Janssen Vaccines & Prevention B.V. *

Nonclinical Overview

MODULE 2.4

VAC31518 (JNJ-78436735)

Rolling Submission Package 1

* Janssen Vaccines & Prevention B.V. is a Janssen pharmaceutical company of Johnson & Johnson and is hereafter referred to as the sponsor.

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LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

Ad26	adenovirus type 26
Ad26.ENVA.01	Ad26 vector encoding the Clade A envelope protein of HIV type 1
Ad26.RSV.preF	Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain
BAL	bronchoalveolar lavage
BALT	bronchial-associated lymphoid tissue
BIDMC	Beth Israel Deaconess Medical Center
CAR	coxsackievirus adenovirus receptor
CBER	Center for Biologics Evaluation and Research
CHMP	Committee for Medicinal Products for Human Use
COVID-19	coronavirus disease 2019
DNA	deoxyribonucleic acid
E1, E3	early gene regions (of Ad26)
EF-PPND	embryofetal and pre- and postnatal development
EMA	European Medicines Agency
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
HIV	human immunodeficiency virus
ICMRA	International Coalition of Medicines Regulatory Authorities
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IM	intramuscular
LLOD	lower limit of detection
LRT	lower respiratory tract
MAA	Marketing Authorisation Application
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	messenger ribonucleic acid
NHP	nonhuman primate(s)
NZW	New Zealand white
OECD	Organization for Economic Co-operation and Development
PBMCs	peripheral blood mononuclear cells
pre-F	pre-fusion conformation-stabilized F protein
ppVNA (pVNA)	pseudotyped virus neutralization assay
q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-qPCR	reverse transcription followed by quantitative polymerase chain reaction
S	Spike
SARS-CoV-1	severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
sgRNA	subgenomic ribonucleic acid
$TCID_{50}$	50% tissue culture infective dose
TetO	tetracycline operon

URT	upper respiratory tract
VAERD	vaccine-associated enhanced respiratory disease
vp	virus particles
(wt)VNA	(wild type) virus neutralization assay
VRBPAC	Vaccines and Related Biological Products Advisory Committee
WHO	World Health Organization

1. OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

1.1. Introduction and Description of Submission Packages

Ad26.COV2.S[1] (also known as VAC31518 or JNJ-78436735) is a monovalent, recombinant, replication-incompetent adenovirus type 26 (Ad26) vectored vaccine encoding a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike (S) protein. It is being developed for prophylactic immunization against coronavirus disease 2019 (COVID-19), which has spread rapidly and globally since its emergence.

This nonclinical overview covers a description of the nonclinical pharmacology testing strategy and a summary of pharmacology studies for which study reports are submitted with rolling submission package 1, together with a complete pharmacokinetic (biodistribution) evaluation of the Ad26 vaccine platform.

In addition, to give a summary of the current status of the nonclinical testing program, this document provides a high level overview of the pharmacology and toxicology studies that are currently ongoing with Ad26.COV2.S (data on file). A detailed assessment of the Ad26.COV2.S specific pharmacology studies that are currently ongoing will be presented in an updated document which will be submitted as part of rolling submission package 2. Furthermore, an assessment of the ongoing Ad26.COV2.S specific toxicology studies, as well as a high-level summary of supportive toxicology data from other vaccines that are based on the Ad26 platform, will also be presented in the updated document as part of rolling submission package 2.

An overview of the nonclinical packages, including status of the reports and timing of submission packages, can be found in Table 1 (pharmacology), Table 2 (pharmacokinetics), and Table 3 (toxicology). An overview of nonclinical studies not included in the final submission dossier, with justification for their absence, will be included as an appendix table at time of finalization of the complete nonclinical package (i.e., Package 3 of rolling submission).

1.2. Pharmacology Testing Strategy

1.2.1. Study Overview and Characteristics

Primary pharmacology studies are listed in Table 1; detailed descriptions are provided in the Pharmacology Written Summary in Mod 2.6.2. To provide an overview of the current status of the nonclinical pharmacology program, interim data from studies where study reports will be provided as part of rolling submission package 2 are presented in Mod2.6.2.

[1] In nonclinical studies performed prior to selection of the Ad26.COV2.S vaccine candidate, Ad26.COV2.S was named Ad26NCOV030 or Ad26COVS1.

Table 1: Overview of Primary Pharmacology Studies

Test Species	Study Identifiers	Study Description	Regimen Dose Level	Study Report
Study Reports Package 1 (current submission)				
mouse	9346-20004 (TV-TEC-171843)	Immunogenicity study to support vaccine candidate selection.	1-dose 10^8, 10^9, 10^{10} vp	Mod4.2.1.1/9346-20004
mouse	9346-20007 (TV-TEC-171881)	Th1/Th2 polarization study to support vaccine candidate selection.	1-dose 10^{10} vp	Mod4.2.1.1/9346-20007
NHP	NHP 20-09 (TV-TEC-175059)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose 10^{11} vp	Mod4.2.1.1/NHP 20-09
Study Reports Package 2				
NZW rabbit	TOX14369 (TV-TEC-175060)	Immunogenicity study to support vaccine candidate selection.	2-dose $5x10^9$, $5x10^{10}$ vp	study ongoing [a)]
Syrian Hamster	TKO 707 (TV-TEC-175626)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose and 2-dose 10^9, 10^{10} vp	study ongoing [a)]
Syrian Hamster	TKO 766 (TV-TEC-176250)	Immunogenicity and efficacy study (vaccine titration study) with histopathology assessment after breakthrough SARS-CoV-2 infection.	1-dose 10^7,10^8, 10^9, 10^{10} vp	study ongoing [a)]

[a)] *To provide an overview of the current status of the nonclinical pharmacology program, interim data from these studies are presented in Mod2.6.2. Study reports of these studies together with updated Module 2 summary documents will be provided in rolling submission package 2.*
NHP: nonhuman primate; NZW: New Zealand white; vp: virus particles.

The nonclinical pharmacology testing of Ad26.COV2.S was guided by the testing principles laid out by the World Health Organization (WHO) Guidelines on Nonclinical Evaluation of Vaccines [26], European Medicines Agency (EMA) Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [4] and Food and Drug Administration (FDA) Guidance on clinical development and licensing of prophylactic Covid-19 vaccines [7]. In addition, the International Coalition of Medicines Regulatory Authorities [ICMRA] convened international regulatory workshops to discuss regulatory considerations related to the development of COVID-19 vaccine candidates, including the type and extent of nonclinical studies required to support proceeding to first-in-human clinical studies (18 March 2020 [13]), and Phase 3 clinical studies (22 June 2020 [14]). A Vaccines and Related Biological Products Advisory Committee (VRBPAC) meeting held on October 22, 2020 included a discussion of assessment and de-risking of vaccine-associated enhanced respiratory disease (VAERD). In these meetings it was acknowledged that there is limited understanding of the value of available nonclinical models in predicting the likelihood of VAERD in humans. However, studies in animal models were considered important to address the potential for VAERD.

The nonclinical pharmacology testing strategy has been presented in previous Scientific Advice interactions to EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Questions 5 and 9) and FDA/Center for Biologics Evaluation and Research (CBER) (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Questions 1 and 5) in the context of the clinical development program. Data presented so far have been considered by multiple agencies as sufficient to support the clinical development up to the currently ongoing phase 3 efficacy study(ies). A meeting with EMA (Co)-Rapporteurs was held on 30 Sep 2020 to outline the nonclinical data package for conditional marketing authorisation application (MAA), and the proposed nonclinical data package for VAERD was considered overall acceptable. An outline of procedural aspects of the nonclinical pharmacology studies is described below.

The primary pharmacology (immunogenicity and efficacy) studies were performed in countries that are part of the Organization for Economic Co-operation and Development (OECD). All of the studies performed under Janssen Vaccines & Prevention supervision were conducted according to good research practices, which included well-documented procedures and quality control of study data. Research practices for studies that were conducted under oversight of the Beth Israel Deaconess Medical Center (BIDMC) have been validated by an audit of study NHP 20-09 by the Sponsor, which did not reveal findings that compromise the integrity and interpretation of the data (audit report in preparation). Information on the Good Laboratory Practice (GLP) status of the pharmacology studies and the location of the test facilities is provided in the Pharmacology Tabulated Summary (Mod2.6.3).

Ad26.COV2.S material used in nonclinical pharmacology studies was produced firstly in a small scale laboratory process (non-Good Manufacturing Practice [non-GMP] research grade material batch P758-028A used in mouse studies 9346-2004 and 9346-20007, rabbit study TOX14369, Syrian hamster study TKO 707, and study NHP 20-09). Later, development grade material generated in a large scale process similar to the 50 L process Phase 1/2a clinical trial material was evaluated (batch P763-004#06: lot E006614 in Syrian hamster study TKO 766).

1.2.2. Animal Model Rationale

Nonclinical pharmacology of Ad26.COV2.S was evaluated in murine, rabbit, Syrian hamster, and nonhuman primates (NHP) (rhesus monkey) animal models for immunogenicity, including assessment of immunological parameters relevant to the theoretical risk of VAERD (see Section 1.2.5). Vaccine efficacy including lung histopathology for assessment of VAERD was evaluated in NHP and Syrian hamsters. The list of all studies intended for evaluation is provided in Table 1. All studies were performed in animals of an age range corresponding to adult age in humans. Female animals were used in the murine and rabbit studies, male animals were used in the Syrian hamster studies, and both male and female animals were used in the NHP study.

The murine model was used to comprehensively examine immunogenicity and Th1/Th2 skewing of the immune response by assessing antibody and cellular immune responses after immunization. In addition, an alternative immunogenicity model was developed in rabbits as

non-rodent species and to verify rabbits as an appropriate species for toxicology studies (Section 1.4).

A SARS-CoV-2 challenge model in rhesus monkeys has been developed by the sponsor [2]. This NHP species recapitulates the mild disease observed in the majority of human cases [21,27]. Previously rhesus monkeys also showed the potential to develop enhanced disease after severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) infection, in the presence of SARS-CoV-1 directed antibodies [19,25]. This is a nonlethal challenge model, applying intranasal and intratracheal inoculation of $1x10^5$ 50% tissue culture infective dose ($TCID_{50}$) SARS-CoV-2 strain USA-WA1/2020, a US isolate with aspartic acid at position 614 of the S protein (D614). In this SARS-CoV-2 challenge model, rhesus monkeys showed modestly decreased appetite and responsiveness suggestive of mild clinical disease; fever, weight loss, and respiratory distress were not observed [2]. Detectable viral replication was predominantly in the upper respiratory tract and lungs, with lung histopathological findings that were consistent with mild human disease. Lower levels of virus were found in the gastrointestinal tract, liver, and kidney. In nonvaccinated animals, peak viral load occurred approximately 2 days after inoculation, and viral clearance in the lower and upper respiratory tract was observed within approximately 3 weeks of inoculation [2]. In addition to the efficacy model, the high level of genetic homology between NHP and people, and their comparative immunology have made NHP the animal model of choice for studies of vaccine immunogenicity [12,15].

With the aim to develop an animal model showing more severe clinical disease than NHP, a SARS-CoV-2 challenge model has also been developed in Syrian hamsters. Syrian hamsters are permissive to SARS-CoV-2 replication and display widespread lung pathology and clinical signs including weight loss. A nonlethal challenge model was used, applying intranasal inoculation of 10^2 $TCID_{50}$ SARS-CoV-2 strain BetaCoV/Munich/BavPat1/2020, a European isolate with glycine at position 614 of the S protein (G614). Peak viral load in the lung measured by $TCID_{50}$ assay was around day 4 with clearance from lung tissue and throat swabs of the hamsters within 7 days.

1.2.3. Vaccine Immunogenicity

Humoral immune responses were measured in mice, rabbits, hamsters, and NHP. Based on experience with SARS-CoV, and in recent SARS-CoV-2 vaccine efficacy studies in NHP, neutralizing antibody responses have been identified as the predominant correlate of protection [20,27]. Neutralizing antibody titers were determined in a virus neutralization assay (wild-type [wt]VNA) with live SARS-CoV-2, or in a pseudotyped virus neutralization assay (ppVNA [pVNA]). In addition, S protein binding antibody titers were determined by enzyme-linked immunosorbent assay (ELISA).

Cellular immune responses were measured in mice, rabbits, and NHP. SARS-CoV-2 specific cellular responses were determined by measuring cytokine production after ex vivo stimulation of splenocytes or peripheral blood mononuclear cells (PBMCs) with peptide pools covering the

complete SARS-CoV-2 S wild type protein sequence. Cytokine production was determined by interferon gamma (IFN-γ) ELISpot.

The immune response to the S protein vaccine antigen was measured post Dose 1, and where tested also post Dose 2, to determine the magnitude of the immune response after vaccination, at the peak of immune response (ie, between 2 and 8 weeks post immunization). While innate responses may contribute to protection early after vaccination, they are not considered to be a major aspect of the protective effect of the vaccine and were therefore not characterized.

1.2.4. Vaccine Efficacy

In the NHP challenge study, the main efficacy readouts were viral load in bronchoalveolar lavage (BAL) and nasal swab samples. The BAL and nasal swab viral loads were determined at 0, 1, 2, 4, 7, 10 and 14 days post inoculation for each NHP, measured by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) of SARS-CoV-2 E gene subgenomic ribonucleic acid (sgRNA).

In the Syrian hamster challenge studies, the main efficacy readouts were viral load in the lung and nasal turbinates 4 days after inoculation, and viral load in throat swab samples taken daily after inoculation. Infectious viral load, ie, the presence of replication competent virus, was measured by $TCID_{50}$ assay, in which samples were titrated on confluent monolayers of Vero E6 cells and the viral titers were calculated.

1.2.5. Assessment of VAERD

There is a theoretical risk of VAERD for SARS-CoV-2 vaccines, based on nonclinical experience with SARS-CoV and MERS-CoV based vaccines. It is acknowledged that there is limited understanding of the value of available nonclinical models in predicting the risk of VAERD in humans. However, studies in animal models are considered important to address the potential for VAERD, in combination with clinical assessments. To the sponsor's knowledge, VAERD for SARS-CoV-2 vaccines has not been observed in nonclinical models yet. Actions taken to mitigate the potential risk of VAERD included immunogenicity assessments to show induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to be beneficial to minimize potential risk of VAERD. In addition, lung histopathology was assessed in Ad26.COV2.S immunized Syrian hamsters and NHP after SARS-CoV-2 challenge and compared to unvaccinated and SARS-CoV-2 infected control animals to evaluate the potential risk of VAERD.

