Review and Comments: Team 5, Tranche 2

Phase 1 / 2 study of Covid-19 RNA vaccine BNT162b1 in Adults
Mulligan, Lyke et. al. Nature Published online 8/12/2020.

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> Phase 1/2 study of COVID-19 RNA vaccine
> BNT162b1 in adults. Nature https://doi.
> org/10.1038/s41586-020-2639-4 (2020).

P1 p2: The authors' put forth the argument that mRNA in BNT162b1 (Note this series used **BNT162b1** not **BNT162b2**) briefly expresses the encoded protein and then is metabolized without being incorporated into the host genome.

"RNA is required for protein synthesis, **does not integrate into the genome, is transiently expressed, and is metabolized and is eliminated by the body's natural mechanisms and, therefore is considered safe.[4, 7]"**

> *[4]Alberer, M. et al. Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: an open-label, non-randomized, prospective, first-in-human phase 1 clinical trial. Lancet 90, 1511-1520 (2017).*
>
> *[7]Sahin, U. e al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature 547, 222-226 (2017).*

However, Zhang et. al. working at MIT demonstrated fragments of SARS-CoV-2 integrated in host DNA in a paper published in 2021, *PNAS* vol. 118, no. 21.

> It will be important, in follow-up studies, to demonstrate the presence of SARS-CoV-2 sequences integrated into the host genome in patient tissues. However, this will be technically challenging because **only a small fraction of cells in any patient tissues are expected to be positive for viral sequences** (61). Consistent with this notion, it has been estimated that only between 1 in 1,000 and 1 in 100,000 mouse cells infected with LCMV either in culture or in the animal carried viral DNA copies integrated into the genome (30). In addition, only a fraction of patients may carry SARS-CoV-2 sequences integrated in the DNA of some cells. However, **with more than 140 million humans infected with SARS-CoV-2 worldwide (as of April, 2021), even a rare event could be of significant clinical relevance.** It is also challenging to estimate the frequency of retro-integration events in cell culture assays since infected cells usually die and are lost before sample collection. For the same reason, no clonal expansion of integrated cells is expected in acute infection experiments. Moreover, **the chance of integration at the same genomic locus in different patients/tissues may be low, due to a random integration process.**

Alden, et. al. reporting in *Current Issues in Molecular Biology* 2022, 44, 1115-1126 found BNT162b2 mRNA is reverse transcribed into host DNA beginning 6 hours after contact with BNT162b2.

> In the BNT162b2 toxicity report, no genotoxicity nor carcinogenicity studies have been provided [26]. Our study shows that BNT162b2 can be reverse transcribed to DNA in liver cell line Huh7, and **this may give rise to the concern if BNT162b2-derived DNA may be integrated into the host genome and affect the integrity of genomic DNA, which may potentially mediate genotoxic side effects**. **At this stage, we do not know if DNA reverse transcribed from BNT162b2 is integrated into the cell genome.** Further studies are needed to demonstrate the effect of BNT162b2 on genomic integrity, including whole genome sequencing of cells exposed to BNT162b2, as well as tissues from human subjects who received BNT162b2 vaccination.

Other studies have shown mRNA from BNT162b2 circulates then may reside longer in host cells. This enhanced stability is the result of N1-methyl-Pseudouridine incorporation into the mRNA.

