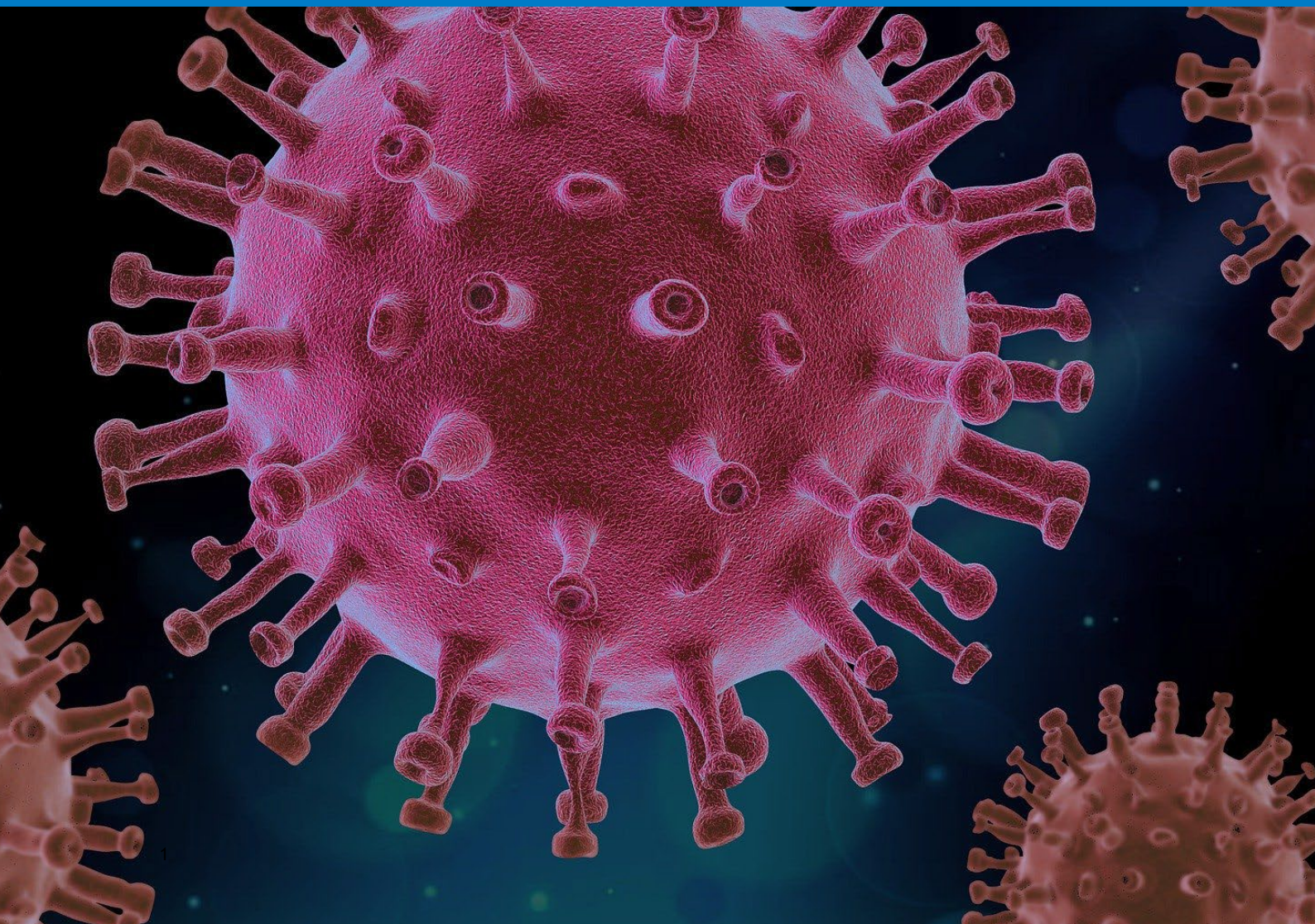
Netherlands National Committee
for the protection of animals
used for scientific purposes

COVID-19: the use of laboratory animals and non-animal methods in the dynamics of a pandemic

Advisory report of the Netherlands National Committee for the protection of animals used for scientific purposes



About NCad

The NCad achieves visible improvements that are specifically related to the Replacement, Reduction and Refinement (3Rs) of animal procedures and to the associated ethical review in scientific research (including applied scientific research) and teaching. Its goal, in doing so, is to minimize laboratory animal use at both national and international level.

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Summary

On 27 October 2020, the Minister of Agriculture, Nature and Food Quality requested that the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) provide an opinion on the effects of COVID-19 on the transition to non-animal innovation and we were asked the following questions:

1. Which animal models have been used around the world in COVID-19 research? What has been the impact of COVID-19 research around the world on the use of laboratory animals?
2. Which alternative (non-animal) methods have been used around the world in COVID-19 research? What has been the effect?
3. Which changes have occurred in the prescribed procedures for vaccine and/or medicine development or in the international adherence to these procedures?
4. With regard to a), b) and c): which lessons or advice can be distilled for the future of the transition to animal-free innovation?

In March 2021, NCad published the interim report '*Leren van Covid-19*' (Learning from COVID-19), in which the initial findings are shared. The report primarily outlines the COVID-19 research landscape from a narrative perspective. This interim report focused on the aspects of research in which the laboratory animals and/or the non-animal method played a key role.
The final report '*COVID-19: het gebruik van proefdieren and proefdiervrije methoden in de dynamiek van een pandemie*' (COVID-19: the use of laboratory animals and non-animal methods in the dynamics of a pandemic) explicitly addresses the 4 questions put to NCad by the Minister of Agriculture, Nature and Food Quality.

Ad Question 1: Which animal models have been used around the world in COVID-19 research? What has been the influence of COVID-19 research around the world on the use of laboratory animals?

Because of the existing system used for annual reporting on laboratory animal use and the research categories applied, both nationally and at European level, it is not possible to get a detailed picture of the extent of this use for COVID-19 research.

Based on the interview conducted it has emerged that COVID-19 research made use of animal models that were also used in the research into SARS and MERS, two diseases caused by related coronaviruses. In general, mice are used for basic scientific research (such as the choice of antigen) and the preparatory research, such as safety and immunogenicity (generating antibodies), hamsters are used mainly for research into the effectiveness of vaccines and medicines, ferrets are used for research into virus transmission and NHPs are used for fine-tuning the information gained from small rodents and for the transition from the preclinical stage to the clinical stage (First-In-Human). In addition, it is striking that Phase 1 studies were sometimes already initiated before all the data from the preclinical research was available (see Table 6). Given the global scope of the research, and into vaccines and medicines in particular, it can be assumed that the use of mice, hamsters, ferrets and NHPs will have increased, with the caveat that research with NHPs has primarily taken place in the US and China.

Ad question 2: Which alternative (non-animal) methods have been used around the world in COVID-19 research? What has been the impact?

At the outbreak of the pandemic, the choice was primarily made in favour of (animal) models with which experience had already been gained. In addition to the urgency of the moment, this will also have been related to sticking with tried and tested systems. However, new projects have been

launched aimed at the development, optimisation and application on non-animal models. Many of these models will not be available for 4 to 5 years, i.e. after the end of the COVID-19 pandemic.

This does not alter that fact that, in addition to animal models, COVID-19 research also uses a wide range of existing non-animal methods.
Physico- and immunochemical methods have been widely used for vaccine development and virus characterisation; in vitro models, both simply cell culture methods and advanced organoid cultures and organs-on-a-chip, have been used for virus characterisation, pathophysiology and for the purposes of developing medicines; pathophysiology has primarily been studied in COVID-19 patients and human volunteers have been used particularly for medicine research. In silico models have been used for virus characterisation purposes, the selection of potential medicines, epidemiology and patient data analysis.

In terms of the use of laboratory animals, the most important reason for using animals in COVID-19 research has been the development of medicines and of vaccines in particular.
The combination of vaccine development based on the latest technologies (vector, RNA/DNA and peptide vaccines), the use of physico and immunochemical techniques and the application of in vitro methods has the potential to lead to a transition in the use of laboratory animals.

One limiting factor in this transition is the lack of crucial in vitro immune parameters in organoid cultures and organs-on-a-chip. This means it is not, or at least not yet, possible to measure the effectiveness of a medicine or vaccine without the use of an animal model. Generating a complex in vitro model, including relevant aspects of the immune system, requires a significant amount of time and resources.

Ad question 3: Which changes have occurred in the prescribed procedures for vaccine and/or medicine development or in the international adherence to these procedures?

As of April 2022, 349 COVID-19 vaccines were in development of which 196 in the pre-clinical stage of research and 153 at the clinical stage of research. The urgency of the pandemic has been a major barrier to the use of new and less trusted models. That sense of urgency has also led – at least for part of the vaccines – to a significant reduction in time from vaccine development to authorisation. This was due to measures such as the overlap between clinical and preclinical research, assessment on the basis of a rolling review and conditional authorisation. These changes have resulted in a higher investment in capacity, and therefore in costs, both in the development stage and in the assessment stage.

The process of saving time did not negatively impact on safety and efficacy; neither in terms of the manufacturer developing the vaccine or medicine, nor in terms of marketing authorisation authorities. This is to say that the quality of the product assessment has not been compromised, with the exception of the studies that are necessary for long-term effects, such as the duration of accrued protection after vaccination, effects on pregnancy. Information on these aspects was provided at a later stage and in the event of the absence of effects, the conditional authorisation was converted to an authorisation.

Ad question 4: What lessons or advice, in the context of the COVID-19 pandemic, can be distilled for the future of the transition to non-animal innovation?

Macro level

One Health is a movement in the medical and biological community based on the correlation and interconnection between humans, animals and nature. Changes in one domain will have consequences for another domain. Research and approaches will therefore have to be multidisciplinary in nature. COVID-19 is a zoonosis: a disease that can be transmitted from animals to humans. The Netherlands has been one of the leaders and pioneers of the One Health movement.

The Minister is requested to continue to promote the relevance and value of One Health and therefore of sustainable and structural solutions to preventing pandemics.
In addition, the Minister is requested to continue the government's commitment to pandemic preparedness within multinational partnerships.

In the context of pandemic preparedness, the government has included an investment sum of 180 million euros for 2022 in the Coalition Agreement, increasing to 300 million euros by 2026.

The Minister is requested to continue to make efforts to earmark part of this investment for the development of innovative non-animal instruments for monitoring, prevention and prophylaxis.

The urgency of the COVID-19 pandemic placed a significant amount of pressure on the vaccine or drug development, primarily in the industry. This frequently involved the use of platform technologies that were developed in an academic setting. Technologies have subsequently been used by the industry for the development of virus-specific vaccines, their optimisation and for the scaling of their production. The pressure on the development of a safe and effective vaccine does not, however, account for the amount of vaccines being worked on. It is plausible that in addition to the public health aspect, economic and national interests may have played a key role.

In the opinion of vaccine experts, vaccine development is not yet possible without the use of laboratory animals. The multitude of vaccines under development have – without evidence to quantify this – led to extensive animal use. In global terms, the WHO has taken on a coordinating role in the domain of COVID-19 research, including through the blueprint group for animal testing, but equally in the development of vaccines and medicines. This role is implemented by means of partnerships with other globally operating organisations, such as the Coalition for Epidemic Preparedness Innovations (CEPI). CEPI is supported by a number of countries, including the Netherlands.

Another global organisation which operates in vaccine development is Gavi, a partnership in the field of vaccines between WHO, UNICEF, the World Bank, the Bill & Melinda Gates Foundation, private vaccine developers and donor countries. Gavi mainly focuses on third-world countries. In an ideal situation, these organisations should be able to make a pre-entry selection for vaccine development, based on needs, development concept and assumed added value in comparison with other products. It is recognised that this infringes on free market thinking and that this free market thinking can drive innovation.

In the context of a restrictive policy on laboratory animals, the Minister is requested to promote alignment and coordination of vaccine development through national contacts with organisations such as the WHO, CEPI and Gavi.

The Minister is also requested to instruct a review into whether an approach in which prototype vaccines are developed for a number of virus families could also yield advantages in terms of the use of laboratory animal, in the context of technical and practical feasibility.

Meso level

It was stated in the interim report that the application of a non-sequential approach of preclinical and clinical research, the assessment of incomplete dossiers at entry level for COVID-19 research (the rolling review) and conditional licensing have led to an acceleration of the assessment process. It is clear from additional interviews that this approach also has its limitations and requires further qualification. The reasons cited in this regard are outlined in Table 6. All this means that the acceleration of the assessment process has an impact of budgets. The introduction of a fast-track process therefore becomes a political decision, based on the willingness to provide additional funding.

As regards the use of laboratory animals, a sequential approach as such will not lead to a reduction in use. Preclinical research will continue to be necessary but will be redistributed over time. A golden standard within the development and production of vaccines and medicines, laid down by law, is that a new product must be safe and effective.

It is not stated anywhere that this must be done on the basis of research involving animals. In the context of the limitations shown by some the animal models used, more can therefore be expected from a study into the relevance of existing animal studies and into the use of time-saving non-animal methods.

The minister is asked to make funds available, in consultation with the Ministry of Health, Welfare and Sport, for conducting a retrospective analysis of the value and limitations of standard animal studies in the research stage and the preclinical trials of vaccines and medicines.

Key factors for the application of non-animal methods in regulatory research include pursuing a policy aimed at the development of these methods, their acceptance by national and international supervisory authorities and the global harmonisation of test guidelines.

In this context, the Minister is requested to promote a guiding policy through existing channels, including in respect of organisations such as the World Health Organization (WHO), the European Medicines Agency (EMA) and the European Pharmacopoeia (Ph.Eur.).

Micro level

Conventional vaccines are based on the inactivation or attenuation of the microorganism or the detoxification of the toxin produced by the microorganism. The latest generation of vaccines, such as the mRNA vaccines and vector vaccines for COVID-19, use molecular biological techniques which were developed at the end of the last century.

The new generation of vaccines requires laboratory animals in the development phase but requires none or very few in routine batch testing. This has everything to do with the fact that these types of vaccines are extensively characterised in the development stage and production is extensively standardised and monitored by means of a set of analytical methods. With a view to a reduction in the use of laboratory animals, it is therefore to be recommended to focus on non-conventional development methods when developing a vaccine.

In order to further reduce the use of laboratory animals, it is recommended that the Minister join up as much as possible with vaccine development at an international level aimed at the development and use of non-conventional methods.

Organ-specific organoids are used for research into the pathogenicity of the virus, while microfluidic cultures of lung tissue are used for research into the kinetics and dynamics of the virus.
Non-animal methods, such as organ-specific organoids, have many advantages, one of which is the reduction of the use of laboratory animals, as well as a reduction in costs and research time. Not all researchers have picked up on these advantages, and it will remain necessary to draw attention to these models. Non-animal methods are also (still) characterised by limitations. One of the key limitations is the limited integration of immune parameters in the in vitro models. The first steps in this field have been taken, however are still at an embryonic stage.

The government's alternative policy focuses primarily on innovative cell culture models. This translates into funding for non-animal research that is often single-mindedly focused on the development of these cell culture models. It should be noted that there is at least as much potential in other non-animal techniques, certainly in the area of infectious diseases. Greater balance and proportionality in the distribution of policy emphasis and available funds would do justice to the importance of these techniques to non-animal research.

The Minister is requested to encourage research into the integration of complex immunological parameters in the new generations of in vitro methods. In addition, the Minister is requested to focus policy emphasis and resource allocation for non-animal research on other techniques and methods in addition to innovative cell culture systems.

Introduction

On 27 October 2020, the Minister of Agriculture, Nature and Food Quality requested the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) to provide an opinion on the effects of COVID-19 on the transition to innovation without laboratory animals, and we were asked the following questions:

1. Which animal models have been used around the world in COVID-19 research? What has been the impact of COVID-19 research around the world on the use of laboratory animals?
2. Which alternative (non-animal) methods have been used around the world in COVID-19 research? What has been the impact?
3. Which changes have occurred in the prescribed procedures for vaccine and/or medicine development or in the international adherence to these procedures?
4. With regard to a), b) and c): which lessons or advice can be distilled for the future of the transition to animal-free innovation?

The minister requested this opinion to gain an overview of and insight into the use of laboratory animals and non-animal methods for research into vaccines and treatment methods for COVID-19 worldwide. On the basis of this, any implications for the transition to animal-free innovations (TPI) can be identified and lessons can be learnt for the future of TPI.

NCad will respond to the above questions below, with the caveat that detailed information and data for some of the questions is not (or not yet) available. Moreover, the pandemic is not yet a concluded period, and future developments may lead to a nuancing of the conclusions and recommendations.

The COVID-19 research project has largely followed the steps that also apply to each other unknown pathogenic microorganism: identification and characterisation of the microorganism on the basis of, among other things, how it spreads; selection of research models (*in vivo* and *in vitro*); research into the pathogenicity of the virus, including the long-term effects; development and assessment of medicines (therapeutics) and vaccines (prophylactics) and the marketing authorisation methods of these products. The difference between this virus and preceding pathogens is that the speed with which the virus has spread globally and the severity of the infection has given unprecedented dynamics to the research area. These dynamics has been amplified even further by the emergence of virulent and infectious variants of the original (Wuhan) virus, of which the principal variants[1] are the Alpha (UK), the Beta (South Africa), the Gamma (Brazil), the Delta (India) and, although less virulent, the Omicron variant.

At present, 507,501,771 million infections have been officially recorded and 6,220,390 million people have died from COVID-19 (WHO data from 14 April 2022[2]). In addition, in the Netherlands, central government expenditure on COVID-19 measures for 2020-2022 is estimated at 89.8 billion euros[3] (spring edition 2022) and at 12.5 x 10^{12} (12.5 thousand billion) dollars[4] worldwide (22 January 2022).

[1] www.hopkinsmedicine.org

[2] Covid19.who.int

[3] https://www.rekenkamer.nl/onderwerpen/corona/nieuws/2022/03/17/coronarekening---voorjaarseditie-2022

[4] Reuters.com

In March 2021, NCad published the interim report 'Leren van Covid-19' (Learning from COVID-19)[5], in which the initial findings are shared.

The most important conclusions from the interim report were that:

- research into the SARS-CoV-2 virus, in terms of both characterisation and medicine/vaccine development, mainly relied on the animal species used in 2002-2003 and 2012 respectively for two other viruses from the family of coronaviruses: the SARS (Severe Acute Respiratory Syndrome) virus and the MERS (Middle East Respiratory Syndrome) virus, mainly mice, hamsters, ferrets and non-human primates;

- the use of non-animal methods rather than animal models was limited in the first instance. It seems that this can be partly ascribed to the urgent nature of the pandemic, which has meant that mainly existing animal models from coronavirus research have been used, and partly to the fact that in vitro models have limitations, such as the lack of integrated aspects of the immune system;

- each model has its limitations, but also offers possibilities in terms of translation to humans. This applies to both animal and non-animal models. In general, animal models were used frequently in vaccine research and *in vitro* models as organoid cultures in the research into pathogenicity;

- a degree of coordination in COVID-19 research was done via the WHO blueprint group[6], where the results of animal research were shared and discussed at an early stage and new research questions were formulated;

- the acceleration in the marketing authorisation process of vaccines and medicines has been achieved by allowing preclinical and clinical studies to partly overlap (the so-called "rolling review") rather than undertaking them sequentially (on the basis of results from previous research). Furthermore, absolute priority was given to the marketing authorisation process of COVID-19 medicines and vaccines, both at the level of the Central Authority for Scientific Procedures on Animals (CCD) and the Central Committee on Research Involving Human Subjects (CCMO) and the level of assessment and marketing authorisation authorities (EMA and the Dutch Medicines Evaluation Board (MEB)). Other factors have also contributed to the quick marketing authorisation of new vaccines (see table 6).

[5]Tussenrapportage 'Leren van Covid-19' | Nieuwsbericht | Nationaal Comité advies dierproevenbeleid (ncadierproevenbeleid.nl), www.ncadierproevenbeleid.nl
[6] https://www.who.int/teams/blueprint/covid-19

The interim report 'Learning from COVID-19' mainly describes the COVID-19 research landscape from a narrative angle. This interim report focused on the aspects of research in which the laboratory animals and/or the non-animal method played a key role.

The final report 'COVID-19: het gebruik van proefdieren and proefdiervrije methoden in de dynamiek van een pandemie' (COVID-19: the use of laboratory animals and non-animal methods in the dynamics of a pandemic) explicitly addresses the 4 questions put to NCad by the Minister of Agriculture, Nature and Food Quality. A further elaboration of the response has taken place by means of literature review and additional interviews with experts involved (please see the list under 'Method' below). Two additional literature reviews have been conducted to elaborate on the question about alternative (non-animal) methods and looking ahead to the future: one at the Amsterdam UMC (in vitro methods) and one at Leiden University (analytical methods). This final report will also discuss the vaccines and medicines for COVID-19 that are being developed in greater detail, including the possibilities and limitations for accelerating the marketing authorisation process.
Finally, recommendations will be made on the basis of a number of lessons that can be learnt from the coronavirus pandemic, which may lead – now or in the future – to a reduction of the use of laboratory animals and the better use of non-animal methods.

Method

For the final report, NCad has held online interviews with experts who are directly or indirectly involved in one or more aspects of the pandemic. To get a picture of the dynamics in the research area, some experts who had been interviewed for the interim report were consulted again. The experts came from academia, with knowledge in the area of virology, immunology, epidemiology, animal models and non-animal models, from the regulatory field, from national and international government, from the animal protection movement, vaccine development and from contract research organisations.

The interviewed experts were working for or were involved with (in alphabetical order):

- WHO blueprint group
- CBG (Dutch Medicines Evaluation Board), preclinical area
- CBG Kwaliteit Geneesmiddelen (MEB Quality Assurance for Medicines)
- CCMO (Central Committee on Research Involving Human Subjects)
- CHMP (Committee for Medicinal Products for Human Use)
- Contract research organisations (CROs)
- Animal welfare organisations (Eurogroup, RSPCA)
- European Commission: DG Research and Innovation
- European Commission: Joint Research Centre Ispra
- FDA (Food and Drug Administration, US)
- Onderzoeksinstelling Primaten (primates research institution)
- RIVM (National Institute for Public Health and the Environment)
- TNO (Netherlands Organisation for Applied Scientific Research)
- University medical research centre (Erasmus MC; Virology department)
- Utrecht University, Faculty of Veterinary Medicine
- Human and veterinary vaccine development
- University of Veterinary Medicine Hanover

On the basis of the information obtained by means of interviews, two literature studies and our own literature study, we will respond in more detail to the minister's questions below.

1. Which animal models have been used around the world in COVID-19 research? What has been the influence of COVID-19 research around the world on the use of laboratory animals?

In the period of the pandemic from January 2020 to April 2022, 18 projects were granted in the Netherlands by CCD, which concerned research into COVID-19 or had an element of research into COVID-19. A total of 120,347 animals were requested for these projects. The animal species and the requested number of each species are listed in the table below. Granted projects mainly involved translational research or a combination of fundamental and translational research.

It should be emphasised that the granted projects give maximum numbers and that projects usually have a duration of five years. Practice shows that often not all granted animals or animal species are used, and this will probably also apply to COVID-19 research. Due to the urgency of the research, some animal species have been requested which have now proved to be less suitable for COVID-19 research. Examples of this are rabbits and rats.

Because in the annual reports on laboratory animal use ('*Zo doende*') COVID-19 research is not a unique objective but data on this research is distributed among different research objectives, it will not be possible to collect reliable information on the use of animals for COVID-19 research per year. Information on this will only be documented in the final report on the completed projects, and only for those projects that have a legal requirement for a retrospective project assessment. This only applies to projects where serious discomfort has occurred among all or some of the animals and for research involving non-human primates.

The COVID-19 projects granted in the Netherlands mainly relate to the development, safety and efficacy of antiviral intervention strategies against the SARS-CoV-2 virus (vaccine and medicine development) in humans and animals. Animals are used to a lesser extent for the characterisation of the SARS-CoV-2 virus and its variants.
The use of animals for research into the pathogenicity of the virus, including long Covid, has only taken place to a very limited extent and seems to have taken place mainly in patient populations (see also the section on CCMO).

Table 1. Requested number of animals in CCD-granted projects for COVID-19 research between January 2020 and April 2022

Animal species	Quantity
Mouse	77,610
Rat	5,000
Hamster	24,019
Rabbit	2,469
Ferret	2,717
Cat	1,870
Dog	301*
Mink	248
Pig	248
Non-human primate	402
Rodents	1,560

* a substantial percentage of these animals was used in a project aimed at serological screening in pets for antibodies against SARS-CoV-2.

Data on laboratory animal use is also available for Germany[7]. This data is not from the government's annual report but from a scientific publication. In the period from 1 February 2020 to 1 August 2021, 4,893 projects involving animal testing were granted, with a total of 7.7 million requested animals. Of these animals, 61,389 are destined for research on SARS-CoV-2: 89.5% mice, 7.3% hamsters and 0.06% non-human primates.

As of 1 January 2021, the Non-Technical Summaries (NTS's) of all European project applications with laboratory animals must be publicised in the publicly accessible database ALURES (Animal Use Reporting, EU System) of the European Commission. [8]this period of the pandemic, this yielded a total of 51 hits when using the search term "Covid-19" and 65 hits when using the search term "SARS-CoV-2".

Data on animal testing in the Netherlands in 2020[9] shows that 448,798 animal tests were carried out in that year. That is 142 more than in 2019. On the basis of Table 1, the annual report for 2020 shows that in comparison with 2019 (the year preceding the COVID-19 pandemic, the number of tests with mice, ferrets and pigs has dropped, while the number of tests with rats (7%), hamsters (317%), rabbits (15%), dogs (46%), cats (253%) and non-human primates (36%) has increased. Not all the numbers can be related to COVID-19 research, but this can be done with regard to the number of hamsters, cats, dogs and non-human primates.

The increase in use of dogs and cats can partly be traced back to a large-scale blood study in pets for antibodies against SARS-CoV-2. As far as a comparison with laboratory animal use in the years 2016 – 2020 is concerned, it is noted that laboratory animal use for 2020 is within the range of use in the pre-coronavirus period for the animal species mentioned most frequently, with the exception of the use of hamsters and cats (see the above comment about cats) and to a lesser extent for NHPs and rabbits. In the case of ferrets, the decrease is most likely related to the fact that research into influenza, for which the ferret is the relevant animal model, was scaled down in the initial phase of the pandemic. The increase as a result of COVID-19 research has been negated by the decrease in the use of ferrets for influenza research.

Table 2. Overview of laboratory animal use for animal species mentioned in relation to COVID-19 research for the period 2016 – 2020

Animal species	Year				
	2020	2019	2018	2017	2016
Mouse	148,291	159,614	155,524	205,993	161,978
Rat	87,169	81,603	91,579	91,537	109,589
Guinea pig	8,537	9,108	11,443	5,816	3,148
Hamster	2,856	684	911	1035	1443
Rabbit	15,373	13,298	13,788	9,764	8,579
Dog	803	550	1016	909	656
Cat	604	171	120	200	89
Ferret	570	641	475	680	294
NHP (macaques)	187	155	160	276	104

An additional question is to what extent the start of the pandemic and the lockdown period that followed have led to the discontinuation of existing tests and/or the scaling down of breeding colonies and the effect of this on laboratory animal use / the number of animals killed prior to the

[7] How many animals are used for SARS-CoV-2 research? Schwedhelm P. et al. EMBO Reports 2021: https://doi.org/10.15252
[8] Webgate.ec.europa.eu
[9] Zo doende 2020 – 2016; annual review of animal testing and laboratory animals of the Netherlands Food and Consumer Product Safety Authority

test. Information on this aspect has been collected by the Netherlands Food and Consumer Product Safety Authority (NVWA) but is not yet available for the year 2021.

However, the not-for-profit organisation Understanding Animal Research in the United Kingdom confirms the scaling down of research alongside sources in the Netherlands[10]('Despite all this research to develop vaccines and treatments for Covid-19, the majority of UK research facilities carried out significantly less research than usual, due to the various national lockdowns').

Animal models

The animal models used have their origin in the research into coronaviruses, in particular SARS (Severe Acute Respiratory Syndrome, 2002/03) and MERS (Middle East Respiratory Syndrome, 2012). As is the case with other models, the animal models in COVID-19 research involve both possibilities and limitations (Table 3).

Table 3. Animal models in SARS-CoV-2 research: possibilities, limitations and objective[11], [12], [13]

	Possibilities	Limitations	Used in
Mouse	• Many reagents present for immunological research • Much is known about the immune system	• Mice do not have ACE2, the enzyme enabling SARS-CoV-2 bonding and absorption into the cell. ACE2 Tg mice do have this enzyme. • Differences in the immune systems of mice and humans • Limited pathology (interstitial pneumonia)	• Fundamental research into the virus • Research into vaccine immunogenicity
Syrian hamster	• Homology with human ACE2 receptor • Susceptible to SARS-CoV-2 virus • Similarity with clinical signs and pathology of humans	• Infection is less severe than in humans • Differences in the immune system of hamsters and humans • Limited availability of specific reagents	• Development of vaccines and medicines
Ferret	• Susceptible to SARS-CoV-2 virus	• Course of the disease less severe than in humans (fever, focal lung pathology) • Virus mainly replicates in upper respiratory tract	• Research into virus transmission
Rat	• Standard model in toxicological research	• Limited pathology, similar to mice	• Used mainly in research into vaccine safety

[10] Understandinganimalresearch.org.uk. Animal research numbers 2020. 15 July 2021.
[11] Ketelaars M.F.M. & Kersten G.F.A. (2021). In vitro vaccine characterisation & animal models in COVID-19 vaccine development. Annex 1 to this report.
[12] How many animals are used for SARS-CoV-2 research? Schwedhelm P. et al. EMBO Reports 2021: https://doi.org/10.15252
[13] Munoz-Fontela C et al. Animal models for Covid-19. Nature (2020) : https://dol.org/10.1038/s41586-020-2787-6

Rabbit	• Standard model in pyrogenicity research	• Rabbit has ACE-2 receptor but susceptibility to SARS-CoV-2 is not clear	• Used in pyrogenicity research of vaccines and in toxicity research
Non-human primates (primarily rhesus and Java macaques)	• Immune system comparable to that of humans • Possibilities for additional and improved immune parameters	• Ethics • Costs and accommodation • Limited symptoms of illness	• Studying virus characteristics • Vaccine development (safety and efficacy) • Pathogenicity

Non-human primates

Within vaccine circles, research with non-human primates (NHPs), mainly Cynomolgus (Java) macaques and rhesus macaques, is often considered a condition for First-In-Human (FIH) trials with a new vaccine or medicine. NHP studies in turn are often the final step after preliminary research in rodents (mice, hamsters), where the safety and efficacy of the product to be researched are the points of focus. NHPs are also used in research into the transmission of the virus and the presence/absence of sterile immunity, which means that a vaccinated animal can still excrete the virus in spite of the vaccination. All vaccines that have been considered in more detail within the context of the annex Analytical models have been tested in primates.

