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# Implications of mutations in the spike protein of Danish mink-derived SARS-CoV-2 isolates for vaccines and monoclonal antibody therapeutics.

## Purpose:

In this text, the data received from SSI regarding neutralization of a mink-derived SARS-CoV-2 isolate carrying 4 simultaneous mutations in the spike protein by convalescent sera from patients early in the Danish epidemic is evaluated, and risks for vaccine efficacy posed by such mink-derived virus strains assessed.

## Summary:

Danish mink-derived SARS-CoV-2 isolates have been reported to carry a number of mutations in the spike protein, with some isolates having 4 simultaneous spike protein mutations.
Some of these mutations have been described as being mink-specific, i.e., as having originated in mink.
However, data from GISAID (https://www.gisaid.org/) , the COVID-19 viral genome analysis pipeline at Los Alamos, as well as scientific publications shows that viruses currently circulating in humans already carry spike protein mutations which are identical or very similar to the spike protein mutations described in Danish mink-derived viruses, albeit at very low frequency (<0.3% of circulating viruses in humans).
Therefore, the spike protein mutations observed in Danish mink-derived viruses should not be termed mink-specific.
The higher frequency of certain spike protein mutations in mink-derived viruses than in human-derived viruses is most likely caused by the virus' adaptation to replication in mink. Thus, the fitness of the mink-derived viruses in humans is most likely reduced.
Only 2 of the mutations in the mink-derived viruses occur at spike protein sites targeted by neutralizing antibodies:

- The receptor-binding site of the spike protein harboring the Y453F mutant is the most critical of these, as it is able to induce particularly potent neutralizing antibody responses.
    - However, the Y453F mutation is conservative, and not expected to have major impact on receptor or antibody binding.
    - In agreement with this, the Y453F spike mutation has been shown to have mild positive effect on binding of the spike protein to the human ACE2 receptor (1,8-fold increase in binding, likely within experimental error).
- The N-terminal domain of the spike protein is known also to be the target for neutralizing antibodies, and the deletions of amino acids 69 and 70 in mink-derived viruses may impact antibody binding.
    - However, residues 69 and 70 in human-derived viruses already carry radical mutations.

In short, based on initial, theoretical/bioinformatical evaluation of the mutations in the spike protein of Danish mink-derived viruses as outlined above, the following can be concluded:

- These mutations do not seem highly likely to raise new issues for vaccine development which do not already exist, due to the well-known and relatively well understood currently ongoing mutation and evolution of SARS-CoV-2 in humans.
- The spike protein mutations are evaluated as not being likely to have substantial impact the efficacy of first-generation vaccines.

In a preliminary experiment, SSI compared the neutralizing activity of 9 human convalescent sera against a mink-derived SARS-CoV-2 isolate containing 4 simultaneous changes in the spike protein (Δ69-70, Y453F, I692V and M1229I) with the neutralizing activity of the same sera against a control SARS-CoV-2 strain without these spike mutations, using a monkey kidney cell line (Vero cells).
On average, these 9 human sera exhibited 2,6-fold reduction in neutralizing activity against the mink-derived SARS-CoV-2 strain containing 4 simultaneous changes in the spike protein, compared to the control virus without these changes. As a rule of thumb, in this type of assay, a 2.6-fold difference would typically be considered to have, at best, low biological significance due to the analytical method variability/inaccuracy, and certainly require a large number of replicates and additional controls to document with acceptable confidence.
Thus, while the serum neutralization data is preliminary and requires follow up (see detailing of data strength/weakness and recommendation for follow-up work in main text), the SSI data nevertheless suggests that the mutations observed in the spike protein of mink-derived viruses are unlikely to markedly impact vaccine efficacy. This DKMA conclusion based on the SSI neutralization data is in agreement with the theoretical/bioinformatic evaluation of the mutations in the spike protein of Danish mink-derived viruses outlined above.
In short, for the reasons above, and with reservations for the preliminary nature of the available data, the following can be concluded :