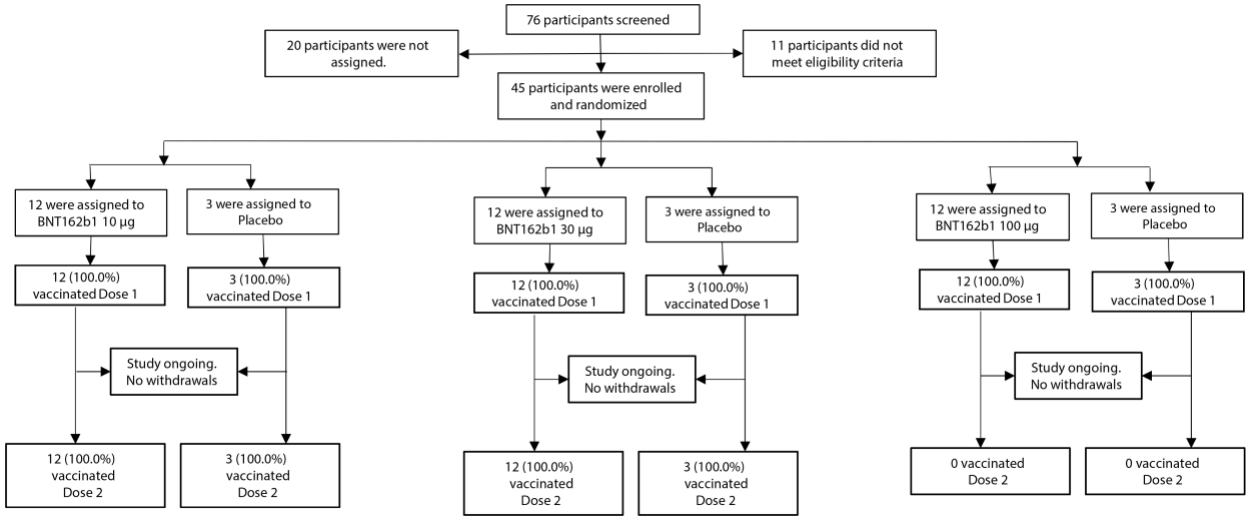
In 2009, Kariko et. al. reported that addition of N1-methyl-Pseudouridine to mRNA "...not only suppresses RNA-mediated immune activation in vitro and in vivo, but also enhances the translational capacity of the RNA."

> [11]*Kariko, K. et al. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol. Ther. 16, 1833-1840 (2008).*

P 1 p3: BNT162b1 was formulated to use N1-methyl-Pseudouridine to stabilize and improve translation. "Vaccine RNA can be modified by incorporating N1-methyl-Pseudouridine which dampens innate immune sensing and increases mRNA translation in vivo.[11]"

"Here, we present available data, through 14 days after a second dose in adults 18 to 55 years of age, from an ongoing Phase I/II vaccine study with **BNT162b1,** which is **also enrolling adults 65 to 85 years of age** (Clinical Trials.gov identifier: NCT04368128)." P2 p1.

Study Design:



- 76 participants screened
- 45 healthy participants randomized into three groups of 12 with 3 placebo groups.
- Mean age 35.4 years, 19-85.
- 51% Male, 49% female.
- Dose levels: 10-µg, 30-µ, 100µ **BNT162b1.**

Page 8 p1: "This study was conducted in healthy men and nonpregnant women 18 to 55 years of age to assess the safety, tolerability, and immunogenicity of ascending dose levels of various BNT162 mRNA vaccine candidates. **In the part of the study reported here, assessment of three dose levels (10-µg, 30-µg, or 100-µg) of the BNT162b1 candidate was conducted at two sites in the United States.** This study utilized a sentinel cohort design with progression and dose escalation taking place after review of data from the sentinel cohort at each dose level."

Endpoints:

- Reporting of solicited local reactions,
- Systemic events,
- Use of antipyretic and/or pain medication within 7 days after vaccination,
- AEs and SAEs (available through up to~45 days after Dose 1)
- Proportion of participants with clinical laboratory abnormalities 1 and 7 days after vaccination
- Shifts in laboratory assessments between baseline and 1 and 7 days after Dose 1 and between Dose 2 and 7 days after Dose 2
- SARS-CoV-2 neutralizing GMT,
- SARS CoV-2 RBD-binding IgG GMCs 7 and 21 days after Dose 1 and 7 and 14 days after Dose 2.