The use of NHPs for COVID-19 research has seen a strong increase globally. In the Netherlands, approximately 200 macaques were used during the pandemic for Covid-related research. Globally, no exact figures are known, but the fact that there has been a strong increase can be deduced from the problems reported in obtaining NHPs. In Europe, a restrictive policy is pursued with regard to research with NHPs, and only F2 animals may be used, which means: offspring of parent animals bred in captivity. Many NHP experiments have taken place and currently take place in countries outside Europe, including the US. American primate centres have said they are experiencing major problems in terms of availability[14].

During the COVID-19 pandemic, NIH (National Institutes of Health, USA) made available an additional subsidy of approximately 35 million dollars to the 7 public primate centres in the US. Information on the use of NHPs for COVID-19 research in the US is not available. It should be noted that in 2019, the year preceding the pandemic, 68,257 NHPs were used in the US[13]. China, one of the largest primate suppliers in the world, has scaled back its export of primates due to the large demand for its own COVID-19 research.

The use of NHPs is seen by a number of the interviewees as ethically questionable but scientifically essential. Although primates, after infection with the SARS-CoV-2 virus, rarely present the severity of the clinical picture that may occur in humans, the infection's pathology is often similar[15]. The immune system of primates and humans are also closely related, and the development of the illness can be monitored by means of non-invasive technologies like CT and PET scans. However, NHP research has limitations in several areas[16]:

- Ethical dilemmas. Ethical dilemmas such as regarding the violation of integrity apply to all research that is carried out using laboratory animals, but in our society this is particularly the case for the use of NHPs. Primates are closest to humans, are highly developed and

[14] Nidhi Subbaraman. The US is boosting funding for research monkey in the wake of COVID. Nature 595, 633-634, 15 July 2021, https://doi.org/10.1038/d41586-021-01894.
[15] Interview Vincent Munster, Opschalen vaccinproductie wordt het grote probleem. Bionieuws, 16 May 2020.
[16] Chatfield K and Morton D. The Use of Non-human Primates in Research. In: Ethics Dumping. Schroeder D et al. (eds), pp 81-90. Springer Link. ISBN 978-3-319-64730-2 (2018)

have a complex, sophisticated social life. The use of NHPs is therefore subject to strict rules, in particular in the EU countries;
- Ecological impact: With the exception of the EU countries, the use of NHPs from the wild is still permitted, including great apes, such as chimpanzees.
- COVID-19 is mainly prevalent among older people and among those with underlying health problems. This is difficult to replicate among laboratory animals in terms of logistics and costs, in particular among NHPs. One of the experts consulted indicated that it is therefore better to use older animals (mice/hamster/rats) in studies into COVID-19 and ageing.
- Practical consequences. Animals infected with SARS-CoV-2 must be accommodated in extra secure rooms (Biosafety Level 3)[17]. This makes the research very expensive.

As regards the questions '*Which animal models have been used around the world in COVID-19 research and what has been the influence of COVID-19 research around the world on the use of laboratory animals?'* the following should be noted:

- Because of the existing system used for annual reporting on laboratory animal use and the research categories applied, both nationally and at European level, it is not possible to get a detailed picture of the extent of this use for COVID-19 research. Nevertheless, some provisional conclusions about the animal models used may be drawn from the 'Zo doende' reports. Moreover, indications can be formulated on the basis of the maximum numbers of requested animals in granted projects. Relevant data from the Netherlands and Germany was available (information scientific publication). These numbers, however, are based on possible use in the course of the project (usually five years) and will be adjusted on the basis of new insights (and this certainly applies to a pandemic such as COVID-19).

- It is clear from the interviews that have been conducted that there is a clear prevalence in the research of animal models that have also been used in the research into SARS and MERS. It is the case, however, that some models, such as mouse and ferret, have turned out to be less suitable for certain purposes. In general, mice are used for basic scientific research (such as the choice of antigen) and the preparatory research, such as safety and immunogenicity (generating antibodies), hamsters are used mainly for research into the effectiveness of vaccines and medicines, ferrets are used for research into virus transmission and NHPs are used for fine-tuning the information gained from small rodents and for the transition from the preclinical stage to the clinical stage (First-In-Human). It is also noted that Stage 1 studies in clinical research were sometimes initiated before all data from preclinical research was available (please see Table 6).

- Given the global scope of the research, in particular into vaccines and medicines, it may be assumed that the use of mice, hamsters, ferrets and NHPs will have increased, in relation to which it should be that use of NHPs primarily took place in US and China.

[17] The Atlantic Sarah Zhang. The Atlantic. August, 31, 2020. COVID-19 Vaccine Research is Facing a Monkey Shortage.

2. Which alternative (non-animal) methods have been used around the world in COVID-19 research? What has been the impact?

In global terms, non-animal methods can be subdivided into biological models and non-biological or analytical models and in silico models. In biological models, the reading parameter is a functional and qualitative response, such as, for example, the neutralisation of the SARS-CoV-2 virus by antibodies. In analytical models, the quantity of the reading parameter is what matters, for example the quantity of antibodies produced by white blood cells, but that quantity does not say anything about the capacity to neutralise the virus. In silico models use computer models for simulation purposes, the creation of databases or modelling studies.

In COVID-19 research, tissue cultures are an example of biological models and an important category of non-animal methods. These methods can be less (such as primary cell cultures) or more (such as organoid cultures and organs-on-a-chip) complex in design. Key analytical methods in COVID-19 research include the physicochemical methods such as mass spectrometry and immunochemical methods such as ELISA (enzyme-linked immunosorbent assay). In silico models, for example, have been used to systematically analyse genetic mutations in the virus or to search repositories specifically for medicines with a potential effect against the SARS-CoV-2 virus.

Two in-depth literature reviews were carried out as part of the response to the questions formulated by the Minister of Agriculture, Nature and Food Quality: one study at Amsterdam UMC into the use of the possibilities and limitations of tissue culture (*in vitro*) methods and another study at Leiden University into the use and possibilities and limitations of analytical methods. In the study into in vitro methods, the emphasis was on innovative methods, in particular organoid cultures and organ(s)-on-a-chip. The final reports of these studies have been included as annexes to these final recommendations. Below is a summary of the results.

2.1 Physicochemical and immunochemical models

The study into analytical methods makes a distinction between physicochemical and immunochemical methods. Physicochemical methods mainly provide insight into the structure (both two- and three-dimensional) and quantity of a molecule/substance. By means of these methods, a product can be adequately characterised, and on the basis of this consistency in production can be ensured. Immunochemical methods involve using immunological techniques to collect valuable information about the immune responses on the basis of aspects such as bonding and bonding strength between antigen and antibody. Examples of applied physicochemical methods in vaccine development are chromatography and spectroscopy; examples of applied immunochemical methods are ELISA (enzyme-linked immunosorbent assay) and Biosensor analysis.

In the literature study by Leiden University, a review was carried out for 12 vaccines, divided among different production methods (RNA and DNA, Vector, Sub-unit and inactivated), either with market authorisation or still in the clinical stage of research, regarding which analytical methods were used in the development stage. The conclusion is that only methods that already existed before the COVID-19 pandemic have been used for COVID-19 research. No physicochemical or immunochemical models have been developed specifically for COVID-19 research. The use of these types of model has not led directly to replacement or reduction in laboratory animal use on a 1-for-1 basis in the development and production of COVID-19 vaccines. However, the combination of well-characterised production platforms that can be used to achieve a more consistent production quality and the physicochemical and immunochemical test modalities that enable good production monitoring can be expected to ultimately lead to vaccine development and non-animal production. In this respect, the report refers to the fact that, within the context of COVID-19 vaccine development, clinical studies took place simultaneously with or even previous to the preclinical studies, which could be an indication that animal research is less relevant for new vaccines than

they were for the previous generation of vaccines. This point will be discussed in greater detail in section 4.2.1. of the report.

2.2 In vitro models

The term '*in vitro'* (Latin for "in glass") refers to the original way in which cells or tissues were cultivated outside the body. Cells can be freshly obtained from humans or animals (primary cell cultures) or can be obtained from transformed and dividing (immortalised) cells (cell lines). These cultures are still in use, but they have now been supplemented with more innovative methods that give more complexity to the cultures. The best known types of cultures are the organoid cultures (mini organs), three-dimensional structures that occur on the basis of the differentiation of stem cells, and the organs-on-a-chip (microfluidic cultures), polymer plates in which a circuit of vessels is lined by specific cells and tissues, which can mimic the dynamics of an organ system to a certain degree on the basis of perfusion systems. Many of these systems work with human cells.

The study of Amsterdam UMC (Annex 2) focused on three subquestions: a) what was the share of *in vitro* methods in global COVID-19 research; b) which research questions involved the use of *in vitro* human airway models and c) what was the role of organoid models in medicine research. Of all COVID-19 publications (accounting for 461,907 publications up to February 2022), 85,077 publications focus on reporting results of studies. The majority of them (56,579) focus on clinical research. Of these publications, 4044 describe animal testing, 2673 describe *in vitro* research and 1193 describe a combination of animal testing and *in vitro* research. *In vitro* models appear to be used in research into the infection capacity of the virus and for research into organ-specific damage and as well as into the study of the effect of medicines in particular.

Innovation in terms of organoid models and organs-on-a-chip in particular offer great potential for replacement of laboratory animal use in a number of areas[18]. Extensive collections of these models are present. The Hubrecht lab in Utrecht has more than 1000 organoid lines in a number of organs and pathologies[19]. The Respiratory data catalogue van EURL-ECVAM (the European Union Reference Laboratory – European Centre for the Validation of Alternative Methods) of the European Commission contains 284 *in vitro* models for respiratory processes and conditions[20]. These examples illustrate the attention for and activity in the field of *in vitro* models, but they also expose a weakness: the fact that many of these models lack standardisation and have only been validated to a limited extent. Focus on these aspects, apart from any technical limitations such as a short lifespan and limited organ functionality, is necessary to enable broad application[21].

2.3 Human studies

Studies with human volunteers form the basis of Stages 1, 2 and 3 of clinical research for the market authorisation of new medicines and vaccines. In that respect, these are not examples of non-animal research, although studies in humans prior to the clinical trials are. Healthy volunteers have only been used to a limited extent outside of clinical trials and in cases where this did occur it was mainly to demonstrate that a potential vaccine was able to elicit an immune response at an early stage of vaccine development: naturally, following the proven safety of the product. According to the information available, there has 'only' been a single study (not in the Netherlands) in which vaccinated subjects were infected with the virulent virus (challenge studies) as part of the

[18] Partho Protim Adhikary et al. (2021). COVID-19 highlights the model dilemma in biomedical research. Nature Review. https://doi.org/10.1038/s41578-021-00305- z

[19] Perrone F & Zilbauer M. Biobanking of human gut organoids for translational research. Experimental & Molecular Medicine 53, 1451-1458 (2021)

[20] Hynes J et al. (2020). Advanced Non-animal Models in Biomedical Research: Respiratory Tract diseases. DOI 10.2760/725821 (data.jrc.ec.europa.eu)

[21] Hofer M and Lutolf P. Engineering organoids. Nature Review Materials (2021), 6, 402 - 420

trial[22]. Prior to this study, an extensive ethical assessment was carried out[23] and the necessary precautions were taken: the trial involved young, healthy volunteers and the challenge virus will have been extensively tested for its virulence in laboratory animals prior to the trial[24]. Based on the results of the human challenge study, the President of the British Society for Immunology concluded that 'in the longer-term, the hope is that the findings of the study will now open up a new research avenue to develop a platform that will allow us to speed up the development of new vaccines, antivirals and diagnostics against COVID-19'.

Many of the studies on the pathophysiology of the various have been conducted in individuals infected with COVID-19, occasionally in combination other underlying conditions. The research questions were highly diverse and could relate to the pathophysiology of an acute infection or of long Covid, with some studies focusing on volunteers with underlying conditions. Examples of these studies are listed below. Human research is subject to strict ethical and scientific rules and must be approved by the Central Committee on Research Involving Human Subjects (CCMO) and the Medical Research Ethics Committee (MREC)[25]. The data of the CCMO[26] listed approximately [27]250 licenced studies for the period of the pandemic based on the search terms COVID 19 and SARS-CoV-2 and corona. Examples of granted studies are:

- SARS-CoV-2 vaccination response in humans living with a SARS-CoV-2 infection
- Early stage of SARS-CoV-2 immune response, including antibodies kinetics in patients with mild COVID-19 symptoms
- Prevalence of asymptotic deep vein thrombosis in admitted patients with COVID-19
- A randomised, double-blind, placebo-controlled single dosage trial to investigate the safety, tolerance, pharmacokinetics and pharmacodynamics of ALKS 6610 in healthy adult volunteers

2.4 In silico models & strategies

In silico methods and strategies make use of computer software and bioinformatics. Application within COVID-19 research is highly diverse and varies from the use of data files to the modelling of processes based on computer programmes[28]. A number of relevant examples include searching for potential COVID-19 medicines in data files of medicines that have been developed for other purposes (the so-called repurpose medicines) or collecting information on the kinetics and dynamics of the virus based on computer modelling. Bioinformatics plays a key role, for example, in using the genome sequence to make statements about new variants of the virus and the spread thereof or in identifying virus epitopes for vaccine development. The processing of data files is essential for epidemiological questions, but equally for a targeted search for relevant literature according to specific criteria (Machine Learning). Many of the areas of application cited have not led directly to a replacement or reduction of animal use, but have indirectly resulted in better and more targeted data availability.

[22] Williams S. First COVID-19 Human Challenge Trial Reveals Uneven Susceptibility. The Scientist, 3 February 2022. www.the-scientist.com

[23] Lambkin-Williams R & DeVincenzo J.P. A COVID-19 human viral challenge model. Learning from Experience.. Influenza Other Respir viruses. Nov.14(6), 747-756. http://doi.org/10.1111/irv.12797

[24] Ramathan R. et al. (2019). Use of controlled human infection models (CHIMS) to support vaccine development in US regulatory considerations. Vaccine. Doi.org/10.1016/j.vaccine.2019.06.009

[25]CCMO decides on clinical studies within the context of vaccine research and MREC decides on clinical studies relating to medicine research.

[26] CCMO website, Public CCMO register: www.ccmo.nl

[27]Not all hits can be traced back to studies COVID-19 research based on the title.

[28] Basu S et al. In silico strategies to combat COVID-19: A comprehensive review. Biotechnol Genet Eng Rev. (2021), 1: 64-81. Doi 10.1080/02648725.2021.1966920

2.5 Multidisciplinary approaches

An application that extends beyond the model is the integration of different non-animal methods into a multidisciplinary study approach. Two examples will be described within the context of research into infectious diseases.
The EURL-Joint Research Centre has started the project Modelling the Pathogenesis of COVID-19 using the Adverse Outcome Pathway (CIAO[29]). This project aims to integrate available data ([30], *in vitro* and clinical) into a coherent picture about the mechanisms involved in the pathogenicity (adverse outcome) of the SARS-CoV-2 virus. The project operates on the basis of a voluntary contribution to the scientific community, where each member shares knowledge and is involved in the integration of that knowledge. Different groups are working on various different biological systems, such as the lungs and airways.
The second example is the project VAC2VAC, which was recently completed and was financed by IMI (Innovative Medicines Initiative). The project has 22 partners from the academic world, regulatory bodies, industry and public research institutions. The project's purpose is to characterise produced vaccine batches, making use of *in vitro* and analytical methods, with the ultimate aim to replace the extensive use of laboratory animals for the batch release required by law (approximately 10% of European laboratory animal use) with non-animal methods. The results will particularly affect the use of laboratory animals for conventionally produced vaccines, such as tetanus and diphtheria vaccines, and the new generation of vaccines such as the mRNA or DNA vaccines and protein subunit vaccines to a lesser extent. Produced batches of this new generation of vaccines are largely released on the basis of physicochemical and immunochemical methods and proven consistency in production.

2.6 Financing of research

It is as yet unclear whether and to what extent the COVID-19 pandemic has led to additional research into non-animal methods in different countries. No statement can be made in this regard based on the available data. Data is available, however, on research financed by the European Commission, in particular the H-2020 Programme and the non-animal research financed by ZonMw (the Netherlands Organisation for Health Research and Development) and in cooperation with the Dutch Society for the Replacement of Animal Testing.

2.6.1 Overview of financing of COVID-19 research by the European Commission (H-2020)

In the course of the pandemic, the European Commission has made available additional funds for COVID-19 research. This research covers a broad range of focus areas: medicine and vaccine research and studying the virus, as well as the social impact of the pandemic and the financial consequences. The largest share of the funds comes from Horizon 2020, the EU funding programme for research and innovation.
Until mid-2021, this COVID-19 part had a total budget of 500 million Euro. H-2020 does not have a specific budget for non-animal research, but subsidies have been earmarked for projects of which non-animal methods form part.

[29] Modelling the pathogenesis of COVID-19 using the Adverse Outcome Pathway Framework (https://europa.eu/newsroom/events)

Table 4. Financing of COVID-19 research by the European Commission (H-2020)

Amount (Euro)	Number of projects	Projects with animal studies	Details
approximately 500 million	> 100	10 (approximately 1 million)	3 projects with NHPs (all focusing on prophylaxis) 1 project with wild animals (bats/mice), focusing on faeces research

Details of H2020 projects

Approximately 10% of the biomedical H2020 projects in the area of COVID-19 used alternatives to animal testing.

- Examples of alternatives used in COVID-19 research:
  - Air-liquid cultures of human airway epithelium
  - Microfluidic cultures of vascular epithelium, brains and neuroepithelium
  - Organoid cultures of the small intestine, liver, lung, heart and kidney

2.6.2 Overview of the financing programme More Knowledge with Fewer Animals of ZonMw.

In the Netherlands, ZonMw is funding (budget of 2.25 million euros) a number of studies aimed at non-animal innovations in COVID-19 research, mainly on the basis of its programme More Knowledge with Fewer Animals. This concerns the following projects:

- Setting up a dynamic cell model in bioreactors based on human airway epithelial cells and blood vessel cells;
- Research in cell models into the effects of a coronavirus infection on various organs, such as the upper and lower airways, intestines and kidneys;
- The use of a blood vessel model on a microchip to research the occurrence of blood clots, a complication occurring among COVID-19 patients;
- Studying lung damage and the underlying pathophysiology in various *in vitro* models such as human cell models, organoid cultures and conventional cultivation models;
- Research in human volunteers into the mechanism behind the preventative effect of heparin in a bond of SARS-CoV-2 to airway epithelial cells.
- ZonMw also finances research into the protective effect of the tuberculosis vaccine BCG against COVID-19 infection, which is done in human volunteers.

These are all studies for which funding began in 2021. Due to the fact that the projects have a duration of approximately 4 years, the results of the studies are not yet available.

2.7 What has been the effect of alternative, non-animal methods?

It is difficult to answer the question of what has been the global effect of non-animal methods on COVID-19 research the question is open to multiple interpretations. There are no concrete figures about the influence of non-animal methods on the use of laboratory animals. Even in the European Union, where monitoring the use of laboratory animals is laid down in regulations, there is no system for monitoring animal use for each individual research project. Moreover, it is clear from the interviews and the literature studies carried out that, because of its urgency, the tendency within COVID-19 research has been to fall back on existing, accepted methods, possibly with the exception of the latest generation of vaccines (RNA/DNA). This conclusion is important, as these are also production platforms that pre-eminently lead to highly defined products for which a good characterisation on the basis of analytical methods may be more important than conducting animal studies.

The impression is also that COVID-19 research may not have led to the development of new non-animal technologies (see also the report on analytical methods), but that recently developed methods that can contribute to a reduction in animal use have been able to prove their worth. This applies, for example, to the physicochemical methods in vaccine production and the use of organoid cultures in pathogenicity research. Furthermore, the COVID-19 pandemic has led to the accelerated development and application of platforms for vaccine production such as the RNA/DNA platforms, and the analytical test methods have proven to be extremely suitable for effective and adequate product control.

In conclusion, as far as the questions "*Which alternative (non-animal) methods have been used around the world in COVID-19 research? And what has been the effect?'* are concerned, it should be noted that:

- As stated in the 'Animal models' section, when the pandemic broke out the tendency was to fall back on existing animal models. In addition to the urgency of the moment, this will also have been related to sticking with tried and tested systems. However, new projects have been launched aimed at the development of non-animal models, but, with a duration of 4 to 5 years, these models will only become available after the pandemic has ended.

- This does not alter the fact that, in addition to animal models, COVID-19 research also uses existing non-animal methods (please also see the Annexes). Physico- and immunochemical methods have been widely used for vaccine development and virus characterisation; in vitro models, both simple cell culture methods and advanced organoid cultures and organs-on-a-chip, have been used for virus characterisation, pathophysiology and for the purposes of developing medicines; pathophysiology has primarily been studied in COVID-19 patients and human volunteers have been used particularly for medicine research. Finally, in silico models have been used for virus characterisation, the selection of potential medicines, epidemiology and analysis of patient data.

- In terms of the use of laboratory animals, the most important reason for using animals in COVID-19 research has been the development of medicines and of vaccines in particular. The combination of vaccine development based on the latest technologies (vector, RNA/DNA and peptide vaccines), the use of physico- and immunochemical techniques and the application of in vitro methods has the potential to lead to a transition in the use of laboratory animals (please see Chapter 3 and the annexes to this opinion for further substantiation).

- A limiting factor in this transition is the absence of in vitro immune parameters in organoid cultures and organs-on-a-chip. This means it is not, or at least not yet, possible to measure the effectiveness of a medicine or vaccine without the use of an animal model. Generating a complex *in vitro* model requires considerable input of time and resources (please also see 4.3.2.).

3. Which changes have occurred in the prescribed procedures for vaccine and/or medicine development or in the international adherence to these procedures?

At the conclusion of this opinion (April 2022), 8 COVID-19 vaccines have been authorised on the market worldwide[31]: 2 RNA vaccines (Pfizer-BioNTech and Moderna(; 2 vaccine based on viral adenovectors (Astra-Zeneca and Janssen Pharmaceuticals), 1 protein subunit vaccine (Nuvaxovid from Novavax) and 3 inactivated vaccines based on Vero cell culture: Sinovac (China) and 2 vaccines from Sinopharm (China).

[31] WHO COVID-19 vaccine tracker and landscape (who.int) (15 April 2022).

In addition, there are ongoing clinical studies for 153 vaccines, and 196 vaccines are still in the preclinical stage of development. At the EMA, 1 vaccine is currently awaiting market authorisation (Vidprevtyn from Sanofi-Pasteur)) and 5 vaccines are subject to rolling review[32]. Different platform technologies are used for the development of vaccines. Table 5 provides an overview of the platform technologies used for the vaccines that are part of clinical studies.

Table 5. Vaccine platforms used in the development of COVID-19 vaccines

Platform technology	Number of vaccines in clinical studies
Protein subunit	51
Viral vectors (non-replicating)	21
DNA	16
Inactivated	21
RNA	28
Virus-like particle	6
Other	10

(WHO COVID-19 vaccine tracker)

The total number of 349 vaccines (preclinical and clinical research) is slightly higher than the number in the interim report (321; 3 May 2021). A certain degree of international supervision in vaccine development has been carried out by WHO, the European Commission and organisations such as CEPI (the Coalition for Epidemic Preparedness Innovations) and Gavi (a vaccine alliance with WHO, UNICEF, the World Bank and the Bill & Melinda Gates Foundation as its most important partners), but it must be concluded that vaccine development was of such an urgent nature that individual countries and companies initiated a development programme immediately at the start of the pandemic and have continued to follow this.

EMA[33] has admitted 8 medicines for COVID-19 to the market (April 2022). Furthermore, two applications are awaiting authorisation. Five of these medicines belong to the category of monoclonal antibodies, and five are pharmaceuticals. Medicines are prescribed as a curative, after diagnosing the infection, but there are also medicines under development that should work as preventative treatment. In addition to these antiviral medicines, research is done into supporting medicines for patients with a COVID-19 infection, such as the use of anti-inflammatories like dexamethasone or convalescent plasma. Finally, medicines have been investigated which have been released by the authorities for other purposes but which were expected to be effective for COVID-19 Examples of these so-called repurposed medicines include the antiparasitic substance Ivermectin and Hydroxychloroquine, a drug against malaria[34]. The data on the safety of repurposed medicines is already available, however additional research (including in animals) is generally required regarding possible protection against COVID-19 infection.

Steps in the development and authorisation of a vaccine or medicine have been described in the interim report. In brief, the development stage (isolation and characterisation of the virus, antigen selection) is followed by preclinical studies in which the safety of the vaccine or medicine is assessed, the pharmacokinetics and dynamics of the vaccine or medicine are studied to determine the efficacy, and information is generated about dosages, the administration route and the number of immunisations/injections required, and in which the product's efficacy is established. This preclinical research partly uses non-animal methods, but laboratory animals are used for a number of studies. In general terms, it can be said that non-animal methods are used in the initial stage of preclinical research, and that animal studies are mainly used in the final stage.

[32] www.ema.europa.eu (15 April 2022)
[33] www.ema.europa.eu (15 April 2022)
[34] The efficacy of both medicines for COVID-19 has been strongly called into doubt.

Pre-clinical animal studies are not prescribed by law but may be included in the marketing authorisation dossier when the vaccine or medicine is authorised, in which case a control test prescribed by law for the routine production of vaccines or medicines must be undertaken. This may be the case for conventionally produced vaccines (inactivated and live, weakened vaccines) but this is rarely the case for more innovative platform technologies such as RNA, DNA and protein subunit vaccines.

The latter products have already been extensively characterised and standardised in the development stage. Control in batch production can then be limited to non-animal methods, which demonstrates consistency in production.

The standard procedure in medicine and vaccine development is that the different steps towards authorisation are undertaken sequentially. his offers the developers the option to take Go/No-Go decisions after each step. Developers may decide to stop further development on the basis of the results of safety studies, or they may decide to adjust the antigen composition on the basis of immunogenicity studies. The assessment authorities will ultimately get the whole marketing authorisation dossier, which offers the possibility to view specific results within the context of the whole. As a consequence, however, the marketing authorisation dossier cannot be assessed until the final stage of product development, which in practice is often after a number of years after the start of development. Because of the urgent aspect of the COVID-19 pandemic, in some cases the standard procedure has been deviated from, some clinical studies started before the preclinical studies had been concluded, and it was decided that the assessment authorities would assess the research data as soon as they became available: the so-called ‘rolling review’. On the basis of the findings of the assessment authorities, the marketing authorisation authority (in Europe the European Medicines Agency, EMA) will decide to release a product for the market.

The non-sequential approach in research and the application of the rolling review for COVID-19 vaccines and medicines has led to a significant reduction in the time between the start of the development and product marketing authorisation. However, the rolling review procedure also has limitations. Normally, the assessment of a product dossier takes 1,200 hours. When the procedure is accelerated, this increases to 2,400 hours. The reason for this is that the documents for assessment presented by the manufacturer do not follow on from each other, additional information must be requested and documents need to be reassessed in the case of a changed context as a result of additional data. This means the burden on the staff of the marketing authorisation authorities increases enormously, and as a consequence the assessment of other dossiers will be delayed (please also see Table 6).

In the interim report, the factors that have led to an acceleration of the vaccine development programme are listed in a table. For the final version of these recommendations, in-depth discussions took place with representatives of the assessment and marketing authorisation authorities. This has led to a number of caveats in respect of the acceleration, which make it less likely that a rolling review procedure will become the standard. The caveats are specified in the table below.

Table 6. Acceleration in vaccine development and authorisation: factors, acceleration and caveats

	Conventional process from development to authorisation	Accelerated COVID-19 vaccine development and authorisation	Additional comments
Factor			
Technology	Conventional vaccines based on inactivation/attenuation of the microorganism, often with the addition of an adjuvant to strengthen the immune response.	Innovative vaccine technologies (RNA and DNA) have been used, which had proved their value in the development of the Ebola vaccine. As information about the efficacy and safety of these technologies was available at the start of the pandemic, the development of these vaccines could be accelerated.	Because the SARS-CoV-2 virus is prone to mutation, vaccine modifications will continue to be necessary. Both from a scientific/societal point of view and from the point of view of laboratory animal use, it is important to use well-characterised vaccine platforms such as the mRNA, DNA, vector or peptide vaccines. Well-characterised means that information about structure, stability and protein-folding is recorded, and production takes place consistently. This prevents a situation where animal testing for safety and efficacy needs to take place routinely for vaccines released in batches. Vaccine development takes time[35]. Antony Fauci, director of US NIAID[36], has suggested to anticipate this and to develop and characterise prototype vaccines for 20 virus families.
Vaccine manufacturers	Often, the market for a new vaccine is limited and the decision to invest in development requires an in-depth cost-benefit analysis.	In the COVID-19 pandemic, however, marketing a vaccine can be a blockbuster for the manufacturer and also boost their reputation.	Developing and marketing these "emergency" products also involves a risk of damage to the manufacturer's reputation, especially if a product turns out to be less effective than anticipated. Manufacturers have taken this risk in the urgent situation of the pandemic, but they will be less inclined to do this for products where speed of development is less of an issue.