The induction of neutralizing antibodies was assessed in every study. A study to assess the Th bias of the immune response induced by Ad26.COV2.S was performed in BALB/c mice (study 9346-20007). For this purpose, a comparison group received recombinant S protein in aluminum phosphate ($AlPO_4$) adjuvant, which is associated with a Th2 biased immune response. Immunologic readouts for Th1/Th2 polarization included IgG subclass binding antibody titers, and cytokine production measured by ELISpot, Multiplex ELISA, and intracellular cytokine staining (ICS).

In addition, an assessment of lung tissue histopathology and other clinical signs of disease was performed in SARS-CoV-2 challenge studies. In 1 Syrian hamster study (TKO 766) lower dose levels of Ad26.COV2.S were included, which induced a suboptimal humoral response allowing breakthrough viral replication in the lungs after SARS-CoV-2 challenge, conditions which are hypothesized to contribute to a risk of VAERD.

During the post inoculation follow-up, the NHP and Syrian hamsters were monitored by clinical observations and viral load levels. At the end of follow-up, gross pathology examination (Syrian hamster only), histopathology evaluation, and immunohistochemistry analysis on selected tissues were performed. Selected respiratory tract tissues were evaluated and assessed (semi-quantitatively graded/scored) for disease-associated pathology findings, such as the severity and extent of alveolar, bronchial, bronchiolar, or tracheal inflammation and/or inflammatory infiltrates, evidence of alveolar edema, or any other degenerative inflammatory changes.

1.3. Pharmacokinetic Testing Strategy

In accordance with the WHO Guidelines on Nonclinical Evaluation of Vaccines [26], pharmacokinetic studies are usually not needed for vaccines. However, in line with the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [4] and the FDA Guidance on considerations for plasmid DNA vaccines for infectious disease indications [6], biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 viral vector platform following intramuscular (IM) injection. The biodistribution profile of the Ad26 vaccine platform has been evaluated in rabbits using an Ad26-based human immunodeficiency virus (HIV) vaccine, i.e., Ad26.ENVA.01 (Ad26 vector encoding the Clade A envelope protein of HIV type 1), and an Ad26-based respiratory syncytial virus (RSV) vaccine, i.e., Ad26.RSV.preF (Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain) (see Table 2).

As explained in Section 3, these vector platform data are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S, for which the same Ad26 vector backbone is used. This position has been confirmed and agreed by the Agency in a Scientific Advice by EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Question 6) and CBER (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Question 2). It is further noted that the same platform biodistribution data were part of the MAA file for the Ebola vaccine component Ad26.ZEBOV (EU/1/20/1444/001). Hence, no pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S specifically.

Table 2: Overview of Biodistribution Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine Regimen, Route, Interval, and Dose Level	GLP	Study Report
Study Reports Package 1 (current submission)				
NZW rabbit	Single dose biodistribution study with a 91-day observation period	Ad26.ENVA.01, IM, single dose, 5×10^{10} vp	Yes	Mod4.2.2.3/ 1645-06074
NZW rabbit	Single dose biodistribution study with a 180-day observation period	Ad26.RSV.preF, IM, single dose, 1×10^{11} vp	Yes	Mod4.2.2.3/ TOX13342

GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

The biodistribution studies with Ad26.ENVA.01 and Ad26.RSV.preF were conducted using the IM route, which is also the intended route for use of Ad26.COV2.S in humans.

The studies were done in rabbits as this is a widely accepted species to assess the nonclinical safety of vaccines. In previous studies, Ad26-based vaccines (including Ad26.COV2.S, Ad26.ENVA.01 and Ad26.RSV.preF) were shown to elicit immune responses in the animals, indicating the rabbit as a relevant nonclinical species [24]. In addition, the rabbits have sufficient muscle mass to receive a full human vaccine dose via the IM route with a single injection. The use of one species is generally considered sufficient to assess the nonclinical safety/biodistribution profile of vaccines [4,26].

The dose levels applied in the biodistribution studies for Ad26.ENVA.01 and Ad26.RSV.preF were similar to, or above the recommended human dose level for Ad26.COV2.S ($5x10^{10}$ vp), hence full human dose levels were tested. Specific quantitative polymerase chain reaction (q-PCR) assays were used to detect and quantify Ad26-vector DNA in various tissues, including the gonads, brain and blood collected at specified time points following vector administration.

The sampling timepoints used in the biodistribution studies with the Ad26 vectors (i.e., Days 11, 61, 91 post-vaccination for Ad26.ENVA.01, and Days 11, 90, 120 or 180 post-vaccination for Ad26.RSV.preF) are in line with biodistribution studies conducted with adenovirus type 5 (Ad5) and type 35 (Ad35) based vaccines as reported by Sheets et al. 2007 [23]. The sampling timepoints for the biodistribution studies were selected to allow sufficient time to confirm clearance (i.e., confirming a downward trend over time) of the Ad26 vector.

The biodistribution studies (with Ad26.ENVA.01 and Ad26.RSV.preF) were conducted as standalone studies (i.e., not integrated in a pivotal nonclinical safety study). For such pharmacokinetics studies, GLP compliance is not generally required. Nevertheless, the biodistribution studies for the Ad26 vector platform were conducted in compliance with GLP.

Nonclinical shedding studies were not conducted for the Ad26 vaccine platform. The risk for shedding of Ad26.COV2.S will be based on clinical shedding data for the Ad26 vaccine platform and is discussed in the Environmental Risk Assessment.

1.4. Toxicology Testing Strategy

The nonclinical safety profile of the Ad26.COV2.S vaccine is being assessed in two pivotal toxicology studies in New Zealand White (NZW) rabbits, i.e., a combined repeated dose toxicity and local tolerance study, and a combined embryofetal and pre- and postnatal development (EF-PPND) toxicity study. The testing is consistent with applicable guidelines, including the WHO Guidelines on Nonclinical Evaluation of Vaccines [26], the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [4], and the FDA Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications [5]. An overview of these ongoing toxicology studies is provided in Table 3.

In addition, the Sponsor has significant nonclinical experience with Ad26-based vaccines using various gene inserts encoding for HIV, malaria, RSV, Ebolavirus, Filovirus, Zika, Influenza and human papillomavirus antigens. More than 10 GLP combined repeat-dose toxicity and local tolerance studies as well as one EF-PPND study (with the Ebola vaccine Ad26.ZEBOV) have been performed in rabbits (or rats) testing the nonclinical safety of these various Ad26-based vaccines.

The study reports and summaries of the ongoing toxicology studies with Ad26.COV2.S, as well as a high-level summary of the supportive toxicology studies with other Ad26-based vaccines will be submitted to the Agency in Package 2 during the second wave of the rolling submission. At that time, the nonclinical overview will be updated with a more extensive description of the Toxicology Testing Strategy, and Toxicology Written and Tabulated Summaries will be provided.

Table 3: Overview of Toxicology Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine Regimen, Route, Interval, and Dose Level	GLP	Study Report
Study Reports Package 2				
NZW rabbit	Combined repeated dose toxicity and local tolerance study	3 injections (Days 1, 15, 29; 2-week interval between doses), IM • Saline • $1x10^{11}$ vp	Yes	TOX14382 ongoing
NZW rabbit	Combined embryofetal and pre- and postnatal development study	3 injections (Day 1[a], GD6 and GD20; 2-week interval between doses), IM • Saline[b] • $1x10^{11}$ vp[b]	Yes	TOX14389 ongoing

[a] Day 1 = 7 days prior to pairing
[b] The study includes 2 subgroups: 1 group consisting of females that are necropsied on GD29 and have a uterine and fetal examination (external, visceral, and skeletal exams), and 1 group consisting of females that are allowed to give birth and in which the survival and development of the kits is evaluated through lactation day 28
GD: gestation day; GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

2. PHARMACOLOGY

2.1. Mechanism of Action

Induction of protective immunity by Ad26.COV2.S is thought to occur through antibody responses, predominantly a neutralizing antibody response, against the S protein. The role of cellular immune responses, particularly during a severe course of infection and for the clearance of virus-infected cells, is currently unclear.

Adenoviruses are non-enveloped viruses composed of an icosahedral nucleocapsid and a single double-stranded linear deoxyribonucleic acid (DNA) genome. There are at least 51 human adenovirus types divided into subgroups A-G based on sequence homology and neutralization by antisera. Overall, adenoviruses, including Ad26 (subgroup D), have been associated with many (mainly mild) clinical syndromes, particularly infections affecting the respiratory tract, the gastrointestinal system, and the eye [11].

To begin the entry process into the host cell, adenoviruses must first initiate contact with the plasma membrane of the target cell. This first recognition is driven by shell-forming polymers of the virion; elongated fibers protrude from the capsid and have a globular “knob” domain at the distal end. The fiber knob functions as the major attachment site for cellular receptors. Currently established receptors on the host cell are cluster of differentiation (CD)46, the coxsackievirus adenovirus receptor (CAR), desmoglein 2, and cell surface sialic acid bearing glycans [1,17]. At the proximal end, the fibers are bound to a pentameric structure, the penton base, that is involved in secondary interactions required for virus entry into the cell [28]. In this secondary interaction, a specialized motif in the penton base protein interacts with an integrin molecule and stimulates endosomal entry of the virion into the host cell [10]. Once the virus has successfully entered the host cell, the endosome acidifies, which leads to a stepwise disassembly of the capsid and release of the virion into the cytoplasm. The virion is then transported to the nuclear pore complex where the viral DNA enters the host nucleus.

Ad26.COV2.S is a monovalent recombinant Ad26-based vector that was rendered replication-incompetent by deletion of the E1 region. The E1 region normally encodes proteins that are involved in transactivation of viral and cellular genes, induction of cellular DNA synthesis, and mitosis. A large part of the E3 region was excised because of the presence of immunomodulatory genes linked to persistence within the host cell and to create sufficient space in the genome for the transgene encoding the full-length SARS-CoV-2 S protein, which was inserted in the E1 region. Ad vectors that have been engineered in this manner can gain entrance into a normal host cell and cause the cell to make proteins encoded by the viral genome, but are unable to spread into new host cells in contrast to a typical infection.

Although unable to replicate in normal cells, these vectors can be produced in the laboratory on genetically engineered cell lines (eg, human PER.C6® and PER.C6 TetR) that complement for the missing E1 region. Since the genes needed for replication are lacking in normal human cells and these genes do not naturally occur in the human genome, this precludes replication of the adenoviral vector following intramuscular administration to humans. Host cell DNA-dependent

RNA polymerase transcribes the transgene encoding S protein into mRNA, which is then translated to produce the S protein. The S protein is then presented on the cell membrane to the host immune system and stimulates an immune response in local lymphoid organs by presentation on antigen presenting cells.

2.2. Study Rationale

The nonclinical pharmacology studies are listed in Table 1. All studies with the exception of Syrian hamster study TKO 766 were performed to inform on vaccine candidate selection for clinical development. The murine studies and rabbit study tested the immunogenicity of the vaccine, and murine study 9346-20007 was specifically designed to determine the Th1/Th2 balance induced by a single dose of vaccine. The Th1/Th2 balance has been linked with the theoretical risk of VAERD. Study NHP 20-09 was performed to assess the immunogenicity and protective efficacy of a single vaccine dose, testing different vaccine candidates including Ad26.COV2.S.

After selection of Ad26.COV2.S for clinical development, Syrian hamster study TKO 766 was performed with lower dose levels of Ad26.COV2.S, which intentionally induced a suboptimal humoral response allowing breakthrough viral replication in the lungs after SARS-CoV-2 inoculation, conditions which are hypothesized to contribute to a risk of VAERD.

2.3. Immunogenicity and Protective Efficacy of Ad26.COV2.S

Murine study 9346-20004 (Mod2.6.2/Sec2.3.1.1) demonstrated the humoral (binding and neutralizing antibody) and cellular immunogenicity of a single dose of Ad26.COV2.S, and also showed the benefit of the stabilizing mutations in increasing humoral immunogenicity compared with an Ad26 vector encoding the wild type S protein.

Murine study 9346-20007 (Mod2.6.2/Sec2.3.1.2) was designed to determine the Th1/Th2 balance induced by a single dose of Ad26.COV2.S. In this study, Ad26.COV2.S was shown to skew towards a Th1 type response. This was measured by the ratio of IFN-γ to the Th2 associated cytokines interleukin-4 (IL-4), IL-5 and IL-10, as well as by the ratio of anti-S protein immunoglobulin G (IgG) antibody subclasses IgG2a and IgG1.

Study NHP 20-09 in rhesus monkeys (Mod2.6.2/Sec2.3.2) was designed to assess the immunogenicity and efficacy of a single dose of $1x10^{11}$ vp Ad26.COV2.S, one of the regimens in clinical study VAC31518COV1001. A single dose of Ad26.COV2.S induced binding and neutralizing antibodies. The NHP were inoculated with SARS-CoV-2 6 weeks after vaccination. In the group immunized with Ad26.COV2.S, viral load in the lower respiratory tract (ie, BAL) was below the lower limit of detection (LLOD) for all NHP (N=6) at all timepoints. Viral load in the upper respiratory tract (ie, nasal swabs) was below the LLOD in 5 out of 6 NHP immunized with Ad26.COV2.S; 1 animal had low viral load detectable at days 1 and 2 post inoculation. In contrast, all sham immunized had high viral loads in the lower respiratory tract (LRT) and upper respiratory tract (URT).

In these studies, immune responses including neutralizing antibody responses were induced as early as 2 weeks post immunization. In addition, a favorable immunogenicity profile was observed in the nonclinical studies, ie, the induction of neutralizing antibodies and a Th1 skewed immune response.

Conclusions on rabbit immunogenicity study TOX14369, and Syrian hamster immunogenicity and efficacy studies TKO 707 and TKO 766, will be added in an update to this document when the study reports are submitted.

2.4. VAERD Risk Monitoring

Nonclinical data have shown the induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to be beneficial to minimize potential risk of VAERD (Section 2.3). VAERD was also monitored in nonclinical efficacy studies by assessment of histopathology in LRT and URT samples (URT samples from Syrian hamster studies only) taken after SARS-CoV-2 inoculation.