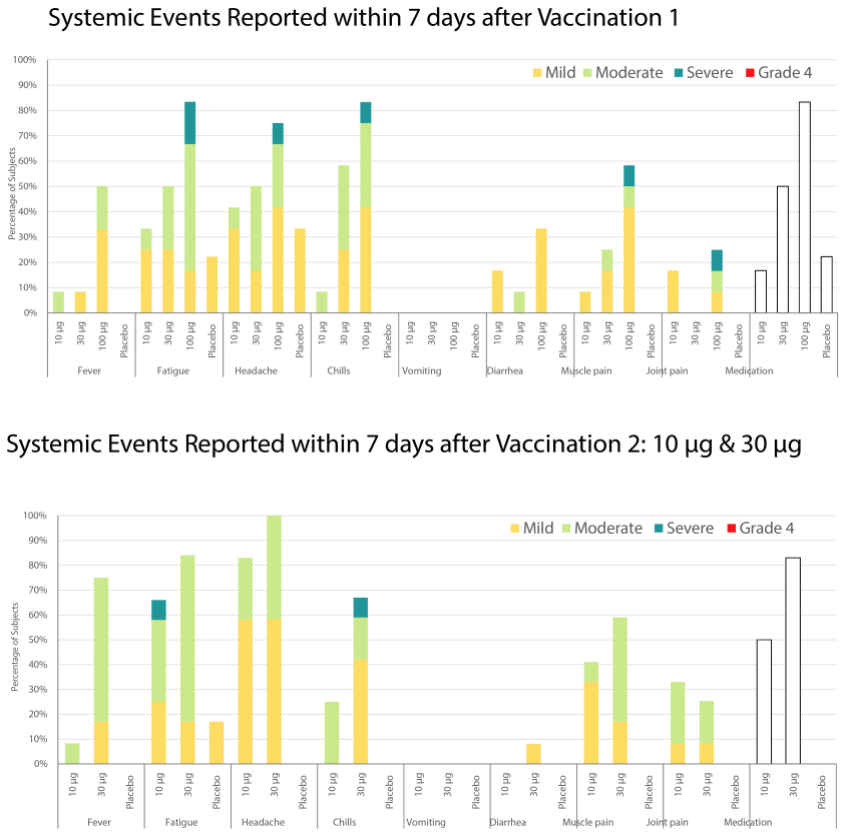
“Hematology and chemistry assessments were conducted at screening, 1 and 7 days after Dose 1, and 7 days after Dose 2.” These data are not reported other than “No Grade 1 or greater change in routine clinical laboratory abnormalities were observed for most participants after either of the BNT162b1 vaccinations. Of those with laboratory changes, the largest changes were decreases in lymphocyte count after Dose 1 in 8.3% (1/12), 45.5% (5/11?), and 50.0% (6/12) of 10 µg, 30 µg and 100 µg BNT162b1 recipients, respectively.” P2 p6.

> Upon request, and subject to review, Pfizer will provide the data that support the findings of this study. Subject to certain criteria, conditions, and exceptions, Pfizer may also provide access to the related individual anonymized participant data. See https://www.pfizer.com/science/clinical-trials/trial-data-and-results for more information. These data are interim data from an ongoing study, with the database not locked. Data have not yet been source verified or subjected to standard quality check procedures that would occur at the time of database lock and may therefore be subject to change.

Note: No data such are immediately available on web site 4/6/2022. (see https://www.pfizer.com/science/clinical-trials/trial-data-and-results/data-requests). What studies were performed? Did they measure d-dimer, il-6, troponin, as well as a complete blood count, electrolytes, renal and hepatic function test? Where are the raw data?

Adverse Event Report:

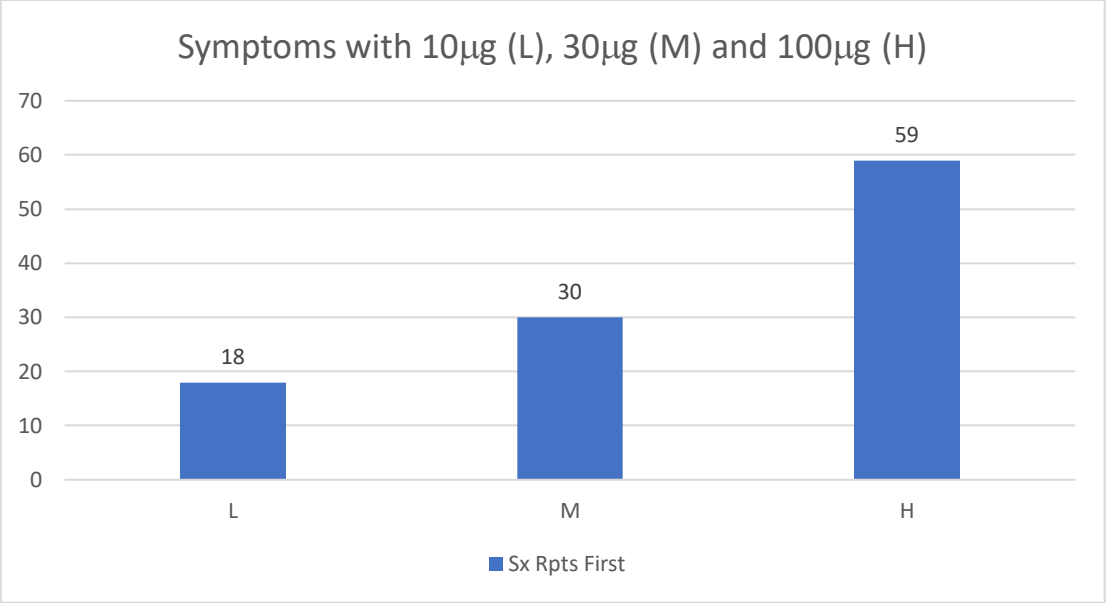
Figure 3:



In these two histogram charts, the x axis reports symptoms, other than the last column, medications. These are subjective complaints, not objective findings. Each active group consists of only 12 subjects, yet the reporting stratifies the data into four different levels of complaints and uses percent rather than raw numbers.

Converting percent back to raw numbers and using a binary reporting for "Yes" symptom is present and "No" symptom is not present, we can covert percentage to raw numbers. Placebo effects were minor and not addressed here.

First Dose	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Jt. Pain	Meds	Total
10	1	4	5	1	2	1	2	2	18
30	1	6	6	7	1	1	2	6	30
100	6	10	9	10	4	7	3	10	59

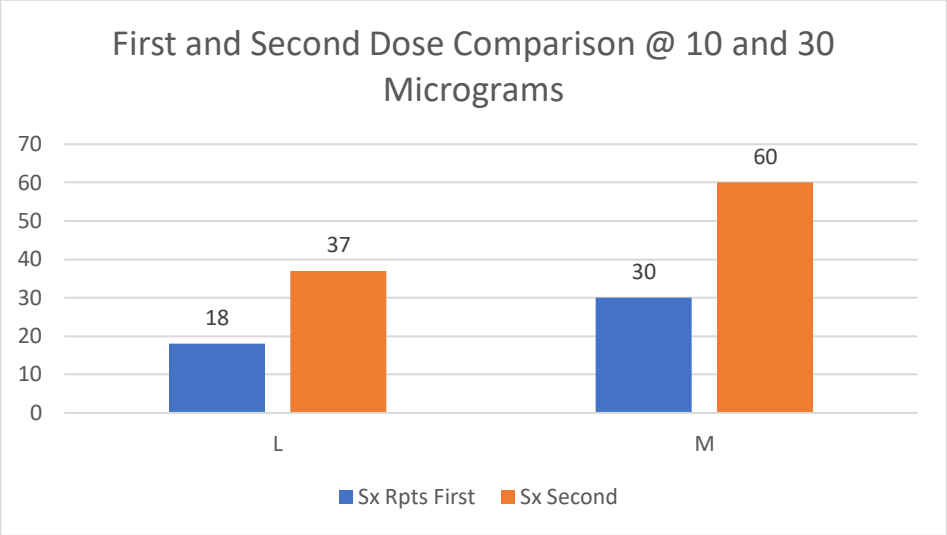


The first dose shows increased symptom reporting associated with increasing dose of mRNA. (L = 10 µg, M = 30 µg and H = 100 µg.) The 100 µg dose was dropped for dose 2.

Comparing 1st and 2nd doses:

30 µg	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Joint Pain	Meds	Total
1st	1	6	6	7	1	3	0	6	30
2nd	9	10	12	8	1	7	3	10	60
Incr.	8	4	6	1	-1	6	1	4	29