[35] To illustrate this point, the first steps towards the development of mRNA vaccines packed in tiny fat balls (liposomes) were taken in the 1960s (Dolgin E., The tangled history of mRNA vaccines. Nature, 16 September 2021, Vol. 597: 318-324

[36] NIAID: National Institute of Allergy and Infectious Diseases, US

	Vaccine development is costly. Numerous go/no-go steps have been built into the development to keep the financial and scientific risks manageable.	Manufacturers made agreements with governments on purchase volumes during the development phase. Financial risks were therefore less of an issue. This meant manufacturers could accelerate the development of promising products considerably. The process was accelerated by carrying out phase 1 and phase 2 trials partly in parallel with preclinical studies (e.g. animal studies in NHPs). The scaling up of vaccine production was already underway before (conditional) authorisation was obtained.	Governments will be less willing or unwilling to make large investments for less urgent vaccine development. The financial risks therefore lie with the industry, which will use strictly defined and time-consuming Go/No Go criteria to minimise these risks. Moreover, vaccine production will not be scaled up until authorisation has been guaranteed. Conducting clinical studies partly in parallel with pre-clinical studies has implications for the required capacity of assessors (please see previous explanation).
	Vaccine development is one of the activities of a manufacturer and 'competes' with other priorities.	Absolute priority was given to the development of a COVID-19 vaccine. This has led to more than 300 candidate vaccines that are more or less advanced in the development process. Support was provided by academic and other research institutions.	Absolute prioritisation will not occur if there is no urgent reason for their development. Collaborations between industry and university institutions will continue, but they will be less dynamic.
Preclinical research	Preclinical studies require prior approval from CCD (after obtaining an opinion from the Animal Tests Committee), and each individual animal study requires prior approval from the Animal Welfare Body.	The procedures and assessment framework have not changed. However, priority has been given to COVID-19 related research and, where possible, CCD has handled dossiers entirely in writing instead of discussing them in meetings held every three weeks.[37]	Here, similarly, a return will be made to the method of the pre-coronavirus period, with no prioritisation taking place.
Clinical research	By law, clinical trials require a prior ethics review by a competent authority (CCMO or METC). There is no prioritisation of dossiers.	Situation in the Netherlands: fast-track procedure for assessment by CCMO (vaccines) and METCs (therapeutics). No difference in assessment framework between COVID-19 research and other	Again, the method in the pre-coronavirus period will be returned to, with no prioritisation being applied.

[37] In the period up to November 2021, CCD handled 17 applications regarding COVID-19 projects.

		research. For therapeutic clinical studies, prioritisation has been carried out by the CoCoN (NFU COVID Committee) of the NFU[38]	
	Clinical studies require sufficiently large cohorts of people to demonstrate the effect of the treatment. The level of protection is a key criterion for vaccines against infectious diseases. In many cases, the infection rate in a population is low, which means that it takes a long time before any effect is demonstrated.	In the case of COVID-19, the infection rate was high, resulting in sufficient information becoming available in a short period of time about control and vaccine groups to demonstrate the protective effect. People's willingness to participate in the trials was also high.	Starting up clinical studies will take longer, because putting together cohorts of test subjects will require more time. It will be more difficult to find test subjects, in particular for phase 1.
Assessment authorities	Assessment of the full marketing authorisation dossier takes place after the manufacturer has completed all studies (preclinical and clinical).	Priority has been given to COVID-19 protocols, for which separate assessment sessions are scheduled. A rolling review procedure was applied, in which interim opinions were given and parts of the dossier were assessed as soon as they were received by the authorities. As a result, clinical trials could be started on the basis of demonstrated safety in preclinical studies.	The sequential research method offers the developers momentum for making go/no-go decisions after each step. The assessment authorities will ultimately get the final marketing authorisation dossier, which offers the possibility to view specific results within the context of the whole. As a consequence, however, the marketing authorisation dossier cannot be assessed until the final stage of product development, which in practice will often be after the clinical study.
	All dossiers presented to the assessment authorities are dealt with. There is no prioritisation.	EMA set up an EMA pandemic Task Force (ETF), which makes a proposal for selection on the basis of the quality and completeness of submitted dossiers. The ETF advises EMA-CHMP.	Due to the urgent nature of the COVID-19 pandemic, the standard procedure was deviated from and the rolling review was used instead. On the basis of the findings of the assessment authorities, the marketing authorisation authority (in Europe the European Medicines Agency, EMA) will decide to release a product for the market.

[38] NFU: Netherlands Federation of University Medical Centres. www.nfu.nl

			The non-sequential approach in research and the application of the rolling review for COVID-19 vaccines and medicines has led to a significant reduction in the time between the start of the development and product marketing authorisation. The rolling review procedure, however, equally has limitations for reasons outlined in the above.
	The dossier is only assessed when **all** results are available, including the results of longevity studies (to determine how long a vaccine provides protection) and reproductive toxicity studies; studies that take longer than six months. Upon submission, three batches of the product have already been commercially manufactured and tested to confirm consistency in the manufacturing process.	A rolling review uses Conditional Marketing Authorisations (CMAs), which means that the developed vaccine is released under conditions. The manufacturer is obliged to supply additional data, such as data on scaling up production results of longevity studies. Failure to comply with this obligation will result in the conditional release being withdrawn. Results of these studies have not yet been included in the marketing authorisation dossier and will be added at a later stage (after marketing authorisation). Upon marketing authorisation, three batches confirming the commercial manufacturing process were not available yet, but a protocol was in place to which the batches had to adhere.	The non-sequential approach in research and the application of the *rolling review* for COVID-19 vaccines and medicines has led to a significant reduction in the time between the start of the development and product marketing authorisation. However, the rolling review procedure also has limitations, as outlined previously. In the case of longevity studies, data is not immediately required for a cost/benefit evaluation, but is required for the potential long-term effects (safety, effectiveness) of the vaccine and there for the ultimate acceptance of the vaccine.

In the summary of steps in the production and authorisation of vaccines and medicines one final step is missing: post-marketing surveillance for possible side effects[39]. This is relevant to the COVID-19 vaccine because the possible side effects of these vaccines are under scrutiny. Post-marketing surveillance is a legal requirement when introducing a vaccine/medicine into the market. The findings of doctors are centrally recorded and assessed for causality. Occasionally findings may lead to additional research, including animal experiments. A well-known example of this is the study that was carried out several years ago into a possible relationship between the occurrence of autism and the presence of mercury in the preservative Thiomersal that can be added to vaccines. Extensive research has shown that no such correlation exists[40].

Side-effects after COVID-19 vaccination have been reported to Lareb, the reporting and knowledge centre for side effects of medicines. Lareb performs part of the legal requirement for pharmacovigilance on behalf of the Medicines Evaluation Board (MEB). Up to 3 April 2022, Lareb received 212,887 reports out of a total of 34.8 million COVID-19 vaccinations administered. As far as can be ascertain, these reports have not led to additional animal experiments. The frequency of occurrence among humans was so low that, statistically, such a side effect would not be displayed among laboratory animals, unless very large numbers of animals were to be used.

In conclusion, as far as the question *'Which changes have occurred in the prescribed procedures for vaccine and/or medicine development or in the international adherence to these procedures?'* is concerned, it should be noted that:

- At the beginning of April 2022, 349 COVID-19 vaccines were in development, with 196 in the pre-clinical stage of research and 153 in clinical trials.

- At the start of the pandemic, conventional (both in vivo and non-animal) models from corona research were used primarily. The urgent nature of the pandemic formed an obstacle to the use of new and less well-trusted models.

- That sense of urgency has also led – at least for part of the vaccines – to a significant reduction in time from vaccine development to authorisation. This was due to measures such as the overlap between clinical and preclinical research, assessment on the basis of a rolling review and conditional authorisation. These adjustments have had consequences for the investment in capacity and therefore the costs, in both the development stage and the assessment stage.

- The process of saving time did not negatively impact on safety and efficacy; neither in terms of the manufacturer developing the vaccine or medicine, nor in terms of marketing authorisation authorities. This is to say that the quality of the product assessment has not been compromised, with the exception of the studies that are necessary for long-term effects, such as the duration of accrued protection after vaccination, effects on pregnancy. Information on these aspects was provided at a later stage and in the event of the absence of effects, the conditional authorisation was converted to an authorisation.

[39] www.lareb.nl
[40] DeStefano F et.al. Principle controversies in vaccine safety in the United States. Clin.Infect.Dis. 2019: 69 (4), 726-731 1

4. What lessons or advice, in the context of the COVID-19 pandemic, can be distilled for the future of the transition to non-animal innovation?

The COVID-19 pandemic arrived unexpectedly at the end of 2019, in a world that was not prepared for such an eventuality and made us realise that a pandemic can happen any time. The impact of COVID-19 was and still is (spring 2022) immense, resulting in an overburdened healthcare system, complex socio-economic problems and costs we can barely even identify or begin to comprehend. Unfortunately, it is not yet possible to give exact figures on the use of laboratory animals, but initial data in the Netherlands and Germany[41] (with the latter based on a scientific publication) shows that although there have been changes in the use of laboratory animals, these changes are not exceptional. Of the 369 applications that were approved by the Central Authority for Scientific Procedures on Animals (CCD) between January 2020 and November 2021, 18 projects related to COVID-19 research (characterisation of the virus, development and control of vaccines and – albeit to a lesser extent – of medicines). From November 2021 to April 2022, no further projects have been approved in relation to COVID-19 research.

The use of animals for COVID-19 research in particular translates into an increasing number of hamsters, mice and non-human primates (NHPs), with the caveat that the increase in mice for COVID-19 in the annual records has been obscured by a scaling down of other research using mice. A finding that the figures on laboratory animal use appear better than expected would, without further information and discussion, be detrimental to the problem of laboratory animal use. In EU legislation, an intrinsic value is attributed to animals. This means that animals are more than just a model for research. Each use requires an ethical assessment, irrespective of the purpose of their use. For that reason, the question in relation to COVID-19 research of whether and how we can replace this use with non-animal methods, either directly or indirectly, is justified.

Below we will address the question of the minister about the lessons that can be learnt from the COVID-19 pandemic in terms of the transition to animal-free innovation. The concept of 'innovation' will be broadly interpreted and will also concern strategies in policy, laws and regulations and research. The classification for Technology Transition will be based on the multilevel perspective. The multilevel perspective distinguishes three different levels: macro, meso and micro level. The macro level represents the regime of global or continental infrastructures, insights or collaborations. The meso level is based on the laws and regulations regime, knowledge structures and international organisations. Finally, the micro level is the niche level, where new developments or insights are allowed to mature.

The recommendations made below relate to the COVID-19 pandemic, however their scope will go beyond this particular area.
Where possible, the lessons learnt will be accompanied by proposals to the minister for promoting the transition to animal-free innovation. These proposals explicitly include preventing the use of laboratory animals during possible future pandemics.

[41] How many animals are used for SARS-CoV-2 research? Schwedhelm P. et al. EMBO Reports 2021: https://doi.org/10.15252

4.1 Macro level

4.1.1 One Health

One Health is a movement in the medical and biological community based on the correlation and interconnection between humans, animals and nature. Changes to one category will have an impact on another category and any research and approaches will therefore have to be multidisciplinary in nature.

COVID-19 is a zoonosis: a disease that can be transmitted from animals to humans. In the cases of certain zoonotic diseases, humans will not spread the disease further as in the case of Q fever, where the pathogen is transmitted from sheep/goats to humans but not from humans to humans. This is different for zoonotic diseases such as influenza and COVID-19, where human-to-human infection will occur. The SARS-CoV-2 has now been detected in various animal species[42],[43] including in mink, ferrets, cats, dogs, bats, chickens, pigs and hamsters.

There is no direct link between One Health and the use of laboratory animals, however there is an indirect link. Preventing a pandemic removes the need to use large numbers of animals for the development of therapeutics and prophylactics.

One possible origin for a pandemic is the way we keep and use animals[44]. Aspects that can be cited in this context include deforestation and climate change, which drive wild animals into inhabited areas, the close coexistence of humans and animals in certain cultures and intensive livestock farming. This means that potential pathogens are able to multiply rapidly and spread around the world in a short period of time due to our travel behaviour. Pandemics based on a zoonosis cannot always be prevented, however the risk can be reduced by tackling environmental problems, changing the way we deal with animals and adapting our lifestyle. All this should be supported by enhanced global monitoring systems for potential zoonotic diseases (pandemic preparedness). One organisation that has built up experience with pandemic preparedness is the World Organization for Animal Health (OIE)[45], including for zoonotic influenza pandemics. Investments in pandemic preparedness systems will be significant but will ultimately be dwarfed by the costs of a pandemic.

The Netherlands has been one of the leaders and pioneers of the One Health movement. The Minister is requested to continue to promote the relevance and value of One Health and therefore of sustainable and structural solutions to preventing pandemics.
In addition, the Minister is requested to continue the government's commitment to pandemic preparedness within multinational partnerships.

In the context of pandemic preparedness, the government has included an investment sum of 180 million euros for 2022 in the Coalition Agreement, increasing to 300 million euros by 2026.[46] The Minister is requested to continue to make efforts to earmark part of this investment for the development of innovative non-animal instruments for monitoring, prevention and prophylaxis.

[42] OIE, COVID-19. www.oie.int
[43] Animal Models and Resources for Coronavirus Research. National Institutes of Health (NIH), Office of Research Structure. www.orip.nih.gov
[44] Laaser U. One Health for One Planet: How to address 21st century education challenges. Business Environment, Health, Policies & Foreign Affairs. SDG Series, Society, Impakter. October 6, 2021.
[45] World Organization for Animal Health (OIE). COVID-19. www.oie.int
[46] Central government. Budgetary annex to the Coalition Agreement, 15 December 2021.

4.1.2 Vaccine R&D

The urgency of the COVID-19 pandemic placed a significant amount of pressure on the vaccine or drug development, primarily in the industry. This frequently involved the use of platform technologies that were developed in an academic setting. Technologies have subsequently been used by the industry for the development of virus-specific vaccines, their optimisation and for the scaling of their production. It is primarily problems relating to the latter aspect that often lead to delay to the delivery date of a new vaccine.

The figures of the WHO COVID-19 vaccine tracker show that eight vaccines were authorised on 3 February 2022, while clinical trials into 153 potential vaccines are taking place and 196 vaccines are at the preclinical trial stage. Broadly speaking, all vaccines can be traced back to 11 types of production platforms, including the RNA, DNA and subunit vaccines. There are differences between the vaccines under development in terms of the choice of antigen, the formulation, the administration route, the use of an adjuvant, etc. The problem of virus mutations likewise has an impact on the development of vaccines and requires vaccines based on conserved antigens, meaning virus antigens that are present in all COVID-19 virus mutations. The pressure on the development of a safe and effective vaccine does not, however, account for the amount of vaccines being worked on. It is plausible that in addition to the public health aspect, economic and national interests may have played a key role.

In the opinion of vaccine experts, vaccine development is not yet possible without the use of laboratory animals. The multitude of vaccines under development has led to extensive animal use. A survey conducted in the UK into the acceptance of animal testing for COVID-19 research found that a majority of citizens that took part in the survey considered the use of animals to be acceptable albeit morally problematic. Or, as one of the interviewees put it pertinently: '*Because I can accept it, doesn't mean that I like it*'[47].

In global terms, the WHO has taken on a coordinating role in the domain of COVID-19 research, including through the blueprint group, but equally in the development of vaccines and medicines. This role is implemented by means of partnerships with other globally operating organisations, such as the Coalition for Epidemic Preparedness Innovations (CEPI). CEPI[48] is supported by a number of countries, including the Netherlands. Another global organisation which is active in vaccine development is Gavi[49], a partnership in the field of vaccines between WHO, UNICEF, the World Bank, the Bill & Melinda Gates Foundation, private vaccine developers and donor countries. Gavi mainly focuses on third-world countries. In an ideal situation, these organisations should be able to make a pre-entry selection for vaccine development, based on needs, development concept and assumed added value in comparison with other products. It is recognised that this infringes on free market thinking and that this free market thinking can drive innovation.

From a rapid vaccine development perspective, an intriguing approach has been put forward by Anthony Fauci[50], the Director of the US National Institute of Allergy and Infectious Diseases (NIAID). His proposal is to develop a prototype vaccine for 20 virus families that could potentially cause a pandemic, have it tested for safety and effectiveness and then realise the conditions required for large-scale production. In the event of a pandemic, the prototype vaccine can be adapted quickly to the specific pathogen causing the pandemic. This vaccine could then be registered by regulatory authorities on the basis of minimal additional data, after which production can be scaled up quickly.

[47] www.understandinganimalresearch.org.uk
[48] www.CEPI.net
[49] www.gavi.org
[50] Kahn L. Future pandemics : act now or pay more later – How One Health and prototype vaccines shift the odds. Health, Politics & Foreign Affairs, Science, Society. Impakter, October 6, 2021

In the context of a restrictive policy on laboratory animals, the Minister is requested to promote alignment and coordination of vaccine development through national contacts with organisations such as the WHO, CEPI and Gavi.

The Minister is also requested to instruct a review into whether an approach in which prototype vaccines are developed for a number of virus families could also yield advantages in terms of the use of laboratory animals, in the context of technical and practical feasibility.

4.2 Meso level

Assessment and authorisation

It was stated in the interim report that the application of a non-sequential approach of preclinical and clinical research, the assessment of incomplete dossiers at entry level for COVID-19 research (the rolling review) and conditional licensing have led to an acceleration of the assessment process. It is clear from additional interviews that this approach also has its limitations and requires further qualification. The reasons cited in this regard are outlined in Table 6.

All this means that the acceleration of the assessment process has an impact of budgets. The introduction of a fast-track process therefore becomes a political decision, based on the willingness to provide additional funding.

As regards the use of laboratory animals, a sequential approach as such will not lead to a reduction in use. Preclinical research will continue to be necessary but will be redistributed over time. This does not change the fact that there are other ways to reduce the use of laboratory animals, both at meso and micro level.

A golden standard within the development and production of vaccines and medicines, laid down by law, is that a new product must be safe and effective. It is not stated anywhere that this must be done on the basis of research involving animals. In the research and pre-clinical stage, developers have the opportunity to use that method or those methods that is or are regarded as most relevant. In spite of the fact that non-animal methods are used in various areas of the research, such as analytical methods, performing standard animal-based studies remains the default. This can partly be understood given that although the animal model has its limitations, it always provides more information than the non-animal approach. However, this reticence is also partly motivated by uncertainty about and/or fear of potentially hidden pitfalls that may present themselves if animal testing has not been carried out, otherwise described as ‘the fear of letting go’.

This could also apply to conducting a study into NHPs prior to the first clinical studies (‘First-In-Human’). This can be illustrated by the fact that all vaccines in Table 4 of the annex ‘Analytical methods’ have been tested in NHPs. A retrospective analysis of the applications submitted to CCMO for clinical studies and dossiers submitted to the assessment authorisation authorities (CBG/EMA) could provide insight into the contribution of used animal studies to the ultimate decision by CCMO to conduct clinical studies and into the dossier assessments by the marketing authorisation authorities. As the responsibility for dossier assessment and product authorisation falls under the Ministry of Health, Welfare and Sport, cooperation with and control by this ministry is necessary.

The key principle of the assessment process is the proven safety and effectiveness of the new vaccine or medicine. In this context, it is doubtful whether assessment via the (expensive) rolling review approach requires less animal testing research than the usual sequential approach. More can be expected from a study into the relevance of existing animal studies (below) and into the use of time-saving non-animal methods (please see 4.3.2.).

The minister is asked to make funds available, in consultation with the Ministry of Health, Welfare and Sport, for conducting a retrospective analysis of the value and limitations of standard animal studies in the research stage and the preclinical trials of vaccines and medicines.

Key factors for the application of non-animal methods in regulatory research include pursuing a policy aimed at the development of these methods, their acceptance by national and international supervisory authorities and the global harmonisation of test guidelines.
In this context, the Minister is requested to promote a guiding policy through existing channels, including in respect of organisations such as the World Health Organization (WHO), the European Medicines Agency (EMA) and the European Pharmacopoeia (Ph.Eur.).

4.3 Micro level

4.3.1 Vaccine platform

Since the introduction of the first vaccine by Jenner (the smallpox vaccine) at the end of the 18th century, vaccine development has evolved. Conventional vaccines are based on the inactivation or attenuation of the microorganism or the detoxification of the toxin produced by the microorganism. Examples of inactivated vaccines are the rabies vaccine and the whooping cough vaccine, attenuated vaccines include the measles and mumps vaccine, and examples of toxoid vaccines are the tetanus and diphtheria vaccine. Subunit protein vaccines have been available since the 1970s. These vaccines only use the parts of microorganism that are important for creating immunity: the antigens. Examples are the acellular whooping cough vaccine, the Hepatitis B vaccine and the pneumococcal vaccine. The latest generation of vaccines, such as the mRNA vaccines and vector vaccines for COVID-19, use molecular biological techniques which were developed at the end of the last century.

The data on laboratory animal use in the EU shows that roughly 10% of all laboratory animals are used for the development, production and testing of organic products[51]. The category is also characterised by the high percentage of animals experiencing severe distress. Further analysis of the numbers shows that the majority of the animals is used for testing the quality of routinely produced vaccine batches for human and veterinary use. These vaccines primarily relate to the conventional vaccines such as for tetanus and diphtheria or the clostridium vaccines for veterinary use. The new generation of vaccines requires laboratory animals in the development phase but requires none or very few in routine batch testing. This has everything to do with the fact that these types of vaccines are extensively characterised in the development stage and production is extensively standardised and monitored by means of a set of analytical methods. With a view to a reduction in the use of laboratory animals, it is therefore to be recommended to focus on non-conventional development methods when developing a vaccine.

In order to further reduce the use of laboratory animals, it is recommended that the Minister join up as much as possible with vaccine development at an international level aimed at the development and use of non-conventional methods.

4.3.2 Optimisation of innovative in vitro methods and focusing on other non-animal methods

Annex 2 'Learning from COVID-2. SARS-CoV-19 and Organoid technology' (report by Amsterdam UMC) presents an overview of innovative *in vitro* methods such as organoid and microfluidic cultures or organs-on-a-chip. These methods have now been incorporated and are also used in COVID-19 research. Organ-specific organoids are used for research into the pathogenicity of the virus, while microfluidic cultures of lung tissue are used for research into the kinetics and dynamics

[51] European Commission. Summary report on the statistics on the use of animals for scientific purposes in the Member States of European Union and Norway in 2018. 14.7.2021 SWD(2021) 204Final

of the virus. These methods have many advantages, including the reduction in laboratory animal use, but also a reduction in costs and research time. Not all researchers have picked up on these advantages, and it will remain necessary to draw attention to these models. This can be aided by clearly showing the advantages of robust and valid *in vitro* methods.

However, these methods are also still characterised by limitations. One of the most important disadvantages in research into respiratory viruses such as SARS-CoV-2 is the limited integration of immune parameters into the *in vitro* models[52]. These limitations are particularly important in research questions where these parameters are essential, such as research into the immunogenicity and effectiveness of vaccines. The immune system is extremely complex and includes various types of immune cells and signalling molecules, each with a specific function and an extensive communication network. Integration of immune parameters into *in vitro* methods as microfluidic cultures will be a difficult task and will require a considerable investment of time, money and cooperation. The first steps in this field have been taken, however are still at an embryonic stage[53].

In addition, aspects such as protocol standardisation and validation are critical to many innovative cell culture methods in order to make these methods attractive for research.

The government's alternative policy focuses primarily on innovative cell culture models. This translates into funding for non-animal research that is often single-mindedly focused on the development of these cell culture models. It should be noted that, particularly in the area of infectious diseases and corresponding vaccine and medicine development, there is at least as much potential in other non-animal methods. Greater balance and proportionality in the distribution of policy emphasis and available funds would do justice to the importance of physico- and immunochemical techniques, research involving human subjects and in silico models for non-animal research.

The minister is requested to encourage research into the integration of complex immunological parameters in the new generations of in vitro methods.

With regard to existing innovative in vitro systems, the Minister is requested to make funds available within research programmes for standardisation and validation of these systems, if possible in an international context.

In addition, the Minister is requested to focus policy emphasis and resource allocation for non-animal research on other techniques and methods in addition to innovative cell culture systems, which includes the analytical techniques, research involving human subjects and in silico techniques.

4.3.3 Shortening the duration of research

In EU Member States, animal testing is subject to conditions. Article 36 van Directive 2010/63/EU on the Protection of animals used for scientific purposes[54] stipulates that projects involving animal studies may only be carried out if they have been authorised by a competent authority. In the Netherlands, this is the Central Authority for Scientific Procedures on Animals (CCD). Authorisation is based on an ethical assessment of the interests of the animals (violation of integrity, discomfort) and the research interests (scientific, social and economic). This assessment process takes time, and is therefore sometimes regarded as a hindrance when it comes to responding quickly to

[52] Bartfeld S. Realizing the potentials of organoids – an interview with Hans Clevers. Journal of Molecular Medicine (2021), 99: 443-447
[53] Bar-Ephraim Y.E., Kretzschmar K. and Clevers H. Organoids in immunological research: Review. Nature Reviews/Immunology (2020), 20: 279-293.
[54] Council Directive 2010/63/EU on the protection of animals used for scientific procedures. Document 32010L0063, 22 September 2010.

biomedical problems such as the COVID-19 pandemic[55]. In general, however, there are no indications that this has been the case. On the contrary. As is the case with the assessment by CCMO in terms of clinical studies and dossier assessment by CBG, CCD has also given priority to the assessment of projects relating to COVID-19 research, making sure assessment takes place with due diligence.

In terms of animal testing, it is not so much the administrative actions of CCD that make it difficult to produce research results within a very short period of time, but rather the logistics of the research (planning, the availability of animals) and the length of the research process (research into the effectiveness of a vaccine by means of animal testing will take at least a couple of months, while research into the duration of immunity will take more than 6 months), which slow down the process. A characteristic of many non-animal methods is that they generate a large volume of research data in a shorter period of time. Analytical methods generally only take a few data, with in vitro methods taking a few weeks. In addition, no authorisation procedure is required for the use of these non-animal methods, which results in additional time savings. Partly because of the above, the importance of the urgent development of non-animal methods is broadly shared[56].

The reduction of the research time will mainly have to be achieved by using faster test methods, which will mainly relate to non-animal methods, both in vitro and analytical. It is recommended that the Minister invest in the development, optimisation and validation of these methods.

4.3.4 Sharing research data

The COVID-19 pandemic has led to a global focus on the virus, the disease caused by the virus, the consequences of the disease and prevention and therapy. The time between the start of the pandemic and the publication of these recommendations is, in terms of how long research tends to take, extremely short. Considering the large number of scientific publications that have appeared since January 2020, there must have been overlaps in research, which has meant that research has been done twice, but also that some of the current studies have been superseded by new insights.

This underlines the importance of a different system for distributing information, avoiding the time-intensive route of peer review, feedback and amendment of a manuscript for publication but a portal for research results which are shared with the field (open science). In our country, the Netherlands Organisation for Scientific Research (NWO) has taken the lead in championing the transition to open science. The NWO regards open science as a movement that represents a more open and participatory research practice in which publications, data, software and other forms of scientific information are shared at the earliest possible stage and made available for reuse[57].

To some extent, this new approach was followed by the WHO blueprint group, where research results were shared and discussed in weekly online meetings in a community of researchers from different forums. The CIAO project of the EURL-Joint Research Centre (please also see 2.5.) is similar in design but more focused on collaboration. Such initiatives deserve support, while proper arrangements should be made in advance about contributing and using information.

[55] Genzel Lisa et al. How the COVID-19 pandemic highlights the necessity of animal research. Current Biology (2020), 30: 1014 – 1018.
[56] OECD. Guidance Document on Good In Vitro Method Practices (GIVIMP). PECD Series on Testing and Assessment. OECD Publishing, 2018. Doi.org/101787/9789264304796-en
[57] NWO. Open Science. nwo.nl

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appendix 1

COVID-19 vaccine development and characterisation: animal models and analytical methods

The potential of analytical methods to reduce, refine & replace animal use

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Abstract

Background. Standard vaccine development takes several years, but during the SARS-CoV-2 pandemic we have seen lightning-fast product development. This was possible because of parallel execution of clinical and preclinical assessment, and commercial production 'at economical risk'. It gives rise to new questions on what we can learn from this pandemic about vaccine development and quality control.

Aim. The aim of this paper is to gather all relevant literature on COVID-19 vaccines in development to provide an overview of the analytical methods used in vaccine development and their potential and limitations in replacing of animal models. Moreover, the role of these methods in SARS-CoV-2 vaccine development and their contribution to reduction, refinement or replacement of animal use will be investigated. Finally, developments in analytical characterisation that may affect animal use in the coming years will be discussed.

Introduction. Vaccines are a heterogeneous product group that are difficult to characterise, and their mechanisms of action are not always fully understood. Hence, each candidate vaccine needs a specific set of analytical methods to characterise and test it. Numerous analytical methods are available that could be part of a set of immunochemical and physico-chemical assays to improve the characterisation without the frequent use of long lasting, expensive, or inaccurate functional immunogenicity studies, partly done in animal models.

Findings. Analytical characterisation techniques used for COVID-19 vaccines were not different from other new generation vaccines, nor did they contribute to reduction of animal use. The SARS-CoV-2 vaccine development as such did not lead to extensive use of new analytics or new characterisation technology, mainly due the urgency of the pandemic which urged scientist to use existing and accepted techniques, rather than using innovative but less established methods. It is expected that the mRNA and vector platforms will be further developed and increasingly applied. The availability of proven vaccine platform technology with respect to safety, efficacy and technical robustness likely will lead to shorter development time for future vaccines and potentially reduced animal use.

Conclusions and perspectives. The analytical toolbox is not expected to change dramatically or replace animal studies soon. Platform technology, highly reproducible manufacturing methods, extensive in vitro characterisation and functional assays may allow for animal free development of vaccines, but this is a long way off. Adaptive immunogenicity cannot be measured in vitro because of the complexity of the immune system. Besides that, animal studies are mandated by regulatory agencies in order to minimize risks during first in human studies. More is expected from better use of the employed animal or human models. Improved analysis of the immune response by applying proteomics and other omics technologies and multi-analyte assays can provide more information from less animals. Human challenge models could measure protection under controlled conditions. Guaranteed product quality using robust analytics could enable these developments since better characterised products increase product knowledge and reduce risks of unexpected events in early clinical development.

Keywords: in vitro characterisation; vaccine development; animal models; COVID-19 vaccines

Introduction

Standard vaccine development is a long process that includes multiple sequential phases. To ensure optimal quality (potency, safety, and stability) and to comply with regulations, the quality control strategy is of crucial importance. In vitro and in vivo studies are used to characterise the vaccine product and to analyse the immunogenicity, before the vaccine can proceed into clinical trials (European Medicines Agency (EMA), 2020).