- The spike protein mutations seen in Danish mink-derived SARS-CoV-2 isolates are evaluated as not being likely to have substantial impact the efficacy of first-generation vaccines and monoclonal antibody therapeutics.
- Specifically, at the population level, the likelihood of spike protein mutations seen in mink-derived SARS-CoV-2 isolates causing vaccine failure is considered to be very low (with the reservation for the preliminary nature of currently available data mentioned below).
- However, it cannot be ruled out that vaccine failure may occur in individual vaccinees infected with such isolates, particularly vaccinees mounting low neutralizing antibody responses to start with.
- This is a preliminary risk assessment, based on available data; further laboratory analyses and scientific studies are needed to better understand the impact of the changes in the spike protein of mink-derived viruses for the transmissibility and neutralization of such viruses in human hosts.

## Mechanisms behind and biological consequences of mutations in spike protein of mink-derived viruses:

Danish mink-derived SARS-CoV-2 isolates have been reported to contain a number of mutations in the spike protein, with some isolates containing 4 simultaneous mutations (Δ69-70, Y453F, I692V, M1229I; ref 16).
Some of these mutations have been described as being mink-specific, i.e., as having originated in mink (for example Y453F; ref 13 and 16).
However, data from GISAID, the COVID-19 viral genome analysis pipeline at Los Alamos as well as scientific publications (14, 15) shows that the Y453F and M1229I were and are already present in SARS-CoV-2 viruses circulating in humans, and as regards the Δ69-70 and I692V, viruses already circulating in humans have comparable changes (e.g. I692F mutation and a variety of radical mutations at spike positions H69 and V70 in human-derived viruses)(table 1). It should also be mentioned that combinations of 2 mutations have already occurred in the spike protein of human-derived viruses, often including the D614G mutation which confers higher transmissibility between humans (8).

- By human-derived viruses is meant SARS-CoV-2 isolates from cases of human-human virus transmission, unrelated to the mink outbreaks in the Netherlands and Denmark in march and august 2020, respectively (6, 13, 16, 18).
- it is recognized that such so-called human-derived viruses very recently crossed into humans from an animal reservoir, likely bats.

Mink were likely infected from humans, and represent naïve and susceptible hosts, with virus spread between mink farms having been rapid. For all these reasons, the mutations in the spike protein of mink-derived viruses are unlikely to represent the result of selective pressure from the immune responses in mink.
More likely, for the reasons above, the mutations seen in the spike protein of mink-derived viruses were caused by the following 2 non-exclusive mechanisms:

- Founder effects: Mutations in the spike protein of human viruses were carried over into mink.
    - This is in fact supported by the available data: The D614G mutation which confers higher transmissibility of virus between humans arose in human-derived virus in China and perhaps Germany around February 2020, and within 3 months outcompeted the original D614 became the dominant virus form globally. The global spread of the D614G mutant through the period February-May 2020 coincided temporally with the disease outbreaks in mink in the Netherlands and Denmark (March and August 2020, respectively; ref 6, 13, 18). This appears to be mirrored in an increased frequency of the D614G mutant in mink-derived virus in Denmark compared to the Netherlands (table 1).
- Adaption to a new host: Mink as well as ferrets are known to be susceptible to SARS-CoV-2 infection, but less so than humans (1, 6, 7). Thus, it is expected that some adaptive changes may occur in the spike protein and other viral proteins after transmission from humans to mink.
    - In human-derived viruses, most mutations in the spike protein, particularly in the receptor-binding part of the protein, reduce the binding to human ACE2 receptor and/or reduce spike protein stability, i.e., have negative effects on virus fitness and transmissibility between humans (8, 9). Further, multiple mutations in spike have a more severe negative effect on ACE2 binding and/or spike stability than single mutations (9).
    - Finally, some mutations in the spike protein increase the binding to the human ACE2 receptor, and viruses carrying these mutations are already circulating between humans; yet, the frequency of such ACE2-binding-enhancing mutations in naturally circulating viruses is very low (<0.3%) and has remained stable over time, most likely because the SARS-CoV-2 virus already has the biologically maximally desirable binding to human ACE2 receptor, acquired already or soon after the species jump to humans, i.e. at the start of the pandemic (8, 9).
    - This is not surprising, given that the binding affinity between the spike protein of SARS-CoV-2 and human ACE2 is in the low pico-molar range (already very strong binding to human ACE2, 10-fold stronger than for SARS-Cov-1; ref 9), and in experiments intentionally selecting S protein mutants for increased binding to human ACE2, this already high binding affinity could be only minimally improved (< 10-fold improvements in binding affinity; ref 9).
    - Thus, for the reasons above, adaptive changes are expected to increase virus fitness in mink, but are likely to be detrimental to virus fitness in humans.