% incr.	800%	67%	100%	14%	-50%	600%	50%	67%	48%



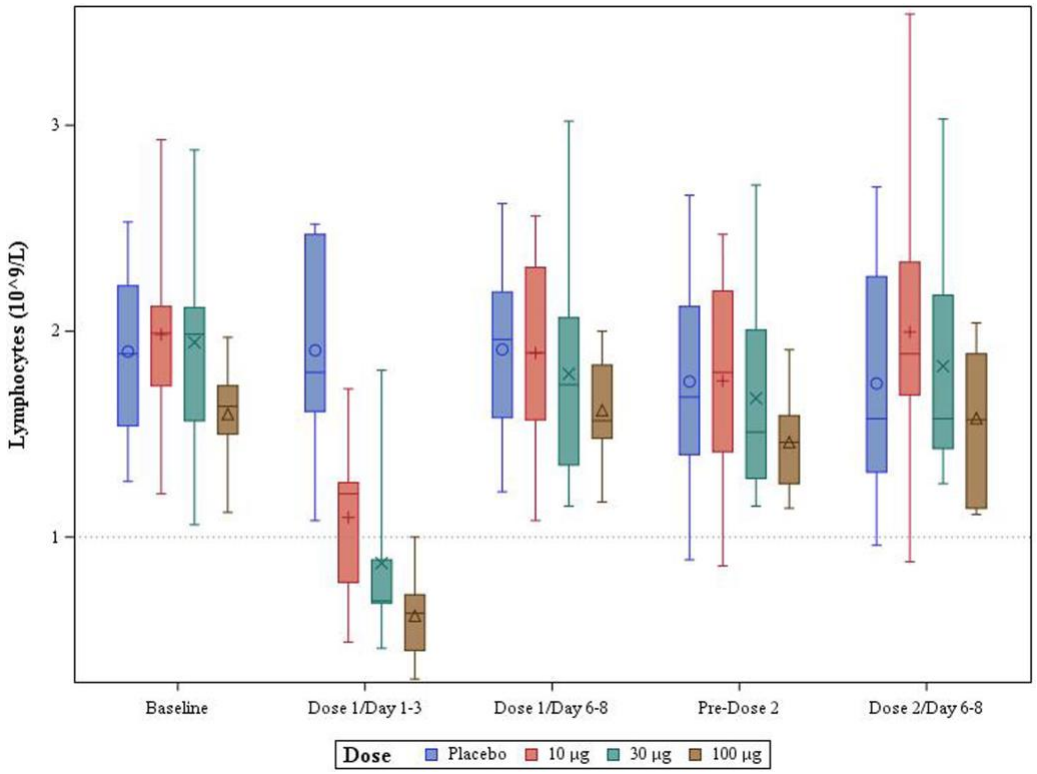
Conclusion: Increased symptoms occur with increased µg dose of BNT162b1. Increased symptoms were reported after the second dose at 10 µg and 30 µg compared with the first dose. The differences in the number of adverse events between different dosages of the "vaccine" other than Placebo versus 10 µg are statistically significant, $p < 0.05$. **(See Appendix).**

Trial #	1	2	1	2	1
Dose	10	10	30	30	100
Pain	7	10	12	12	12
Redness	0	0	2	2	4
Swelling	0	0	0	2	5

Pain, redness and swelling was reported but was not very useful other than a dose effect may be present for pain at the site of injection. Redness can be very subjective and swelling is very difficult to determine.

Objective findings including blood pressure, heart rate, fever, temperature, respiratory rate, physical examinations and complete laboratory findings were not reported and are not available on the Pfizer web site.

Extended Data Figure 1: Lymphocyte changes following three dosing levels as a function of time