During the SARS-CoV-2 pandemic we have seen lightning-fast product development. While traditionally it took years or decades to produce an efficacious and safe vaccine, a global united effort has led to the conditional marketing authorisation of several vaccines within just over a year. The unprecedented speed of these vaccines can partially be credited to the parallel execution of clinical trials and preclinical assessment as well as commercial production 'at economic risk', i.e. preparations for vaccine production, including the construction of manufacturing sites and actual production of bulk vaccine under Good Manufacturing Practices. Although all mandatory steps for market authorisation have been executed for all licenced vaccines, this gives rise to new questions on what lessons can be learned from this pandemic about vaccine development and quality control.

There are five main vaccine categories in the global effort to combat SARS-CoV-2: protein subunit vaccines (including virus like particles), DNA & RNA vaccines, vector vaccines, inactivated vaccines, and live attenuated vaccines. In this report, the latter will not be discussed since the number of live attenuated COVID-19 vaccines is very limited. The WHO keeps an overview of all COVID-19 vaccines in clinical and preclinical development (WHO, 2021b).

Vaccines are a highly heterogenous product group that are often difficult to characterise when compared to other protein-based biologics. The large diversity of pathogens makes vaccines a very diverse product group of which the different mechanisms of action are not always fully understood. Hence, each candidate vaccine needs its own specific set of analytical methods to accurately define it and test its quality (Metz et al., 2009). The selection of these methods depends on the required information and nature of the vaccine. There are now numerous analytical methods available that could potentially be part of a set of immunochemical and physico-chemical assays to improve the characterisation by fingerprinting of the product without the frequent use of long lasting, expensive, or inaccurate functional (in vivo) immunogenicity studies and thus increase effectivity of vaccine development.

To find out what lessons can be learned from the application of characterisation techniques in COVID-19 vaccine development, how animal models were used, and to further investigate what the potential of in vitro assays is in replacing animal studies, literature on the development of COVID-19 vaccines was studied. The Vaccine Tracker by the WHO served as a starting point for relevant literature (WHO, 2021b). For each vaccine category, four vaccines were selected that were in the most advanced stage of research on August 3rd, 2021. The clinical and preclinical reports were gathered from ClinicalTrials.gov and PubMed (Dai et al., 2020a; S. G. Smith, Smits, Joosten, Van Meijgaarden, et al., 2015a). Since the vaccines were still in development, once weekly, the WHO Vaccine Tracker was screened for updates and additional searches were carried out if necessary. Due to the vast amount of literature on the subject and the continuous publication of new material during the project, this is not a

systematic review. Also, analytical methods are often used as tools and marginally described. In literature databases papers using certain methods and assays are hard to find in a systematic way. And finally, only a relatively small fraction of the vaccine development work is reported; industry is usually reluctant to disclose data which may have impact on their position relative to competitors. Literature was collected until December 1st, 2021. From the identified literature, an overview of all reported characterisation assays and animal models was made. In some cases, not all information was published. For example, for one vaccine that has proceeded to phase III, no animal studies have been reported. Moreover, routine quality control tests were possibly not performed, available or reported because they were considered of minor importance at the time of reporting, potentially causing underreporting. Also, some information is proprietary knowledge.

Using full analytical capacity to improve the characterisation and quality control of vaccine further could potentially help to reduce, refine, and replace animal studies. Animal studies are still considered as valuable tests by many vaccine developers and regulators. There is a wide variety of available animal models, but their potential to be predictive for performance in humans is not clear in the case of emerging infectious diseases such as COVID-19. In this review, the use of non-animal analytics in COVID-19 vaccine development is evaluated. Also, drawbacks and opportunities for each available animal model are discussed and their predictive value towards human responses is evaluated.

The objective of the report is to shed light on the following questions:

- Which analytical methods are used in vaccine development and what potential and limitations do these methods have in replacement of animal models?
- What was the role of these methods in SARS-CoV-2 vaccine development and did these methods contribute to reduction or replacement of animal use?
- In the coming years, what developments in analytical characterisation are expected that may affect animal use?

List of abbreviations

AF4	Asymmetrical flow field-flow fractionation
API	Active pharmaceutical ingredient
AUC	Analytical ultracentrifugation
BAL	Bronchoalveolar lavage
BALB-c mouse	Immunodeficient laboratory-bred strain of the house mouse
BLI	Biolayer interferometry
c-EM	Cryogenic electron microscope
CE	Capillary electrophoresis
CPE	Cytopathic effect
DLS	Dynamic Light Scattering
ELISA	Enzyme-linked Immuno Sorbent Assay
EM	Electron microscope
GMT	Geometric mean titre
hACE2	Human angiotensin-converting enzyme 2
IEF	Isoelectric focusing
IgG	Immunoglobin G
LC-MS	Liquid-chromatography/mass spectrometry
LNP	Lipid nanoparticle
MALS	Multi-angle Light Scattering
NAb	Neutralising antibody
NHP	Non-human primate
PAGE	Polyacrylamide gel electrophoresis
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RBD	Receptor binding domain
S-protein	SARS-CoV-2 Spike protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	size exclusion chromatography
SPR	Surface plasmon resonance
UV	Ultraviolet
UV/Vis	UV–visible spectrophotometry
VNT	Virus neutralisation titre

Overview of studied vaccines

Table 1. Overview of the studied vaccines. Vaccine group, WHO Tracker ID number, name and developer are shown (WHO, 2021a).

Vaccine group	ID	Vaccine name	Vaccine developer
Protein subunit	#11	ZF2001	Anhui Zhifei Longcom Biopharmaceutical
	#21	VAT00002	Sanofi Pasteur + GSK
	#28	SCB-2019	Clover Biopharmaceuticals Inc./GSK/Dynavax
	#8	SARS-CoV-2-S	Novavax
DNA/RNA	#10	BNT162b2	Pfizer/BioNTech
	#15	INO-4800	Inovio Pharmaceuticals
	#39	ArCoV	Academy of Military Science (AMS), Walvax Biotech.
	#9	mRNA-1273	Moderna
Vector	#25	GRAd-COV2	ReiThera + Leukocare + Univercells
	#4	ChAdOx1nCov-19	AstraZeneca + University of Oxford
	#5	Ad5 v. COVID Vacc.	CanSino Biological Inc./Beijing Institute of Biotech.
	#7	Ad26.COV2.S	Janssen Pharmaceutical
Inactivated	#1	CoronaVac	Sinovac Research and Development Co., Ltd
	#19	BBV152	Bharat Biotech International Limited
	#2	SinoPharm-1	Sinopharm
	#3	BBIBP-CorV	Sinopharm

1.1 Product characterisation

Product characterization is defined here as non-animal testing of the product, either drug product or intermediate products like drug substance. It provides mainly information about the chemical and physical state of the product. These methods do not a priori provide information about functional properties of the antigen or vaccine, i.e. potency and safety. For these latter characteristics in vivo data are needed (animal or human) or an assay with a proven and validated correlate with biological activity.

Electrophoresis & Western blotting

Electrophoresis is used to assess protein or RNA/DNA composition and integrity. With respect to protein antigens, it can give information on covalent modifications and size, as well as the isoelectric point, purity and aggregation and degradation of a protein (Metz et al., 2009). This includes detection of glycosylation and the presence of charge variants in protein antigen. In vaccine development, generally four types of electrophoresis can be distinguished: capillary electrophoresis (CE), polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and 2d-electrophoresis (2d-E), the latter combining IEF and PAGE, i.e., charge and size-based separation. IEF and 2d-E were not reported in the identified literature while PAGE was frequently described. CE was only reported once, for the characterisation of DNA/RNA vaccines. Vogel et al. (2021) describe the use of microfluidic CE to determine the purity and integrity of the SARS-CoV-2 RNA in the BNT162b1/2 mRNA vaccines. By comparing the CE peaks to the calculated lengths of the RNA, the method can be used to confirm purity and integrity of the mRNA.

More commonly described in COVID-19 vaccines is PAGE. Most virus and protein based COVID-19 vaccines undergo (SDS-)(PAGE) at some point during their development. Different modes can be used to analyse proteins. Native, non-reducing to assess non-covalent protein interactions and apparent size based on protein folding or sodium dodecyl sulfate treated and chemically reduced PAGE, assessing molecular weight of individual proteins. Western blotting using SDS-PAGE and detection with antigen-specific antibodies can be used to confirm the identity of separated proteins, like the SARS-CoV-2 S-protein and RBD structures used in subunits protein vaccines (Dai et al., 2020b; Liang et al., 2021; Tian et al., 2021). In DNA/RNA vaccines it can also be used to verify the expression (in vitro, as a quality control (QC) test) of the desired antigens, such as the SARS-CoV-2 RBD (Vogel et al., 2021). In vector vaccine development western blotting is also used to confirm the expression of desired antigens. To do so, in vitro, cells are infected with the vector. Subsequently, cells are lysed, proteins are separated on gel and using labelled antigen-specific antibodies, mRNA gene product is detected (Capone et al., 2021; Q. Guo et al., 2018; Mercado et al., 2020). Finally, western blotting can also be used in inactivated vaccines to identify epitopes such as S1 and S2, RBD and N proteins (Ganneru et al., 2020; Gao et al., 2020). Scanning densitometry can be used to measure the density of electrophoresis bands and therefore serves, in this case, as a sequential step to electrophoresis to obtain semi-quantitative data. It was used once in the identified literature, to determine the purity of the S-protein in a protein subunit vaccine (Tian et al., 2021).

Chromatography

Chromatographic techniques, such as size exclusion chromatography (SEC-HPLC) and ion exchange chromatography, are commonly used in the production (purification) of vaccines

and were regularly reported in the literature. Because these methods rely on size- and charge based separation respectively, they are also regularly used in combination. Such tools can also be used in QC to verify the purity, but this application was scarce in the identified literature. SEC-HPLC was the only chromatographic method that was used for the characterisation of the COVID-19 vaccines. SEC-HPLC can be used to study the active pharmaceutical ingredient, but also particular delivery systems for the API such as lipid nanoparticles (LNPs). SEC-HPLC can be used to determine the purity, integrity, and stability of the product. By analysing the fraction of proteins at specific molecular weights, it is possible to determine the relative amount of protein in a vaccine (Cao et al., 2021; Vogel et al., 2021). This is only useful for simple protein mixtures because SEC-HPLC lacks specificity for more complex protein mixtures. Apart from analysing physical integrity of proteins, RNA/DNA, or delivery systems, using SEC-HPLC, the complete protein composition of the vaccine can be detected and quantified. This includes for example specifically cleaved S-proteins in a subunit vaccine (Liang et al., 2021). Liang et al. motivated the choice of their S-protein subunit vaccine candidate by demonstrating it was cleaved at the S1/2 junction, which is similar to the cleaving of wild type SARS-CoV-2 S-Trimer. S1/S2 cleavage is necessary to prepare the S-protein for membrane fusion. This is the only example from the identified literature of this application of SEC-HPLC.

Spectroscopy

The field of spectroscopy covers a broad range of techniques that are important in determining protein and nucleic acid concentration, protein higher order structure (UV, fluorescence, circular dichroism). This is important since the recognition sites of antibodies (B-cell epitopes) often depend on conformation of protein antigens. Moreover, techniques such as light scattering techniques (DLS, MALS, UV/vis) and imaging techniques (EM) give information on size, number and structure of nanoparticles such as virus-like particles, lipid nanoparticles as well as detection and quantification of unwanted (protein) aggregates which may form during manufacturing or storage. Spectroscopic methods and cryo-EM can confirm the structure of proteins in candidate vaccines, which is an important step in vaccine characterisation as well as in batch verification.

UV absorbance spectroscopy

UV absorbance spectroscopy is a quantitative technique that is used to determine protein concentration, especially in protein subunit vaccines (Guirakhoo et al., 2020; Sanyal et al., 2021). Today, direct particle counting techniques, such as nanoparticle tracking analysis (NTA) based on light scattering, are available (Filipe et al., 2010), but were not reported yet in the identified literature. UV Absorbance spectroscopy can be used to measure protein concentration at 280 nm and RNA at 260 nm. Absorbance spectroscopy at wavelengths above 300 nm (UV/Vis), in contrast, can be applied to detect protein aggregates. UV/Vis was used once, to measure the total number of viral particles in an adenovirus-vectored vaccine (S. Wu et al., 2020). The total number of viral particles can be calculated from the UV absorbance assay using the method described by Maizel et al. (1968), who determined that, for adenoviruses, there are 1.1×10^{12} particles per OD_{260} unit.

Electron microscopy (EM) based techniques

Cryogenic EM (c-EM) has proven to be a valuable tool in early SARS-CoV-2 research, since it was used to determine the structure of the full trimeric S-protein in detail. Current cryo-EM technology and imaging software enables the assessment of the structure of proteins as

detailed as more conventional X-ray diffraction, but without the need to produce crystals, which is more complicated and potentially introduces artefacts. Using Cryo-EM, Wrapp et al. gathered crucial information on SARS-CoV-2's differences with other corona viruses and the conformation of its RBD, providing a promising starting point for the rapid development and evaluation of medical countermeasures against the virus (Wrapp et al., 2020). c-EM gives detailed information on the conformation of, for example, the S-protein and its RBD trimers. The S-protein exists in different conformations: a prefusion and postfusion state. This refers to the membrane fusion that is necessary for the virus to enter the host cell. Furthermore, in the prefusion conformation the monomers (three per intact S-protein) can have an 'open' or 'closed' conformation in which the receptor is in accessible or inaccessible states, respectively. For vaccines, a closed prefusion state must be maintained (Xiong et al., 2020). Hence, c-EM can validate the shape, size and integrity of inactivated vaccines (Gao et al., 2020) and subunit vaccines. Additionally, by determining the structure of antigen/antibody complexes, useful information on the binding process and epitope availability can be obtained, which is important in understanding the impact of novel SARS-CoV-2 strains on for instance vaccine efficacy (Cao et al., 2021). Lastly, this method can be used to determine the morphology of lipid nanoparticles and confirm the presence of RNA based on this morphology (N. N. Zhang et al., 2020).

Like c-EM, negative-stain transmission electron microscopy (TEM) can be used to verify the size and shape of viral particles and identify its spikes in subunit and inactivated vaccine candidates (Ganneru et al., 2020; Meyer et al., 2020; Vogel et al., 2021; Wang et al., 2020). Moreover, visualisation of receptor and antibody binding and structural analyses can confirm if antigens in vaccine candidates are as accessible as in live virus (Liang et al., 2021; Vogel et al., 2021). This is crucial information because the visualisations of S-protein, associated (or not) with inactivated virus or virus like particles can guide predictions on stability and immunogenicity. Other methods to assess structural elements in SARS-CoV-2 vaccines that were described in the identified literature included atomic force microscopy (AFM) and scanning electron microscopy (SEM) (Hanifehnezhad et al., 2020).

Dynamic Light scattering

Dynamic light scattering (DLS) is a technique that measures the fluctuations in scattered light by molecules or particles between 1 and 1000 nm in size, as determined by their Brownian (random) motion. Subsequently, the nanoparticle diffusion coefficient is calculated which can be used to determine the hydrodynamic diameters of the particles (Caputo et al., 2019). In COVID-19 vaccines it is mainly used to define the size of nanoparticles (<1000 nm) or proteins (N. N. Zhang et al., 2020). Moreover, based on the polydispersity index, the distribution of size between proteins (Tian et al., 2021) and nanoparticles can be compared. Also, light scattering techniques can be used to monitor non-specific protein aggregation in VLPs (Sanyal et al., 2021).

Furthermore, the application of DLS is important in mRNA vaccines because mRNA is delivered via LNPs. The size is a critical parameter because it can have impact on the in vivo processing and as a result on immunogenicity (Hassett et al., 2019; Sanyal et al., 2021). Furthermore, most vaccines are subject to a 0.2 μm sterile filtration step and a mean particle size of above 150 nm can be detrimental to product recovery. An advantage of DLS is that it requires no accurate knowledge of the sample concentration, but only values of the solvent viscosity and

temperature (Wyatt Technology Corporation, n.d.). While DLS is also relatively inexpensive and simple in use, it should not be used as the only method for characterisation of complex products, because it has a relatively low resolution. An important disadvantage is its inability to distinguish between small aggregates and large proteins and it cannot produce reliable measurements in the presence of plasma proteins (Caputo et al., 2019). These problems can be overcome by using other sizing techniques such as Nano Tracking Analysis or first separating the suspension based on size, such as SEC-HPLC or Asymmetrical flow field-flow fractionation (AF4).

Other spectroscopic methods

Circular dichroism is a method that can be used to study protein conformation (Michiels et al., 2020; Sanyal et al., 2021). In the identified literature on COVID-19 vaccines, however, no application of circular dichroism was found. Similarly, fluorescence spectroscopy, that can be used to study protein folding and higher order structures, was not found in the identified literature. Nuclear magnetic resonance & infrared were also not reported.

Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) is a technique for the quantitative analyses of macromolecules and particles in a solution. AUC is not often applied because of the cost of the equipment and expertise that is necessary to conduct these analyses. It works well on heterogeneous samples and characterises solutions in their biologically relevant conditions. The technique has two distinct applications: sedimentation velocity, which gives information on the size and shape of molecules and particles, and sedimentation equilibrium, which gives for example information on solution stoichiometries (relationship between the quantities of reactants and products in reactions) and association constants between macromolecules, such as antigen and antibodies (Cole et al., 2008). In COVID-19 research, AUC was used to determine and compare the weights of stabilized (tandem repeat single chain) RBD dimer of MERS-CoV, SARS-CoV and SARS-CoV-2 (Dai et al., 2020b). Dai et al. used this in their strategy to design vaccines against COVID-19 and SARS.

Immunochemical assays for antigen characterisation

ELISA

Enzyme-linked Immuno Sorbent Assay (ELISA) is an immunochemical technique that can be widely applied, e.g., for analysis of antigens as well as measuring antibody responses in serum (see section 1.2). In the context of vaccine development, it is in the first place used to determine antigen concentrations, binding specificity, and for epitope identification (Liang et al., 2021; Richmond et al., 2021). In protein subunit and inactivated vaccine candidates for example, the reactivity and specificity of S-protein and RBD based subunit vaccines to hACE2 were tested using ELISA (Cao et al., 2021; Tian et al., 2021). These analyses showed that the vaccine proteins bound specifically to hACE2 with higher affinity than wild type SARS-CoV-2. In mRNA vaccines, RBD expression in vitro can be determined by ELISA (N. N. Zhang et al., 2020).

Biosensors

Biosensor analyses can give more insight in the binding kinetics between virus particles or viral antigens and receptors or antibodies to determine epitope integrity. Biosensors are also used to measure antigen concentration as an alternative for ELISA type of antigenicity assays.

Biolayer interferometry (BLI) is one example of a biosensor analysis. It is a label-free technology for measuring biomolecular interactions. It consists of a protein coated biolayer surface to which analytes bind, becoming immobilised. The interference pattern of light reflected from these two layers is analysed in real time, allowing accurate measurement of binding specificity, rates of association and dissociation, or concentration with precision and accuracy.

BLI was an important tool in early SARS-CoV-2 research. It was used to compare the binding affinities of SARS-CoV-2 and SARS-CoV viral structures to the hACE2 receptor and helped identify the most important binding sites of the SARS-CoV-2 Spike protein (Casalino et al., 2020; Walls et al., 2020). Walls et al. assessed if the most important binding sites from the SARS-CoV (F. Li et al., 2005) were conserved in SARS-CoV-2. This way, they found eight positions that are key for S/ACE2 binding that were strictly conserved between the two viruses. Casalino et al. used BLI to identify important sites that are essential for the recognition of the ACE2 receptor by SARS-CoV-2 (Casalino et al., 2020). They compared the binding kinetics of the wild-type SARS-CoV-2 with a recombinant virus where glycans were deleted at 2 positions. BLI confirmed that the deletion of the glycans resulted in a decrease in ACE2-binding. Similarly, BLI was used to determine the binding affinity of vaccine candidate S-proteins, S-Trimers and RBDs to hACE2 (Liang et al., 2021; Robbiani et al., 2020; Tian et al., 2021; N. N. Zhang et al., 2020). These papers mention higher or similar affinity when compared to wild type viral proteins.

Surface plasmon resonance (SPR) is another label-free biosensor analysis that is similar to BLI but uses refractive index changes at the binding surface to study antigen binding to surface-immobilized antibodies at the sensor surface. Because the refractive index change depends on the mass that is bound to the sensor, SPR is especially useful for the analysis of proteins and high-abundance samples (Singhal et al., 2010). An advantage of the SPR technology is the ability to screen several proteins in parallel because the sensor contains four flow cells (Navratilova & Hopkins, 2010). SPR has been used to determine the binding kinetics between antibodies and SARS-CoV-2 RBD (Dai et al., 2020b; Du et al., 2020; Vogel et al., 2021). SPR results showed that the RDB-sc dimer of Dai et al. had comparable but slightly higher binding affinity to its receptor than the RBD monomer. SPR is a highly sensitive method, but changes in the refractive index caused by solution flowing over the sensor, make it susceptible to interference. The risk of interference is lower for BLI, but compared to SPR, it is not as sensitive (Shah & Duncan, 2014).

Other methods

Mass spectrometry is a technique that measures the charge-to-mass-ratio of ions. Liquid-chromatography/mass spectrometry (LC-MS) is a related method that combines separation (by LC) with mass spectrometry. It allows for unambiguous protein identification and quantification of molecules. Hence, it was used to determine the N-terminus of the S-protein in a vector vaccine (Bos et al., 2020) and the quantification of lipids in an mRNA vaccine (Hassett et al., 2019).

Peptide sequencing via Edman degradation is a method to sequence peptides by breaking it down, one amino acid at a time. It was described only once in the identified literature and was used to confirm the desired S1/S2 cleavage in a protein subunit vaccine (Liang et al., 2021).

Flow cytometry is a conventional technique that can be used for a wide variety of applications. For a vector vaccine, it was used to assess transgene antigenicity of the Ad26 vector with five DNA constructs (Bos et al., 2020). Patel et al. conducted a flow cytometry-based assay to study the ACE2 receptor binding of the SARS-CoV-2 Spike protein (2020). Apart from T-cell response, Vogel et al. also used flow cytometry to confirm RBD expression by transfected cells and for vaccine antigen detection (2021).

1.2. Characterisation of the immune response

An essential step in vaccine development is demonstrating its potency. In some cases, e.g., live concepts such as attenuated vaccines and vector vaccines, the potency correlates with the amount of viable virus. In the development phase this is often not yet clear and in vivo dose-response studies are needed. Also, in vivo studies are still needed to analyse the immune mechanism in detail with respect to innate, antibody and T-cell responses. Ideally, also the protection against the pathogen is measured. Since this is rather difficult, there are numerous other methods to measure the immune response as induced by a vaccine. These assays include serological assays to measure antibodies as well cell-based assays. Proper use of these assays is highly important both in the context of animal studies in vaccine development as well as in clinical trials. To develop and select only the most relevant and appropriate assays is important in order to minimize expensive and time consuming animal studies (NC3Rs, 2020). Here, we discuss the most important techniques and assays to characterise the immune response. An overview of the assays used per vaccine is provided in **Table 2. Overview of assays used to characterise the immune response against SARS-CoV-2 virus, by vaccine group and vaccine.** This table represents the assays that were reported. If an assay was not reported – for any reason – this does not mean that it was not done.

Antibody ELISA

As previously mentioned, ELISA is an important tool in the characterisation of vaccine, but in vaccine development ELISA is equally important for serological analysis. It is frequently used for the detection of antigen-specific (SARS-CoV-2 S-protein or RBD) antibodies after immunisation with the experimental vaccines or in SARS-CoV2 infected patients (Amanat et al., 2020). In early phases, usually, the sera of vaccinated mice or non-human primates and convalescent patients are used for these experiments (Gary et al., 2021; Liang et al., 2021; Yadav, Ella, et al., 2021). In later phases, the same principle is applied using human sera from (early) clinical trials (Cao et al., 2021; Stephenson et al., 2021; Yang et al., 2021; Y. Zhang et al., 2021).

Other studies demonstrated the potential of an ELISA based neutralisation assay as an additional assay to determine the immune status of COVID-19 infected or vaccinated individuals (Meyer et al., 2020). This is a serological assay to detect immunodominant neutralizing antibodies that target the SARS-CoV-2 S-protein. The principle of the test is based on antibody-mediated blockage of the ACE2/RBD interaction that takes place in an ELISA plate well (Tan et al., 2020). In such a surrogate neutralisation assay, no live SARS-CoV-2 is needed, which is an important advantage compared to regular neutralisation assays.

Chemiluminescence immunoassay

Chemiluminescence immunoassay (CLIA) is a highly specific technique to measure antibody binding. It has a wide dynamic range, high degree of automation and the ability to run many antibody tests, including different isotypes. The addition of enhancers like ferrocyanide leads to exceptional analytical sensitivity which is superior to methods like ELISA (Cinquanta et al., 2017). CLIA was used for one vaccine candidate, where it was used in clinical studies to measure IgG binding to S1-S2 (Lanini et al., 2021).

Bead-based (multiplex) immunoassays

Luminex multiplex assays can simultaneously detect and quantitate multiple proteins, such as antibodies with different antigen specificities, cytokines and growth factors, or expressed genes. This high-throughput technology produces results comparable to conventional assays such as ELISA and qPCR, but potentially with greater efficiency and throughput (Thermo Fisher Scientific, 2021). Also, less material is needed, and assay variability is reduced because many analytes (e.g., cytokines) are measured in one sample using mixtures of beads functionalized with different ligands (e.g., anti-cytokines). The Luminex assay was used to detect relative quantity of antigen-specific antibody titres in pre-clinical studies for a vector vaccine candidate (Mercado et al., 2020; Tostanoski et al., 2020). Secreted cytokines from

Table 2. Overview of assays used to characterise the immune response against SARS-CoV2 virus, by vaccine group and vaccine. Assays that were applied in both clinical and animal studies are marked [1], those only in clinical studies are marked [2] and those that were only used in animal studies are marked [3].

		Antibody assays		Cellular response			*References*
	ID	ELISA	NA	ELISpot	ICS	Histology	
Protein subunit	8	X[1]	X[1]	X[3]	X[3]	X[3]	(Guebre-Xabier et al., 2020; Madhi et al., 2021; Tian et al., 2021)
	11	X[1]	X[1]	X[1]	X[3]	X[3]	(An et al., 2021; Dai et al., 2020b; Yang et al., 2021; N. N. Zhang et al., 2020)
	21	X[2]	X[2]		X[2]		*(Goepfert et al., 2021)*
	28	X[1]	X[1]	X[3]	X[2]	X[3]	*(Liang et al., 2021; Richmond et al., 2021)*
DNA & RNA	15	X[1]	X[1]	X[1]	X[2]	X[3]	*(Gooch et al., 2021; Patel et al., 2020; T. Smith et al., 2020; Tebas et al., 2021)*
	39	X[3]	X[3]	X[3]		X[3]	*(N. N. Zhang et al., 2020)*
	9	X[1]	X[1]		X[1]	X[3]	*(Chu et al., 2021; Corbett, Flynn, et al., 2020; Jackson et al., 2020)*
	10	X[3]	X[1]	X[1]	X[1]	X[3]	*(Frenck et al., 2021; Sahin et al., 2020; Vogel et al., 2021; Walsh et al., 2020)*
Vector	25	X[3]	X[2]	X[1]	X[2]	X[3]	*(Agrati et al., 2021; Capone et al., 2021; Lanini et al., 2021)*
	7	X[1]	X[1]	X[1]	X[1]	X[3]	(Mercado et al., 2020; Sadoff, Le Gars, et al., 2021; Stephenson et al., 2021; Tostanoski et al., 2020)
	4	X[1]	X[1]	X[1]	X[3]	X[3]	*(Folegatti et al., 2020; Marsh et al., 2021; van Doremalen et al., 2020; Voysey et al., 2021)*
	5	X[1]	X[1]	X[1]	X[1]		*(S. Wu et al., 2020; Zhu, Guan, et al., 2020; Zhu, Li, et al., 2020)*
Inactivated	1	X[1]	X[1]	X[2]		X[3]	(Cao et al., 2021; Gao et al., 2020; Y. Zhang et al., 2021)
	2	X[1]	X[1]				(Al Kaabi et al., 2021; Duan et al., 2020; Fischinger et al., 2019; W. Guo et al., 2021)
	3		X[1]			X[3]	*(Al Kaabi et al., 2021; Wang et al., 2020)*
	19	X[1]	X[1]	X[1]	X[1]	X[3]	*(Ganneru et al., 2020; Mohandas et al., 2021; Yadav, Ella, et al., 2021)*

Note. Vaccine ID corresponds with the ID numbers from table 1 and are derived from the WHO Vaccine Tracker (WHO, 2021b). *Abbreviations: ELISA = Enzyme-linked Immuno Sorbent Assay; NA = neutralisation assay; ELISpot = enzyme-linked immune absorbent spot; ICS = intracellular cytokine staining.*

mice vaccinated with an mRNA vaccine were also measured and analysed using the multiplex bead-based Luminex assay (Corbett, Edwards, et al., 2020). Another multiplex assay by the same producer was used to measure concentrations of cytokines is the ProcartaPlex multiplex immunoassay, which was also used in a preclinical study (Vogel et al., 2021).

Using the Cytometric Bead Array (CBA), cytokines as a function of innate immunity activation by inactivated antigens and adjuvants were measured (Yadav, Ella, et al., 2021). CBA allows, for example, for the identification of the critical role of IFNα in both the antiviral and proinflammatory cytokine functions and linking the innate immunity to the adaptive immunity (Ganneru et al., 2020). These types of assays increase our understanding of the mechanism of action of vaccines. This is mandatory for regulatory approval. These assays only need small sample quantities and usually produce many high-quality data. This results in better understanding of the immune mechanism of vaccines, which allows for a more informed decision to move to clinical development. In addition, small blood samples are needed which makes it possible to use these analytics in clinical studies enabling better comparison with results from preclinical studies.

Neutralisation assays

Although immunoassays like antibody ELISAs are important to assess the immunogenicity of a vaccine, they do not provide information about the functionality of the antibodies. To further investigate this, their ability to neutralize the virus can be determined in vitro using a virus neutralisation assay. The advantage of this approach is that there is no need to challenge susceptible animals with infectious virus. Moreover, these assays can be used in experimental animals that are not susceptible to the virus, but nevertheless will produce an antibody response when in contact with the virus.