In short:

- It does not appear that SARS-CoV-2 spike protein has acquired unique mutations in mink.
- For this reason, the spike protein mutations observed in mink-derived viruses should not be termed mink-specific.
- Thus, the spike protein mutations observed in mink-derived viruses do not appear to raise new issues for vaccine and monoclonal antibody development which do not already exist due to the well-known and relatively well understood currently ongoing mutation and evolution of SARS-CoV-2 in humans (see for example refs 3, 8, 9, 10, 11, 14, 15, 17).

Only 2 of the mutations in the mink-derived viruses occur at spike protein sites targeted by neutralizing antibodies (table 1):

- The receptor-binding site of the spike protein harboring the Y453F mutant is the most critical of these, as it is able to induce particularly potent neutralizing antibody responses (9).

    - However, the Y453F mutation is conservative, and not expected to have major impact on receptor or antibody binding.
    - In agreement with this, the Y453F spike mutation having been shown to have mild positive effect on binding of the spike protein to the human ACE2 receptor (1,8-fold increase in binding, likely within experimental error; ref 9).
- The N-terminal domain of the spike protein is known to also be the target for neutralizing antibodies, and the deletions of amino acids 69 and 70 in mink-derived viruses may impact antibody binding.
    - However, residues 69 and 70 in human-derived viruses already carry radical mutations (table 1).

In short:

- The several mutations observed in the spike protein mink-derived viruses are already present in human-derived viruses circulating between humans.
- For this reason, and the reasons detailed in the text above, the mutations observed in the spike protein of mink-derived viruses are evaluated as not being likely to have substantial impact the efficacy of first-generation vaccines and monoclonal antibody therapeutics.
- This assessment is based on available data, which is limited.
- Thus, unquestionably, studies are warranted and required to better understand the properties and pathogenicity of mink derived viruses, as well as the implications of the changes present in the spike protein of mink-derived viruses for SARS-CoV-2 serodiagnostics, monoclonal antibody therapeutics and vaccines in development.

**Table 1: Summary of aminoacid mutations in spike protein of mink-derived SARS-CoV-2 in Denmark and the Netherlands, based on publicly available data**

| Mutation in spike protein of mink-derived virus, compared to human-derived virus | Frequency of mutation | Location of mutation in spike protein | Spike mutation already present in human-derived viruses ? | Comments |
|---|---|---|---|---|
| Δ69-70 (deletion of aminoacids 69 and 70) | * Not known | N-terminal domain. | H69D, H69Q, H69R, H69Y, V70A, V70F and V70I mutations present in human-derived viruses (rare, <0.3%). | N-terminal domain is surface-exposed, and known to be target for neutralizing antibodies in humans. |
| Y453F | 5/13 NL sequences. 8/12 DK sequences. | Receptor-binding domain. | Yes (rare, <0.3%). | Receptor-binding domain is surface-exposed. Most of the known highly potent neutralizing antibodies target the receptor-binding domain. The Y453F mutation is conservative, and not expected to have major impact on receptor or antibody binding. This is in agreement with the Y453F spike mutation having been shown |