In study NHP 20-09 (Mod2.6.2/Sec2.3.2) the following LRT histopathological parameters were assessed: alveolar edema, inflammation interstitial/septal thickening, mononuclear cell infiltrates in perivascular/ peribronchiolar space, macrophage infiltrates in alveolar space, macrophage infiltrates in bronchiolar space, neutrophil infiltrates in alveoli, bronchioloalveolar hyperplasia, bronchial-associated lymphoid tissue (BALT) hyperplasia. No signs of VAERD were observed in this NHP challenge study, either when assessing the cumulative histopathology score or any individual score compared with the unvaccinated control group. The histopathology scores were reduced in Ad26.COV2.S immunized animals, when compared with the mock immunized animals. Conclusions on viral load are included in Section 2.3.

Conclusions on VAERD risk monitoring in Syrian hamster studies TKO 707 and TKO 766 will be added in an update to this document when the study reports are submitted.

3. PHARMACOKINETICS

Biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 vaccine platform following IM injection in NZW rabbits using Ad26.ENVA.01 and Ad26.RSV.preF. No pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S.

The Ad26 vector contains deletions in the early region (E1) of the Ad26 genome, rendering it replication-incompetent. Ad26-based vaccines, including Ad26.COV2.S, require recombinant E1 complementing cell lines, like the PER.C6 (TetR) cells, for virus replication. Outside of these specific cellular environments, Ad26-based vaccines, including Ad26.COV2.S, cannot replicate or reproduce and are therefore expected to show a limited distribution and persistence following administration. This is confirmed by the biodistribution studies with Ad26.ENVA.01 (study 1645-06074; Mod2.6.4/Sec4.1) and Ad26.RSV.preF (study TOX13342; Mod2.6.4/Sec4.2). In these studies, animals were sacrificed on Days 11, 61, or 91 (Ad26.ENVA.01), and on Days 11, 90, 120 or 180 (Ad26.RSV.preF) following single IM injection at a dose level of 5×10^{10} vp

(Ad26.ENVA.01) or 1×10^{11} vp (Ad26.RSV.preF). Tissues from these animals were harvested for analysis of Ad26 vector DNA using q-PCR. As a general pattern, both Ad26 vectors showed a limited biodistribution profile following IM administration, as they were primarily detected at the site of injection, regional (iliac) lymph nodes and (to a lesser extent) the spleen. No Ad26 vector DNA was detected in the gonads or in the brain. Comparing the various necropsy timepoints following IM administration (i.e., Days 11, 61, and 91 for Ad26.ENVA.01; Days 11, 90, 120 and 180 for Ad26.RSV.preF), a downward trend in the number of positive tissues, and/or vector copy number present in those positive tissues was observed, to levels close to, or below the respective limits of detection, indicating clearance of the Ad26 vector. These data further indicate that the Ad26 vector does not replicate and/or persist in the tissues following IM injection. Despite differences in the expressed transgene insert, both Ad26 vectors showed a similar pattern of biodistribution and clearance when delivered via the IM route at full human dose levels in the rabbit.

The Ad26 vector backbone used for Ad26.COV2.S is identical to the vector backbone of the Ad26-based vaccines that were tested in the available biodistribution studies (i.e., Ad26.ENVA.01 and Ad26.RSV.preF). The only difference between the vectors, apart from the encoded antigen transgene, is the insertion of a tetracycline operon (TetO) motif in the cytomegalovirus promoter sequence of the transgene expression cassette of Ad26.COV2.S. The TetO motif was included in the Ad26 vector to improve vector bioreactor yields, as described in Mod3.2.S.2.3 Control of Materials – Source, History, and Generation of the Pre-Master Virus Seed.

The insertion of the TetO sequence in the transgene expression cassette is not considered to impact the biodistribution profile of the Ad26 vector. Adenoviruses are non-enveloped viruses whose cell entry, and therefore tropism, is dictated via interactions of structural capsid proteins (mainly the fiber and penton base) with specific cellular receptors [22]. The adenoviral capsid is a highly complex and organized structure [18] which does not easily allow for the introduction or exchange of other proteins. The transgene expression cassette itself, which is inserted into the site where the early E1 gene was previously located, is thus not considered to impact on the formation or the composition of the Ad26 vector capsid, and hence tropism of the vector. As a consequence, the biodistribution profile of the Ad26 vector is considered independent of the transgene/expression cassette, which is supported by the comparable biodistribution profile observed for Ad26.ENVA.01 and Ad26.RSV.preF. The results with the Ad26 vector are in line with a study from Sheets et al, 2009 [23], which concluded that the biodistribution profile for Ad5 and Ad35 vectors was consistent, regardless of differences in transgene inserts.

Adenoviral vectors are considered non-integrating because they lack the machinery to integrate their genome into the host chromosomes (eg. EMA Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors [4]; FDA Guidance for Industry, Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events [8]). As such, upon transduction of a cell, the adenoviral DNA does not integrate into the host genome, but rather resides episomally in the host nucleus [9,16] Therefore, and also supported by the results from the biodistribution studies where clearance of the vector was shown, the risk of integration of

genetic material from an Ad26 vector in the human host is unlikely. This, together with the fact that the Ad26 biodistribution studies did not show a signal in the gonads, excludes the need for dedicated nonclinical germline transmission studies.

In conclusion, the biodistribution data obtained with Ad26.ENVA.01 and Ad26.RSV.preF are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same route of administration (IM) and at a comparable dose level ($5x10^{10}$ vp to 1×10^{11} vp). The results from these studies show a limited distribution profile and indicate clearance over time of the Ad26 vector following IM injection.

4. TOXICOLOGY

A combined repeated dose toxicity and local tolerance study as well as a combined embryofetal and pre-and postnatal development toxicity study in rabbits are ongoing. The reports and summaries from these studies will be submitted to the Agency in Package 2 and will be accompanied by a high-level summary of supportive toxicology data from other vaccines that are based on the Ad26 platform.

5. INTEGRATED OVERVIEW AND CONCLUSIONS

Pharmacology

Immunogenicity data from studies in mice and NHP show that a single dose of Ad26.COV2.S induces humoral and cellular immune responses as early as 2 weeks post immunization. Ad26.COV2.S induces neutralizing antibodies and a Th1 skewed immune response, factors that are thought to be beneficial to minimize potential risk of VAERD.

In an NHP SARS-CoV-2 challenge study, a single dose of $1x10^{11}$ vp Ad26.COV2.S fully protected 6 out of 6 NHP from viral replication in the lung, and protected 5 out of 6 NHP from viral replication in the nose. At this time, the protective efficacy of Ad26.COV2.S in humans is not known.

In an NHP SARS-CoV-2 challenge study, no signs of VAERD were observed based on histopathologic assessment of lung tissue and absence of vaccine related clinical observations. The theoretical risk of Ad26.COV2.S VAERD in humans is unknown.

Pharmacology studies in rabbits and Syrian hamsters are ongoing.

Biodistribution

Biodistribution studies in rabbits showed a limited distribution profile of the Ad26 vector. Clearance (i.e., reflected by a downward trend in number of positive tissues and vector copies over time, to levels close to, or below the respective detection limits) of the Ad26 vector was observed following IM injection, indicating that the vector does not replicate and/or persist in the tissues. As biodistribution is considered dependent on the viral vector platform and not on the transgene insert, the biodistribution results obtained with Ad26.ENVA.01 and Ad26.RSV.preF

are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same (i.e., IM) route.

Toxicology

Toxicology studies in rabbits with Ad26.COV2.S are ongoing. The reports and summaries of these studies will be submitted to the Agency in Package 2 and will be accompanied by a high-level summary of supportive toxicology data from other vaccines that are based on the Ad26 platform.

Overall Conclusion

To be completed.

6. LIST OF LITERATURE CITATIONS

Literation citations are located in Mod4.3.

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2.4. Nonclinical Overview

Drug Substance	AZD1222
ANGEL ID	Doc ID-004365565
Date	30 September 2020

2.4 Nonclinical Overview AZD1222

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1 OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

In partnership with the University of Oxford, AstraZeneca (the Sponsor) is developing AZD1222 for the prevention of coronavirus disease-2019 (COVID-19). AZD1222 is a recombinant chimpanzee adenovirus (ChAd) expressing the severe respiratory syndrome-coronavirus-2 (SARS CoV-2) spike (S) surface glycoprotein. Development of AZD1222, previously referred to as ChAdOx1 nCoV-19, was initiated by the University of Oxford with subsequent transfer of development activities to the Sponsor.

AZD1222 is a recombinant replication-defective ChAd vector expressing the SARS CoV-2 S surface glycoprotein, driven by the human cytomegalovirus major immediate early promoter that includes intron A with a human tissue plasminogen activator (tPA) leader sequence at the N terminus. Spike (S) is a type I, trimeric, transmembrane protein located at the surface of the viral envelope, giving rise to spike shaped protrusions from the SARS-CoV-2 virion. The S protein's subunits are responsible for cellular receptor angiotensin-converting enzyme 2 (ACE-2) binding via the receptor-binding domain and fusion of virus and cell membranes, thereby mediating the entry of SARS-CoV-2 into the target cells. The S protein has an essential role in virus entry and determines tissue and cell tropism, as well as host range. The roles of the S in receptor binding and membrane fusion make it a desirable target for vaccine and antiviral development. AZD1222 expresses a codon-optimised coding sequence for S protein from the SARS-CoV-2 genome sequence accession MN908947.

The ChAdOx1 platform technology was used to support the first-in-human (FIH) and other early clinical AZD1222 studies. This approach of using platform data to support a FIH clinical study is consistent with the views expressed by global regulators at the International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 (ICMRA 2020).

To date, immunology and biological activity studies (including prime boost vaccination) of AZD1222, have been conducted in mice, non-human primates, ferrets and pigs (Table 1).

Biodistribution studies with AZD1222 have not been performed based upon previously generated biodistribution data with similar replication-defective ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice that showed no evidence of replication of the virus or presence of disseminated infection after intramuscular (IM) injections (Table 2). Studies evaluating any toxicity due to AZD1222 have not been conducted to date. Toxicology studies on a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein, as well as other ChAd vaccines (AdCh63 MSP-1, ChAd OX1 NP+M1) are shown as a reference (Table 3). Ongoing or planned nonclinical studies are listed in Table 4.

All pivotal nonclinical safety studies were conducted in OECD member countries and in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP), and according to relevant International Conference on Harmonisation guidelines.

Table 1 List of Nonclinical Pharmacology Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Source	GLP Y/N
Murine Immunogenicity (Van Doremalen et al 2020)	Balb/C and CD-1 mice	Single dose, IM 6×10^9 vp AZD1222	Jenner Institute - Oxford University / NIH	N
Non-human Primate Efficacy and Immunogenicity (van Doremalen et al, 2020)	Rhesus macaques	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University / NIH	N
Non-human Primate Efficacy and Immunogenicity (6284)	Rhesus macaques	Single dose, IM 2.5×10^{10} vp AZD1222	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N
Ferret Efficacy and Immunogenicity (20-01125)	Ferret	Single dose, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University / CSIRO, Australia	Y
Ferret Efficacy (6285)	Ferret	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N
Porcine Immunogenicity (Graham et al 2020)	White-Landrace-Hampshire cross-bred pigs	Day 0 and 28, IM 5.12×10^{10} vp AZD1222	Jenner Institute - Oxford University / Pirbright Institute, UK	N

CSIRO = Commonwealth Scientific and Industrial Research Organisation, Geelong, Australia; IM = intramuscular; NIH = National Institute of Health

Table 2 List of Nonclinical Distribution Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
AdCh63 MSP-1 and MVA MSP-1 Tissue Distribution Study By Intra-Muscular Administration To Mice (Report UNO0014/RMBBioDIST-001)	Balb/C mice	Day 1, IM 1×10^{10} vp	Huntingdon Life Sciences, UK	Y
AdCh63ME-TRAP Tissue Distribution Study By Intra-Dermal Administration To Mice (UNO0009/MAB-001)	Balb/C mice	Day 1, IM $3.3x\ 10^{9}$ vp	Huntingdon Life Sciences, UK	Y

Table 3 List of Nonclinical Toxicology Studies with Similar Replication-defective ChAd Vaccines

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
Mouse Toxicity ChAdOx1 Chik Vaccine or ChAdOx1 MERS (QS18DL)	Balb/C mice	Day 1 and 15, IM 1×10^{10} vp	Envigo CRS Limited UK	Y
ChAd OX1 NP+M1 and MVA NP+M1: Toxicity Study by Intramuscular Administration to Mice (XMM0003)	Balb/C mice	Day 1, IM ChAd OX1 NP+M1 1×10^{10} vp and Day 15, IM MVA NP+M1 1.5×10^{7} pfu	Envigo CRS Limited UK	Y
Mouse Toxicity AdCh63 MSP-1 and MVA MSP-1 or a Combination of AdCh63 ME-TRAP and MVA ME-TRAP (UNO0013)	Balb/C mice	Day 1, IM ChAd63ME-TRAP 1×10^{10} vp Day 15, IM ChAd63ME-TRAP/ MVA ME TRAP 0.78×10^{10} vp / 6.85×10^{7} pfu	Envigo CRS Limited UK	Y

IM = intramuscular

Table 4 List of Ongoing and Planned Nonclinical Studies with AZD1222

Study (Report Number)	Species	Status	GLP Y/N
ChAdOx-1 HBV and MVA-HBV Biodistribution Study in BALB/c Mice with Shedding Assessment (0841MV38.001)	Balb/C mice	Initiated Audited Draft November 2020	Y
Cardiovascular and Respiratory Assessment Following Intramuscular Administration to Male Mice (617078)	Balb/C mice	Initiated Audited draft October 2020	Y
Mouse Repeat-Dose Toxicity Study (513351)	Balb/C mice	Initiated Audited draft February 2021	Y
Mouse Development and Reproductive Toxicology Study - preliminary (490838)	Balb/C mice	Initiated Audited draft October 2020	Y
Mouse Development and Reproductive Toxicology Study – main	Balb/C mice	Planned Audited draft February 2021	Y

2 PHARMACOLOGY

2.1 Primary Pharmacodynamics

Immunogenicity studies in animal models responsive to AZD1222 were conducted to evaluate the immunologic properties of this COVID-19 vaccine candidate to support FIH clinical trials. AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. These studies included evaluation of humoral, cellular and functional immune responses. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated enhanced respiratory disease (ERD). A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (Van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

2.2 Secondary Pharmacodynamics

Secondary pharmacodynamic studies have not been conducted with AZD1222.