A conventional type of neutralisation assay is based on the cytopathic effect (CPE). CPE can be studied using replication competent virus or using pseudoviruses. Replication competent virus yields better and more easily reproducible results but using live SARS-CoV-2 requires biosafety level 3 laboratories. The practical limitations of these laboratories can be partially overcome using pseudoviruses in biosafety level 2 laboratories (Riepler et al., 2021). To assess the potency of a vaccine candidate, sera from vaccinated animals or humans are diluted and incubated with SARS-CoV-2 or pseudovirus (Chen and Zhang Int J Biol Sci 2021). Subsequently, Vero E6 cells are added and incubated. Virus replication will result in CPE (Tian et al., 2021). CPE was also be used to verify the inactivation process in inactivated vaccine candidates, since the absence of CPE proves the absence of live virus (Ganneru et al., 2021). While CPE can be used to gather information on various important characteristics, the method is inherently slow. It can take up to a week to produce definitive indications of CPE (Sanyal et al., 2021).

Another method for detecting neutralising antibodies is the plaque reduction neutralisation assay (PRNT). In this assay, virus-antibody interaction occurs on a microtiter plate after which the antibody effect on viral infectivity is measured. The destruction of cells that are infected by the virus leads to visible a plaque (absence of cells) that can be detected in a variety of ways. The plaques are counted and the reduction of these plaques due to antibody presence can be used as a measure of neutralisation. A disadvantage of the all virus neutralisation assays is that they are labour intensive and takes time to conduct, making it less suitable for high throughput research (the Department of Immunization Vaccines and Biologicals, 2014).

Nonetheless, it yields valuable information, hence PRNT was broadly used in the preclinical and clinical phases of COVID-19 vaccine research. In all types of vaccine candidates, it was described at least once in the identified literature, in most cases to assess the neutralisation capacity of the vaccine and to measure the neutralising antibody titres (Gooch et al., 2021; T. Smith et al., 2020; S. Wu et al., 2020; Yadav, Ella, et al., 2021). In several cases, complementary to PRNT for the detection of neutralizing antibodies, ELISA was used to detect binding antibodies (W. Guo et al., 2021; Tebas et al., 2021). Lanini et al. also reported using the PRNT to detect neutralising antibodies and found that this technique was considerably more sensitive than a microneutralization assay based on CPE. Moreover, they conclude that the lack of standardised assays for vaccine performance complicates the comparison between COVID-19 vaccines that are in development (Lanini et al., 2021). This was also reported by Yang et al. (2021).

There is a variety of similar (micro)neutralisation assays that are comparable to PRNT. Folegatti et al. used PRNT and a microneutralisation assay to assess the reduction of microplaques after immunizing humans with the ChAdOx1 nCoV-19 vector vaccine (2020). This method was also used for the detection of neutralising antibodies in the development of a subunit vaccine and a DNA vaccine in non-human primates and ferrets (Liang et al., 2021; Riddell et al., 2021). To increase throughput as well as assay robustness, the microneutralisation assays was also performed by measuring the optical density in stained Vero E6 cells (S. Wu et al., 2020). The alternative to virus assays, pseudovirus neutralisation assays, were also often reported. This entails assays in which a replication incompetent pseudovirus is created (Nie et al., 2020). This can be done using, for example, the full SARS-CoV-2 spike protein in lentiviruses or other viruses (Capone et al., 2021; Gary et al., 2021; S. Wu et al., 2020). Often, live SARS-CoV-2 and pseudovirus neutralization assays were conducted complementary to each other, for example to determine the 50% and 90% neutralising titre (Dai et al., 2020b).

While developing an mRNA vaccine, Jackson et al. found a strong correlation between live virus and pseudovirus neutralisation assay, suggesting that – when sufficiently validated – a pseudovirus neutralisation assay has the potential to serve as a surrogate for live virus neutralisation assays (Jackson et al., 2020). These neutralisation assays give important information on the performance of a vaccine candidate. Interestingly, Amanat et al. found a strong correlation between these assays and ELISA binding results (2020).

ELISpot

In the animal studies as well as clinical phases of vaccine development the enzyme-linked immune absorbent spot assay (ELISpot), plays in important role because it provides information cellular responses (including B-cell responses). For ELISpot analyses, usually splenocytes are used in animal models and peripheral mononuclear cells (PBMCs) in clinical studies. It is mostly used to study the T-cell response and cytokine production in vaccinated animals or humans (Gooch et al., 2021; van Doremalen et al., 2020; Vogel et al., 2021). This method is often accompanied by intracellular cytokine staining, using flowcytometry for identification and quantification.

Flow cytometry

A widely used assay to assess T-cell immune response is intracellular cytokine staining (ICS) followed by flow cytometry. Usually done with peripheral blood mononuclear cells from clinical studies or blood, spleen or lymph nodes from animal studies, ICS can detect immunological biomarkers in the form of expressed cytokines. An advantage of ICS over ELISpot is the ability to detect subsets of responder cells such as CD4 and CD8 T-cells and associated markers of differentiation (S. G. Smith, Smits, Joosten, Meijgaarden, et al., 2015). The method was used in all vaccine types to measure the T-cell response (Dai et al., 2020b; Ganneru et al., 2021; Gary et al., 2021; Mercado et al., 2020).

FC was used for intracellular cytokine staining analysis in numerous studies, but also to measure the SARS-CoV-2 specific cytokine production and T-cell responses (Corbett, Flynn, et al., 2020; Tebas et al., 2021; Yadav, Ella, et al., 2021; N. N. Zhang et al., 2020; Zhu, Guan, et al., 2020). Moreover, it was used to quantify INFγ and B-cells (Corbett, Flynn, et al., 2020; Gary et al., 2021). In one study, it was used to quantify the lymphocyte percentage and blood lymphocyte subset distribution (Fischinger et al., 2019; Wang et al., 2020).

Signs of infection and disease after challenge; histopathology

In animal studies involving challenge, a number of assays were used to measure protection. For challenge studies in non-human primates these can include body temperature and viral load from nose and rectal swabs by titration while alive (Wang et al., 2020). After euthanasia as well as for challenge studies in smaller experimental animals such as mice (using mouse adapted challenge strains), histology of the lungs was often done to detect pneumonia on tissue level (Wang et al., 2020) and in some cases to detect virus or viral RNA (N. N. Zhang et al., 2020). Since symptoms of disease are relatively mild in most experimental animals, histopathology is a relevant ex vivo assay to determine protection against disease.

1.3. Essential assays per vaccine type

An important aspect for implementing the 3R principle is the availability of quality indicating in vitro assays. In vitro characterisation, using a fingerprint approach, can potentially demonstrate batch consistency with no or limited use of animal studies, even in the development phase. Given the fact that during COVID-19 vaccine development, a lot of clinical studies were performed simultaneously with or before animal studies, perhaps the need for animal studies is not as evident as it used to be with more traditional vaccine and less advanced assays. In an attempt to explore this, all assays that were reported in the identified literature have been documented and can be compared between vaccine groups. Table 3 provides an overview of assays that were used for each vaccine (category). It is, however, important to note that the (lack of) reporting of assays in the literature does not mean that those were the only, or best, assays to be used.

Universal techniques

When looking at Table 3, two techniques can be identified as universal for the characterisation of all vaccine groups: ELISA and PAGE. These are conventional assays and give important information about the antigen concentrations, binding specificity, and the identity of epitopes and separated proteins. While the information gathered with these applications can be different for vaccine groups – Western blotting using SDS-PAGE was for example used to confirm the identity of separated proteins in subunits protein vaccines and to verify the expression of antigens in DNA/RNA vaccines – they are universally applied and would be a very good standard candidate for fingerprint sets of assays.

It is notable that very few studies in the identified literature described the use of chromatography. Since chromatographic assays are generally widely used, the absence of them in the literature might be attributed to lack of reporting, rather than a lack of application.

Protein subunit

In subunit protein vaccines, the target antigen and adjuvants are key attributes of vaccine quality. Hence, antigen characterisation is specifically important. This includes (SDS-)PAGE, but also immunoassays with antigen specific (preferably monoclonal) antibodies to determine antigenicity. These assays include ELISA, BLI or SPR. A correlation between antigen ELISA (antigenicity) and in vivo production of antibodies (immunogenicity) has been reported for other vaccines but needs to be established on a case-by-case basis. Apart from antigen characterisation, the adjuvant content and interaction between antigen and adjuvant, if any, are essential information. This is unique compared to the other vaccine types. In the identified literature, effects of the adjuvants were mainly assessed by in vivo comparing adjuvanted vaccine with unadjuvanted vaccine, for example by measuring ACE2 competitive titres and neutralising antibody titres (Liang et al., 2021). The adjuvant/vaccine production processes were occasionally described, but because most adjuvants are commercially available, production and characterisation of the adjuvant alone was not. In Figure 1, the key aspects of quality control for subunit protein vaccines and mRNA vaccines are shown. It shows the unique attributes of both vaccines and highlights the need to not only identify and study the final product, but also the target antigen, adjuvant, mRNA bulk and nanoparticle carrier.

Table 3. Overview of assays used to characterise the candidate vaccine, by vaccine group and vaccine.

		Chromatography			Immunochemical					Spectroscopy					Electroph.		FC	MS	DSC	*References*
	ID	Rph	IEx	SEC	BLI	ELISA	IB	IFA	CD	TEM	cEM	FM	DLS	UV	CE	PAGE				
Protein subunit	8				X	X				X			X			X			X	*(Tian et al., 2021)*
	11					X					X					X				(Dai et al., 2020b; Du et al., 2020)
	21																			-
	28			X	X					X						X		EDM		*(Liang et al., 2021)*
DNA & RNA	15																			-
	39					X	X	X			X	X	X			X				*(N. N. Zhang et al., 2020)*
	9					X							X							*(Hassett et al., 2019)*
	10			X	X			X		X	X	X			X	X	X			*(Vogel et al., 2021)*
Vector	25																			-
	7				X	X										X	X	X		*(Bos et al., 2020)*
	4																			-
	5																			-
Inacti-vated	1			X		X														(Cao et al., 2021)
	2																			-
	3									X						X				*(Wang et al., 2020)*
	19									X						X				*(Ganneru et al., 2021)*

Note. Vaccine ID corresponds with the ID numbers from table 1 and are derived from the WHO Vaccine Tracker (WHO, 2021b). *Abbreviations: Rph = reversed phase chromatography; IEx = ion exchange chromatography; SEC = size exclusion chromatography; BLI = biolayer interferometry; ELISA = enzyme linked immunosorbent assay; IB = immunoblotting; IFA = immunofluorescence assay; CD = circular dichroism; TEM = transmission electron microscope; cEM = cryogenic electron microscope; DLS = dynamic light scattering; UV = ultraviolet absorbance spectroscopy; CE = capillary electrophoresis; PAGE = (sodium dodecyl sulphate)–polyacrylamide gel electrophoresis; FC = flow cytometry; MS = mass spectrometry; EDM = Edman degradation; DSC = differential scanning calorimetry.*

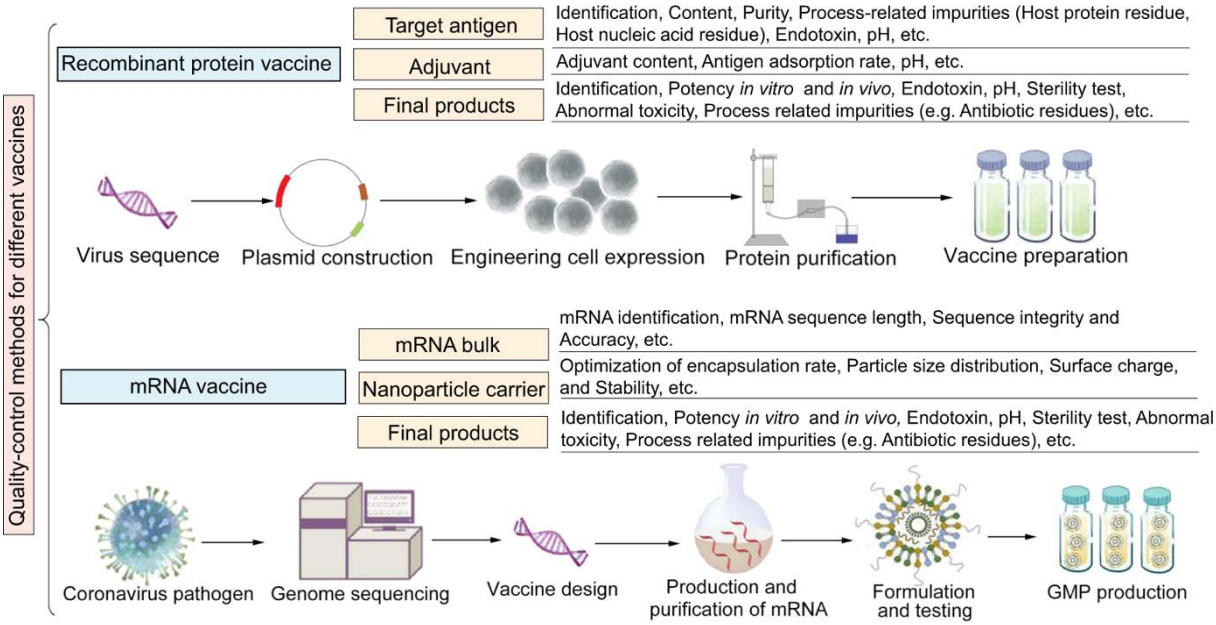

Figure 1. Reprinted from Mao et al. (2021). Key points of quality control for COVID-19 vaccines developed by different platforms.

RNA/DNA

Because RNA/DNA vaccines are the only non-proteinaceous vaccines, their characterisation and quality control are unique. After manufacturing and formulation of mRNA into LNP, the expression of the target antigen is confirmed in vitro, using for example immunoblotting (N. N. Zhang et al., 2020). Subsequently, the expressed protein can be subjected to binding analyses to ensure its quality. More quality control steps include mRNA identification and verification (Mao et al., 2021). As highlighted in Figure 1, the quality of the nanoparticle carrier is also critical for the effectiveness and safety of the vaccine. In order to ensure high quality of the nanoparticles, factors like encapsulation rate, particle size distribution, surface charge, stability and immunogenicity, should be carefully considered (Mao et al., 2021). In the identified literature, researchers were often brief about these processes: “Formulations were tested for particle size, RNA encapsulation, and endotoxin. All LNPs were found to be between 50 and 142 nm in size by dynamic light scattering and with greater than 69% encapsulation and <3 EU/mL endotoxin. Lead lipids selected for further evaluation were between 66 and 107 nm, with greater than 72% encapsulation” (Hassett et al., 2019).

Vector

Vector vaccines are dosed based on the virus titre. This makes titration especially important for this vaccine group. Moreover, the expression of the transgene is essential for vector vaccines, which can be confirmed for example by flow cytometry (Bos et al., 2020). Since most vector vaccines are replication deficient viruses, the genetic stability is especially important in this group. It is essential that the vector remains unable to replicate to ensure the safety of the vaccine. The stability is also important in ascertaining the presence of the transgene to optimise efficacy. Another unique characteristic of vector vaccines is that people (or animal models) can already have antibodies against the vector itself by natural introduction or previous vaccination with the same vector. One example of this was found in the study reported by Wu et al. in which they found a lot of participants with pre-existing immunity to

their Ad5 vector (2020). They noted that “the high pre-existing immunity did weaken the humoral and cellular immune response in some clinical trials”. With regard to the characterisation of a vaccine, this concern should be taken into account. That means that, especially with older vectors, immunogenic assays should be conducted for the vector as well as the vaccine. It is essential that neutralising antibodies against the vector do not compromise the efficacy of the vaccine.

Inactivated vaccines

Inactivated vaccines are produced by inactivating virulent virus. This can be done using for example heat or β-propiolactone (Hanifehnezhad et al., 2020; Wang et al., 2020). The verification of inactivation is of crucial importance in this vaccine group. This can be done by inoculating appropriate host cells and incubating them with the inactivated virus for several days. Absence of CPE can verify the successful inactivation of the virus. For one inactivated vaccine, it was reported that “other conventional methods were also used for characterisation of viruses required by Chinese regulatory authorities” (Meyer et al., 2020). These probably include (SDS-)PAGE, ELISA, and electron microscopy because they are important tools to verify the quality and purity of the vaccine.

2.1 Animal models

There are several animal models that are used in COVID-19 vaccine development. The animal model should yield sufficiently valuable and predictable data for clinical studies, hence there are some key attributes that should be considered. Firstly, in order to demonstrate protection, the animal model should exhibit relevant characteristics of the human disease after being exposed to the challenge pathogen. Also, immune markers should reflect the protective immune response that would be generated by humans and the immune response should be reflective of that in humans. Moreover, the immunological assays should be species-independent to allow for accurate predictions (Golding et al., 2018). Table 4. gives an overview of the animal models that were used for each vaccine type. In this section, these animal models will be discussed, including their advantages and disadvantages. Also, the animal models that were used as a challenge model will, if possible, be compared to other challenge models and clinical trials.

Table 4. Overview of all animal models used in the identified literature, by vaccine group and vaccine.

	ID	Rabbit	Ferret	Hamster	GP	Rat	Mouse	NHP	*References*
Protein subunit	8						X	X	*(Guebre-Xabier et al., 2020; Tian et al., 2021)*
	11			X			X	X	*(An et al., 2021; Du et al., 2020)*
	21								-
	28						X	X	*(Liang et al., 2021)*
DNA & RNA	15		X		X		X	X	*(Patel et al., 2020; Riddell et al., 2021; T. Smith et al., 2020)*
	39						X	X	*(N. N. Zhang et al., 2020)*
	9					X	X	X	*(Corbett, Flynn, et al., 2020; Hassett et al., 2019)*
	10						X	X	*(Vogel et al., 2021)*
Vector	25						X	X	*(Capone et al., 2021)*
	7			X			X	X	*(Bos et al., 2020; Mercado et al., 2020; Tostanoski et al., 2020)*
	4						X	X	*(Marsh et al., 2021; van Doremalen et al., 2020)*
	5		X				X		*(Q. Guo et al., 2018; S. Wu et al., 2020)*
Inacti-vated	1					X	X	X	*(Gao et al., 2020)*
	2								-
	3	X			X	X	X	X	*(Wang et al., 2020)*
	19	X		X		X	X	X	*(Ganneru et al., 2021; Mohandas et al., 2021)*

Note. Vaccine ID corresponds with the ID numbers from the WHO Vaccine Tracker (Golding et al., 2018). *For vaccine #21, no animal studies have been reported to the time of writing. Abbreviations: GP = guinea pigs; NHP = non-human primates.*

Seven models

When considering the relevance of animal models, and challenge studies specifically, it is essential to look at the predictive value they have. Ideally, an animal challenge study is 100% predictive for the effect in humans, both with respect to potency and safety. Selecting the right animal studies, however, is complicated. Different pathogens require different models, and some animals have limited availability, and some are more expensive than others. Apart from comparability to human responses, availability of the animals, costs in case less expensive animal models can be used, and availability of immunological read-outs, affect the selection. In COVID-19 vaccine development, seven animal models are generally used: non-human primates (mostly rhesus macaques), (human ACE2 transgenic) mice, rats, guinea pigs, hamsters, rabbits, and ferrets. These models are selected for various reasons, and all have their advantages and disadvantages.

The most popular animal model in COVID-19 vaccine development is the mouse. Several mouse models were used, such as the hACE2 transgenic BALB-c mice that were initially developed for SARS-CoV research. Mice, including hACE2 mice, do not develop severe COVID-19 like humans do. They do however, “developed interstitial pneumonia characterised with inflammatory cell infiltration, alveolar septal thickening, and distinctive vascular system injury, which recapitulated the clinical features in most COVID-19 patients” (Sun et al., 2020).

Apart from mice, rhesus macaques and cynomolgus macaques are most frequently used in SARS-CoV-2 infection and vaccination studies. According to Salguero et al. “both macaque species authentically represent mild to moderate forms of COVID-19 observed in the majority of the human population and both species should be used to evaluate the safety and efficacy of interventions against SARS-CoV-2” (2021). In the identified literature, rhesus macaques were used 12 times, while cynomolgus macaques were used 6 times.

The use of rats was also reported in the identified literature. They were mostly used in inactivated vaccine development and once in a protein subunit vaccine. Two types of rats were reported: Sprague – Dawley (SD) rats and Wistar rats. Rats were mainly used for toxicity studies and occasionally for humoral immunogenicity (Ganneru et al., 2021; Hassett et al., 2019). Rats are equally susceptible to SARS-CoV-2 as mice, but their larger size is a minor advantage as it allows for repetitive bleeding in experiments.

Guinea pigs have been used in the development of many vaccines, including those against influenza, tuberculosis, diphtheria, but also for Ebola research (Yadav, Sapkal, et al., 2021). Guinea pigs are especially ideal to study dermal vaccination, since, “unlike other small animal models such as the mouse, the guinea pig's skin possesses a defined epidermis” (Schultheis et al., 2017). Smith et al. observed SARS-CoV-2-S binding antibody titres and the blocking of ACE2/S protein interaction in guinea pigs after dermal vaccination with a DNA vaccine (T. Smith et al., 2020).

Syrian golden hamsters are a more permissive model for SARS-CoV-2 because their ACE2 receptor is homologous to the human ACE2 receptor, transmission between animals has been reported and they show symptoms that are similar to human COVID-19 (Chan et al., 2020; Sia et al., 2020). Moreover, research has shown that old male hamsters have a higher chance of showing signs of disease, which is comparable to humans as well (Osterrieder et al., 2020). However, the lack of research tools, such as immunologic reagents, is recognised as a major drawback. To overcome this obstacle, more antibodies and microarrays need to be developed or transgenic hamsters are needed for better evaluation of this model. CRISPR/Cas9 technologies have led to more possibilities for the latter (R. Li et al., 2018).

SARS-CoV-2 is capable of interacting with the rabbit ortholog of the ACE2 receptor (L. Wu et al., 2020), but other than that there is little literature on rabbits and SARS-CoV-2. New Zealand white rabbits were used by Ganneru et al. for a repeated dose toxicity study (2021). Male chinchilla rabbits and Japanese white rabbits were used in two immunogenicity studies (Meyer et al., 2020; Wang et al., 2020).

Ferrets are commonly used as a model animal for respiratory diseases, such as influenza, and are susceptible to SARS-CoV-2 infection, but this species does not develop severe disease.

However, SARS-CoV-2 can replicate in the upper respiratory tract of ferrets for up to 8 days and transmission between ferrets has been observed (Shi et al., 2020). Hence, ferrets are useful to study the transmission of SARS-CoV-2. By assessing the level of viral shredding, the ferret model is regarded as a relevant model for testing SARS-CoV-2 vaccine candidate safety, immunogenicity, and efficacy. It most closely models asymptomatic or mild infection in humans (Marsh et al., 2021).

It is notable that while numerous scholars have reported the urgent need to identify suitable small animal models for preclinical evaluation of COVID-19 vaccines (Chan et al., 2020; Dinnon et al., 2020; Imai et al., 2020; Ryan et al., 2021; Sia et al., 2020), they have hardly been used in the identified literature, apart from mice. A possible explanation could be that the development of these vaccines was initiated early in the pandemic, when the pathogenesis and transmission of SARS-CoV-2 in animals such as hamsters, ferrets and guinea pigs was not extensively researched yet. On the other hand, hamsters have been used as a suitable animal model in SARS-CoV research before (Roberts et al., 2008).

2.2 Challenge studies

Macaques

Non-human primates are the most described animal challenge model in the identified literature on COVID-19 vaccine development. They were used in 13 of the 16 vaccines that were included in this search. Rhesus macaques were used 9 times, while cynomolgus macaques were used in 5 studies. In one study, both species were used; the cynomolgus macaques were used in immunogenicity and safety studies, while the rhesus macaques were used as challenge model (Wang et al., 2020). Here, we discuss the results from the challenge studies and compare them to (relevant) results from clinical studies.

#10: BNT162b2 (mRNA vaccine)

Vogel et al. describe the rhesus macaques challenge study for their two mRNA vaccine candidates (in a later stage, BNT162b2 was selected over BNT162b1 because of greater tolerability with comparable immunogenicity in clinical trials) (2021). Six macaques were immunised in two dose groups for each vaccine and a control group. None of the rhesus macaques that were challenged (immunized or not) showed any signs of illness. Evidently, none of the macaques died during this challenge study. Viral RNA was not detected in any of the BNT162b2 immunised and SARS-CoV-2 challenged animals, while multiple animals in the control group had detectable viral RNA in bronchoalveolar lavage fluid. In nasal, oropharyngeal, and rectal swabs, viral RNA was detected more often in the control group than the vaccine group.

In the phase III clinical study that was described by Thomas et al., COVID-19 was established in 77 vaccine recipients and 850 placebo recipients (42.094 total participants), corresponding to a vaccine efficacy of 91,3% (2015b). So, the vaccine provides high protection against virus infection in NHP as well as protection in humans. However, these numbers are hard to compare, which is also stated by Vogel et al., “the 2–4-year-old male-macaque challenge model is primarily a model of SARS-CoV-2 infection rather than a model of COVID-19 disease” (2021). Nevertheless, it is interesting to compare the serological data of the NHP study with clinical studies, such as the phase I study reported by Walsh et al. (2020). Vogel et al. made the comparison between macaque geometric mean antibody titres with convalescent human sera. Antibody titres in vaccinated NHP were higher as compared to human convalescent sera (2021). It could also be interesting to compare the values from the macaques to those from the phase I study by Walsh et al. Despite measuring different IgG geometric means (Vogel et al. measured the RBD-binding IgG GMT, while Walsh et al. measured the S1-binding IgG GMT), a comparison between these numbers can give a general idea of the predictive value of antibody responses and VNT in macaques. These values can be found in Figure 2 and Figure 3 . The data show that responses are different (antibody titres as well as VNT are higher in NHP at 30 µg dose), but also that data cannot be compared because there are too many variables. Studies in NHP give a good impression on the potency, but not more than that.

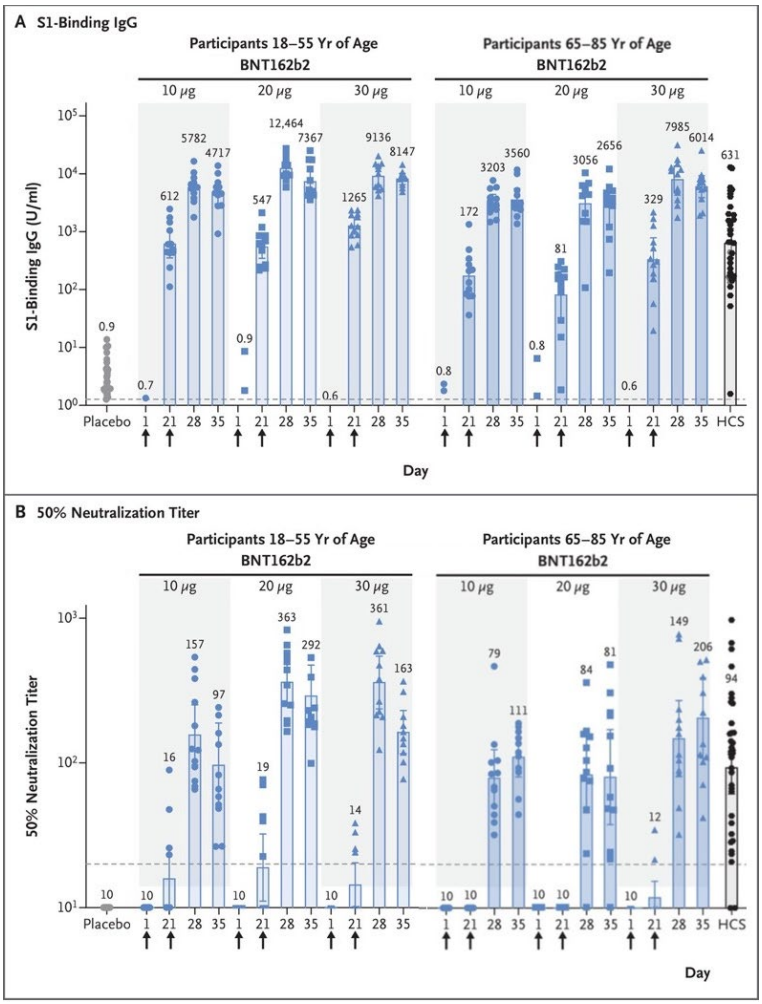

Figure 2. Immunogenicity of BNT162b2. Adapted from Walsh et al. (2020). *Participants in groups of 15 received an injection with the indicated dose levels of the BNT162 vaccine candidates (12 participants) or placebo (3 participants) on days 1 and 21. Arrows indicate days of vaccination. Responses in the placebo recipients in each of the dose-level groups are combined. Serum samples were obtained before injection (on day 1) and on days 21, 28, and 35 after the first dose. The blood samples obtained on days 28 and 35 are those obtained 7 days and 14 days, respectively, after the second dose. Human coronavirus disease 2019 (Covid-19) or SARS-CoV-2 infection convalescent serum (HCS) samples were obtained from 38 donors at least 14 days after polymerase chain reaction–confirmed diagnosis and at a time when the donors were asymptomatic. Panel A shows the geo metric mean concentrations of recombinant S1-binding IgG (lower limit of quantitation, 1.267; dashed line), and Panel B the 50% SARS-CoV-2–neutralizing geometric mean titres (lower limit of quantitation, 20; dashed line).*

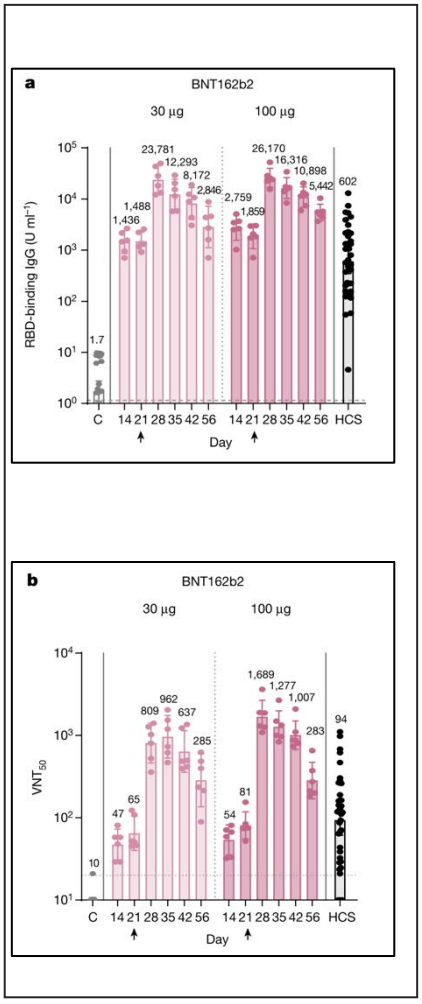

Figure 3. Macaque immunogenicity. Adapted from Vogel et al. (2021). Male macaques (2–4 years old) were injected on day 0 and day 21 (arrows below the x-axes indicate the day of second injection) with 30 μg or 100 μg BNT162b2 (n = 6 each). Additional macaques received saline (control (C), n = 9)). Human convalescent sera (HCS) were obtained from patients infected with SARS-CoV-2 at least 14 d after PCR-confirmed diagnosis and at a time when acute COVID-19 symptoms had resolved (n = 38). The HCS panel is a benchmark for serology studies. a, Concentrations (in arbitrary units) of IgG that binds recombinant SARS-CoV-2 RBD (lower limit of detection (LLOD) = 1.72 U ml−1). b, SARS-CoV-2 50% virus-neutralization titres (VNT50) (LLOD = 20).