| Mutation in spike protein of mink-derived virus, compared to human-derived virus | Frequency of mutation | Location of mutation in spike protein | Spike mutation already present in human-derived viruses ? | Comments |
|---|---|---|---|---|
| | | | | to have mild positive effect on binding of the spike protein to the human ACE2 receptor (1,8-fold increase in binding, likely within experimental error; ref 9). |
| D614G | 7/13 NL sequences. 12/12 DK sequences. | D-domain of S1 subunit (carboxy-terminal part of S1 subunit) | Yes (currently dominant in human epidemic, globally). | Mutant considered to be associated with higher transmissibility between humans, and higher susceptibility to neutralization by antibodies. The apparent increase in frequency of the mutant in Danish samples mirrors its increased dominance in human-derived viruses through the period when the mink outbreaks occurred in the Netherlands and Denmark (march through august 2020; see ref. 17 and references therein). |
| I692V | * Not known | Seven aminoacids downstream from furin cleavage site between S1 and S2 subunits (i.e. in N-terminus of S2 subunit). | I692 F mutation in human-derived viruses (rare, <0.3%). | Spike protein location not surface-exposed, unlikely to be target for neutralizing antibodies.<br><br>Mink mutation is more conservative than mutant already present in humans. |
| M1229I | * Not known | Transmembrane part | Yes (rare, <0.3%).<br>In addition, M1229L and M1229/ mutations are also seen in humans (also rare, <0.3%). | Spike protein location not surface-exposed , unlikely to be relevant for antibody neutralization of virus. |

The data in the table is based on publicly available spike protein sequences and information from SSI (16).
Information regarding whether spike protein mutations in mink-derived viruses are already present in human-derived viruses is from GISAID, the COVID-19 viral genome analysis pipeline at Los Alamos, and scientific publications (14, 15).
Mutations are reported in single-letter aminoacid code, using the following convention: Residue in SARS-CoV-2, Wuhan-Hu-1, followed by residue number in the spike protein, followed by residue in mutates spike protein.

Accession numbers:

- Reference spike protein sequence, human-derived SARS-CoV-2, Wuhan-Hu-1 (first whole-genome SARS-CoV-2 sequence): Accession number YP 009724390.
- Mink-derived spike protein sequences from the Netherlands: Accession numbers QJS39496, QJS39507, QJS39519, QJS39531, QJS39543, QJS39555, QJS39567, QJS39579, QJS39591, QJS39603, QJS39615, QJS39627, and QJF11995 (13 in total).
- Mink-derived spike protein sequences from Denmark: QNJ45106, QNJ45118, QNJ45130, QNJ45142, QNJ45154, QNJ45166, QNJ45178, QNJ45190, QNJ45202, QNJ45214, QNJ45226 and QNJ45238 (12 in total).

* These mutations do not appear in the publicly available sequences.

# Initial experimental work done at SSI to evaluate the implications of spike protein mutations in mink-derived viruses for countermeasure development:

The data discussed in the following is from the first serum neutralization experiment of mink-derived virus with human convalescent sera, carried out by Dr Anders Fomsgaard and Maria Magdalena Lassauniére (SSI report dated 02.11.2020; ref 16).
The neutralizing activity of human convalescent sera against a mink-derived SARS-CoV-2 isolate containing 4 simultaneous changes in the spike protein (Δ69-70, Y453F, I692V and M1229I; see table 1) was compared to the neutralizing activity of the sera against a control SARS-CoV-2 strain without these spike mutations. Serial dilutions of convalescent sera were pre-incubated with fixed amounts of mink-derived or control virus for 1h, followed by inoculation on Vero cells (African green monkey kidney cell line) and incubation for 24h. Neutralization titers were defined as serum dilutions causing 50% reduction in the levels of nucleocapsid protein production ($NT_{50}$ values), determined by immunostaining of fixed cells (exact experimental and analysis details lacking).
A total of 9 human convalescent sera were tested, all from patients early in the Danish epidemic.