2.3 Safety Pharmacology

A cardiovascular and respiratory assessment following IM administration of AZD1222 to male mice was initiated on the 8th August 2020 (Study 617078). An audited draft is due October 2020.

2.4 Pharmacodynamic Drug Interactions

Pharmacodynamic drug interaction studies have not been conducted with AZD1222.

3 PHARMACOKINETICS

3.1 Absorption

Absorption studies evaluations are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional absorption, distribution, metabolism, and excretion (ADME) evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune responses.

3.2 Distribution

Distribution studies are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional ADME evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune response.

AZD1222 is replication-incompetent in human cells due to a block in gene expression caused by the deletion of the E1 genes. Therefore, after the initial infection of the cells that the virus enters, there will be no further infection and no spread of the virus within the body. Given all these data on biodistribution of replication incompetent chimpanzee adenoviral vectored vaccines, it was not considered appropriate to repeat an in vivo study with AZD1222 in which the insert does not affect viral replication cells. Therefore, no AZD1222 biodistribution study was performed. Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injection. A biodistribution study using the ChAdOx1 vector with an hepatitis B virus (HBV) insert after IM injection has being conducted in mice. An audited draft report is due end November 2020.

Intramuscular administration of AZD1222 is expected to minimise risk of systemic exposure. The biodistribution of AZD1222 following intramuscular administration is expected to be similar to that of AdCh63, confined to the site of injection and draining lymph nodes.

3.3 Metabolism

Metabolism studies have not been conducted with AZD1222. The expected consequence of metabolism of biotechnology-derived vaccines is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood.

3.4 Excretion

Excretion studies have not been conducted with AZD1222. No virus excretion is expected with AZD1222 as it is a non-replicating vaccine vector. Biodistribution studies with related platform vectors have shown no distribution beyond the site of injection or draining lymph nodes.

3.5 Pharmacokinetic Drug Interactions

Pharmacokinetic drug interaction studies have not been conducted with AZD1222.

3.6 Other Pharmacokinetic Studies

Other pharmacokinetic studies have not been conducted with AZD1222.

4 TOXICOLOGY

4.1 SINGLE DOSE TOXICITY

No single dose toxicity studies have been performed with AZD1222.

4.2 REPEAT DOSE TOXICITY

A repeat-dose GLP toxicity study with AZD1222 in mice was initiated on the 9th September 2020, with an audited draft report due February 2021 (Study 513351).

As the ChAdOx1 platform technology utilized for AZD1222 is well characterized, toxicology data with ChAdOx1 MERS-CoV vaccine expressing the full-length Spike protein in mice (Report QS18DL), was used to support first in human (FIH) clinical trials for AZD1222 (International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 [ICMRA 2020]). In addition, toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) are also discussed.

The mouse was chosen as the test species because of its acceptance by regulatory agencies and develops appropriate immune responses following immunisation with AZD1222. The Balb/c strain was used as this was the relevant strain for the vaccine construct.

A brief summary of the key findings from the ChAdOx1 MERS vaccine toxicology study in in mice is provided below.

- Changes at the intramuscular injection sites (inflammatory cell infiltrates) were observed in the majority of females and in several males.
- Histopathological changes in the spleen (increased germinal centre development) correlated with an increased spleen weight in females. Increased germinal centre development of the right lumbar lymph nodes (draining lymph node), correlated macroscopically with enlargement, was observed for the majority of treated animals. Slightly higher circulating white blood cell numbers were observed.
- At the end of the study treatment there was a slightly lower than control body weight gain for treated males and females. For males this was due mainly to slightly lower than control weight gains during Days 15 to 18 however for females this was due mainly to small weight losses during this period. Mice were dosed on Day 1 and 15, with necropsy on day 28.
- Slightly lower group mean liver weight for males and females (0.92X and 0.90X control), higher phosphorus concentration for females (1.2X control) or lower triglyceride concentration for males and females (0.56X and 0.64X) were observed. There was no correlation with histopathological changes.

The spectrum and severity of these changes were consistent with the administration of an antigenic substance such as ChAdOx1 MERS, and were considered to be non-adverse.

Results from the toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) were consistent with ChAdOx1 MERS and were well tolerated with no associated adverse effects. The toxicity data (and toxicity in the target organs) from the ChAdOx1 and ChAd63 based vaccines follows the same pattern, where findings were consistent with a predicted response to vaccine administration.

4.3 Toxicokinetics

Toxicokinetic studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Pharmacokinetic studies (eg, for determining serum or tissue concentrations of vaccine components) are normally not needed.

4.4 Genotoxicity (Mutagenicity)

Genotoxicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines, (WHO 2005), genotoxicity studies are normally not required for the final vaccine formulation and therefore have not been conducted.

4.5 Carcinogenicity

Carcinogenicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Carcinogenicity studies are not required for vaccine antigens. AZD1222 is a replication deficient, non-integrating

adenovirus vector so there is no risk of carcinogenicity. To date there have been no clinical reports of chromosomal vector integration following adenovirus vector-mediated gene transfer.

4.6 Developmental and Reproductive Toxicity

An evaluation of the impact of AZD1222 on embryo-foetal development is being conducted in a dose-range study initiated on 30 June 2020 with an audited draft due October 2020 (Study 490838). The main GLP embryo-foetal development study is scheduled to start at the end of November 2020 with an audited draft due February 2021.

4.7 Local Tolerance

A dedicated local tolerance study with AZD1222 was not conducted. However, local tolerance was evaluated as part of a repeat dose GLP toxicology study in mice with the related ChAdOx1 MERS vaccine (Report QS18DL). Changes related to treatment with ChAdOx1 MERS vaccine were seen in the tissues of the intramuscular injection site, the right lumbar lymph node (draining lymph node) and the spleen of mice. The inflammatory cell infiltrate seen in the tissues of the intramuscular injection sites (infiltrates of lymphocytic/mononuclear inflammatory cells) were caused by the intramuscular injection of the vaccine with the increased germinal centre development of the right lumbar lymph node caused by immune stimulation of the lymphatic drainage from this area and are not considered adverse.

4.8 Other toxicity Studies

No other toxicity studies with AZD1222 were conducted.

5 INTEGRATED OVERVIEW AND CONCLUSIONS

AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated ERD. A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (Van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injections. WHO guidelines on nonclinical evaluation of vaccines states that pharmacokinetic studies (eg, for determining serum or tissue

concentrations of vaccine components) are normally not needed and specific studies should be considered on a case-by-case basis (eg, when using novel adjuvants or alternative routes of administration).

Biodistribution studies are more informative when a replication-competent virus is administered since the amount of virus present in the subject (experimental animal or human volunteer) will increase following injection, and some viruses have a known propensity to accumulate in particular organs. For example, Vaccinia virus may be found at high titres in the ovaries and adenovirus accumulates in the liver. However, replication-deficient viruses are known to infect cells at the injection site, and although some infectious viral particles may drain to local lymph nodes and travel through the blood to other sites in the body, concentrations of virus at these sites are so low after dilution in the blood and other tissues that they are not reliably detected in the lymph nodes and never detected elsewhere in the body. A biodistribution study would demonstrate if, unexpectedly, viral replication was taking place after injection. However, this is not an appropriate assay to use to detect replication competent virus, which is tested for in an in vitro assay which has much greater sensitivity for detecting even small amounts of replication competent virus in the vaccine preparation. AZD1222 is made using a platform technology utilized for other previously studied investigational vaccines and is sufficiently characterized to use toxicology data with other vaccines that use the same platform (Development and Licensure of Vaccines to Prevent COVID-19, FDA Guidance for Industry, June 2020 FDA 2020). Administration of a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein was associated with treatment related changes in the right lumbar lymph node, spleen and intramuscular injection site. The spectrum and severity of the changes were consistent with the administration of an antigenic substance such as ChAdOx1 MERS which were considered to be non-adverse. This was also true for the similar replication-defective ChAd vaccines, ChAd OX1 NP+M1 and AdCh63 MSP-1.

In conclusion, similar ChAd vaccines are well tolerated and are not associated with any adverse effects in mice. Further, similar ChAd vaccines show no evidence of replication or dissemination after IM injection in mice. AZD1222 is immunogenic in mice, ferrets, NHP and pig models inducing humoral and cellular immune responses. Vaccination with AZD1222 significantly reduced viral load following a SARS-CoV-2 challenge in rhesus macaques with no evidence of ERD.

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Janssen Vaccines & Prevention B.V. *

Nonclinical Overview

MODULE 2.4

VAC31518 (JNJ-78436735)

* Janssen Vaccines & Prevention B.V. is a Janssen pharmaceutical company of Johnson & Johnson and is hereafter referred to as the sponsor.

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LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

Ad26	adenovirus type 26
Ad26.COV2.S	Ad26 vector encoding a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike protein
Ad26.ENVA.01	Ad26 vector encoding the Clade A envelope protein of HIV type 1
Ad26.RSV.preF	Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain
Ad26.ZEBOV	recombinant, replication-incompetent, adenovirus type 26 (Ad26) vector encoding the GP of EBOV Mayinga variant
BAL	bronchoalveolar lavage
BALT	bronchial-associated lymphoid tissue
BIDMC	Beth Israel Deaconess Medical Center
CAR	coxsackievirus adenovirus receptor
CBER	Center for Biologics Evaluation and Research
CHMP	Committee for Medicinal Products for Human Use
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
DNA	deoxyribonucleic acid
E1, E3	early gene regions (of Ad26)
EF-PPND	embryo-fetal and pre- and postnatal development
EMA	European Medicines Agency
ELISA	enzyme-linked immunosorbent assay
EUA	Emergency Use Authorization
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
HIV	human immunodeficiency virus
HPV	human papillomavirus
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ICMRA	International Coalition of Medicines Regulatory Authorities
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IM	intramuscular
LLOD	lower limit of detection
LRT	lower respiratory tract
MAA	Marketing Authorization Application
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	messenger ribonucleic acid
MVA	Modified Vaccinia Virus
MVA-BN	Modified Vaccinia Ankara-Bavarian Nordic A/S vector backbone
MVA-BN-Filo	Recombinant, non-replicating in human cells, Modified Vaccinia Ankara - Bavarian Nordic (MVA-BN®) vector encoding the GP of EBOV Mayinga, SUDV Gulu, MARV Musoke, and the TAFV nucleoprotein
NHP	nonhuman primate(s)
NZW	New Zealand white
OECD	Organization for Economic Co-operation and Development
PBMCs	peripheral blood mononuclear cells

PCR	polymerase chain reaction
PDCO	Pediatric Committee
PIP	Pediatric Investigation Plan
PSP	Pediatric Study Plan
pre-F	pre-fusion conformation-stabilized F protein
PSP	Pediatric Study Plan
ppVNA (pVNA)	pseudotyped virus neutralization assay
q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-qPCR	reverse transcription followed by quantitative polymerase chain reaction
S	Spike
SARS-CoV-1	severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
sgRNA	subgenomic ribonucleic acid
$TCID_{50}$	50% tissue culture infective dose
TetO	tetracycline operon
TetR	tetracycline repressor
URT	upper respiratory tract
VAERD	vaccine-associated enhanced respiratory disease
vp	virus particles
(wt)VNA	(wild type) virus neutralization assay
VRBPAC	Vaccines and Related Biological Products Advisory Committee
WHO	World Health Organization

1. OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

1.1. Introduction

Ad26.COV2.S[1] (also known as Ad26COVS1, VAC31518, or JNJ-78436735) is a monovalent vaccine composed of a recombinant, replication-incompetent human adenovirus type 26 (Ad26) vector, constructed to encode the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike (S) protein, stabilized in its prefusion conformation. It is being developed for prophylactic immunization against coronavirus disease 2019 (COVID-19), which has spread rapidly and globally since its emergence.

Ad26.COV2.S encodes a full-length S protein, derived from a SARS-CoV-2 clinical isolate (Wuhan, 2019, whole genome sequence NC_045512), with 2 amino acid changes in the S1/S2 junction that knock out the furin cleavage site, and 2 proline substitutions in the hinge region. Prior to selection of Ad26.COV2.S for clinical development, the sponsor evaluated different vaccine constructs with design elements previously shown to be successful for other coronavirus S protein based vaccines, eg, prefusion-stabilizing substitutions and heterologous signal peptides [27,38]. In vitro characterization of these SARS-CoV-2 S protein constructs demonstrated that the introduction of stabilizing substitutions (ie, furin cleavage site mutations and two consecutive prolines in the hinge region of S2) increased the ratio of neutralizing versus non-neutralizing antibody binding, suggestive of a benefit of the prefusion conformation of the S protein for improved antigenicity. Furthermore, the wild-type signal peptide was best suited for the correct cleavage needed for a natively folded protein. The combination of the wild-type signal peptide, the furin cleavage site mutations and the proline substitutions translated into superior immunogenicity in mice [2]. In addition, several vaccine constructs were tested for immunogenicity and efficacy in NHP. The in vivo testing in NHP showed that the design of Ad26.COV2.S was optimal in inducing robust neutralizing antibody responses as well as protection following SARS-CoV-2 challenge [25]. The increase in neutralizing antibodies in mice, rabbits, hamsters and NHP, and the increase in protective efficacy in hamsters and NHP, induced by Ad26.COV2.S compared with an Ad26 vector encoding wild-type S protein (Ad26NCOV006) is shown in nonclinical studies in this document, and in the articles by Mercado et al [25], Bos et al [2], and van der Lubbe et al [35]. The selection of the S protein stabilized in its prefusion conformation was further substantiated in additional ongoing nonclinical studies [32,34].

The proposed clinical vaccine regimen consists of a single vaccination with Ad26.COV2.S at a dose level of $5x10^{10}$ virus particles (vp). The vaccine is a suspension for intramuscular (IM) injection provided in multi-dose vials. The dose volume is 0.5 mL. The vaccine composition is described in Table 1.

[1] In nonclinical studies performed prior to selection of the Ad26.COV2.S vaccine candidate, Ad26.COV2.S was named Ad26NCOV030. The name Ad26.S.PP is used in some publications.

Table 1: Ad26.COV2.S Vaccine Composition

Vaccine	Nature and Composition
Ad26.COV2.S	Adenovirus type 26 (Ad26) vectored vaccine encoding the SARS-CoV-2 S protein. Produced in the human PER.C6® TetR cell line. $5x10^{10}$ vp as a 0.5 mL single dose. Excipients: sodium chloride, citric acid monohydrate, polysorbate 80, 2 hydroxypropyl-β-cyclodextrin, ethanol, sodium hydroxide, water for injection.

