24 h	227766,667			7,310667e+008
48 h	139995			2,1038333e+008
72 h	132585			7,8667e+007
6 d	162383,333			2920333,333
9 d	76573,333			509000

Please note that commas are used as decimal separators.

Descriptive statistics, bioluminescence assay, area under the curve

	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
Number of values	3			3
Minimum	657790			1352000000
Maximum	889908			1848000000
Range	232118			496000000
Mean	765040			1652666667
SD	117058			264244836
SEM	67583			152561827

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	80,68
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0,9680

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
		(b) (4)			
		(b) (4)			
Buffer control vs. modRNA-Luciferase LNP8	-1651901626	-2072407467 to -1231395786	Yes	****	<0,0001
		(b) (4)			
		(b) (4)			
		(b) (4)			

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

Luciferase-Specific ELISA

Descriptive statistics, luciferase-specific ELISA, day 9

	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
Number of values	3			3
Minimum	0,0110			0,0100
Maximum	0,0190			0,0220
Range	0,00800			0,0120
Mean	0,0153			0,0177
SD	0,00404			0,00666
SEM	0,00233			0,00384

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	0,4597
P value	0,6520
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R square	0,1209

No post-test for non-significant main test.

ELISpot analysis

Descriptive statistics, ELISpot analysis, day 9

	Buffer control	(b) (4)	(b) (4)	modRNA-Luciferase LNP8
Number of values	3			3
Minimum	4,00			381
Maximum	148			749
Range	144			368
Mean	53,0			519
SD	82,3			201
SEM	47,5			116

Please note that commas are used as decimal separators. SD: Standard deviation. SEM: Standard error of the mean.

One-way ANOVA with Tukey's multiple comparisons post-test, bioluminescence assay, area under the curve

ANOVA summary	
F	19,90
P value	0,0005
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R square	0,8819

Tukey's multiple comparisons test	Mean diff.	95,00% CI of diff.	Significant?	Summary	Adjusted P value
		(b) (4)			
		(b) (4)			
Buffer control vs. modRNA-Luciferase LNP8	-465,7	-769,0 to -162,3	Yes	**	0,0051
		(b) (4)			
		(b) (4)			
		(b) (4)			

Please note that commas are used as decimal separators. F: F-statistic. P values ≤ 0.05 indicate statistically significant difference. R square: Coefficient of determination. CI: Confidence interval. n.s.: Not significant.

(b) (6)

Von: (b) (6)
Gesendet: Freitag, 27. November 2020 07:24
An: (b) (6)
Cc: (b) (6)
Betreff: signatures: R-20-0072v3.0 (BNT162)
Anlagen: R-20-0072 Report V3.0 Bioluminescence in vivo_final_sig.pdf

Kennzeichnung: Zur Nachverfolgung
Kennzeichnungsstatus: Gekennzeichnet

Hello (b) (6)

With this email I'm giving my approval for the R&D report R-20-0072 version 3 update.

Best,
(b) (6)

(b) (6)
BioNTech SE
(b) (6)