In the following, all interpretations of the SSI data represent evaluation done at the DKMA.
The $NT_{50}$ titers of the 9 human convalescent sera against the control SARS-CoV-2 strain ranged from 39 to >1280 (table 2). Four of these sera had neutralization titers < 80, considered to be low titers, and for the group as a whole, the geometric mean titer was 168 (table 1). This wide range of neutralization titers as well as relatively high frequency of low-end NT50 values has been reported by others for SARS-CoV-2 convalescent sera, and was as such not surprising (2, 4, 5). However, with the reservation that neutralization assays are not standardized and neutralization titer values difficult to compare between datasets, these patient sera appeared to exhibit lower neutralization titers than expected in vaccinees.
For the mink-derived strain, neutralization titers ranged from 5 to >1280, with a geometric mean of 64 (table 2).
Thus, on average, these 9 human sera exhibited a 168/64=2.6-fold reduction in neutralizing activity against the mink-derived SARS-CoV-2 strain containing 4 simultaneous changes in the spike protein, compared to the control virus without these changes.
Neutralizing titers against control virus exhibited a good linear correlation with neutralization titers against mink-derived virus (figure 1, R2>0,9), with inclinations of the regression lines were 0,74 to 0,97 (figure 2).
Based on the regression equations shown in figure 2, a neutralization titer of for example 160 against human-derived virus corresponds to a neutralization titer of 0.75 x 160 - 41 = 79 (figure 2 panel A) to 0.98 x 160 - 76 = 80 (figure 2 panel B) against mink-derived virus.
Thus, based on regression analysis, human sera exhibited 160/80 = 2-fold to 160/79 = 2-fold reduction in neutralizing activity against the mink-derived SARS-CoV-2 strain containing 4 simultaneous changes in the spike protein, compared to the control virus without these changes, in agreement with the results based on simple geometric group means detailed above.
Neutralization assays are known to exhibit a relatively high level of variability, and as a simplified, general rule of thumb, differences in neutralization titers of 10-fold or more are considered to be high and likely biologically significant (see for example ref. 3 and 8), whereas the biological significance of neutralization titer differences of less than 10-fold are less likely to have biological significance, and differences of 2 fold or less would typically be considered to have at best low biological significance, and

certainly require a large number of replicates and additional controls to document with acceptable confidence (8).
Thus, the observed reduction in mean neutralization activity of human convalescent sera against mink-derived virus (average 2,6-fold reduction for group of 9 convalescent sera) is considered to be of low significance, given the known variability of neutralization assays, and preliminary nature of the data (see section on data strength and recommendations for follow-up experiments below).
Sera which were low-titered against the control virus exhibited the largest reductions in titer against the mink-derived virus, and this correlation was significant (figure 1; P=0,0182). Thus, the potential risk of failure of COVID-19 vaccines against mink-derived cluster 5 strains is likely highest in vaccinees exhibiting low vaccine responses to start with.
At the same time, of the 4 sera with titers <80 (sera 1 through 4, table 2), neutralization of mink virus varied from 7% to 72% of the neutralization of control virus (table 2), and in this (admittedly very small) sub-group of sera with low neutralization titers, the correlation between neutralization of human and mink virus was less clear (figures 1 and 2; not detailed). This is not surprising, because the distribution of circulating polyclonal neutralizing antibodies in patients against different parts of the S protein (for example balance between amounts of circulating polyclonal neutralizing antibodies against N-terminal domain versus receptor-binding domain) is known to be idiosyncratic amongst patients (3, 4, 5), and also, affinities of neutralizing polyclonal anti-S protein antibodies differ between patients (4); these idiocyncrasies in circulating polyclonal neutralizing antibody responses are likely due to factors such as subtle differences in how the spike protein was presented to patient B-cells, age of patients, MHC-II haplotype of patients, etc.
In short:

- The serum neutralization data is preliminary and has several shortcomings requiring follow up (see detailing and recommendation for follow-up work above).
- Nevertheless, the data suggests that the mutations observed in the spike protein of mink-derived viruses are unlikely to impact vaccine efficacy.
- This conclusion based on evaluation of SSI data is in agreement with the conclusion drawn based on bioinformatic analysis and theoretical considerations in the section above, titled "Mechanisms behind and biological consequences of mutations in spike protein of mink-derived viruses".