This nonclinical overview covers a description of the nonclinical pharmacology testing strategy, a summary of immunogenicity and efficacy data, and an assessment of the theoretical risk of vaccine-associated enhanced respiratory disease (VAERD). In addition, this overview covers a description of the pharmacokinetic (biodistribution) and toxicology testing strategy. An integrated assessment of the biodistribution profile of the Ad26 vector, as well as the nonclinical safety profile of Ad26.COV2.S, based on an Ad26.COV2.S-specific repeat-dose and local tolerance study, and a developmental and reproductive toxicity study are presented. To further support the toxicological evaluation of Ad26.COV2.S, a high-level summary of toxicology data from other Ad26-based vaccines is provided.

Overall, Ad26.COV2.S was immunogenic and protected animals from SARS-CoV-2 infection in nonclinical studies. The available nonclinical data did not show any adverse vaccine-related effects and support the use of Ad26.COV2.S in humans. The data further suggest that the theoretical risk of VAERD with Ad26.COV2.S is low.

An overview of the nonclinical packages can be found in Table 2 (pharmacology), Table 4 (pharmacokinetics), and Table 5 (toxicology). An overview of nonclinical studies not included in the final submission dossier, with justification for their absence, is included in Appendix 1.

1.2. Pharmacology Testing Strategy

1.2.1. Study Overview and Characteristics

Primary pharmacology studies are listed in Table 2; detailed descriptions are provided in the Pharmacology Written Summary in Mod 2.6.2.

Table 2: Overview of Primary Pharmacology Studies

Test Species	Study Identifiers	Study Description	Regimen Dose Level	Study Report
mouse	9346-20004 (TV-TEC-171843)	Immunogenicity study to support vaccine candidate selection.	1-dose 10^8, 10^9, 10^{10} vp	Mod4.2.1.1/9346-20004
mouse	9346-20007 (TV-TEC-171881)	Th1/Th2 skewing study to support vaccine candidate selection.	1-dose 10^{10} vp	Mod4.2.1.1/9346-20007
NHP	NHP 20-09 (TV-TEC-175059)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose 10^{11} vp	Mod4.2.1.1/NHP 20-09
NZW rabbit	TOX14369 (TV-TEC-175060)	Immunogenicity study to support vaccine candidate selection.	2-dose $5x10^9$, $5x10^{10}$ vp	Mod4.2.1.1/TOX 14369
Syrian Hamster	TKO 707 (TV-TEC-175626)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose and 2-dose 10^9, 10^{10} vp	Mod4.2.1.1/TKO 707
Syrian Hamster	TKO 766 (TV-TEC-176250)	Immunogenicity and efficacy study (vaccine titration study) with histopathology assessment after breakthrough SARS-CoV-2 infection.	1-dose 10^7,10^8, 10^9, 10^{10} vp	Mod4.2.1.1/TKO 766
NHP	NHP correlate analysis	Across-study report, correlation of immunogenicity with protection in nonhuman primates		Mod4.2.1.1/NHP correlate analysis

NHP: nonhuman primate; NZW: New Zealand white; vp: virus particles.

The nonclinical pharmacology testing of Ad26.COV2.S was guided by the testing principles laid out by the World Health Organization (WHO) Guidelines on Nonclinical Evaluation of Vaccines [37], European Medicines Agency (EMA) Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7], Food and Drug Administration (FDA) Guidance on development and licensing of prophylactic Covid-19 vaccines [10] and FDA Guidance for Industry for emergency use authorization for vaccines to prevent COVID-19 [11]. In addition, the International Coalition of Medicines Regulatory Authorities [ICMRA] convened international regulatory workshops to discuss regulatory considerations related to the development of COVID-19 vaccine candidates, including the type and extent of nonclinical studies required to support proceeding to first-in-human clinical studies (18 March 2020 [17]), and Phase 3 clinical studies (22 June 2020 [18]). A Vaccines and Related Biological Products Advisory Committee (VRBPAC) meeting held on October 22, 2020 included a discussion of assessment and de-risking of VAERD. In these meetings it was acknowledged that there is limited understanding of the value of available nonclinical models in predicting the likelihood of VAERD in humans. However, studies in animal models were considered important to address the potential for VAERD.

The nonclinical pharmacology testing strategy has been presented in previous Scientific Advice interactions to EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Questions 5 and 9) and FDA/Center for Biologics Evaluation and Research (CBER) (PTS

5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Questions 1 and 5) in the context of the clinical development program. Data presented so far have been considered by multiple agencies as sufficient to support the clinical development including the currently ongoing phase 3 efficacy studies. The proposed nonclinical data package, including data to assess a theoretical VAERD risk, was considered overall acceptable in a meeting with EMA (Co)-Rapporteurs was held on 30 September 2020 to outline the nonclinical data package for conditional marketing authorization application (MAA). FDA feedback on 09 January 2021 stated that proposed nonclinical Emergency Use Authorization (EUA) data packages showing a low theoretical risk of VAERD are sufficient to support the review of the EUA application. An outline of procedural aspects of the nonclinical pharmacology studies is described below.

The primary pharmacology (immunogenicity and efficacy) studies were performed in countries that are part of the Organization for Economic Co-operation and Development (OECD. All of the non-GLP studies performed under Janssen Vaccines & Prevention supervision were conducted according to good research practices at facilities with suitable expertise. Janssen Preclinical QA conducted an audit of a selection of data for study NHP 20-09 conducted by the Beth Israel Deaconess Medical Center (BIDMC) and for Syrian hamster study TKO 707 conducted at [redacted] The audits focused on data traceability (retrieval, reconstruction, full disclosure, safe storage and review) and data integrity (data consistency and risk for bias). Through Janssen's assessment of the available data and study reports, several data handling and source verification gaps were noted, however the audits did not reveal any findings that compromise the integrity or interpretation of the data. Information on the Good Laboratory Practice (GLP) status of the pharmacology studies and the location of the test facilities is provided in the Pharmacology Tabulated Summary (Mod2.6.3).

Ad26.COV2.S material used in nonclinical pharmacology studies was produced firstly in a small scale laboratory process (non-Good Manufacturing Practice [non-GMP]). This research-grade material (batch P758-028A) was used in mouse studies 9346-2004 and 9346-20007, rabbit study TOX14369, Syrian hamster study TKO 707, and study NHP 20-09). Later, development-grade material generated in a large scale process similar to the 50 L process used for Phase 1/2a clinical trial material was evaluated (batch P763-004#06: lot E006614 in Syrian hamster study TKO 766 and lot E006613 in study NHP 20-14 described as part of the NHP correlates analysis report).

Data from ongoing studies

Additional studies with Ad26.COV2.S are ongoing from which interim data have been published. The data from these ongoing studies support the results presented in this submission, and are cited where appropriate. In addition, interim study data from study NHP 20-14 are presented in an across-study report of immune correlates of protection (Mod4.2.1.1/NHP correlate analysis). These ongoing studies are summarized in Table 3.

Table 3: Ongoing Pharmacology Studies With Data Referenced in the Submission

Test Species	Study Identifiers	Study Description	Regimen Dose Level	Reference
Syrian Hamster	Hamster 20-09 (TV-TEC-180196)	Immunogenicity and efficacy study to support vaccine candidate selection (high inoculum study). Lung histopathology assessment.	1-dose and 2-dose 10^9, 10^{10} vp	Tostanoski et al [34]
NHP	VH808.681 (TV-TEC-182907)	Immunogenicity and efficacy in aged NHP. Lung histopathology assessment.	1-dose and 2-dose $5x10^{10}$, 10^{11} vp	Solforosi et al [32]
NHP	CRL 2020-3373 (TV-TEC-179493)	Durability of immune response and protection (6 and 12 months after first vaccination). Includes assessment of cellular immunogenicity. Lung histopathology assessment.	1-dose and 2-dose $5x10^{10}$ vp (2-dose), 10^{11} vp (1-dose)	Solforosi et al [32][a]
NHP	NHP 20-14 (TV-TEC-176763)	Immunogenicity and efficacy study (vaccine titration study). Lung histopathology assessment.	1-dose $2x10^9$, $1.125x10^{10}$, $5x10^{10}$, 10^{11} vp	Mod4.2.1.1/NHP correlate analysis

[a] At this time, immunogenicity data up to 14 weeks post immunization are published.
NHP: nonhuman primate; vp: virus particles.

1.2.2. Animal Model Rationale

Nonclinical pharmacology of Ad26.COV2.S was evaluated in murine, rabbit, Syrian hamster, and nonhuman primate (NHP) (rhesus monkey) animal models for immunogenicity, including assessment of immunological parameters relevant to the theoretical risk of VAERD (see Section 1.2.5). Vaccine efficacy including clinical signs and lung histopathology for assessment of VAERD was evaluated in Syrian hamsters and NHP. The list of all studies intended for evaluation is provided in Table 2. All studies were performed in animals of an age range corresponding to adult age in humans. Female animals were used in the murine and rabbit studies, male animals were used in the Syrian hamster studies, and both male and female animals were used in the NHP study.

The murine model was used to comprehensively examine immunogenicity and Th1/Th2 skewing of the immune response by assessing antibody and cellular immune responses after immunization. In addition, an alternative immunogenicity model was developed in rabbits as non-rodent species and to verify rabbits as an appropriate species for toxicology studies (Section 1.5).

A SARS-CoV-2 challenge model has been developed in Syrian hamsters, which are permissive to SARS-CoV-2 replication and display widespread lung pathology and body weight loss upon infection. A nonlethal challenge model was used, applying intranasal inoculation of 10^2 $TCID_{50}$ SARS-CoV-2 strain BetaCoV/Munich/BavPat1/2020, a European isolate with glycine at position 614 of the S protein (G614). Peak viral load in the lung measured by $TCID_{50}$ assay was around day 4 with clearance from lung tissue and throat swabs of the Syrian hamsters within 7 days [35] In addition, SARS-CoV-2-induced lung pathology was also evident 4 days post inoculation, and therefore a 4 day follow-up was selected for these studies. Body weight loss is transient in Syrian hamsters after SARS-CoV-2 infection, with peak body weight loss typically at Day 6 post infection, and is a clinical sign of disease.

A SARS-CoV-2 challenge model in rhesus monkeys has also been developed by the sponsor [4]. In this challenge model, sites of viral replication are predominantly the upper and lower respiratory tract, leading to lung histopathology consistent with mild human disease. This NHP species recapitulates asymptomatic to moderate disease observed in the majority of human cases [4,26,28], and other SARS-CoV-2 vaccines with proven efficacy in phase 3 clinical trials were previously assessed in comparable rhesus monkey challenge studies [5,36]. This is a nonlethal challenge model, applying intranasal and intratracheal inoculation of $1x10^5$ 50% tissue culture infective dose ($TCID_{50}$) SARS-CoV-2 strain USA-WA1/2020, a US isolate with aspartic acid at position 614 of the S protein (D614). In this SARS-CoV-2 challenge model, rhesus monkeys showed modestly decreased appetite and responsiveness suggestive of mild clinical signs of disease; fever, weight loss, and respiratory distress were not observed [4]. Lower levels of virus were found in the gastrointestinal tract, liver, and kidney. In nonvaccinated animals, peak viral load occurred approximately 2 days after inoculation, and viral clearance in the lower and upper respiratory tract was observed within approximately 2 weeks of inoculation [4 and study NHP 20-09]. This is very similar to the kinetics of SARS-CoV-2 clearance in humans [3]. A comparable SARS-CoV-2 challenge model has been developed by the sponsor in aged rhesus monkeys using a G614 virus variant that showed a significant rise in body temperature after virus inoculation and is reported in Solforosi et al [32].

In addition to the efficacy model, the high level of genetic homology between NHP and humans, and their comparative immunology have made NHP the animal model of choice for studies of vaccine immunogenicity [14,19].

1.2.3. Vaccine Immunogenicity

Humoral immune responses were measured in mice, rabbits, Syrian hamsters, and NHP. Based on experience with SARS-CoV, and in recent SARS-CoV-2 vaccine efficacy studies in NHP, binding and neutralizing antibody responses have been identified as the predominant correlates of protection [24,34,39], and the correlation has also been confirmed for Ad26.COV2.S (NHP correlate analysis, and van der Lubbe et al [35]). Neutralizing antibody titers were determined in a virus neutralization assay (wild-type [wt]VNA) with live SARS-CoV-2, or in a pseudotyped virus neutralization assay (ppVNA [pVNA]). In addition, binding antibody titers to the full length ectodomain of the S protein were determined by enzyme-linked immunosorbent assay (ELISA).

Cellular immune responses were measured in mice, rabbits, and NHP. SARS-CoV-2 specific cellular responses were determined by measuring cytokine production after ex vivo stimulation of splenocytes or peripheral blood mononuclear cells (PBMCs) with peptide pools covering the complete SARS-CoV-2 S wild type protein sequence. Cytokine production was determined by interferon gamma (IFN-γ) ELISpot.

While innate responses may contribute to protection early after vaccination, they are not considered to be a major aspect of the protective effect of the vaccine and were therefore not characterized.

1.2.4. Vaccine Efficacy

In the Syrian hamster challenge studies, the main efficacy readouts were SARS-CoV-2 viral load in throat swab samples taken daily after inoculation and in lung and nasal turbinate tissue samples collected at day 4 after inoculation. Infectious viral load, ie, the presence of replication competent virus, was measured by $TCID_{50}$ assay, in which samples were titrated on confluent monolayers of Vero E6 cells and the viral titers were calculated. Additionally, body weight loss was monitored daily during a 4 day follow up period.

In the NHP challenge study, the main efficacy readouts were viral load in bronchoalveolar lavage (BAL) and nasal swab samples. The BAL and nasal swab viral loads were determined at 0, 1, 2, 4, 7, 10 and 14 days post inoculation for each NHP, measured by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) of SARS-CoV-2 E gene subgenomic ribonucleic acid (sgRNA).