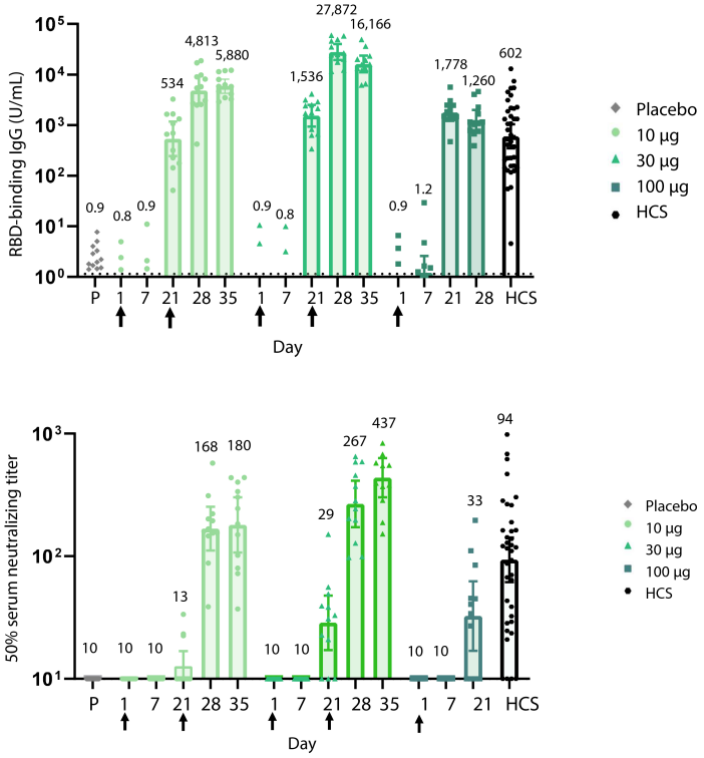
Lymphocytopenia on Days 1-3 after the first dose occurred in 1/12, 5/11, 6/12 for 10 µg, 30 µg and 100 µg respectively. **No lymphocyte reporting is given following the second dose during the comparable interval, Day 1-3, which after the first dose produced substantial drops in lymphocytes.** 1/12 (10 µg), 1/11 (30 µg) and 4/12 (100 µg) had Grade 3 decreases in lymphocytes. Neutropenia occurred in two subjects, one each in the 10 µg and 30 µg groups. No explanation for the decrease in lymphocytes and neutrophils is given. The reporting of raw data is required here, not a verbal description.

There is a lymphocyte measurement for a second dose @100 µg whereas the text "Based on the reactogenicity reported after the first dose of 100 µg and the second dose of 30 µg participants who received an initial 100 µg dose **did not receive a second 100 µg dose**." P2 p4

The schematic in Figure 1 also indicates no second 100 µg doses were given. Yet, there is a plot of second dose of 100 µg as indicated by the brown data candle plot on the far right. Was a second 100 µg dose given or not?

Finally, the variance in lymphocyte counts in the second dose 30 µg group appears to be very high. Was there a lymphocytosis as well as lymphocytopenia? Why? We need the actual data here. What caused the lymphocytopenia and were these cases associated with lymphadenopathy and or splenomegaly?

Immune Response:



Immune response was assessed using geometric mean titers of RBD-binding IgG concentrations at baseline, 7 and 21 days after dose 1 and at 7 and 14 days after dose two in the 10 and 30 µg groups. The 100 µg dose was given only once according to this chart. A second assessment using neutralizing titers showed increases after the second dose.

Discussion:

"Our study had several limitations. **While we used convalescent sera as a comparator, the kind of immunity (T cells versus B cells or both) and level of immunity needed to protect from COVID-19 are unknown."**

"Further, this analysis of available **data did not assess immune responses or safety beyond 2 weeks after the second dose of vaccine. Both are important to inform the public health use of this vaccine. "**

"Follow -up will continue for all participants and will include collection of SAEs for 6 months and COVID-19 infection and multiple additional immunogenicity measurements through up to two years."

"The clinical testing of BNT162b1 described here has taken place in the context of a broader, ongoing COVID-19 vaccine development program. That **program includes the clinical testing of three additional vaccine candidates** including candidates encoding the full-length spike, and a parallel trial in Germany, in which additional immune responses including neutralizing

responses against variant strain and cell-mediated responses are being assessed (US manuscript in preparation).[24]"

"The clinical findings for the BNT162b1 RNA-based vaccine candidate are encouraging and strongly support accelerated vaccine candidate development, including efficacy testing, and at-risk manufacturing to maximize the opportunity for the rapid production of a SARS-CoV-2 vaccine to **prevent** COVID-19."

Comments/Questions:

BNT162b1 not BNT162b2 was used in this Phase I/II clinical trial. What are the differences between the two? Was there a Phase I/II trial for BNT162b2? Why was the substitution made?

Was the 100 μg dose repeated or not? Extended Data Figure 1 shows a data plot for the 100 μg dose at Dose 2 Day 6-8.