#9: mRNA-1278 (mRNA vaccine)

Rhesus macaques were also used as a challenge model for the mRNA-1278 vaccine, as reported by Corbett et al. (2020). The results are more informative about SARS-CoV-2 infection than COVID-19 disease (prevention), similar to the results that were described for the BNT162b2 vaccine.

Corbett et al. reported increased VNT (50% inhibitory dilution) between 2 weeks after first and two weeks after second vaccination (GMT 63 versus 103), but do not mention of any clinical symptoms or adverse events. Two days post challenge, one of eight macaques in the two vaccinated groups had detectable subgenomic RNA in BAL fluid, compared to all eight animals in the control group. On the same day, subgenomic RNA was detected in none of the nasal swabs from the highest dose vaccinated group and in five specimens from the lower dose. All control animals had detectable RNA in their nasal swabs. The S-specific IgG binding was 5 times higher than serum from convalescent patients, while neutralising GMT saw a 15-fold increase compared to the convalescent serum (2020). So, similar to study #10, vaccinated NHP developed higher humoral responses than infected humans.

Peak anti-SARS-CoV-2-spike binding antibodies GMT in a phase II study increased to a maximum of 239 µg/mL, while the peak level in convalescent COVID-19 patients was 48 µg/mL (Chu et al., 2021). The vaccine efficacy was 92.3% for preventing SARS-CoV-2 infection. Vaccine efficacy in preventing severe Covid-19, a key secondary end point, was 98.2% (El Sahly et al., 2021). Corbett et al. reported no evidence of viral RNA or viral antigen in the vaccinated group at day 8 after challenge (2020).

Hamsters

Non-human primates were by far the most used challenge model in COVID-19 vaccine research. However, several other models are available, and some may be better suitable. The golden Syrian hamster, for example, can be very well used as a challenge model. Unlike macaques, hamsters do develop severe COVID-19.

#7: Ad26.COV2.S (Vector vaccine)

The Ad26.COV2.S vector vaccine was tested in multiple animal species. Apart from mouse studies, two challenge models were used: rhesus macaques and hamsters (Mercado et al., 2020; Tostanoski et al., 2020). When comparing the results from these two challenge models, it is evident that, unlike the macaques, hamster do develop clinical signs after challenge. Tostanoski et al. report (severe) weight loss and (partial) mortality (2020). This shows that hamsters are a better challenge model to study the protection against (severe) illness and mortality. Moreover, they found no mortality in the vaccinated group and vaccinated hamsters showed less weight loss, indicating protection against severe clinical disease as well as mortality. Vaccinated animals did lose some weight, indicating a lesser degree of protection against mild disease.

On the contrary, but in line with other research, Mercado et al. reported “minimal clinical disease” in macaques, vaccinated or not (2020). This, once again, highlights the main disadvantage of this animal model. They did, however, find no detectable virus in lung lavages in the vaccinated macaque group, whereas the control group were infected and had viral RNA in their lungs. Finally, Mercado et al. reported no increase in T-cell response after challenge in

the vaccinated group and a high increase after challenge in control animals, which "suggests minimal to no virus replication" in the vaccinated group (2020). These data are not comparable to the hamster model, as the T-cell response in hamster was not assessed. In a phase II clinical study, the majority of vaccinated participants did show an increase in T-cell activity (Sadoff, Le Gars, et al., 2021).

In the phase III clinical study, the overall efficacy of the vaccine was determined at 85.4% against severe/critical COVID-19 (Sadoff, Gray, et al., 2021). There were three deaths in the vaccine group, but none of these was COVID-19 related. So, the vaccine was safe and induced high protection against disease both in hamsters as well as in humans.

#19: BBV152 (Inactivated vaccine)

Mohandas et al. also reported the use of hamsters as a challenge model (2021). They observed significant weight loss in vaccinated as well as placebo groups. However, contradictory to the previously discussed hamster challenge model, Mohandas et al. did not observe any other clinical signs and no hamster met the Institutional Animal Care and Use Committee humane euthanasia criteria. SARS-CoV-2 viral genomic RNA was significantly higher in the control group than in the vaccinated group, indicating protection against infection. Mohandas et al. conclude: "Lower viral load, absence of lung pathology, and high titres of neutralizing antibodies post-infection demonstrate the protective efficacy" (2021).

The BBV152 vaccine was studied by Yadav et al. in a rhesus macaque challenge model (2021). They concluded that the vaccine has protective efficacy in the non-human primate because of "the presence of subgenomic RNA in the BAL fluid and lung tissue (at necropsy) of animals from placebo group I at 7 days post challenge and absence in the vaccinated animals". Moreover, four out of five animals of the placebo group had detectable gRNA and sgRNA in the lungs, while all animals from the vaccinated groups had none. In the control group, Yadav et al. report clinical signs but not in the vaccinated group. The antigen specific IgG levels and neutralising antibody titres after challenge were of comparable levels between the hamster and macaque study.

The vaccine's overall efficacy in a phase III clinical study with 24,419 participants was 77.8% and the efficacy against severe COVID-19 was 93,4% (Ella et al., 2021). Unsolicited adverse events were reported in 1.7% of the vaccinated group and 1.8% in the control group, hence "no safety concerns were raised".

Ferrets

#5: Ad5-nCov (Vector vaccine)

Since ferrets do not develop severe COVID-19 like disease, they are regarded as a good way to model asymptomatic or mild infections in humans. It is known that SARS-CoV-2 can replicate effectively in the upper respiratory tract of ferrets, but not in the lungs. Wu et al. used a ferret challenge model and evaluated the protective efficacy of the Ad5-nCov vaccine in the upper respiratory tract (S. Wu et al., 2020). All vaccinated animals produced systemic S-specific IgG and virus neutralising antibodies, while control animals did not. The vaccinated group had no virus in the nose washes, in contrast to the control group. Wu et al. did not

investigate transmission between the ferrets. They did find a significant increase in T-cell activity in the vaccinated group, as compared to the control group.
In a phase II clinical trial reported by Zhu et al., a high neutralising antibody titre was found in the vaccinated cohort after 28 days (2020). 52% of the participants had pre-existing antibodies to the Ad5 vector. These participants had RBD-specific ELISA antibody and neutralising antibody levels that were approximately two-times lower than those without pre-existing immunity. The phase 3 study has not been reported yet, so vaccine efficacy is not yet known.

#4 ChAdOx1 nCoV-19 (Vector vaccine)

Marsh et al. report another ferret challenge study (2021). Vaccination with half the human dose led to a sharp increase in neutralising antibody levels in all animals 7 days after the second vaccination, which declined prior to the challenge. Administering a booster dose led to another sharp increase in neutralising antibodies. The vaccinated and challenged animals showed lower levels of virus shedding in nasal-wash and oral swabs than the control group. This indicates that the vaccine does not induce sterilising immunity, which is consistent with observations in humans.

Marsh et al. (2021) observed no clinical symptoms in immunised ferrets. None of the ferrets developed fever or weight loss and all animals remained alert and responsive throughout the study. Immediately following the virus challenge, two control animals that received placebo vaccination were euthanised due to a severe unexpected reaction. Moreover, several animals in the vaccinated group had acute unexpected reactions of which one was reason to euthanise. Histopathological assessment of tissues suggested an allergic reaction resulting in respiratory distress. After the first vaccination, animal in the intramuscular administration group had reliable neutralising antibody titres, while the animals in the intranasal group did not. A second vaccination led to sharp increases in neutralising antibodies for both groups. No antibodies were detected in the control group (2021).

A phase I/II study by Folegatti et al. conclude that unsolicited adverse events that were possibly related to the vaccine were mild and moderate in nature and resolved within the follow-up period (2020). In a phase III study, Voysey et al. reported two deaths in the vaccinated group, but these were considered unrelated to vaccination. The overall efficacy of the vaccine against symptomatic COVID-19 was 70.4% (2021).

Conclusions and future perspectives

This report provides an overview of the analytics of COVID-19 vaccine concepts, published in scientific literature. Also, a brief overview of the used animal models for immunogenicity measurements is given. Given the enormous volume of studies published it is not a systematic review. Besides, it is likely that not all techniques and studies were reported because they were considered of minor importance in comparison to the overall objective of the study. Every vaccine type (e.g., mRNA, vector, subunit, inactivated, live inactivated) was and is used for SARS-CoV-2 vaccine development. Each category has its critical quality attributes and, as a result, a different set of assays is used for each vaccine type. However, substantial overlap between the groups was observed. Nonetheless, there are many valuable assays that yield a vast amount of data on stability, identity, safety, and efficacy.

The objective of the report was to address the following questions:

- Which analytical methods are used in vaccine development and what potential and limitations do these methods have in replacement of animal models?
- What was the role of these methods in SARS-CoV-2 vaccine development and did these methods contribute to reduction or replacement of animal use?
- In the coming years, what developments in analytical characterisation are expected that may affect animal use?

The first two questions were addressed simultaneously. Analytical development is progressing continuously, but it seems that the analytical toolkit used for SARS-CoV-2 vaccines is not more extensive than or as advanced as in other vaccine development projects. Perhaps it is even more conservative. This may have to do with the time pressure, leading to the use of proven technology. Even vaccines based on mRNA and viral vectors were already in development for years and have been tested in the clinic before the COVID-19 pandemic. As a result, the analytical toolbox for these vaccines was ready to use. This toolbox consisted for a considerable part of analytics which also has been used for other vaccines. For instance, chromatography, electrophoresis and particle size analysis for mRNA vaccines and virus titration for vector vaccines.

The number of animal studies performed to test SARS-CoV-2 vaccines is probably lower than for other vaccines, simply because time was lacking to perform more. In fact, in some cases animal studies were done overlapping with clinical studies. Apparently, the vaccine developers and ethical committees granting permission for clinical studies were sufficiently confident that these early clinical studies could be done with safe and potent vaccine concepts. Indeed, the animal studies that have been published, all showed that the tested vaccines were immunogenic and adverse effects were not reported. There seemed to be no need to optimise the vaccine concepts in an iterative manner; it was mostly “first time right”. We think that this is because the platforms had already been shown to be safe and often efficacious in clinical trials for other infectious diseases. The characterisation of these vaccines was excellent, but not better or different than other vaccine concepts that took longer to develop.
So, in short, the use of true vaccine platforms has led to a fast progression to clinical trials and eventually (emergency) licensing. That said, the many SARS-CoV-2 vaccine concepts that are not yet licensed may have encountered problems, such as lack of immunogenicity, production problems or stability issues. This may result in additional characterisation, both in vitro as well as in vivo. These negative results are usually not reported and probably never will be. Many

of these vaccines are not developed via a platform (except perhaps DNA vaccines) with ready-to-use and/or modular production steps but using trial-and-error approaches and may thus encounter the same problems as any other classical vaccine.

The third question was about the expected developments in the short term and long term. The SARS-CoV-2 vaccine development did not lead to extensive use of new analytics or new characterisation technology. There was no time to do so and using the existing set of analytical techniques allowed the swift development of several safe and effective vaccines. It is expected that the mRNA and vector platforms will be exploited further in a high pace. Improved, variant adapted SARS-CoV-2 vaccines are already in the clinic and many other vaccines based on these platforms are under development. The coming years, it will become clear whether SARS-CoV-2 was an easy target, and we were just lucky, or that mRNA and vector vaccines are indeed generally applicable. The analytical development will follow suit. It is expected that in the short run, the analytical toolbox will not change dramatically or replace animal studies. However, it may be possible to reduce animal testing in the case of product improvement. A thermostable mRNA formulation (e.g., lyophilized) – although this is a major change in the production process – may not need in vivo testing in animals, but a small bridging clinical trial may be sufficient. Robust in vitro characterisation, providing analytical fingerprints of vaccine batches, can guarantee product quality without the need for animal testing. An adaptation of the antigen to achieve a better match with circulating viral strains may even be possible without animal studies. For routine batch release, mRNA vaccines are a prime candidate for release procedures without the use of animals because the assays are available and the product and production process are relatively well defined.

For preclinical studies with new platform-based vaccines (such as influenza), the use of animals will depend on the type of vaccine or pathogen that is targeted. In situations less urgent than the COVID-19 pandemic, it is likely or even mandatory to do the preclinical assessment before phase I studies will start. The use of transgenic animals to have a better comparator with humans with regard to adaptive immune responses will likely increase. When safe and effective platforms are available, the focus will be on the effects of the antigen. It may be possible to assess innate immune activation with *in vitro* cell-based functional tests, but adaptive immunity can – for the time being - only be measured in intact immune systems of animals and humans because the frequency of naive B- and T-cells of a certain antigen specificity is very low and the immune system consists of many cells and molecules which interact at many different sites of the body.

On the long term, the combination of platform technology, highly reproducible manufacturing methods, extensive in vitro characterisation and *in vitro* functional assays may allow animal free development of vaccines, but this is a long way off. However, it is possible and also recommended by NGOs to do batch release on the basis of *in vitro* potency assays (Sanyal et al., 2021; WHO, 2021c). For mRNA vaccines this could be the amount of mRNA combined with an *in vitro* translation assay. For vector vaccines this could be a virus titration for viable virus and a cell based assay for the transgene expression. With respect to animal use during the development phase more is expected from better use of the employed animals or humans as model. Improved analysis of the immune response, using systems vaccinology (i.e. using transcriptomics, proteomics and network analysis to analyse wanted and unwanted vaccine responses), can provide more valuable information from less animals. Human challenge

models would allow to measure protection under controlled conditions. These developments are possible because product quality can be guaranteed using robust analytics to monitor both product and production process.

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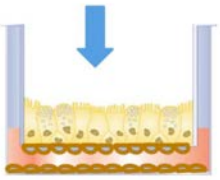
appendix 2

Learning from COVID-19
SARS-CoV-2 and Organoid technology


January 2022

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Abstract

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic has generated a large amount of research into SARS-CoV-2 and its effects, to find answers and save lives. This is a unique opportunity to take advantage of the huge progress made in science over the last decades. Innovations have been made that were unimaginable over 70 years ago, when animal models became a golden standard for medical research, such as Organs on a Chip and Organoid models. Use of animal models in research is becoming less self-evident, not only because of public concern for animal and human welfare, but increasingly from a scientific point of view as well, emphasizing the importance of innovation and implementation of non-animal methods to improve biomedical research. With the current volume of research on SARS-CoV-2, an important question is 'has the scientific community seized this opportunity to make more use of innovative, animal free, research methods?'. This report presents the results of a literature search to identify different research models used in SARS-CoV-2 research and to make recommendations on how the proportion of innovative non-animal models can be improved, specifically in virological research.

Introduction

SARS-CoV-2, the cause of the COVID-19 pandemic is a zoonotic beta-coronavirus first discovered in China in 2019. Initial symptoms were acute respiratory distress syndrome (ARDS); however, it has since been discovered that the virus can cause multiple pathologies in different organs. Outcomes for patients range from none or mild symptoms, to death or chronic issues. Since the start of the pandemic research from all over the world has resulted in an enormous number of studies into the virus itself and the interaction with humans, in the quest for finding a cure and prophylaxis. [1, 2]

Animal models became a golden standard for medical research over 70 years ago, but since then it became increasingly clear that responses in animals do not fully represent disease in humans. Pathogen tropism (the preference of a pathogen for a host) and differences in pathology between humans and animals complicates translation of data from animal studies to humans. For this reason, and because animal welfare is becoming increasingly more important in society, animal use in science is under scrutiny. [3, 4, 5]

These developments emphasize the importance of innovation and implementation of non-animal methods to improve both scientific research and welfare for human and non-human animals. Over the past decades new innovative methods to study disease in humans have been developed to enable this. One of these innovations to improve translation of research outcomes to humans and to reduce and replace animal use in research, is the use of organoids as a model for human disease. Organoids are three-dimensional, organ-like structures that are grown from either adult stem cells or pluripotent stem cells. Under specific controlled conditions in culture, the stem cells can generate organ-specific cell types. These can self-organize and display functionality of the organ, albeit in a limited way, which can be used as a human model for medical research. Further progress in organoid-model techniques has resulted in the development of two-dimensional organotypic cultures in which both the basal side and the lumen of the mimicked organ are accessible by culturing differentiated stem cells in an air-liquid interface. This enables detailed research into infection dynamics and pathways by identifying receptors and reactions of the cells involved. Development of co-cultures to research different organ systems and their interactions improves representation of human in vivo processes even more. Organoid models are particularly useful in virology research, as viruses are obligate intracellular pathogens that can only replicate in living host cells. Interactions of viruses with the host cell and subsequent reactions can cause pathology and the study of these processes is therefore of great importance for both prevention and cure of viral infections. [6, 7]

Project

The Minister of Agriculture, Nature and Food Quality has requested information on the effects of coronavirus disease of 2019 (COVID-19) on the transition to innovation without laboratory animals (NCad 2021). The main question is: “What are the proportions of models used in SARS-CoV-2 research and how can the proportion of innovative non-animal models be improved?" [8]

To answer this question, the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) initiated this project and our part is to take a closer look at the proportions of organoid type models used in SARS-CoV-2 research, specifically human airway models. Virological research at the OrganoVIR Labs, Department of Medical Microbiology, Amsterdam UMC, is performed with organoid and organotypic cultures, therefore this department was approached by NCad to investigate the role of organoid technology in SARS-CoV-2 research.

A preliminary study done by this department earlier this year found that most of the studies on SARS-CoV-2 with human-based organoid technology focused on pathogenesis, followed by antiviral therapy. Over 50% of the studies on pathogenesis with organoid technology used human airway models. [9]

To further investigate these results and assess the proportion of human airway models used compared to animal models and human patient data a literature search on SARS-CoV-2 studies was conducted, focusing on use and type of models, lung pathology/pathogenesis and antiviral therapy.

The project is divided into three separate research questions (RQ) to evaluate the use of *in vitro* stem cell based human airway models, such as lung organoids and Air-Liquid-Interface/Human Airway Epithelium (ALI/HAE) in SARS-CoV-2 research.

1. Investigate the proportions of Animal, *In vitro* and Clinical publications on SARS-CoV-2 research: What are the numbers and proportions of animal models versus *in vitro* human airway models used in SARS-CoV-2 research.

2. Categorize the research questions of SARS-CoV-2 lung pathology research and specify the *in vitro* human airway models used: Which areas in SARS-CoV-2 lung pathology have been researched and what were the predominantly used models to study these questions?

3. Identify drugs and organoid models used in SARS-CoV-2 antiviral therapy research: Which drugs have been investigated *in vitro* with organoids in SARS-CoV-2 antiviral therapy research and which type of models are mainly used?

The results will be presented in this report along with recommendations on how to improve implementation of this technology to accelerate moving towards non-animal research in science, particularly the use of *in vitro* human airway models in virology research.

Methods

The three separate research questions to evaluate the use of *in vitro* stem cell based human airway models were performed in the CAMARADES COVID-19 SOLES database. This is a COVID-19 Systematic Online Living Evidence Summary (SOLES) based on primary research studies, developed by the CAMARADES research group at Edinburgh University for stakeholders in COVID-19 research.
The CAMARADES group performs a weekly search, without language restrictions, on primary research on COVID-19 disease or the SARS-CoV-2 virus in PubMed, Embase, Web of Science, the WHO database of publications on coronavirus disease (COVID-19), BioRxiv and MedRxiv. The results are added on a weekly basis to the COVID-19 SOLES database after deduplication with The Automated Systematic Search Deduplicator (ASySD) tool. Primary research is defined as original clinical, in vivo non-human animal, *in vitro*, and in silico research in which data are gathered, reported, analyzed, and interpreted in that research article and original results are presented. This includes Peer-reviewed studies, pre-prints, systematic reviews, and conference abstracts related to primary research. Secondary publications such as, narrative reviews, opinion pieces, etc., and publications not related to COVID-19 or SARS-CoV-2 are excluded. [10, 11]

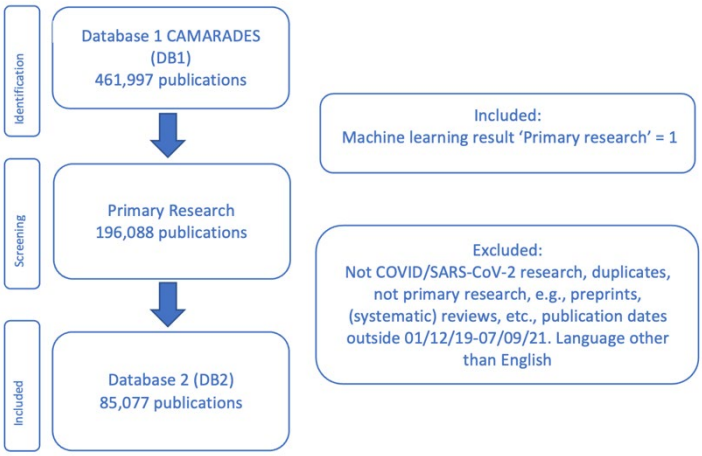

Figure 1 Overview publications screened

For this research a csv (comma separated values) copy of the full COVID-19 SOLES database (last updated 7th Sep 2021, 461,997 publications) was supplied by University of Edinburgh CAMARADES team. [12] Included in this database were machine learning (ML) results of three trained algorithms, created to include or exclude the studies in the following categories: Primary research, Animal research *and In vitro* research. All the studies in the database were scored by the algorithm. Based on a set threshold value publications were either in- or excluded, whereby '1' indicates the study was included in said categories, and '0' indicates it was excluded. The sensitivity was preset to 95%, leaving a 5% margin of error, as this was deemed to be an estimated average sensitivity of dual human screening. The Primary research algorithm includes all studies with an abstract reporting original COVID-19 research. The Animal research algorithm includes all studies using any animal species or model and the *In vitro* research algorithm includes all studies that conduct interventional experiments using any *in vitro* or ex vivo model. [13]

The supplied database with machine learning results (DB1 461,997 publications) was uploaded in R-Studio (R version 4.1.1 (2021-08-10) and filtered on ML primary research. This subset was subsequently further deduplicated, filtered on publication date between 2019-2021 (no relevant results in Dec 2019), and English language only, followed by removal of systematic reviews, preprints, etc., resulting in DB2 with 85,077 publications. Further in/excluding and data management was done with Rayyan and Monday.com. See figure 1 and search details and publications in appendix.

Total number and proportions of models used in SARS-CoV-2 research

To get the numbers and proportions of models used in SARS-CoV-2 research for research question 1, subsets were created in DB2 by filtering on machine learning (ML) results for Animal research and *in vitro* research as well as publications included in both categories by ML. Searches were performed to identify Clinical studies and the proportion of organoid studies (all organs), specifically publications with Human Airway Model (airway organoid/organotypic cell cultures). Search terms were partly based on the MeSH terms used by Kroon [9], and a check of abstracts was done to identify additional terminology. Another search in DB2 was done to find categories for the remainder of the subset, such as surveys, questionnaires, mathematical/computational models, and epidemiological studies, not taking overlap into account. Publications from the results of Kroon and publications in a subset that was not categorized as primary research by ML were checked, missing publications were added to the result. An additional search was done in DB2 for publications with animal models, as well as separate searches for studies with non-human primates (NHP), hamsters, and mice, these results were further broken down to Lung pathology research for RQ2, and treatment and vaccine research for RQ3. Finally, a search on Remdesivir and (hydroxy)chloroquine in DB2 (85,077 publications) was analyzed for number of organoid, animal and clinical studies.

SARS-CoV-2 pathology studies, research questions and models used (RQ2)

For research question 2, which areas in SARS-CoV-2 lung pathology have been researched and what were the predominantly used models to study these questions, the organoid subset created for RQ1 (418 publications) was used for further analysis for lung pathology research questions (RQ2). The research questions of the publications found were divided into three categories: Model development, Receptor & entry, and Host (cell) & immune response. The results were also categorized by cell types used for the organoid models. Publications with non-human primates, hamsters and mice were extracted with a search done in DB2, and research questions were categorized as the organoid subset, with one additional category, (re)infection and transmission.

SARS-CoV-2 antiviral therapy studies, drugs investigated, and models used (RQ3)

To identify drugs investigated *in vitro* with organoids, related search terms were used to find relevant publications. The results were summarized by type of organoid model and results found.

Results

One of the positive outcomes of the Sars-CoV2 pandemic is the worldwide collaboration of researchers to obtain knowledge on this rapidly spreading virus. Many publications are published online, after rapid peer reviewing processes or as preprints, and are being shared as open data. The disadvantage is that this 'data-avalanche' makes it progressively harder to find relevant data, in the commonly searched databases such as PubMed, that is reliable and valid. Over the past two years, nearly half a million publications on Sars-CoV2 were added to the COVID-19 SOLES database by the Camarades team, with the aim to better categorize publications on Sars-CoV2. This is a record in number of publications on a topic in such a short time span. [14] The COVID-19 SOLES database is compiled of search results of four databases (PubMed, Embase, Web of Science and WHO) saving us searching multiple databases. The ML provided by the team enabled filtering on primary research, improving the data clean-up.

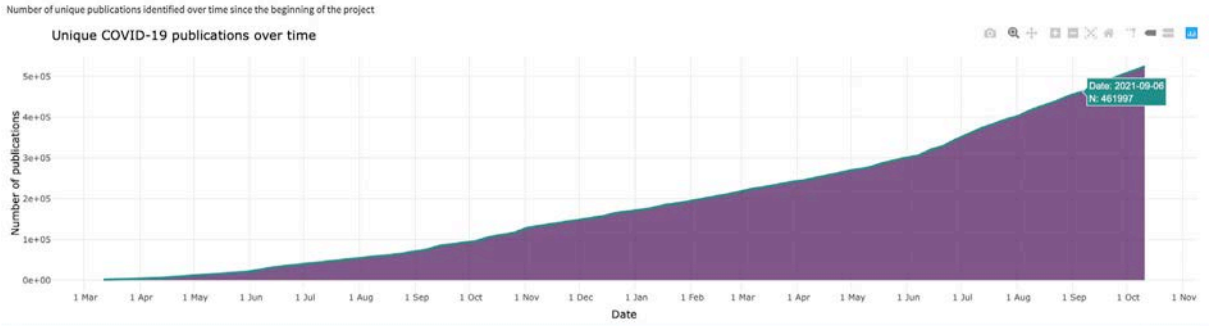

Figure 2 Number of COVID-19 publications over time from COVID-19 SOLES website

Total number and proportions of models used in SARS-CoV-2 research

Over 460,000 papers were found in the CAMARADES COVID-19 SOLES database on Sars-CoV2 (table 1 and figure 1); Database 1 (DB1) filtered by machine learning (ML) for Primary Research resulted in 196,088 publications. After deduplication and clean-up 85,077 publications remained in subset Database 2 (DB2). ML was applied for categorizing DB2 into Animal research, *in vitro* research, and overlap of these two categories, resulting in 4,044 (4.8%), 2,673 (3.1%) and 1,193 (1.4%) publications, respectively. The search for Clinical studies with search terms resulted in 56,579 publications which comprises 66.5% of all publications in DB2, and 1,400 publications in this subset were categorized as *in vitro* research by ML as well.

A search in DB2 to find categories for the remainder of the subset resulted in 21,663 publications (25.5%) on surveys, questionnaires etc., 6,631 publications (7.8%) with mathematical/computational models and 4,186 publications (4.9%) on epidemiology, not taking overlap into account. The rest of DB2, 13,190 publications (Other, 15.5%), was not categorized further yet. Searching DB2 for research done with organoid models (all organs) resulted in 418 publications, with 119 publications using Human Airway Models (airway organoid/organotypic cell cultures). In conclusion, only 0.5% of all research publications on Sars-CoV2 in our database DB2 containing over 85,000 publications could be labeled as research with organoids, and 0.14% of the total number of publications in DB2 was performed with a human airway model.

Table 1 Categorized SARS-CoV-2 publications in CAMARADES COVID-19 SOLES DB

Database / subset	Publications	Percentage
Database 1	**461,997**	**% of 461,997**
Primary research (ML)	196,088	42.44%
Database 2	85,077	18.42%
Database 2	**85,077**	**% of 85,077**
Animal research (ML)	4,044	4.75%
In vitro research (ML)	2,673	3.14%
Overlap Animal/In vitro research (ML)	1,193	1.40%
Clinical research (search)	56,579	66.50%
Overlap Clinical/In vitro research	1,400	1.65%
Organoids (search)	418	0.49%
Human Airway Models	119	0.14%
Surveys	21,663	25.46%
Computational	6,631	7.79%
Epidemiological	4,186	4.92%
Other	13,190	15.50%

Database 2: total amount of publications on Sars-CoV2 primary research after filtering.
Categorization of DB2 by machine learning (ML) or with search terms (search)
Note that not all overlap has been analyzed in these results, the % will therefore not add up to 100%

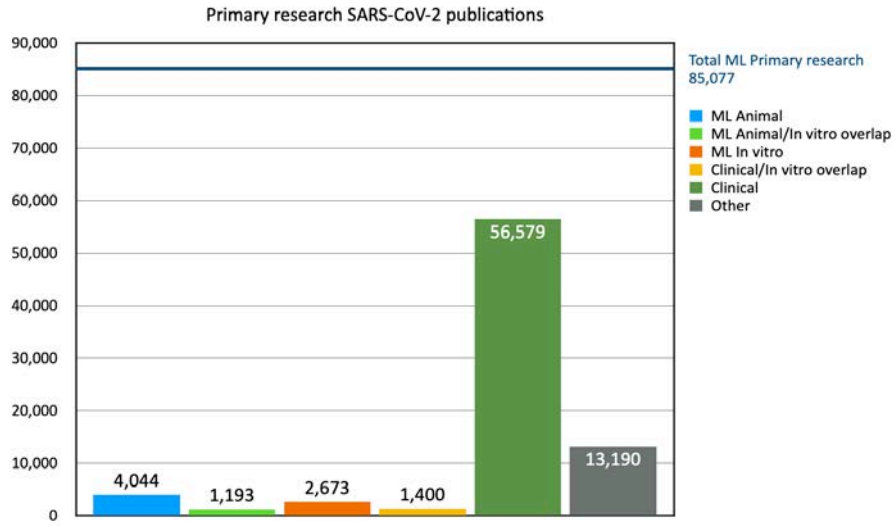

Figure 3 Numbers of SARS-CoV-2 publications in CAMARADES COVID-19 SOLES DB.
Animal & In vitro subsets were obtained with ML, Clinical and other subsets with search terms.