1.2.5. Assessment of VAERD

There is a theoretical risk of VAERD for SARS-CoV-2 vaccines, based on nonclinical experience with SARS-CoV and MERS-CoV based vaccines. It is acknowledged that there is limited understanding of the value of available nonclinical models in predicting the risk of VAERD in humans. However, studies in animal models are considered important to address the potential for VAERD, in combination with clinical assessments. To the sponsor's knowledge, VAERD for SARS-CoV-2 vaccines has not been observed in nonclinical models or in human efficacy studies. Actions taken to assess the potential risk of VAERD included lung histopathology assessment in Ad26.COV2.S immunized Syrian hamsters and NHP after SARS-CoV-2 challenge in comparison with unvaccinated and SARS-CoV-2 infected control animals. In 1 Syrian hamster study (TKO 766) lower dose levels of Ad26.COV2.S were included, which induced a suboptimal humoral immune response allowing breakthrough viral replication in the lungs after SARS-CoV-2 challenge, conditions which are hypothesized to contribute to a risk of VAERD. Increased clinical signs of disease and viral load are thought to be evidence for an increased risk of VAERD, therefore these factors have been assessed. In addition, immunogenicity was assessed to show induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to minimize the potential risk of VAERD.

The induction of neutralizing antibodies was assessed in each nonclinical pharmacology study. A study to assess the Th skewing of the immune response induced by Ad26.COV2.S was performed

in BALB/c mice (study 9346-20007). For this purpose, a comparison group received recombinant S protein in aluminum phosphate ($AlPO_4$) adjuvant, which is associated with a Th2 skewed immune response. Immunologic readouts for Th1/Th2 skewing included IgG subclass binding antibody titers, and cytokine production measured by ELISpot, Multiplex ELISA, and intracellular cytokine staining (ICS).

To assess signs for general vaccine associated enhanced disease (VAED), viral load and signs of clinical disease have been monitored in Syrian hamsters and in NHP during the post inoculation follow-up. At the end of follow-up, a histopathology evaluation was conducted in all efficacy studies, and for Syrian hamsters, a gross pathology examination and immunohistochemistry analysis of selected respiratory tissues for the presence of SARS-CoV-2 nucleoprotein, was performed. Selected respiratory tract tissues were evaluated and assessed (semi-quantitatively graded/scored) for disease-associated pathology findings, such as the severity and extent of alveolar, bronchial, bronchiolar, or tracheal inflammation and/or inflammatory infiltrates, evidence of alveolar edema, or any other degenerative inflammatory changes.

1.3. Safety Pharmacology Testing Strategy

Safety pharmacology studies have not been performed with Ad26.COV2.S, since data (e.g., detailed clinical observations) from repeat-dose toxicity studies with Ad26.COV2.S (see Section 4.1.1) and other Ad26-based vaccines (see Section 4.1.2) did not suggest that these vaccines have a significant impact on physiological functions (e.g., central nervous system, respiratory, and cardiovascular functions) other than those of the immune system. Therefore, and in line with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], stand-alone safety pharmacology studies are not deemed necessary.

1.4. Pharmacokinetic Testing Strategy

In accordance with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], pharmacokinetic studies are usually not needed for vaccines. However, in line with the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7] and the FDA Guidance on considerations for plasmid DNA vaccines for infectious disease indications [9], biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 viral vector platform following IM injection. The biodistribution profile of the Ad26 vaccine platform has been evaluated in rabbits using an Ad26-based human immunodeficiency virus (HIV) vaccine, i.e., Ad26.ENVA.01 (Ad26 vector encoding the Clade A envelope protein of HIV type 1), and an Ad26-based respiratory syncytial virus (RSV) vaccine, i.e., Ad26.RSV.preF (Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain) (see Table 4).

As explained in Section 3, these vector platform data are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S, for which the same Ad26 vector backbone is used. This position has been confirmed and agreed by the Agency in a Scientific Advice by EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Question 6) and CBER (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Question 2). It is further noted that the same platform biodistribution data were part of the MAA

file for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001), which was approved in 2020. Hence, no pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S specifically.

Table 4: Overview of Biodistribution Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine, Route, Interval, and Dose Level	GLP	Study Report
NZW rabbit	Single dose biodistribution study with a 91-day observation period	Ad26.ENVA.01, IM, single dose, 5×10^{10} vp	Yes	Mod4.2.2.3/ 1645-06074
NZW rabbit	Single dose biodistribution study with a 180-day observation period	Ad26.RSV.preF, IM, single dose, 1×10^{11} vp	Yes	Mod4.2.2.3/ TOX13342

GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

The biodistribution studies with Ad26.ENVA.01 and Ad26.RSV.preF were conducted using the IM route, which is also the intended route for use of Ad26.COV2.S in humans.

The studies were done in rabbits as this is a widely accepted species to assess the nonclinical safety of vaccines. In available nonclinical (safety) studies, Ad26-based vaccines (including Ad26.COV2.S, Ad26.ENVA.01 and Ad26.RSV.preF) were shown to elicit immune responses against the vaccine antigen in the animals, indicating the rabbit as a relevant nonclinical species [33]. In addition, the rabbits have sufficient muscle mass to receive a full human vaccine dose via the IM route with a single injection. The use of one species is generally considered sufficient to assess the nonclinical safety/biodistribution profile of vaccines [7,37].

The dose levels applied in the biodistribution studies for Ad26.ENVA.01 and Ad26.RSV.preF were similar to, or above the human dose level for Ad26.COV2.S ($5x10^{10}$ vp), hence full human dose levels were tested. Specific quantitative polymerase chain reaction (q-PCR) assays were used to detect and quantify Ad26-vector DNA in various tissues, including the gonads, brain and blood collected at specified time points following vector administration.

The sampling timepoints used in the biodistribution studies with the Ad26 vectors (i.e., Days 11, 61, 91 post-vaccination for Ad26.ENVA.01, and Days 11, 90, 120 or 180 post-vaccination for Ad26.RSV.preF) are in line with biodistribution studies conducted with adenovirus type 5 (Ad5) and type 35 (Ad35) based vaccines as reported by Sheets et al. 2008 [30]. The sampling timepoints for the biodistribution studies were selected to allow sufficient time to confirm clearance (i.e., confirming a downward trend over time) of the Ad26 vector.

The biodistribution studies (with Ad26.ENVA.01 and Ad26.RSV.preF) were conducted as standalone studies (i.e., not integrated in a pivotal nonclinical safety study). For such pharmacokinetics studies, GLP compliance is not generally required. Nevertheless, the biodistribution studies for the Ad26 vector platform were conducted in compliance with GLP.

Nonclinical shedding studies were not conducted for the Ad26 vaccine platform. The risk for shedding of Ad26.COV2.S will be based on clinical shedding data for the Ad26 vaccine platform and is discussed briefly in Section 3.

1.5. Toxicology Testing Strategy

The nonclinical safety profile of the Ad26.COV2.S vaccine has been assessed in two pivotal toxicology studies in New Zealand white (NZW) rabbits, i.e., a combined repeat-dose toxicity and local tolerance study, and a combined embryo-fetal and pre- and postnatal development (EF-PPND) toxicity study. The testing is consistent with applicable guidelines, including the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7], the ICH-S5 Guideline on Detection of Reproductive and Developmental Toxicity for Human Pharmaceuticals [16], and the FDA Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications [8]. An overview of these toxicology studies is provided in Table 5.

In addition, the Sponsor has significant nonclinical experience with Ad26-based vaccines using various gene inserts encoding for HIV, malaria, RSV, Zika virus, Filovirus (Ebolavirus, Marburgvirus), influenza and human papillomavirus (HPV) antigens. More than 10 GLP combined repeat-dose toxicity and local tolerance studies as well as one EF-PPND toxicity study (with the Ebola vaccine component Zabdeno [Ad26.ZEBOV-GP [recombinant]]) have been performed in rabbits (or rats), testing the nonclinical safety of these various Ad26-based vaccines. A high-level summary of these supportive data is given in Sections 4.1.2 and 4.2.2 and in Appendix 2.

In the two pivotal GLP toxicology studies (TOX14382 and TOX14389), 3 doses of Ad26.COV2.S at $1x10^{11}$ vp/dose were administered IM with a 14-day interval period between the injections. The vaccine dose, the applied dose volume (1 mL) and the number of doses (3) administered in the toxicology studies cover the proposed clinical regimen of the vaccine ($5x10^{10}$ vp, administered as a single dose in 0.5 mL). The IM route is the intended route for use in humans.

The use of one species is considered sufficient to assess the nonclinical safety profile of a vaccine [37]. A relevant animal is defined as a species capable of mounting an immune response to the vaccine antigen [33]. The rabbit was considered a relevant toxicological species to assess the nonclinical safety profile of the Ad26.COV2.S vaccine since it was shown to elicit specific immune responses against the SARS-CoV-2 S protein (see Sections 2.3, 4.1.1 and 4.2.1). The use of the rabbit is further supported by the fact that it is a species that is widely used to assess preclinical toxicity and local tolerance of vaccine candidates, and for which sufficient historical control data exist. In addition, in contrast to rodents (e.g., rats, mice), rabbits have sufficient muscle mass to receive a full human dose via the IM route, which increases the relevance of this species to assess local tolerance of vaccines.

Table 5: Overview of Toxicology Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine, Route, Interval, and Dose Level	GLP	Study Report
NZW rabbit	Combined repeat-dose toxicity and local tolerance study	3 injections (Days 1, 15, 29; 2-week interval between doses), IM • Saline • $1x10^{11}$ vp	Yes	Mod4.2.3.2/ TOX14382
NZW rabbit	Combined embryo-fetal and pre- and postnatal development study	3 injections (Day 1[a], GD6 and GD20; 2-week interval between doses), IM • Saline[b] • $1x10^{11}$ vp[b]	Yes	Mod4.2.3.5.2/ TOX14389

[a] Day 1 = 7 days prior to pairing
[b] The study includes 2 subgroups: 1 group consisting of females that are necropsied on GD29 and have a uterine and fetal examination (external, visceral, and skeletal exams), and 1 group consisting of females that are allowed to give birth and in which the survival and development of the kits is evaluated through lactation day 28
GD: gestation day; GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

The repeat-dose toxicity (including local tolerance) and EF-PPND toxicity studies were conducted with the Phase 1/2a clinical Ad26.COV2.S material which is representative for the material that is used in the (Phase 3) clinical studies, as well as for commercial batches (refer to respective quality sections 3.2.S.2.6-Manufacturing Process Development-Comparability and 3.2.P.2.3–Manufacturing Process Development-Comparability). Actual and potential (process-related) impurities present in the Ad26.COV2.S drug product have been toxicologically assessed and do not raise a safety concern at the maximum anticipated levels per vaccine dose (refer to respective quality sections 3.2.S.2.6 - Impurity Criticality Evaluation).

The toxicology studies were conducted in conformance with GLP, 21 CFR, Part 58 and/or OECD Principles of GLP in a country that is part of the OECD Mutual Acceptance of Data Process and include the appropriate documentation. The immunogenicity assessment which was included in the toxicology studies was not conducted in compliance with GLP but was conducted and documented by qualified staff in accordance with good scientific practices and standard operating procedures. Since these data are not generally considered a pivotal safety endpoint, but are mainly included as a pharmacodynamic endpoint to confirm responsiveness of the test species to the vaccine administration, this GLP exception does not affect the quality and integrity of the study (conclusions), and overall GLP status of the study.

The local tolerance of the IM administered Ad26.COV2.S vaccine was evaluated as part of the pivotal toxicity studies (i.e., reactions at the injection site were assessed by Draize scoring in the repeat-dose and EF-PPND toxicity studies and by histopathological evaluation in the repeat-dose toxicity study). Therefore, no standalone local tolerance study was conducted. Also, a separate study to determine single dose toxicity was not performed. Possible signs of acute toxicity were monitored following the first vaccination in the repeat-dose toxicity study.

In accordance with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], genotoxicity and carcinogenicity studies have not been performed. Also, the risk for genotoxicity and carcinogenicity is deemed low since adenoviral vectors are classified as non-integrating because they lack the machinery to integrate their genome into the host chromosomes (eg., *"EMA Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors,*

2006" [6]; *"Long Term Follow-Up After Administration of Human Gene Therapy Products, 2020" [12]*). As such, upon transduction of a cell, the adenoviral DNA does not integrate into the host genome, but rather resides episomally in the host nucleus. Such episomal transduction reduces the risk of insertional mutagenesis [13,21].

Dedicated fertility studies are not routinely required for vaccines and have not been performed because histopathology data from the (repeat-dose) toxicity study are considered to provide sufficient relevant information concerning a possible impact of the vaccine regimen on the integrity of the reproductive organs [33]. Histopathology data from the Ad26.COV2.S repeat-dose toxicity study (see Section 4.1.1) did not reveal any effects on male or female sex organs that would impair male or female fertility. Also, the histopathology data from the available repeat-dose GLP toxicity studies for the Ad26 platform in rabbits (see Section 4.1.2) do not raise any concerns that Ad26-based vaccines adversely affect male or female reproductive organs. This is further supported by results from the available biodistribution studies (see Section 3 and Mod2.6.4), showing that the Ad26 vector does not distribute to the gonads (testes, ovaries) following IM injection. An assessment of female fertility was included in the EF-PPND toxicity study with first vaccination in the premating period (study TOX14389). Based on the above, further dedicated male or female fertility studies are not deemed necessary. This position was confirmed by CHMP (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, CHMP Response to Sponsor Question 8) and CBER (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Question 4).

Studies in juvenile animals were not performed because the repeat-dose toxicity and local tolerance study and the combined EF-PPND toxicity study are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children from birth onwards (see Section 4.3 for discussion). During the assessment procedure of the Pediatric Investigation Plan (PIP) and the Pediatric Study Plan (PSP), the Pediatric Committee (PDCO) and CBER, respectively, confirmed that no further nonclinical studies were deemed necessary to support the pediatric development. Final approval of both documents is pending.

Neurovirulence testing was not done since (1) Ad26.COV2.S is replication-incompetent, (2) distribution into the brain was not seen for the Ad26 vector following IM injection as explained in Section 3, and (3) Ad26.COV2.S, as well as other Ad26-based vaccines, did not show any adverse effects on nervous tissues in the available repeat-dose toxicity studies (see Mod2.6.6/Sec3 and 5.2).

The nonclinical safety package presented for Ad26.COV2.S (i.e., Ad26.COV2.S specific repeat-dose toxicity, and developmental and reproductive toxicity studies, as well as biodistribution studies covering the Ad26 vector) is similar to the package that was submitted and approved (in 2020) under the MAA for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001).

2. PHARMACOLOGY

2.1. Mechanism of Action

Induction of protective immunity by Ad26.COV2.S is thought to occur through antibody responses, predominantly a neutralizing antibody response, against the S protein. The role of cellular immune responses, particularly during a severe course of infection and for the clearance of virus-infected cells, is currently unclear.