**Table 2: Raw serum neutralization data.**

| **Patient** | **Neutralization titer ($NT_{50}$)** | | **Titer ratio mink virus/control virus** |
|---|---|---|---|
| | Control virus isolate | Mink-derived cluster 5 virus isolate | |
| 1 | 39 | 28 | 0,72 |
| 2 | 68 | 5 | 0,07 |
| 3 | 54 | 9 | 0,17 |
| 4 | 67 | 30 | 0,45 |
| 5 | 186 | 67 | 0,36 |
| 6 | >1280 | >1280 | 1,00 |
| 7 | 846 | 606 | 0,72 |
| 8 | 186 | 54 | 0,29 |
| 9 | 291 | 177 | 0,61 |
| | | | |
| * Descriptive statistics, all 9 sera: | | | |
| *mean:* | *335* | *251* | *0,49* |
| *median:* | *186* | *54* | *0,45* |
| *geometric mean:* | *168* | *64* | *0,38* |
| | | | |
| *Descriptive statistics, excl. patient 6:* | | | |
| *mean:* | *217* | *122* | *0,42* |
| *median:* | *127* | *42* | *0,40* |

| | | | |
|---|---|---|---|
| *geometric mean:* | *142* | *52* | *0,36* |

The data is from the first neutralization experiment, carried out by Dr Anders Fomsgaard and Maria Magdalena Lassauniére (SSI report dated 02.11.2020; ref 16).
* The patient 6 serum with a titer of >1280 was assigned the value 1280.
The geometric mean is usually used in evaluating results from serological assays such as this; for convenience, mean, median and geometric mean values are all shown.

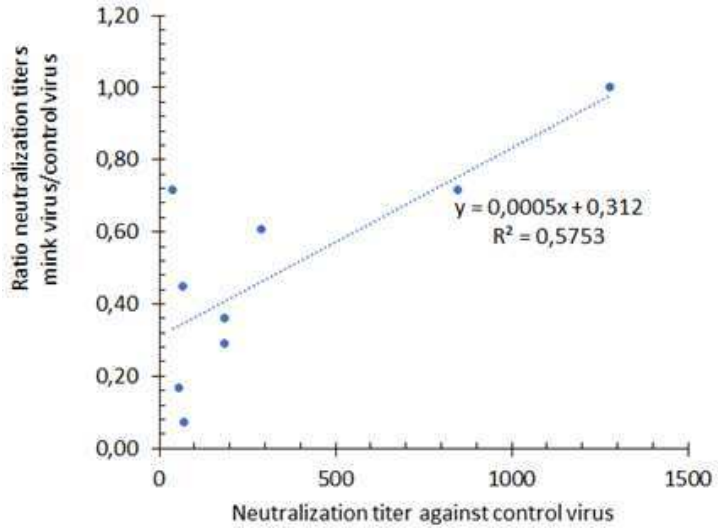


**Figure 1: Relationship between magnitude of anti-SARS-CoV-2 antibody response in patients against homologous (human-derived) virus, and failure to neutralize mink-derived virus**
The inclination of the regression line was significantly different from 0 (P=0,182; GraphPad QuickCalc software, available online https://www.graphpad.com/quickcalcs/linear2/)