The researchers erroneously believed that the mRNA in BNT162b1 would be transient, briefly producing spike protein then being metabolized and gone with no translation into host DNA. There is now concern that BNT162b2 mRNA code may be incorporated into the host genome based on a study by Alden, et. al. (See page 1 for the citation). Similar concerns were raised by Zhang, et. al. with regard to SC2 viral mRNA.

Clinical findings reported in this paper are deficient in presenting adequate detailed findings and should have body weight changes, appetite, and symptom changes during the reporting intervals, vital signs, physical findings and complete laboratory results.

This study was published in August 2020. Where are the reports noted as pending in the paper?

What role did N1-methyl-Pseudouridine (1MP) have in the unexpectedly long bioavailability of mRNA products? If not, what is the mRNA longevity attributable to? Does this enhanced stability have anything to do with dropping the lymphocyte counts noted in the Pre Clinical studies?

The 100 μg dose not only suppressed lymphocytes, but had a marked decline in immune response compared with immune sera and lower doses of BNT162b1. How and why did this happen? Is BNT162b1/BNT162b2 toxic to lymphocytes?

The objective of the vaccine was to prevent COVID-19. This product failed to prevent COVID-19. This product failed to prevent illness, hospitalization and death from COVID-19.

Was a risk benefit analysis performed? If so, where can the document be found?

Appendix: Statistical Analysis

How to interpret results:

The first two tests are Chi Square test. The left most numbers are:
1 10 μg Yes (number of adverse events)
2 10 μg No (number of without adverse events)
3 30 μg Yes
4 30 μg No
5 100 μg Yes
6 100 μg no

A simple data transformation was required to use the Chi Square test. All numbers were multiplied by 10.

The first number under each AE category is the number of events (X10)
The second number under each AE category is the expected number of events
The third number is the Chi Square statistic.

The larger the Chi Square statistic, the more unusual the event.

The p values of both dose 1 and dose 2 Chi Square test are less than 0.05 and therefore the test is statistically significant.

The six other tests are Test of Proportions. It is the total number of all categories of AE divided by the total number of events.

All but the first (10 μg vs. placebo), are statically significant.

Chi-Square Test: Fever, Fatigue, Headache, Chills, Diarrhea, Muscle Pain, Joint

1st Dose without placebo X 10

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Joint Pain
1	10	40	50	10	20	10	20
	22.50	22.50	22.50	22.50	22.50	22.50	22.50
	6.944	13.611	33.611	6.944	0.278	6.944	0.278
2	110	80	70	110	100	110	100
	97.50	97.50	97.50	97.50	97.50	97.50	97.50
	1.603	3.141	7.756	1.603	0.064	1.603	0.064
3	10	60	60	70	10	30	0
	37.50	37.50	37.50	37.50	37.50	37.50	37.50
	20.167	13.500	13.500	28.167	20.167	1.500	37.500
4	110	60	60	50	110	90	120
	82.50	82.50	82.50	82.50	82.50	82.50	82.50
	9.167	6.136	6.136	12.803	9.167	0.682	17.045
5	60	100	90	100	40	70	30
	73.75	73.75	73.75	73.75	73.75	73.75	73.75
	2.564	9.343	3.581	9.343	15.445	0.191	25.953
6	60	20	30	20	80	50	90
	46.25	46.25	46.25	46.25	46.25	46.25	46.25
	4.088	14.899	5.709	14.899	24.628	0.304	41.385
Total	360	360	360	360	360	360	360

	Medication	Total
1	20	180
	22.50	
	0.278	
2	100	780
	97.50	
	0.064	
3	60	300
	37.50	
	13.500	
4	60	660
	82.50	
	6.136	
5	100	590
	73.75	
	9.343	
6	20	370
	46.25	
	14.899	
Total	360	2880