The S protein on the surface of SARS-CoV-2 binds to the ACE2 receptor of a host cell, allowing the virus to infect the cell. Vaccination with Ad26.COV2.S leads to humoral and cellular immune responses directed against the S protein. The production of neutralizing and other functional S-specific antibodies may block ACE2 receptor binding to the S protein, thereby inhibiting viral entry into host cells, and mediate cellular effector mechanisms via Fc function, leading to clearance of SARS-CoV-2 virus particles and infected cells. Cellular immune responses may further contribute to protection by clearing SARS-CoV-2 infected cells via cytotoxic mechanisms.

Although unable to replicate in normal cells, Ad26.COV2.S can be produced in the laboratory on genetically engineered cell lines (eg, human PER.C6® and PER.C6 tetracycline repressor [TetR] cell lines) that complement for the missing E1 region. Since the genes needed for replication are lacking in normal human cells and these genes do not naturally occur in the human genome, this precludes replication of Ad26.COV2.S following IM administration to humans. Upon IM administration of Ad26.COV2.S, binding of the Ad26 vector to cellular receptors is mediated by fibers on the capsid. Currently suggested receptors on the host cell are cluster of differentiation (CD)46, the coxsackievirus adenovirus receptor (CAR), desmoglein 2, and cell surface sialic acid bearing glycans [1,22]. After transduction of the cell, vector DNA enters the nucleus without integrating into the genome, and is driving cellular production of the S protein. The S protein is then presented on the cell membrane to the host immune system and stimulates an immune response in local lymphoid organs by presentation on antigen presenting cells.

2.2. Study Rationale

The nonclinical pharmacology studies are listed in Table 2. All studies with the exception of Syrian hamster study TKO 766 were performed to inform on vaccine candidate selection for clinical development. The murine studies and rabbit study tested the immunogenicity of the vaccine, and murine study 9346-20007 was specifically designed to determine the Th1/Th2 balance induced by a single dose of vaccine. The Th1/Th2 balance has been linked with the theoretical risk of VAERD. Study NHP 20-09 was performed to assess the immunogenicity and protective efficacy of a single vaccine dose, testing different vaccine candidates including Ad26.COV2.S. Study TKO 707 in Syrian hamsters was performed to assess immunogenicity and protective efficacy of different vaccine candidates, in both 1-dose and 2-dose regimens.

After selection of Ad26.COV2.S for clinical development, Syrian hamster study TKO 766 was performed with lower dose levels of Ad26.COV2.S, which intentionally induced a suboptimal humoral response allowing breakthrough viral replication in the lungs after SARS-CoV-2 inoculation, conditions which are hypothesized to contribute to a risk of VAERD.

Additional studies are ongoing where interim data has been or will be published in support of Ad26.COV2.S development, or interim data is presented. These studies are listed in Table 3: Ongoing Pharmacology Studies With Data Referenced in the Submission.

2.3. Immunogenicity and Protective Efficacy of Ad26.COV2.S

Murine study 9346-20004 (Mod2.6.2/Sec2.3.1.1) demonstrated the humoral (binding and neutralizing antibody) and cellular immunogenicity of a single dose of Ad26.COV2.S, and also showed the benefit of the stabilizing mutations in increasing humoral immunogenicity compared with an Ad26 vector encoding the wild type S protein.

Murine study 9346-20007 (Mod2.6.2/Sec2.3.1.2) was designed to determine the Th1/Th2 balance induced by a single dose of Ad26.COV2.S. In this study, Ad26.COV2.S was shown to skew towards a Th1 skewed response. This was measured by the ratio of the Th1 associated cytokine IFN-γ, over the Th2 associated cytokines interleukin-4 (IL-4), IL-5 and IL-10, as well as by the ratio of anti-S protein immunoglobulin G (IgG) antibody subclasses IgG2a and IgG1.

Study TOX14369 in New Zealand White rabbits (Mod2.6.2/Sec2.3.2) demonstrated humoral (binding and neutralizing antibody) and cellular immunogenicity of a single dose of Ad26.COV2.S, and showed that binding and neutralizing antibody titers and cellular immune responses were further increased after a second dose of Ad26.COV2.S. This study also showed the benefit of the stabilizing mutations in increasing humoral immunogenicity compared with an Ad26 vector encoding the wild type S protein.

Syrian hamster study TKO 707 (Mod2.6.2/Sec2.3.3.1) assessed the immunogenicity, efficacy, and histopathology of 2 dose levels (10^9 and 10^{10} vp) of Ad26.COV2.S in a 1-dose regimen, and in a 2-dose regimen with a 4-week interval. A single dose of Ad26.COV2.S was immunogenic, and a second dose of Ad26.COV2.S further increased binding and neutralizing antibody titers. Ad26.COV2.S given in 1-dose and 2-dose regimens resulted in a significantly lower infectious viral load in lung tissue samples after intranasal SARS-CoV-2 challenge compared with Ad26.Empty mock-immunized animals. Overall, the majority of animals immunized with Ad26.COV2.S showed undetectable infectious viral load in the lungs. Ad26.COV2.S induced significantly higher antibody titers than the Ad26 vector encoding the wild type S protein, and a significant difference in infectious viral load in the lung was observed after challenge in the single dose regimen.

Syrian hamster study TKO 766 (Mod2.6.2/Sec2.3.3.2) assessed a single dose immunization regimen and included also lower dose levels of Ad26.COV2.S (dose level range 10^7 to 10^{10} vp). Ad26.COV2.S induced dose level-dependent S protein binding and SARS-CoV-2 neutralizing antibody titers. The study confirmed robust protective efficacy of Ad26.COV2.S against SARS-CoV-2 challenge at high vaccine doses, which is consistent with data from study TKO 707, while lower vaccine dose levels resulted in dose-dependent reduction of efficacy and partial breakthrough infection.

Study NHP 20-09 in rhesus monkeys (Mod2.6.2/Sec2.3.4) was designed to assess the immunogenicity and efficacy of a single dose of $1x10^{11}$ vp Ad26.COV2.S, one of the regimens in clinical studies VAC31518COV1001 and VAC31518COV2001. A single dose of Ad26.COV2.S induced binding and neutralizing antibodies. The NHP were inoculated with SARS-CoV-2 6 weeks after vaccination. In the group immunized with Ad26.COV2.S, viral load in the lower respiratory tract (ie, BAL) was below the lower limit of detection (LLOD) for all NHP (N=6) at all timepoints. Viral load in the upper respiratory tract (ie, nasal swabs) was below the LLOD in 5 out of 6 NHP immunized with Ad26.COV2.S; 1 animal had low viral load detectable at days 1 and 2 post inoculation. In contrast, all sham immunized NHP had high viral loads in the lower respiratory tract (LRT) and upper respiratory tract (URT) that persisted up to 14 days post inoculation .

In these studies, immune responses including neutralizing antibody responses were induced as early as 2 weeks post immunization. In addition, a favorable immunogenicity profile was observed in the nonclinical studies, ie, the induction of neutralizing antibodies and a Th1 skewed immune response. In both Syrian hamsters and NHP SARS-CoV-2 specific binding and neutralizing antibodies significantly correlate with protection from infection with SARS-CoV-2 (NHP correlate analysis and van der Lubbe et al [35]).

Similar results have been observed in ongoing studies. The increase in neutralizing antibody titers after a second dose of Ad26.COV2.S and the Th1 skewing of the immune response has been confirmed in NHP [32]. Antibody titers persisted for at least 14 weeks after a single dose of $5x10^{10}$ vp or $1x10^{11}$ vp [32]. The protective efficacy of Ad26.COV2.S in Syrian hamsters has been demonstrated in an additional, more stringent SARS-CoV-2 challenge model using a D614 variant of the virus [34], complementing current data with a G614 SARS-CoV-2 variant (studies TKO707 and TKO766). Interim data from an ongoing study in aged NHP showed that a single dose of $1x10^{11}$ vp Ad26.COV2.S afforded protection from challenge with $1x10^{5}$ $TCID_{50}$ of the G614 variant strain SARS-CoV-2/human/NLD/Leiden-0008/2020 three months post single dose immunization [32]. In the ongoing dose level titration study NHP 20-14, dose levels of $1x10^{11}$ vp and $5x10^{10}$ vp Ad26.COV2.S afforded complete protection from viral load in the lung, and lower dose levels of $1.125x10^{10}$ vp and $2x10^{9}$ vp afforded protection in 4 out of 5 animals (Mod2.6.2/Sec2.4).

2.4. VAERD Risk Monitoring

Nonclinical data have shown the induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to minimize potential risk of VAERD (Section 2.3). VAERD was also monitored in nonclinical efficacy studies by assessment of histopathology in respiratory tract tissues taken after SARS-CoV-2 inoculation and by clinical monitoring of infected animals.

In Syrian hamster studies TKO 707 (Mod2.6.2/Sec2.3.3.1) and TKO 766 (Mod2.6.2/Sec2.3.3.2) the following histopathological parameters were assessed: alveolitis, extent of alveolitis/alveolar damage, alveolar edema, alveolar hemorrhage, type II pneumocyte hyperplasia, bronchitis, bronchiolitis, peribronchial and perivascular cuffing, tracheitis and rhinitis. Study TKO 766 included lower dose levels of Ad26.COV2.S (dose level range 10^{7} to 10^{10} vp), which resulted in

dose-dependent reduction in protective efficacy against SARS-CoV-2 challenge and breakthrough infection in the lung. In these studies, there was no evidence of VAERD, including Syrian hamsters with breakthrough infection in the lung (total 28 out of 56 immunized hamsters).

In study NHP 20-09 (Mod2.6.2/Sec2.3.4) the following LRT histopathological parameters were assessed in Ad26.COV2.S immunized and sham-treated animals after SARS-CoV-2 challenge: alveolar edema, inflammation interstitial/septal thickening, mononuclear cell infiltrates in perivascular/ peribronchiolar space, macrophage infiltrates in alveolar space, macrophage infiltrates in bronchiolar space, neutrophil infiltrates in alveoli, bronchioloalveolar hyperplasia, bronchial-associated lymphoid tissue (BALT) hyperplasia. No signs of VAERD were observed in this NHP challenge study, either when assessing the cumulative histopathology score or any individual score compared with the unvaccinated control group. The histopathology scores were reduced in Ad26.COV2.S immunized animals, when compared with the mock immunized animals. Conclusions on viral load are included in Section 2.3. In this study, breakthrough infection in the lung was not detected in Ad26.COV2.S immunized NHP.

The Th1 skewing of the immune response has been confirmed in interim data from an ongoing study in NHP [32]. Moreover, interim data from an ongoing studies in Syrian hamsters and in (aged) NHP showed that Ad26.COV2.S afforded protection from challenge with SARS-CoV-2, with no histopathologic evidence of VAERD [32,34].

We have shown in Ad26.COV2.S immunized Syrian hamsters and NHP absence of enhanced lung pathology and clinical signs of disease compared with controls after SARS-CoV-2 inoculation, even under suboptimal immunity allowing breakthrough infection. Together with induction of neutralizing antibodies and a Th1 skewed immune response after Ad26.COV2.S dosing these data suggest that the theoretical risk of VAERD and VAED for Ad26.COV2.S is low.

3. PHARMACOKINETICS

Biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 vaccine platform following IM injection in NZW rabbits using Ad26.ENVA.01 and Ad26.RSV.preF. No pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S.

The Ad26 vector contains deletions in the early region (E1) of the Ad26 genome, rendering it replication-incompetent. Ad26-based vaccines, including Ad26.COV2.S, require recombinant E1 complementing cell lines, like the PER.C6 (TetR) cells, for virus replication. Outside of these specific cellular environments, Ad26-based vaccines, including Ad26.COV2.S, cannot replicate or reproduce and are therefore expected to show a limited distribution and persistence following administration. This is confirmed by the biodistribution studies with Ad26.ENVA.01 (study 1645-06074; Mod2.6.4/Sec4.1) and Ad26.RSV.preF (study TOX13342; Mod2.6.4/Sec4.2). In these studies, animals were sacrificed on Days 11, 61, or 91 (Ad26.ENVA.01), and on Days 11, 90, 120 or 180 (Ad26.RSV.preF) following single IM injection at a dose level of 5×10^{10} vp (Ad26.ENVA.01) or 1×10^{11} vp (Ad26.RSV.preF). Tissues from these animals were harvested for analysis of Ad26 vector DNA using q-PCR. As a general pattern, both Ad26 vectors showed a

limited biodistribution profile following IM administration, as they were primarily detected at the site of injection, regional (iliac) lymph nodes and (to a lesser extent) the spleen. No Ad26 vector DNA was detected in the gonads or in the brain. Comparing the various necropsy timepoints following IM administration (i.e., Days 11, 61, and 91 for Ad26.ENVA.01; Days 11, 90, 120 and 180 for Ad26.RSV.preF), a clear downward trend in the number of positive tissues, and/or vector copy number present in those positive tissues was observed, to levels close to, or below the respective limits of detection towards the end of the observation period, indicating clearance of the Ad26 vector. The data further indicate that the Ad26 vector does not replicate and/or persist in the tissues following IM injection. Despite differences in the expressed transgene insert, both Ad26 vectors showed a similar pattern of biodistribution and clearance when delivered via the IM route at full human dose levels in the rabbit. This pattern of clearance and (limited) distribution as observed for the Ad26 vector is in line with results from other (replication-incompetent) adenoviral vectors, including Ad5 and Ad35 [30].

The Ad26 vector backbone used for Ad26.COV2.S is identical to the vector backbone of the Ad26-based vaccines that were tested in the available biodistribution studies (i.e., Ad26.ENVA.01 and Ad26.RSV.preF). The only difference between the vectors, apart from the encoded antigen transgene, is the insertion of a tetracycline operon (TetO) motif in the cytomegalovirus promoter sequence of the transgene expression cassette of Ad26.COV2.S. The TetO motif was included in the Ad26 vector to improve vector bioreactor yields, as described in Mod3.2.S.2.3 Control of Materials – Source, History, and Generation of the Pre-Master Virus Seed.