SARS-CoV-2, research questions and models used for airway pathology studies

For RQ2 we performed a search to further categorize studies with animal models and organoids by three research topics: Lung Pathology, Treatment, and Vaccines. By search terms, the number of publications with animal models (1,758) was lower than found by ML in Table 1. We decided to continue with the search by terms, as for the organoid research in this database ML cannot be applied. Publications with animal models were further divided into research with non-human primates (NHP), Hamster and Mouse models as being the most used animal models as per the search results. The majority of the papers in the animal model subset was performed with mice (710 publications, 40%) followed by NHP (307, 18%). Division into research topics showed that publications with animal models were mainly on Treatment and Vaccines and less on Lung pathology. In contrast, publications in the organoid subset (418) were almost equally divided over Lung pathology and Treatment. There were no publications on vaccines found in the organoid subset.

To understand when organoid technology was applied, we studied what research questions were addressed in the organoid subset on lung pathology, which were a total of 58 publications (13.9%). These publications were assigned to one or more categories based on the main topics of the research questions mentioned in the abstracts, per year (figure 4) and per organoid type used (figure 5). Thirty-six publications (62.1%) were assigned to the Host and Immune response category (#host(cell)_/_immune_response). These included research into how the host cells respond to the virus, like the rapid remodeling of diverse host systems found after infection with SARS-CoV-2 by Hekman et al. [15] Twenty publications (34.5%) were assigned to the category Receptor/Entry (#receptor_/_entry), studying which type of cells are most prone to infection and which receptors and pathways are important in this process, e.g., the study done by Clausen et al. identifying cellular heparan sulfate as a necessary co-factor for SARS-CoV-2 infection. [16] Twenty-two publications (37.9%) were included in the Model development category (#model_dev_/_check). This was research done to provide evidence organoids can be infected with SARS-CoV-2 and can therefore be used as a model to study certain aspects of COVID-19 pathogenesis and pathology, like the study done by Xia et al. [17] As these processes are all intertwined, several studies were included in more than one category described above. A breakdown of the airway organoid types showed that bronchial cells were the most used (figure 5).

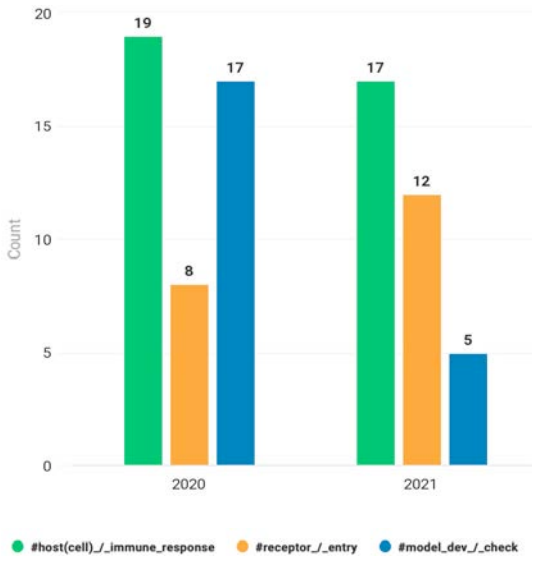

Figure 4 Organoid research question category per year

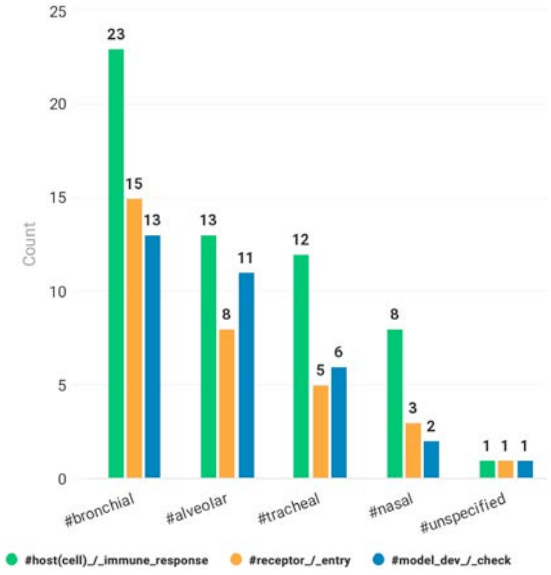

Figure 5 Organoid research question category per cell type

Next, we compared the research question categories studied in the organoid subset with the animal model search subset. Compared to the research question categories in organoids, another category was identified in the animal model studies, namely (re)infection_/_transmission (figure 6). [18] This category was not identified in the organoid subset. In the animal models, the topic host cell/immune response stood out as being the most frequently studied, while the topic receptor/entry was studied to a lesser extent than in the organoid subset. The topic model development was mainly found in studies with mice.

Table 2 Comparison of research topics studied in animal models and organoids in DB2

	Non-human primate (NHP)	Hamster	Mouse	Total AM 1.758	Organoids 418
Animal models DB2 (AM) (search terms)	307	168	710	1,185 (67.4%)	
Lung pathology (RQ2)	25	27	46	98 (5.6%)	58 (13.9%)
Treatment (RQ3)	110	77	369	556 (31.6%)	47 (11.2%)
Vaccines (RQ3)	90	72	254	416 (23.7%)	0

Results NHP, hamster, mouse (in Animal research search subset) and Organoids for lung pathology, treatment & vaccine. Other results organoids (not analyzed): not human airway model, other pathology, comorbidities, non-antiviral treatments.

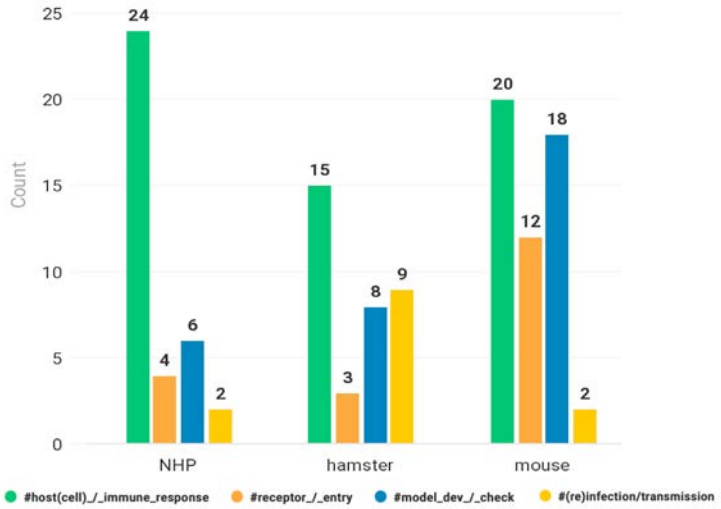

Figure 6 Research question category for NHP, hamster, mouse in lung pathology (Animal research ML)

SARS-CoV-2 antiviral therapy studies, drugs investigated, and organoid models used.

Organoid models are used in testing antiviral therapy, as shown by a search on antiviral therapy tested with organoid models that resulted in 47 publications (table 2) with 68 drugs studied (see appendix). To study the contribution of organoid models to antiviral research, we searched our database DB2 specifically for Remdesivir and (Hydroxy)Chloroquine as the most studied compounds for Sars-CoV2. As expected, most publications were from studies with patients (2,054, 83.1%), while the amount of animal studies published with these compounds was relatively low (182, 7.4%). As expected, the number of publications with organoids was lowest (37, 1.5%) (table 3).

Table 3 Publications on Remdesivir and (Hydroxy)Chloroquine

	Remdesivir	Chloroquine	Total
Total hits	902	1,569	2,471
Organoid	21 (2.3%)	16 (1.0%)	37 (1.5%)
Animal	90 (10.0%)	92 (5.9%)	182 (7.4%)
Clinical	703 (77.9%)	1,351 (86.1%)	2,054 (83.1%)

Publications on Remdesivir and (Hydroxy)Chloroquine studied with organoid, animal, and clinical models

Most of the antiviral drug research in human organoid models was done with airway organoid models (figure 7). In 86% of the studies, the drugs tested were successful in inhibiting Sars-CoV2 replication in the human airway models (figure 8).

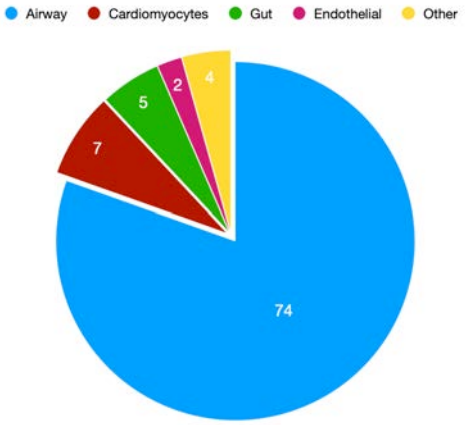

Figure 7 Number of organoid types used (Other = kidney, liver, skin, oral epithelium)

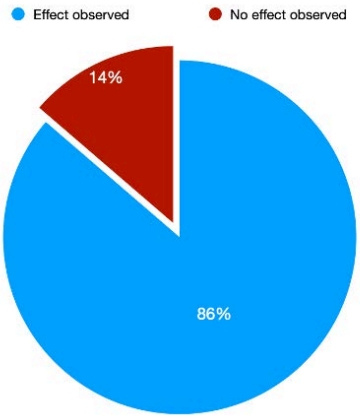

Figure 8 Drug effects observed in airway organoid types

Conclusion and Discussion

In our report we used the COVID-19 SOLES database by the Camarades team, which after filtering contained 85,077 primary research publication on Sars-CoV2, to study the proportion of clinical studies, animal models and the contribution of organoid models in SARS-CoV-2 research. Clinical research stood out with 66.5% of the publications, which is to be expected in the middle of a pandemic with large numbers of COVID-19 patients. The percentage of research publications with animal models was 4.75%, comparable to the number of publications with *in vitro* research (3.14%). In our earlier search (see Kroon et al) we reported that 60% of the COVID research was done with animal models. [9] By taking a closer look at how these figures were obtained in PubMed we found that the search filter for animal research contained the MeSH term "Primates". As humans are categorized under this MeSH term, the original search contained clinical studies labeled as animal research. Rerunning the filter without this MeSH term resulted in 2,012 hits for the period researched by Kroon and 3,389 for the period of this study. This is more in line with the machine learning results of 4,044, and the search term results on animal models (1,758), in the COVID-10 SOLES database, which is the result of 4 database searches (PubMed, Embase, Web of Science and WHO). Running the Syrcle Animal research filter [19] in combination with the SARS-CoV-2 filter from Kroon resulted in 5,690 hits, also more in line with our results. This underlines the difficulties encountered in searching for reliable research data in the commonly used databases. Publications of Sars-CoV2 research performed with organoid models was only 0.5% of the total amount of primary research publications in our database. A reason for this could be that in case of an acute health crisis, like the current pandemic, researchers use models that are familiar and available, such as *in vitro* cell lines and animal models. Organoid and other innovative non-animal research require specific knowledge, which may also be a limiting factor for researchers to switch. However, it could be that the contribution of organoid technology to COVID research is higher than in other virology fields. Our study is the first to compare the proportion of animal models, clinical research, and *in vitro* research, and it is currently unknown if our results represent a general picture of the state of organoid use in virology. To understand how organoid technology is applied, we compared the number of publications with animal models and organoid models for lung pathology, treatment, and vaccine development. Since we did not find studies on SARS-CoV-2 vaccine development with organoids, we focused on lung pathology and treatment development to elucidate the kind of research questions where organoid technology is used compared to the kind of questions where animal models are being used. The main topics where organoid technology was used were model building, studying host(cell) response and immune response, and looking into interaction and entry of the virus with and into the cell. The proportion of studies on model development decreased, while those on receptor interaction increased from 2020 to 2021 (figure 4). For research done with animal models we could identify another category of research, namely on re-infection and transmission. Results of lung pathology research question categories for non-human primates, hamsters, and mice showed that the number of publications assigned to research question category "model development & check" was considerably higher in mouse models compared to hamsters and non-human primates. This could be explained by the fact that laboratory mice are not spontaneous animal models for SARS-CoV-2 infections, unlike e.g., hamsters, macaques, and ferrets. Genetic alteration of both the virus and the animal have been performed to increase susceptibility of mice to SARS-CoV-2. [20] Again, this could be explained by the fact that researchers stick to models that are familiar and available to them, but this would need further investigation. Hamsters were used for investigating reinfection and transmission research questions, most likely as they are spontaneous models for SARS-CoV-2 infections and easier and cheaper to use compared to non-human primates. Both non-human primates and hamster models were used to study the host response to infection. [21,22] Interestingly, a few studies did use organoids and co-cultures with immune cells to study innate immune reactions. [23, 24, 25] Adaptive immunity is harder to study ex vivo, however, tonsil organoids may prove useful as a model for human adaptive immune responses, and this is worth looking into further. [26, 27, 28]

One of the aims of this project was to study the contribution of organoid technology in antiviral therapy development. Antiviral therapy development is an important field where organoid technology could play a significant role in both increasing efficacy of testing as well as replacing animal models.
In this report we showed that organoid technology was applied for antiviral research. However, the overwhelming number of publications came from clinical studies. In addition, the amount of animal model studies was still higher than organoid model studies.

An additional result of this study is that finding relevant research has proven to be a huge challenge. Results of publications found not only depend on the databases and search terms used, but also heavily rely on the quality of the abstract of the publications, and on terminology used. There seems to be a lack of worldwide standardized requirements for relevant information that needs to be described in the abstract. Since title and abstract are the two main parts of a publication used for searching in the primary stages of literature research, this creates uncertainty about whether all relevant research is or can be found. The sheer numbers of COVID-19 research make it impossible to go through all the research without the help of IT-solutions. Machine learning algorithm results were useful to define primary, animal, or *in vitro* research categories, but due to lack of a specific machine learning result for publications with clinical or organoid models, these searches were done with regex search terms in R-studio. This was also used for animal models for RQ2 and 3. Searching the data in this manner has drawbacks, however it was the best way available to analyze this database, due to the fact the dataset used was comprised of search results of four different databases. This method differs from searching with MeSH terms in PubMed used by Kroon. [9] Finding the most relevant search terms to use is more difficult in regex as there are no predetermined categories, like MeSH terms in PubMed. This also means that search terms mentioned in the abstract maybe random, e.g., when searching on vaccine, the abstract may only mention that there is no vaccine yet, but the actual study may be unrelated to vaccine research. However, MeSH terms are only applicable in PubMed and have their own limitations; publications need to be assigned appropriate MeSH terms, which can take several months, and the MeSH database is updated yearly, but publications with MeSH terms already assigned are not checked if they have been affected by the database update.

The results of this study show the proportion of the different models used in primary SARS-CoV-2 research and the relatively modest contribution or organoid technology to the overall number of studies. Our study also clearly shows that organoid technology has a place in virology research. Multiple models are being used to study research questions that were previously only possible to study with *in vitro* studies or animal models. We suspect, based on the overlapping lung pathology research question categories, that more organoid technology could be implemented in this field. However, we do not have specific data to draw a final conclusion. It would be interesting to further investigate this to improve implementation of animal free techniques in virology research.

Importance

The results of our study show a small but substantial contribution of organoid technology in primary SARS-CoV-2 research. Therefore, there is still a large need to stimulate non-animal research. The current pandemic can still be an opportunity to improve the use of non-animal models. The need to go beyond the old familiar models and find ways to get results that are more translatable to humans is crucial. This still can, and needs to be emphasized and promoted, as we still have a lot to learn about SARS-CoV-2 and other viruses. Organizing how we collect data and designing solutions to find relevant data in an expedited and uncomplicated way, will not just be necessary in the current pandemic, it is also imperative for other and future research. To find missing and vital pieces in the pathogenesis and treatment of known viral disease, and equally important, to be prepared for new outbreaks. Helping researchers with this mind shift could be achieved by finding out which factors are significant in choosing a research model and addressing those factors.

Recommendations

Encouraging TPI in virology: creating a mind-shift

To improve implementation of non-animal solutions for biomedical research a mind shift is necessary. We need more knowledge about crucial factors involved in the researcher's decision what model to use for which research questions, so we can try to influence this. By researching the researchers, we can investigate these factors and identify more precisely what is required to improve and accelerate the transition to non-animal models. This could be done by interviewing researchers from different fields within virology.

Using existing data to help improve the implementation of animal-free innovations

For existing data to be of value, people need to be able to find and access it. To achieve this, we must improve finding and open sharing of information and knowledge, and promote standardization of publications, especially abstracts. We can do this by creating search filters for non-animal model publications to search the main existing databases (PubMed & Embase), like the Syrcle Animal research filters [19, 29] and developing Machine Learning/algorithms for finding and categorizing non-animal model research. Other options would be, investigating available online resources and combining those where possible to build a knowledge hub to facilitate sharing knowledge and collaboration on projects. And, to create a Systematic Online Living Evidence Summary (like Camarades DB) platform with non-animal model virus research publications. Using existing data is important to help convince researchers organoid technology can render valid results. For analysis of publications to compare outcomes of studies with organoid models versus animal models or clinical data, such as drug trials. And, to evaluate if and how research questions investigated with animal models, or parts thereof, can be replaced by organoid models. Such an analysis would be especially feasible for publications on antiviral treatment, with data on efficacy of antiviral compounds from human clinical trials used as comparison. That way, it can be determined whether data from organoids perform better than from animal models in predicting antiviral responses in patients. In addition, data on the efficacy of clinically approved drugs such as chloroquine for a new application could be done without animal studies, as such drugs can be tested in clinical trials without the need for safety studies. The question remains what knowledge is gained from animal studies with approved clinical drugs that cannot be obtained by clinical trials, or maybe with organoid technology. By increasing knowledge and availability of organoid technology and investigating what factors drive the choice for a specific research model, an effort could be made to replace or seriously reduce screening and testing of drugs on animals.

Development of complex human models

We need to identify necessary innovations in non-animal models to be able to study virus research questions that still require animal models, e.g., adaptive immune system. Today, organoid technology cannot replace animal studies on vaccine development as that would require a model where complex interactions take place of the immune system with a pathogen. However, parts of such a response could be simulated in human models that are currently becoming available. Organoids interacting with immune cells and pathogens have already been published.[30] In addition, complex model systems such as organ-on-chips, where different organs are connected by fluids simulating the blood flow, are already available.[31] Such devices could further be developed by incorporating endothelial cells and immune cells, thereby creating a more complex physiological environment to study human host-pathogen interactions. This requires intensive collaborations of researchers from both academia and industry with different backgrounds, such as on virology, immunology, vaccinology, tissue engineering, drug development, cell biology, system biology, modelling and physics. Encouraging researchers to collaborate on development of complex human models by funding of international consortia would be helpful in taking steps towards development of innovative human models for the future.

Recommended follow-up

1. Research the factors involved in choice of research model; what does the researcher need to choose organoid models

2. Compare organoid results with animal models & clinical data; which questions in virus research can be studied with organoids

3. Investigate innovations necessary; what is the next step in organoid technology for virus research

4. Facilitate findability and accessibility with IT; what can IT do for researchers

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Appendix

1) Regex search terms

Organoid model: "organoid|air-liquid|air liquid|human airway epitheli|\\bHAE\\b|\\bALI\\b|transwell|seeded|self organiz| self-organis|mesenchymal|embryonic |stem cell|pluripotent|progenitor|iPSC|human airway model| enteroid|colonosphere|colonoid |mini-gut|gastroid|pancreatoid|hepatoid|spheroid enterosphere| neurosphere"
(Excl.) "hemopoietic|haemopoietic|transplant|limb ischemia|hereditary|acute lung injury"
Human Airway Model: "human airway epitheli|\\bHAE\\b|\\bALI\\b|human airway model|lung organoid"

Animal models total:
"animal model|fish model|zebrafish|zebra fish|guppy|guppies|gallus|quail|poultry|chick|fowl|\\bbird\\b| chicken|\\bpig\\b|scrofa|swine|piglet|sheep|goat|camelid|llama|alpaca|\\bdog\\b|\\bdogs\\b|\\bcat\\b|\\bcats\\b|canis lupus| felis catus|\\brat\\b|\\brats\\b|ferret|\\bmink\\b|mustel|Neogale|weasel| \\bermine|guinea pig|cavia|marmoset|chinchilla|gerbil|rodent|squirrel|chipmunk|rabbit|\\bhare\\b| hamster|hamsters|Cricetinae|Mesocricetus auratus| golden syrian|transgenic|humanized|humanised|\\bmouse\\b|\\bmice\\b|murine|musculus|non-human primate|non human primate|nhp|macaque| macaca|fascicularis|mulatta| Cynomolgus|Chlorocebus| monkey|baboon"

Animal models NHP, hamster, mouse:
NHP: "non-human primate|non human primate|nhp|macaque|macaca|fascicularis|mulatta|Cynomolgus|Chlorocebus|monkey|baboon"
Hamster: "hamster|hamsters|Cricetinae|Mesocricetus auratus|golden syrian"
Mouse: "transgenic|humanized|humanised|\\bmouse\\b|\\bmice\\b|murine|musculus"

Clinical model: "case|patient|hospital|intensive care|clinical|general practitioner|general practice|family doctor|post mortem|autopsy"

Other models: "mathematic|computation|in silico|in-silico|simulate|simulation|(deep|machine) learning"; "survey|questionnaire|questionaire|cross-sectional|cross sectional|interview|mental health|depression|telemedicine|tele-medicine|telehealth|tele-health"; "epidemiol"

Pathology: "lung|airway|respiratory tract|pulmorary|alveol|acinus|bronch|pneumocyte|endothel"
Combined with "patholog|pathogenesis|pathogenicity|pathophysiologic|comorb|autopsy|post mortem"

Antivirals: "treatment|therap|inhibit|drug"

2) Research question 1, 2 & 3 article list (Organoid models)

Title	Authors (year)	Organoid model
"Lung Time No See": SARS-Cov-2 Spike Protein Changes Genetic Expression in Human Primary Bronchial Epithelial Cells After Recovery	Evans N, et al. (2021)	airway
3C-like protease inhibitors block coronavirus replication in vitro and improve survival in MERS-CoV-infected mice	Rathnayake A, et al. (2020)	airway
A comparative analysis of SARS-CoV-2 antivirals characterizes 3CLpro inhibitor PF-00835231 as a potential new treatment for COVID-19.	De Vries M, et al. (2021)	airway
A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures.	Dinnon KH, et al. (2021)	airway
A robust SARS-CoV-2 replication model in primary human epithelial cells at the air liquid interface to assess antiviral agents.	Nguyen Dan Do T, et al. (2021)	airway
A treatment that eliminates SARS-CoV-2 replication in human airway epithelial cells and is safe for inhalation as an aerosol in healthy human subjects.	Davis MD, et al. (2020)	airway
Actionable Cytopathogenic Host Responses of Human Alveolar Type 2 Cells to SARS-CoV-2.	Hekman RM, et al. (2020)	airway
Adult Stem Cell-derived Complete Lung Organoid Models Emulate Lung Disease in COVID-19.	Tindle C, et al. (2020)	airway
Alpha-1 antitrypsin inhibits TMPRSS2 protease activity and SARS-CoV-2 infection.	Wettstein L, et al. (2021)	airway
An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice.	Sheehan T, et al. (2020)	airway
An organoid-derived bronchioalveolar model for SARS-CoV-2 infection of human alveolar type II-like cells.	Lamers MM, et al. (2021)	airway
An organotypic airway culture model for studying SARSCoV- 2 infection.	Becker M, et al. (2020)	airway
Androgen Signaling Regulates SARS-CoV-2 Receptor Levels and Is Associated with Severe COVID-19 Symptoms in Men	Samuel RM, et al. (2020)	airway
Antihypertensive drug treatment and susceptibility to SARS-CoV-2 infection in human PSC-derived cardiomyocytes and primary endothelial cells	Iwanski J, et al. (2021)	endothelial, cardiomyocytes
Aprotinin Inhibits SARS-CoV-2 Replication.	Bojkova D, et al. (2021)	airway
AT-527, a double prodrug of a guanosine nucleotide analog, is a potent inhibitor of SARS-CoV-2 in vitro and a promising oral antiviral for treatment of COVID-19.	Good SS, et al. (2021)	airway
Berberine and Obatoclax Inhibit SARS-Cov-2 Replication in Primary Human Nasal Epithelial Cells In Vitro.	Varghese FS, et al. (2020)	airway
C5aR inhibition of non-immune cells suppresses inflammation and maintains epithelial integrity in SARS-CoV-2-infected primary human airway epithelia.	Posch W, et al. (2021)	airway
Carrageenan containing over-the-counter nasal and oral sprays inhibit SARS-CoV-2 infection of airway epithelial cultures.	Schutz D, et al. (2021)	airway
Characterization and Treatment of SARS-CoV-2 in Nasal and Bronchial Human Airway Epithelia	Pizzorno A, et al. (2020)	airway
Characterization of the SARS-CoV-2 Host Response in Primary Human Airway Epithelial Cells from Aged Individuals.	Bharathiraja S, et al. (2021)	airway
Cholesterol 25-Hydroxylase inhibits SARS-CoV-2 and other coronaviruses by depleting membrane cholesterol.	Wang S, et al. (2020)	airway
Cigarette smoke exposure increases ace-2 expression and sars-cov-2 infection severity in human and ferret airways and induces apoptotic cell injury in vitro.	Hussain SS, et al. (2020)	airway
Cigarette Smoke Specifically Affects Small Airway Epithelial Cell Populations and Triggers the Expansion of Inflammatory and Squamous Differentiation Associated Basal Cells.	Wohnhaas C, et al. (2020)	airway
Clinical analysis and pluripotent stem cells-based model reveal possible impacts of ACE2 and lung progenitor cells on infants vulnerable to COVID-19	Zhang Z, et al. (2021)	airway
Coldzyme maintains integrity in sars-cov-2-infected airway epithelia.	Posch W, et al. (2021)	airway
Common genetic variation in humans impacts in vitro susceptibility to SARS-CoV-2 infection.	Dobrindt K, et al. (2021)	airway
Comparison of anti-SARS-CoV-2 activity and intracellular metabolism of remdesivir and its parent nucleoside	Toa S, et al. (2021)	airway

Coupled CRC 2D and ALI 3D Cultures Express Receptors of Emerging Viruses and Are More Suitable for the Study of Viral Infections Compared to Conventional Cell Lines.	Xia S, et al. (2020)	airway
Cystic fibrosis airway cells are less susceptible to sars-cov-2 infection and have altered ph regulatory protein expression.	Saunders J, et al. (2021)	airway
Development of alveolar and airway cells from human iPS cells: toward SARS-CoV-2 research and drug toxicity testing.	Tsjuji K, et al. (2020)	airway
Diesel Particulate Matter 2.5 Induces Epithelial-to-Mesenchymal Transition and Upregulation of SARS-CoV-2 Receptor during Human Pluripotent Stem Cell-Derived Alveolar Organoid Development.	Jung-Hyun K, et al. (2020)	airway
Direct Exposure to SARS-CoV-2 and Cigarette Smoke Increases Infection Severity and Alters the Stem Cell-Derived Airway Repair Response.	Purkayastha A, et al. (2020)	airway
Discovery of SARS-CoV-2 antiviral drugs through large-scale compound repurposing.	Riva L, et al. (2020)	airway
Disparate temperature-dependent virus-host dynamics for SARS-CoV-2 and SARS-CoV in the human respiratory epithelium.	Vkovski P, et al. (2021)	airway
Drug inhibition of SARS-CoV-2 replication in human pluripotent stem cell-derived intestinal organoids	Kruger J, et al. (2020)	gut
DT388-c-peptide: Novel recombinant antiviral for prevention and early treatment of SARS-CoV-2 infection.	Predella C, et al. (2021)	airway
Dynamic innate immune response determines susceptibility to SARS-CoV-2 infection and early replication kinetics.	Cheemarla, NR, et al. (2020)	airway
Early nasal type I IFN immunity against SARS-CoV-2 is compromised in patients with autoantibodies against type I IFNs.	Lopez J, et al. (2021)	airway
Effect of therapeutics on the modulation of ACE2 expression in airway epithelium: Implications for covid-19.	Singhera GK, et al. (2021)	airway
Effects of Asthma and Human Rhinovirus A16 on the Expression of SARS-CoV-2 Entry Factors in Human Airway Epithelium.	Murphy RC, et al. (2020)	airway
Electronic Cigarette Aerosol Is Cytotoxic and Increases ACE2 Expression on Human Airway Epithelial Cells: Implications for SARS-CoV-2 (COVID-19)	McAlinden K, et al. (2021)	airway
Ethacridine inhibits SARS-CoV-2 by inactivating viral particles.	Xiaoquan L, et al. (2020)	airway
Experimental and natural evidence of SARS-CoV-2 infection-induced activation of type I interferon responses.	Banerjee A, et al. (2021)	airway
Expression of the SARS-CoV-2 ACE2 Receptor in the Human Airway Epithelium.	Rostami ZH, et al. (2020)	airway
Fighting the storm: could novel anti-TNFα and anti-IL-6 C. sativa cultivars tame cytokine storm in COVID-19?	Kovalchuk A, et al. (2021)	skin
Gene expression and in situ protein profiling of candidate SARS-CoV-2 receptors in human airway epithelial cells and lung tissue.	Aguiar JA, et al. (2021)	airway
High-content screening of Thai medicinal plants reveals Boesenbergia rotunda extract and its component Panduratin A as anti-SARS-CoV-2 agents.	Kanjanasirirat P, et al. (2020)	airway
High-throughput human primary cell-based airway model for evaluating influenza, coronavirus, or other respiratory viruses in vitro.	Gard AL, et al. (2021)	airway
Host and viral determinants for efficient SARS-CoV-2 infection of the human lung.	Chu H, et al. (2021)	airway
Host metabolism dysregulation and cell tropism identification in human airway and alveolar organoids upon SARS-CoV-2 infection.	Rongjuan P, et al. (2020)	airway
Host-Pathogen Responses to Pandemic Influenza H1N1pdm09 in a Human Respiratory Airway Model	Pharo EA, et al. (2020)	airway
HTCC as a polymeric inhibitor of SARS-CoV-2 and MERS-CoV.	Milewska A, et al. (2021)	airway
Human coronaviruses 229E and OC43 replicate and induce distinct anti-viral responses in differentiated primary human bronchial epithelial cells.	Loo SL, et al. (2020)	airway
Human embryonic stem cell-derived cardiomyocyte platform screens inhibitors of SARS-CoV-2 infection.	Williams TL, et al. (2021)	cardiomyocytes
Human Induced Pluripotent Stem Cell-Derived Lung Epithelial System for SARS-CoV-2 Infection Modeling and Its Potential in Drug Repurposing.	Surendran H, et al. (2020)	airway
Human Lung Stem Cell-Based Alveolospheres Provide Insights into SARS-CoV-2-Mediated Interferon Responses and Pneumocyte Dysfunction.	Katsura H, et al. (2020)	airway
Humanized COVID-19 decoy antibody effectively blocks viral entry and prevents SARS-CoV-2 infection.	Huang KY, et al. (2020)	airway