Chi-Sq = 496.633, DF = 35, P-Value = 0.000

Results for: 2nd Dose wo placebo x 10

Chi-Square Test: Fever, Fatigue, Headache, Chills, Diarrhea, Muscle Pain, Joint

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Joint Pain
1	10	80	100	30	0	50	40
	46.25	46.25	46.25	46.25	46.25	46.25	46.25
	28.412	24.628	62.466	5.709	46.250	0.304	0.845
2	110	40	20	90	120	70	80
	73.75	73.75	73.75	73.75	73.75	73.75	73.75
	17.818	15.445	39.174	3.581	29.004	0.191	0.530
3	90	100	120	80	10	70	30
	75.00	75.00	75.00	75.00	75.00	75.00	75.00
	3.000	8.333	27.000	0.333	56.333	0.333	27.000
4	30	20	0	40	110	50	90
	45.00	45.00	45.00	45.00	45.00	45.00	45.00
	5.000	13.889	45.000	0.556	93.889	0.556	45.000
Total	240	240	240	240	240	240	240

```
        Medication   Total
    1           60     370
              46.25
              4.088

    2           60     590
              73.75
              2.564

    3          100     600
              75.00
              8.333

    4           20     360
              45.00
             13.889

Total          240    1920

Chi-Sq = 629.452, DF = 21, P-Value = 0.000
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Test and CI for Two Proportions 10 μg vs p

Sample	X	N	Sample p
1	18	96	0.187500
2	7	72	0.097222

Difference = p (1) - p (2)
Estimate for difference: 0.0902778
95% CI for difference: (-0.0135438, 0.194099)
Test for difference = 0 (vs not = 0): Z = 1.70 P-Value = 0.088

Test and CI for Two Proportions 30 μg vs p

Sample	X	N	Sample p
1	30	96	0.312500
2	7	72	0.097222

Difference = p (1) - p (2)
Estimate for difference: 0.215278
95% CI for difference: (0.100039, 0.330516)
Test for difference = 0 (vs not = 0): Z = 3.66 P-Value = 0.000

Test and CI for Two Proportions 100 μg vs p

Sample	X	N	Sample p
1	59	96	0.614583
2	7	72	0.097222

Difference = p (1) - p (2)
Estimate for difference: 0.517361
95% CI for difference: (0.398360, 0.636362)
Test for difference = 0 (vs not = 0): Z = 8.52 P-Value = 0.000

Test and CI for Two Proportions 30 μg vs 10 μg

Sample	X	N	Sample p
1	30	96	0.312500
2	18	96	0.187500

Difference = p (1) - p (2)
Estimate for difference: 0.125
95% CI for difference: (0.00378499, 0.246215)
Test for difference = 0 (vs not = 0): Z = 2.02 P-Value = 0.043

Test and CI for Two Proportions 100 μg vs 10 μg

Sample	X	N	Sample p
1	59	96	0.614583
2	18	96	0.187500

Difference = p (1) - p (2)
Estimate for difference: 0.427083
95% CI for difference: (0.302286, 0.551881)
Test for difference = 0 (vs not = 0): Z = 6.71 P-Value = 0.000

Test and CI for Two Proportions 100 μg vs 30 μg

Sample	X	N	Sample p
1	59	96	0.614583
2	30	96	0.312500

Difference = p (1) - p (2)
Estimate for difference: 0.302083
95% CI for difference: (0.167638, 0.436528)
Test for difference = 0 (vs not = 0): Z = 4.40 P-Value = 0.000

Dose 1:

Dosage - Reaction	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Joint Pain	Meds	Total
10 μg Yes	1	4	5	1	2	1	2	2	18
10 μg No	11	8	7	11	10	11	10	10	78
30 μg Yes	1	6	6	7	1	3	0	6	30
30 μg No	11	6	6	5	11	9	12	6	66
100 μg Yes	6	10	9	10	4	7	3	10	59
100 μg No	6	2	3	2	8	5	9	2	37
p Yes	0	2	3	0	0	0	0	2	7
p No	9	7	6	9	9	9	9	7	65
Total	45	45	45	45	45	45	45	45	360

Dose 2:

Dosage - Reaction	Fever	Fatigue	Headache	Chills	Diarrhea	Muscle Pain	Joint Pain	Meds	Total
10 μg Yes	1	8	10	3	0	5	4	6	37
10 μg No	11	4	2	9	12	7	8	6	59
30 μg Yes	9	10	12	8	1	7	3	10	60
30 μg No	3	2	0	4	11	5	9	2	36
p Yes	0	2	0	0	0	0	0	0	2
p No	6	4	6	6	6	6	6	6	46
Total	30	30	30	30	30	30	30	30	240