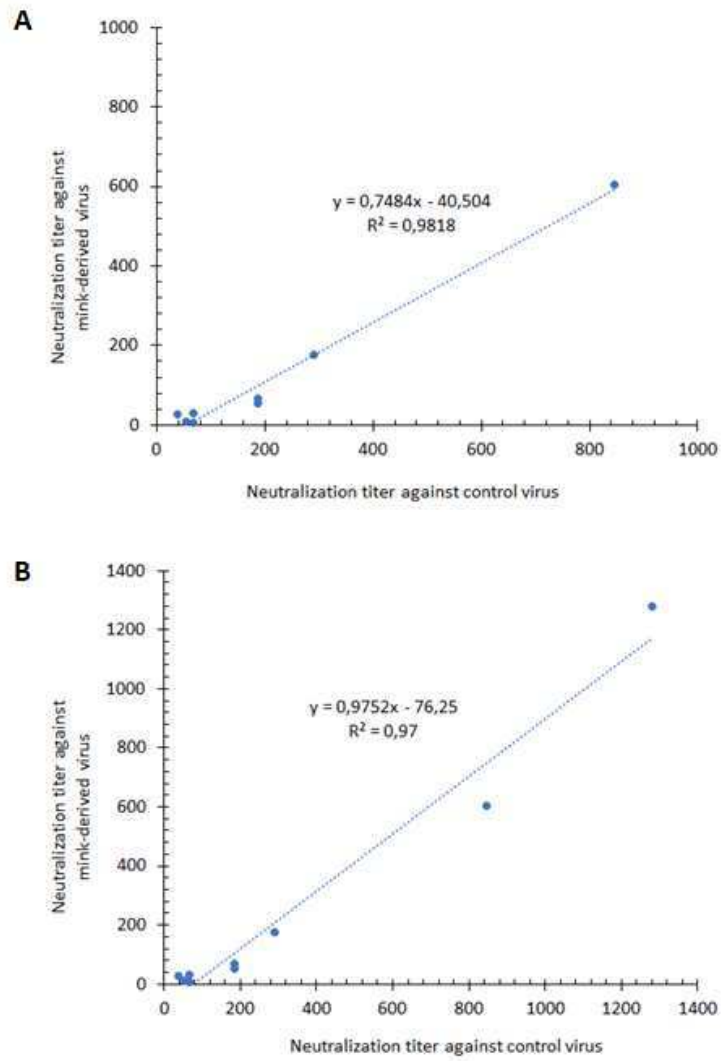


**Figure 2: Ability of convalescent human sera to neutralize mink-derived SARS-CoV-2 cluster 5 isolate with 4 simultaneous changes in spike (Δ69-70, Y453F, I692V, M1229I)**
Panel A, serum with a titer of >1280 is excluded.
Panel B, serum with a titer value of >1280 is included, and assigned a titer value of 1280.

## Strength of data from initial studies, and recommendations for follow-up experiments:

All interpretations of the SSI data below represent evaluation done at the DKMA.
The data has the following shortcomings:

- The cell culture system used for neutralization assays is permissive for SARS-CoV-2 replication (6), but is non-human, and represents a cell type which is not a primary target for infection in humans (Vero cells; African green monkey kidney cell line). The ACE2 receptor expressed by Vero cells has been reported to differ by approx. 5% from human ACE2 protein at the amino acid level (1), and while Vero cells are often used for in vitro studies of SARS-CoV-2 virus, typically human cell lines are also included (6), and results obtained using Vero cells and human cells may differ (6).
- For neutralization results to be valid, identical amounts of mink-derived and control virus should be used, and these 2 viruses should exhibit identical or very comparable replication kinetics in Vero cells. It is stated in the SSI report that same amounts of mink-derived and control virus

were used, but it is not known which methods were used to quantitate these 2 virus isolates. Comparison of replication kinetics in Vero cells for the 2 viruses is not provided.

- With the reservation that neutralization assays are not standardized and neutralization titer values are difficult to compare between datasets, the patient serum panel appeared to be low titered compared to expected neutralization titers in vaccinees.
- The serum neutralization data represents a single experiment, the number of replicate determinations is not reported, and the overall variability of the neutralization assay is not known; such assays are known to be quite variable.
- The technical performance of the neutralization assay is not known; specifically, the cutoff level between positive and negative sera, and the linearity (dose-response) of the assay are of interest.

For the reasons above, the strength of the data is evaluated as low (preliminary results), as is also acknowledged in the SSI report.

For initial confirmatory follow-up experiments, the following is recommended:

- Clinical data should be provided for patient sera (e.g. patient age, and time interval since infection).
- Sera which are more representative of vaccinees should be included.
- More virus strains should be included.
- More technical information should be provided for the variability of the neutralization assay, controls used, and the performance of the assay (see above).
- The virus neutralization/serology work should be expanded to include determination of the affinity of the mutated spike protein in mink-derived cluster 5 strain(s) for human ACE2 receptor.

It should also be mentioned here that even the relatively modest and focused follow-up work outlined above represents a relatively large effort.

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