Hydroxychloroquine use against SARS-CoV-2 infection in non-human primates	Maisonnasse P, et al. (2020)	airway
Identification of Cross-Reactive CD8+ T Cell Receptors with High Functional Avidity to a SARS-CoV-2 Immunodominant Epitope and Its Natural Mutant Variants.	Chao H, et al. (2020)	airway
Identification of SARS-CoV-2 inhibitors using lung and colonic organoids.	Han Y, et al. (2021)	airway
In search of preventive strategies: novel high-CBD Cannabis sativa extracts modulate ACE2 expression in COVID-19 gateway tissues.	Wang B, et al. (2020)	airway, gut, oral epithelium
In vitro Characterisation of SARS-CoV-2 and Susceptibility of Domestic Ferrets (Mustela putorius furo).	Marsh GA, et al. (2021)	airway
In vivo antiviral host transcriptional response to SARS-CoV-2 by viral load, sex, and age.	Lieberman NAP, et al. (2020)	airway
In well-differentiated primary human bronchial epithelial cells, TGF-?1 and TGF-?2 induce expression of furin.	O'Sullivan MJ, et al. (2021)	airway
Inactivation of Material from SARS-CoV-2-Infected Primary Airway Epithelial Cell Cultures.	Barrow KA, et al. (2021)	airway
Infection of human Nasal Epithelial Cells with SARS-CoV-2 and a 382-nt deletion isolate lacking ORF8 reveals similar viral kinetics and host transcriptional profiles.	Gamage AM, et al. (2020)	airway
Influenza virus infection increases ACE2 expression and shedding in human small airway epithelial cells.	Schweitzer KS, et al. (2020)	airway
Inhaled corticosteroids downregulate the SARS-CoV-2 receptor ACE2 in COPD through suppression of type I interferon.	Finney LJ, et al. (2020)	airway
Inhibition of Coronavirus Entry In Vitro and Ex Vivo by a Lipid-Conjugated Peptide Derived from the SARS-CoV-2 Spike Glycoprotein HRC Domain.	Outlaw VK, et al. (2020)	airway
Inhibition of coronavirus infection by a synthetic STING agonist in primary human airway system.	Zhu Q, et al. (2021)	airway
Inter-subject variation in ACE2 protein expression in human airway epithelia and its relationship to SARS-CoV-2 infection.	Li K, et al. (2021)	airway
Isolation of human coronaviruses OC43, HKU1, NL63, and 229E in Yamagata, Japan, using primary human airway epithelium cells cultured by employing an air-liquid interface culture.	Komabayashi K, et al. (2021)	airway
JAK inhibitors dampen activation of interferon-stimulated transcription of ACE2 isoforms in human airway epithelial cells.	Lee HK, et al. (2021)	airway
Long-Term Modeling of SARS-CoV-2 Infection of In Vitro Cultured Polarized Human Airway Epithelium.	Hao S, et al. (2020)	airway
Lung cancer models reveal SARS-CoV-2-induced EMT contributes to COVID-19 pathophysiology.	Stewart CA, et al. (2020)	airway
Mechanism of baricitinib supports artificialintelligence-predicted testing in COVID-19 patients	Stebbing J, et al. (2020)	liver
Morphogenesis and cytopathic effect of SARS-CoV-2 infection in human airway epithelial cells	Zhu N, et al. (2020)	airway
Morphological Cell Profiling of SARS-CoV-2 Infection Identifies Drug Repurposing Candidates for COVID-19.	Mirabelli C, et al. (2020)	airway
Neutrophil Extracellular Traps Induce the Epithelial-Mesenchymal Transition: Implications in Post-COVID-19 Fibrosis.	Pandolfi L, et al. (2020)	airway
Neutrophils significantly enhance pro-inflammatory cytokine release from airway epithelial cells in response to sars-COV-2 infection.	Calvert BA, et al. (2020)	airway
Off-target In Vitro Profiling Demonstrates that Remdesivir Is a Highly Selective Antiviral Agent.	Xu Y, et al. (2021)	airway
Polyphenylene carboxymethylene (PPCM) microbicide repurposed as antiviral against SARS-CoV-2. Proof of concept in primary human undifferentiated epithelial cells.	Escaffre O, et al. (2021)	airway
Preclinical evaluation of Imatinib does not support its use as an antiviral drug against SARS-CoV-2.	Touret F, et al. (2021)	airway
Progenitor identification and SARS-CoV-2 infection in long-term human distal lung organoid cultures.	Salahudeen AA, et al. (2020)	airway
Protease Inhibitors: Candidate Drugs to Inhibit Severe Acute Respiratory Syndrome Coronavirus 2 Replication	Yamaya M, et al. (2020)	airway
Remdesivir Inhibits SARS-CoV-2 in Human Lung Cells and Chimeric SARS-CoV Expressing the SARS-CoV-2 RNA Polymerase in Mice	Pruijssers AJ, et al. (2020)	airway
Replication of SARS-CoV-2 in human respiratory epithelium.	Milewska A, et al. (2020)	airway

Replication of Severe Acute Respiratory Syndrome Coronavirus 2 in Human Respiratory Epithelium.	Milewska A, et al. (2020)	airway
Resveratrol and Pterostilbene Inhibit SARS-CoV-2 Replication in Air-Liquid Interface Cultured Human Primary Bronchial Epithelial Cells.	Ter Ellen BM, et al. (2021)	airway
Rethinking Remdesivir: Synthesis, Antiviral Activity and Pharmacokinetics of Oral Lipid Prodrugs.	Schooley RT, et al. (2020)	airway
Revealing Tissue-Specific SARS-CoV-2 Infection and Host Responses using Human Stem Cell-Derived Lung and Cerebral Organoids	Tiwari SK, et al. (2021)	airway
Role of Cigarette Smoke on Angiotensin-Converting Enzyme-2 Protein Membrane Expression in Bronchial Epithelial Cells Using an Air-Liquid Interface Model.	Caruso M, et al. (2021)	airway
SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor	Hoffmann M, et al. (2020)	airway
SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo.	Hou YJ, et al. (2020)	airway
SARS-CoV-2 entry into human airway organoids is serine protease-mediated and facilitated by the multibasic cleavage site.	Mykytyn AZ, et al. (2021)	airway
SARS-CoV-2 induces double-stranded RNA-mediated innate immune responses in respiratory epithelial derived cells and cardiomyocytes.	Li Y, et al. (2020)	airway
SARS-CoV-2 Infection Depends on Cellular Heparan Sulfate and ACE2.	Clausen TM, et al. (2020)	airway
SARS-CoV-2 Infection of Pluripotent Stem Cell-derived Human Lung Alveolar Type 2 Cells Elicits a Rapid Epithelial-Intrinsic Inflammatory Response.	Huang J, et al. (2020)	airway
SARS-CoV-2 infection of primary human lung epithelium for COVID-19 modeling and drug discovery.	Mulay A, et al. (2021)	airway
SARS-CoV-2 infection rewires host cell metabolism and is potentially susceptible to mTORC1 inhibition.	Mullen PJ, et al. (2021)	airway
SARS-CoV-2 productively infects human gut enterocytes.	Lamers MM, et al. (2020)	airway
SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues.	Ziegler CGK, et al. (2021)	airway
SARS-CoV-2 triggers an MDA-5-dependent interferon response which is unable to control replication in lung epithelial cells.	Rebendenne A, et al. (2021)	airway
SARS-CoV-2-mediated dysregulation of metabolism and autophagy uncovers host-targeting antivirals.	Gassen NC, et al. (2020)	airway, gut
Single cell resolution of SARS-CoV-2 tropism, antiviral responses, and susceptibility to therapies in primary human airway epithelium.	Fiege JK, et al. (2021)	airway
Single-cell longitudinal analysis of SARS-CoV-2 infection in human airway epithelium identifies target cells, alterations in gene expression, and cell state changes.	Ravindra NG, et al. (2021)	airway
Structure-based phylogeny identifies avoralstat as a TMPRSS2 inhibitor that prevents SARS-CoV-2 infection in mice.	Sun YJ, et al. (2021)	airway
Suramin inhibits SARS-CoV-2 infection in cell culture by interfering with early steps of the replication cycle	Da Silva CS, et al. (2020)	airway
The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets.	Peacock TP, et al. (2021)	airway
The New Generation hDHODH Inhibitor MEDS433 Hinders the In Vitro Replication of SARS-CoV-2 and Other Human Coronaviruses.	Calistri A, et al. (2021)	kidney
The potential involvement of JAK-STAT signaling pathway in the COVID-19 infection assisted by ACE2.	Luo J, et al. (2020)	airway
The rocaglate CR-31-B (-) inhibits SARS-CoV-2 replication at non-cytotoxic, low nanomolar concentrations in vitro and ex vivo.	Muller C, et al. (2020)	airway
The SARS-CoV-2 Cytopathic Effect Is Blocked by Lysosome Alkalizing Small Molecules.	Gorshkov K, et al. (2020)	airway
The SARS-CoV-2 Transcriptome and the Dynamics of the S Gene Furin Cleavage Site in Primary Human Airway Epithelia.	Zou W, et al. (2021)	airway
Three-Dimensional Human Alveolar Stem Cell Culture Models Reveal Infection Response to SARS-CoV-2.	Youk J, et al. (2020)	airway
TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human airway cells	Bestle D, et al. (2020)	airway
Transcriptional Profiling of Immune and Inflammatory Responses in the Context of SARS-CoV-2 Fungal Superinfection in a Human Airway Epithelial Model.	Nicolas de Lamballerie C, et al. (2020)	airway

Transmission, infectivity, and neutralization of a spike L452R SARS-CoV-2 variant.	Deng X, et al. (2020)	airway
Type 2 inflammation modulates ACE2 and TMPRSS2 in airway epithelial cells.	Kimura H, et al. (2020)	airway
Type 2 inflammation reduces ACE2 protein in bronchial and nasal epithelial cells	Kimura H, et al. (2021)	airway
Type I and Type III IFN Restrict SARS-CoV-2 Infection of Human Airway Epithelial Cultures	Vanderheiden A, et al. (2020)	airway
Type I interferon susceptibility distinguishes SARS-CoV-2 from SARS-CoV.	Lokugamage KG, et al. (2020)	airway
Virucidal and antiviral activity of astodrimer sodium against SARS-CoV-2 in vitro.	Paull JRA, et al. (2021)	airway
Visualization of SARS-CoV-2 using Immuno RNA-Fluorescence in Situ Hybridization.	Kula-Pacurar A, et al. (2020)	airway

3) Research question 2b article list (Animal models)

Title	Authors	Year	Animal model
A Human-Immune-System mouse model for COVID-19 research (DRAGA mouse: HLA-A2.HLA-DR4.Rag1KO.IL-2Rc KO.NOD)	Brumeanu T, et al.	2020	mouse
A Mouse Model of SARS-CoV-2 Infection and Pathogenesis	Sun S, et al.	2020	mouse
A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures	Dinnon K, et al.	2020	mouse
A SARS-CoV-2 Infection Model in Mice Demonstrates Protection by Neutralizing Antibodies	Hassan A, et al.	2020	mouse
Age-Dependent Progression of SARS-CoV-2 Infection in Syrian Hamsters	Osterrieder N, et al.	2020	hamster
Age-determined expression of priming protease TMPRSS2 and localization of SARS-CoV-2 in lung epithelium.	Schuler B, et al.	2020	mouse
Cell-Type Apoptosis in Lung during SARS-CoV-2 Infection.	Liu Y, et al.	2021	NHP
Cellular events of acute, resolving or progressive COVID-19 in SARS-CoV-2 infected non-human primates.	Fahlberg M, et al.	2020	NHP
Cellular tropism of SARS-CoV-2 in the respiratory tract of Syrian hamsters and B6.Cg-Tg(K18-ACE2)2Prlmn/J transgenic mice.	Yen H, et al.	2021	hamster, mouse
Characterization of an attenuated SARS-CoV-2 variant with a deletion at the S1/S2 junction of the spike protein.	Wang P, et al.	2021	hamster, mouse
Characterization of Virus Replication, Pathogenesis, and Cytokine Responses in Syrian Hamsters Inoculated with SARS-CoV-2.	Yang S, et al.	2021	hamster
Co-infection by severe acute respiratory syndrome coronavirus 2 and influenza A(H1N1) pdm09 virus enhances the severity of pneumonia in golden Syrian hamsters.	Zhang A, et al.	2020	hamster, mouse
Comparative analysis of ACE2 protein expression in rodent, non-human primate, and human respiratory tract at baseline and after injury: A conundrum for COVID-19 pathogenesis.	Soni S, et al.	2021	NHP, mouse
Comparison of rhesus and cynomolgus macaques as an infection model for COVID-19.	Salguero F, et al.	2021	NHP
Comparison of SARS-CoV-2 Variants of Concern 202012/01 (U.K. Variant) and D614G Variant Transmission by Different Routes in Syrian Hamsters.	Mohandas S, et al.	2021	hamster
Contribution of SARS-CoV-2 Accessory Proteins to Viral Pathogenicity in K18 Human ACE2 Transgenic Mice.	Silvas J, et al.	2021	mouse
Disruption of Adaptive Immunity Enhances Disease in SARS-CoV-2 Infected Syrian Hamsters.	Brocato R, et al.	2020	hamster
Effects of cigarette smoking on SARS-CoV-2 receptor ACE2 expression in the respiratory epithelium?	Heijnk I, et al.	2020	mouse
Endothelial cell damage is the central part of COVID-19 and a mouse model induced by injection of the S1 subunit of the spike protein.	Nuovo G, et al.	2020	mouse
Endothelial cell infection and dysfunction, immune activation in severe COVID-19.	Qin Z, et al.	2021	NHP, mouse

Establishment of an African green monkey model for COVID-19 and protection against re-infection.	Woolsey C, et al.	2020	NHP
Expressions and significances of the angiotensin-converting enzyme 2 gene the receptor of SARS-CoV-2 for COVID-19	Zhou F, et al.	2020	mouse
Gender associates with both susceptibility to infection and pathogenesis of SARS-CoV-2 in Syrian hamster.	Yuan L, et al.	2021	hamster
Generation of a Broadly Useful Model for COVID-19 Pathogenesis Vaccination and Treatment	Zhuang S, et al.	2020	mouse
H1N1 exposure during the convalescent stage of SARS-CoV-2 infection results in enhanced lung pathologic damage in hACE2 transgenic mice.	Li H, et al.	2021	mouse
Hamster and ferret experimental infection with intranasal low dose of a single strain of SARS-CoV-2.	MonchatreLeroy E, et al.	2021	hamster
High levels of soluble CD25 in COVID-19 severity suggest a divergence between anti-viral and pro-inflammatory T-cell responses.	Xie M, et al.	2021	mouse
High-Fat High-Sucrose Diet Increases ACE2 Receptor Expression in Lung and Pancreatic Islets in SIV-Infected Rhesus Macaques: Implications for Increased Risk for SARS-CoV-2 Infection	Levitt D, et al.	2021	NHP
Highly susceptible SARS-CoV-2 model in CAG promoter-driven hACE2 transgenic mice.	Asaka M, et al.	2021	mouse
Human angiotensin-converting enzyme 2 transgenic mice infected with SARS-CoV-2 develop severe and fatal respiratory disease	Golden J, et al.	2020	mouse
Hypoxia induces expression of angiotensin-converting enzyme II in alveolar epithelial cells: Implications for the pathogenesis of acute lung injury in COVID-19.	Sturrock A, et al.	2021	mouse
IFN signaling and neutrophil degranulation transcriptional signatures are induced during SARS-CoV-2 infection.	Rosa B, et al.	2021	NHP
Induction of Pro-Inflammatory Cytokines TNF, IL-6, and HMGB1 by SARS-CoV-2 Spike Proteins in Mice and in Murine Macrophage Cultures	Sam G, et al.	2021	mouse
Infection with novel coronavirus (SARS-CoV-2) causes pneumonia in Rhesus macaques	Shan C, et al.	2020	NHP
Infectious Clones Produce SARS-CoV-2 That Causes Severe Pulmonary Disease in Infected K18-Human ACE2 Mice.	Liu X, et al.	2021	mouse
Intranasal exposure of African green monkeys to SARS-CoV-2 results in acute phase pneumonia with shedding and lung injury still present in the early convalescence phase	Cross R, et al.	2020	NHP
Is Cross-Reactive Immunity Triggering COVID-19 Immunopathogenesis?	Beretta A, et al.	2020	NHP
K18-hACE2 mice develop respiratory disease resembling severe COVID-19.	Yinda C, et al.	2021	mouse
Lung expression of human angiotensin-converting enzyme 2 sensitizes the mouse to SARS-CoV-2 infection.	Han K, et al.	2021	mouse
Mice with induced pulmonary morbidities display severe lung inflammation and mortality following exposure to SARS-CoV-2.	Ralach R, et al.	2021	mouse
Multi-organ histopathological changes in a mouse hepatitis virus model of COVID-19.	Paidas M, et al.	2021	mouse
Myeloid cell interferon responses correlate with clearance of SARS-CoV-2.	Singh D, et al.	2021	NHP
Neuroinvasion and Encephalitis Following Intranasal Inoculation of SARS-CoV-2 in K18-hACE2 Mice.	Kumari P, et al.	2021	mouse
Oral SARS-CoV-2 inoculation establishes subclinical respiratory infection with virus shedding in golden Syrian hamsters.	Lee A, et al.	2020	hamster
Overexpression of the SARS-CoV-2 receptor ACE2 is induced by cigarette smoke in bronchial and alveolar epithelia.	Liu A, et al.	2020	mouse
Pathogenesis and transmission of SARS-CoV-2 in golden hamsters.	Sia S, et al.	2020	hamster
Pathogenesis of SARS-CoV-2 in Transgenic Mice Expressing Human Angiotensin-Converting Enzyme 2	Jiang R, et al.	2020	mouse
Predictive Value of Precision-Cut Lung Slices for the Susceptibility of Three Animal Species for SARS-CoV-2 and Validation in a Refined Hamster Model.	Gerhards N, et al.	2021	hamster
Prior aerosol infection with lineage A SARS-CoV-2 variant protects hamsters from disease, but not reinfection with B.1.351 SARS-CoV-2 variant.	Yinda C, et al.	2021	hamster
Pro-inflammatory microenvironment and systemic accumulation of CXCR3+ cell exacerbate lung pathology of old rhesus macaques infected with SARS-CoV-2.	Zheng H, et al.	2021	NHP

Protection against reinfection with D614- or G614-SARS-CoV-2 isolates in golden Syrian hamster.	Brustolin M, et al.	2021	hamster
Pulmonary fibroproliferative response in old mice that survive acute lung injury by mouse-adapted sars-cov-2.	Montgomery S, et al.	2021	mouse
Q493K and Q498H substitutions in Spike promote adaptation of SARS-CoV-2 in mice.	Huang K, et al.	2021	mouse
Quantitative proteomics of hamster lung tissues infected with SARS-CoV-2 reveal host factors having implication in the disease pathogenesis and severity.	Suresh V, et al.	2021	hamster
Rescue of SARS-CoV-2 from a Single Bacterial Artificial Chromosome	Ye C, et al.	2020	hamster
Respiratory disease in rhesus macaques inoculated with SARS-CoV-2	Munster V, et al.	2020	NHP
Responses to acute infection with SARS-CoV-2 in the lungs of rhesus macaques, baboons and marmosets.	Singh D, et al.	2021	NHP
Role of the neutrophil chemoattractant CXCL5 in the SARS-CoV-2 infection-induced lung inflammatory innate immune response determined using an in vivo hACE2 transfection mouse model.	Liang Y, et al.	2020	mouse
SARS-CoV-2 B.1.1.7 Infection of Syrian Hamster Does Not Cause More Severe Disease, and Naturally Acquired Immunity Confers Protection	Nuñez I, et al.	2021	hamster
SARS-CoV-2 Bearing a Mutation at the S1/S2 Cleavage Site Exhibits Attenuated Virulence and Confers Protective Immunity.	Sasaki M, et al.	2021	hamster
SARS-CoV-2 Causes a Systemically Multiple Organs Damages and Dissemination in Hamsters.	Song Z, et al.	2020	hamster
SARS-CoV-2 envelope protein causes acute respiratory distress syndrome (ARDS)-like pathological damages and constitutes an antiviral target.	Xia B, et al.	2021	mouse
SARS-CoV-2 infection aggravates chronic comorbidities of cardiovascular diseases and diabetes in mice.	Ma Y, et al.	2021	mouse
SARS-CoV-2 infection dynamics in lungs of African green monkeys	Speranza E, et al.	2020	NHP
SARS-CoV-2 infection protects against rechallenge in rhesus macaques.	Chandrashekar A, et al.	2020	NHP
SARS-CoV-2 infection, neuropathogenesis and transmission among deer mice: Implications for spillback to New World rodents.	Fagre A, et al.	2021	mouse
SARS-CoV-2 Infects Endothelial Cells In Vivo and In Vitro.	Liu F, et al.	2021	NHP, mouse
SARS-CoV-2 rapidly adapts in aged BALB/c mice and induces typical pneumonia.	Zhang Y, et al.	2021	mouse
SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues	Ziegler C, et al.	2020	NHP
SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues	Ziegler C, et al.	2020	mouse
Sex Differences in Lung Imaging and SARS-CoV-2 Antibody Responses in a COVID-19 Golden Syrian Hamster Model.	Dhakal S, et al.	2021	hamster
Single intratracheal exposure to SARS-CoV-2 S1 spike protein induces acute lung injury in K18-hACE2 transgenic mice	Pavel S, et al.	2021	mouse
Single-cell RNA analysis on ACE2 expression provides insights into SARS-CoV-2 potential entry into?the bloodstream and heart injury	Wei G, et al.	2020	mouse
Single-cell RNA sequencing reveals SARS-CoV-2 infection dynamics in lungs of African green monkeys.	Speranza E, et al.	2021	NHP
Single-cell transcriptomic atlas of primate cardiopulmonary aging.	Ma S, et al.	2020	NHP
Spike mutation D614G alters SARS-CoV-2 fitness and neutralization susceptibility	Shi P, et al.	2020	hamster
Spike protein of SARS-CoV-2 activates macrophages and contributes to induction of acute lung inflammation in male mice.	Cao X, et al.	2021	mouse
Spike Protein of SARS-CoV-2 Activates Macrophages and Contributes to Induction of Acute Lung Inflammations in Mice	Cao X, et al.	2021	mouse
STAT2 signaling restricts viral dissemination but drives severe pneumonia in SARS-CoV-2 infected hamsters.	Boudewijns R, et al.	2020	hamster, mouse
Surgical mask partition reduces the risk of non-contact transmission in a golden Syrian hamster model for Coronavirus Disease 2019 (COVID-19)	Chan J, et al.	2020	hamster

Synergism of TNF-? and IFN-? Triggers Inflammatory Cell Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes.	Karki R, et al.	2020	mouse
Syrian hamsters as a small animal model for SARS-CoV-2 infection and countermeasure development	Iwatsuki I, et al.	2020	hamster
The expression of SARS-CoV-2 receptor ACE2 and CD147, and protease TMPRSS2 in human and mouse brain cells and mouse brain tissues.	Qiao J, et al.	2020	mouse
The G614 pandemic SARS-CoV-2 variant is not more pathogenic than the original D614 form in adult Syrian hamsters.	Stauft C, et al.	2021	hamster
The gastrointestinal tract is an alternative route for SARS-CoV-2 infection in a nonhuman primate model.	Jiao L, et al.	2020	NHP
The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice.	Deng B, et al.	2020	mouse
The Post-Acute Phase of SARS-CoV-2 Infection in Two Macaque Species Is Associated with Signs of Ongoing Virus Replication and Pathology in Pulmonary and Extrapulmonary Tissues.	Böszörményi K, et al.	2021	NHP
Tissue Distribution of ACE2 Protein in Syrian Golden Hamster (Mesocricetus auratus) and Its Possible Implications in SARS-CoV-2 Related Studies.	Suresh V, et al.	2020	hamster
Vascular Disease and Thrombosis in SARS-CoV-2-Infected Rhesus Macaques	Aid M, et al.	2020	NHP
Vasculitis and Neutrophil Extracellular Traps in Lungs of Golden Syrian Hamsters With SARS-CoV-2.	Becker K, et al.	2021	hamster
Virulence and pathogenesis of SARS-CoV-2 infection in rhesus macaques: A nonhuman primate model of COVID-19 progression.	Zheng H, et al.	2020	NHP

4) Research question 3 overview Organoid types and effects for drugs screened

Drug	Action	Organoid model used
3C-like protease inhibitors	Protease inhibitor	airway
ACE2 antibody	ACE2 antibody	cardiomyocytes
ACE2-Fc fusion protein	ACE2 antibody	airway
Alpha-1 antitrypsin	Protease inhibitor	airway
Amiodarone (hydrochloride)	K & Ca Channel inhibitor	airway
Apilimod	Immunomodulatory	airway
Aprotinin	Protease inhibitor	airway
Astodrimer sodium	Virucidal	airway
AT-511 (free base form of AT-527)	Guanine analog	airway
AT-527	Guanine analog	airway
Avoralstat	Protease inhibitor	airway
Baricitinib	Kinase inhibitor	liver
Benztropine	Dopamine inhibitor	cardiomyocytes
Berberine	Phytoalexin	airway
Boesenbergia rotunda extract	Anti-inflammatory	airway
Bosutinib	Kinase inhibitor	airway
Bovine lactoferrin	Dopamine inhibitor	airway
Camostat	Protease inhibitor	cardiomyocytes

Cannabis sativa extracts	Cannabinoid	airway, gut, oral epithelium
Cannabis sativa extracts	Cannabinoid	skin
Carrageenan	Cell entry blocker	airway
Chloroquine	Immunomodulatory	airway
Cholesterol-25-hydroxylase (CH25H)	Cell entry blocker	airway
Clofazimine	Bacterial inhibitor	airway
Clomipramine	Immunomodulatory	airway
Coldzyme	Blocks viral binding	airway
Corticosteroids	Immunomodulatory	airway
CR-31-B	RNA helicase inhibitor	airway
dimeric amidobenzimidazole (diABZI)	STING receptor agonist	airway
Domperidone	Dopamine inhibitor	airway
DX600	ACE2 blocker	cardiomyocytes
E64d	Protease inhibitor	cardiomyocytes
EIDD-1931 (nucleoside molnupiravir)	viral RNA polymerase inhibitor	airway
Entecavir (hydrate)	Transcription inhibitor	airway
Ethacridine	Antiseptic	airway
Famotidine	Histamine blocker	gut
Fedratinib	Kinase inhibitor	airway
Gilteritinib	Kinase inhibitor	airway
HTCC	Cell entry blocker	airway
Hycanthone	Immunomodulatory	airway
Hydroxchloroquine	Immunomodulatory	airway
Hydroxychloroquine	Immunomodulatory	airway
Hydroxychloroquine	Immunomodulatory	airway
IFN (β1 and λ1)	Immunomodulatory	airway
IFNB1	Immunomodulatory	airway
Imatinib	Kinase inhibitor	airway
Imatinib	Kinase inhibitor	airway
Interferon-λ1a (IFN-λ1a)	Immunomodulatory	airway
Ipratropium bromide	Muscarinic receptor agonist	airway
Lipid-Conjugated Peptide	Cell entry blocker	airway
Lisinopril	ACE2 blocker	endothelial, cardiomyocytes
Lomitapide	Kinase inhibitor	airway

Losartan	Angiotensin receptor blocker	endothelial, cardiomyocytes
Lufotrelvir	Protease inhibitor	airway
MDL 28170	Cathepsin inhibitor	airway
MEDS433	hDHODH inhibitor	kidney
Metoclopramide	Dopamine inhibitor	airway
MK-2206	Kinase inhibitor	airway, gut
Molnupravir (NHC/EIDD-2801)	viral RNA polymerase inhibitor	airway
Mycophenolic acid (MPA)	IMPDH inhibitor	airway
Niclosamide	Antiparasitic	airway, gut
Obatoclax	Bcl2 inhibitor	airway
ONO 5334	Cathepsin inhibitor	airway
Optate	Blocks viral replication	airway
Polyphenylene carboxymethylene	Virucidal	airway
Pterostilbene	Phytoalexin	airway
Quinacrine dihydrochloride	Immunomodulatory	airway
Rapamycin	mTor inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	gut
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir	viral RNA polymerase inhibitor	airway
Remdesivir (nucleosides)	viral RNA polymerase inhibitor	airway
Resveratrol	Phytoalexin	airway
ROC-325	Immunomodulatory	airway
S1RA	Sigma-1 receptor antagonist	airway
SERPINA1/alpha-1 antitrypsin	Protease inhibitor	airway
Suramin	Antiparasitic	airway
Thioguanine	Guanine analog	airway
Verteporfin	Immunomodulatory	airway
Z-FA-FMK	Protease inhibitor	airway