The insertion of the TetO sequence in the transgene expression cassette is not considered to impact the biodistribution profile of the Ad26 vector. Adenoviruses are non-enveloped viruses whose cell entry, and therefore tropism, is dictated via interactions of structural capsid proteins (mainly the fiber and penton base) with specific cellular receptors [29]. The adenoviral capsid is a highly complex and organized structure [23] which does not easily allow for the introduction or exchange of other proteins. The transgene expression cassette itself, which is inserted into the site where the early E1 gene was previously located, is thus not considered to impact on the formation or the composition of the Ad26 vector capsid, and hence tropism of the vector. As a consequence, the biodistribution profile of the Ad26 vector is considered independent of the transgene/expression cassette, which is supported by the comparable biodistribution profile observed for Ad26.ENVA.01 and Ad26.RSV.preF. This is in line with a study from Sheets et al, 2008 [30], which concluded that the biodistribution profile for Ad5 and Ad35 vectors was consistent, regardless of differences in transgene inserts.

As described above (see Section 1.5), adenoviral vectors are classified as non-integrating because they lack the machinery to integrate their genome into the host chromosomes (e.g., EMA Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors [6]; FDA Guidance for Industry, Long Term Follow-Up After Administration of Human Gene Therapy Products [12]). As such, upon transduction of a cell, the adenoviral DNA does not integrate into the host genome, but rather resides episomally in the host nucleus [13,21]. Therefore, and also supported by the results from the biodistribution studies where clearance of the vector was shown, the risk of integration of genetic material from an Ad26 vector in the human host genome and

potentially associated delayed/long-term adverse events is unlikely. In fact, clinical data from long-term follow-up observations of subjects who have received adenovirus (gene-therapy) products in trials conducted over the last years, further support the assessment of low risk for delayed/long term side effects of these products [12].

Ad26 vectors show limited shedding following intramuscular injection in clinical trials. Shedding studies with Ad26.RSV.preF, Ad26.ZEBOV and Ad26.Mos4.HIV showed that Ad26 vector DNA is rarely found and disappears quickly over time in secreted body fluids after vaccination. No replication-competent virus could be detected in samples positive for vector DNA (Environmental Risk Assessment).

In a human shedding study performed with Ad26.RSV.preF (study VAC18193RSV1005), vector DNA was observed in a single semen specimen at a single timepoint (Day 8 post vaccination), but all subsequent samples up to 183 days post vaccination (i.e., covering 2 sperm cycles) from this subject were negative for vector DNA (Environmental Risk Assessment). This, together with the results from the nonclinical biodistribution studies described above, in which no Ad26 vector DNA was detected in the gonads (i.e., testes and ovary), indicates that it is unlikely that the Day 8 positive semen sample represents association of the Ad26.RSV.preF vector with the sperm cells, and that the Ad26 vector does not persist in the semen. In addition, no infectious virus was detected in the culture from this sample, indicating that no replication of vaccine vector or adenovirus was found in this single semen sample. Overall, considering the non-integrating characteristic of adenoviral vectors, together with the fact that the nonclinical biodistribution studies did not show any dissemination/persistence of the Ad26 vector in the male and female gonads, there is no need to conduct dedicated nonclinical germline transmission studies.

Dissemination of the Ad26 vector to breast milk or to/across the placenta has not been specifically assessed in the available nonclinical biodistribution studies. However, considering the pattern of limited biodistribution (as summarized above), and in line with the limited shedding as observed in clinical studies (Environmental Risk Assessment), Ad26.COV2.S is unlikely to be excreted in breastmilk. Even if a small quantity of the vector would be (transiently) excreted via the milk, it would not be considered a risk to lactating newborns, specifically with regard to infections, as Ad26.COV2.S is replication-incompetent and does not encode a complete SARS-CoV-2 virus. In addition, Ad26.COV2.S is unlikely to be disseminated to the placenta considering its non-replicating characteristic, and consistent with the limited distribution as observed following IM injection. In line with the above, even if a small quantity of the (non-replicating) vector would be (transiently) disseminated to/across the placenta, it would not be considered a risk for the growing fetus. It is further noted that the pivotal combined EF-PPND toxicity study in rabbits (study TOX14389, see Section 4.2.1) did not indicate any signs of reproductive toxicity following maternal immunization with Ad26.COV2.S during pregnancy.

In conclusion, the biodistribution data obtained with Ad26.ENVA.01 and Ad26.RSV.preF are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same route of administration (IM) and at a comparable dose level ($5x10^{10}$ vp to $1x10^{11}$ vp). The results from these studies show a pattern of limited distribution and indicate clearance over

time of the Ad26 vector following IM injection. The data do not indicate any dissemination/persistence of the Ad26 vector to or in the male and female gonads of the rabbits.

4. TOXICOLOGY

The nonclinical safety profile of the Ad26.COV2.S vaccine was assessed in a pivotal repeat-dose toxicity study, including local tolerance, as well as in a combined EF-PPND toxicity study. In addition, a high-level summary of supportive toxicology data with other Ad26-based vaccines using various antigen transgenes is described.

4.1. General Toxicity and Local Tolerance

4.1.1. Ad26.COV2.S

In the pivotal repeat-dose toxicity and local tolerance study in rabbits (study TOX14382; Mod2.6.6/Sec 3), Ad26.COV2.S was well tolerated when administered on three occasions over 4 weeks (i.e., every 2 weeks) at 1 x 10^{11} vp/dose. The observed changes were related to a normal, anticipated (local and systemic) immunologic response to vaccination, and consisted clinically of (rare) transient local injection site dermal reactions, with transient minimal hyperthermia and minimal body weight loss or lower body weight gain after injection. This was associated with a transient (acute phase/immune) response in clinical pathology parameters, characterized by increases in plasma proteins (C-reactive protein [CRP], fibrinogen and globulins), and white blood cell counts (monocytes and lymphocytes). Microscopic pathology findings of minimal to slight inflammation and hemorrhage were observed at the injection sites, along with increased lymphoid cellularity of germinal centers in popliteal and iliac lymph nodes and the spleen, which is consistent with an immune response to the vaccine administration. Overall, the findings were considered non-adverse and were partially or completely reversible after a 3-week treatment-free period. All vaccinated animals developed an antibody response against SARS-CoV-2 S protein, confirming responsiveness of the rabbits to the vaccine.

The target organs or tissues identified in this study (i.e., injection site, [draining] lymph nodes and spleen) were consistent with the tissues that tested mostly positive for Ad26 DNA in the biodistribution studies for the Ad26 platform (Section 3).

Immunogenicity of Ad26.COV2.S was tested in mice, rabbits, Syrian hamsters, and NHPs, and vaccine efficacy was tested in Syrian hamsters and NHPs (see Section 2). Ad26.COV2.S was tested up to a dose level of 1×10^{10} vp in mice and Syrian hamsters, 5×10^{10} vp in rabbits, and 1×10^{11} vp in NHP. No vaccine-related adverse effects were noted in these non-GLP immunogenicity/efficacy studies.

4.1.2. Ad26-Platform: Supportive Toxicity Data

The Sponsor has significant nonclinical experience with Ad26-vectored vaccines using various transgenes encoding for HIV, malaria, RSV, Zikavirus, Filovirus (Ebolavirus, Marburgvirus), influenza (Universal influenza) and HPV antigens. More than 10 GLP combined repeat-dose toxicity and local tolerance studies as well as one EF-PPND toxicity study (with Ad26.ZEBOV; see Section 4.2.2) have been performed in rabbits (or rats) testing the nonclinical safety of these

various Ad26-based vaccines either alone or in combination with various other vaccine modalities, including Ad35-based vaccines, Modified Vaccinia Virus Ankara (MVA)-based vaccines and/or (glyco)proteins with or without an aluminum phosphate adjuvant. In these studies, up to 5 sequential IM dose administrations have been tested at levels up to 4×10^{11} vp. The tested vaccine regimens in these supportive Ad26-platform studies were well tolerated. Irrespective of the antigen transgene used, the vaccine-related effects observed in these supportive studies were largely similar across studies and considered to be reflective of a normal, immunologic response to the administered vaccines, similar to the responses noted with Ad26.COV2.S in TOX14382. No vaccine-related adverse effects were observed. Therefore, these studies with other Ad26-based vaccines are considered to provide supportive repeat-dose toxicity (including local tolerance) data for Ad26.COV2.S, which is based on the same Ad26 vector backbone.

An overview of the completed GLP toxicology studies testing various Ad26-based vaccines and/or vaccine regimens is provided in Appendix 2.

4.2. Reproductive Toxicity

4.2.1. Ad26.COV2.S

Female reproductive toxicity and fertility were assessed in a combined EF-PPND toxicity study in the rabbit using a 3-dose vaccine regimen (study TOX14389; Mod2.6.6/Sec5.2). In this study, a first vaccine dose of Ad26.COV2.S at $1x10^{11}$ vp was administered 7 days prior to mating (i.e., Day 1) with untreated male rabbits to ensure induction of a maternal immune response during mating and early gestation.

In order to evaluate potential direct embryotoxic effects of the components of the vaccine formulation and to ensure high antibody titers during early gestation, a second vaccine dose was administered 6 days after mating (i.e., on Gestation Day [GD] 6), corresponding to the start of organogenesis, around implantation in rabbits. This was followed by a third vaccination on GD20, in order to assess the (direct) effects of the vaccine during late gestation and to ensure a sustained maternal immune response during late gestation as well as during lactation.

This dose scheme allows an assessment of potential effects of the vaccine and/or the maternal immune response induced by the vaccine on the various stages of pregnancy. The design of this study has been discussed with CHMP and CBER and was found acceptable (EMA/CHMP/SAWP/221031/2020, dated 24 April 2020, CHMP response to Sponsor Question 10; PTS 5513, CRMTS 12536, CBER feedback dated 5 June 2020, CBER Response to Sponsor Question 10).

There was no adverse effect of Ad26.COV2.S on reproductive performance, fertility, ovarian and uterine examinations, parturition, or macroscopic evaluations in parental females. In addition, there was no adverse effect of vaccination on fetal body weights, external, visceral and skeletal evaluations, or on postnatal development of the offspring (sex ratios, survival, body weights, clinical findings, developmental evaluations, and macroscopic evaluations). The parental females as well as their fetuses and offspring (kits) exhibited SARS-CoV-2 S protein-specific antibody titers, indicating that maternal antibodies were transferred to the fetuses during gestation.

Overall, the EF-PPND toxicity study with Ad26.COV2.S did not reveal any evidence of impaired female fertility and did not indicate harmful effects with respect to reproductive toxicity. In addition, the combined repeat-dose toxicity and local tolerance study with Ad26.COV2.S as well as the general (supportive) toxicity studies with other Ad26-based vaccines have not revealed any effects on male sex organs that would impair male fertility.

4.2.2. Ad26-Platform: Supportive Reproductive Toxicity Data

As part of the Ad26 platform, a supportive EF-PPND toxicity study (TOX11212) has been conducted in rabbits using an Ad26-based Ebola vaccine, i.e., Ad26.ZEBOV (Ad26 vector encoding the glycoprotein of Zaïre ebolavirus Mayinga variant; 1×10^{11} vp) in a 2-dose homologous regimen, or in a 2-dose heterologous regimen with MVA-BN-Filo (3.61×10^{8} Inf U). This study is considered to provide supportive reproductive and developmental toxicity data for Ad26.COV2.S, as Ad26.ZEBOV is produced from the same Ad26 vector backbone as Ad26.COV2.S. Similar to the EF-PPND toxicity study with Ad26.COV2.S, the vaccine regimens in this study with Ad26.ZEBOV did not induce maternal or developmental toxicity in rabbits following maternal exposure during the premating and gestation period. This study (TOX11212) was part of the MAA file for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001).

4.3. Juvenile Toxicity

Studies in juvenile animals were not performed. During the assessment procedure of the PIP and the PSP, the PDCO and CBER, respectively, confirmed that no further nonclinical studies were deemed necessary to support the pediatric development. Final approval of both documents is pending.

All vaccine-related effects noted in the dedicated Ad26.COV2.S studies, as well as in the supportive studies with other Ad26-based vaccines (see Appendix 2) in (young) adult rabbits were considered to reflect a non-adverse, immunologic response to the injection of a vaccine. These studies did not indicate any other target organs than those anticipated based on the pharmacological mode of action, i.e., the immune system. Hence, the main consideration when immunizing infants or children would be any untoward effect associated with the immune system.

It is noted that immune system development is significantly more advanced in humans at birth when comparing with routine preclinical species [15,31]. Therefore, from a preclinical perspective, vaccine studies in juvenile animals (i.e., very young or newborn animals) would not yield any additional relevant information and have therefore not been conducted. Interpretation of any findings derived from such models would pose challenges, especially since it may not be possible to extrapolate immune system developmental stages from the animal model to humans [33].

In the repeat-dose toxicity and local tolerance studies with Ad26.COVS2.S and other Ad26-based vaccines, the lower age range of the NZW rabbits used was approximately 12 weeks at start of the study, which is an age range equivalent to young adolescent age in humans [20]. These animals were confirmed to be immunologically responsive to the vaccine, i.e., in all toxicology studies a

specific antibody response was detected. This immune response was not associated with any adverse effects in the animals.

In the EF-PPND toxicity study with Ad26.COV2.S, fetuses and offspring (kits) from vaccinated parental females exhibited SARS-CoV-2 S protein-specific antibody titers, indicating that maternal antibodies were transferred to the fetuses during gestation. No adverse effects from exposure to these (maternal) antibodies were observed on fetal or postnatal development in the rabbit.

Overall, the available (general) repeat-dose toxicology studies with Ad26.COV2.S and Ad26-based vaccines are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children/adolescents. There were no findings in these nonclinical studies that would indicate a concern for the use of the vaccine in infants/children/adolescents.

5. INTEGRATED OVERVIEW AND CONCLUSIONS

Pharmacology

Immunogenicity data from studies in mice, rabbits, Syrian hamsters, and NHP show that a single dose of Ad26.COV2.S induces humoral and cellular immune responses as early as 2 weeks post immunization. Ad26.COV2.S induces neutralizing antibodies and a Th1 skewed immune response, factors that are thought to be beneficial to minimize potential risk of VAERD.

In a Syrian hamster SARS-CoV-2 challenge model, Ad26.COV2.S provided dose-level dependent protection from infectious viral load in the lung. In a SARS-CoV-2 NHP challenge model, immunization with Ad26.COV2.S at dose levels of 1×10^{11} vp and 5×10^{10} vp fully protected 16 out of 16 NHP from viral replication in the lung and protected 14 out of 16 NHP from viral replication in the nose (studies NHP 20-09 and NHP 20-14). In both Syrian hamsters and NHP SARS-CoV-2 specific binding and neutralizing antibodies significantly correlate with protection from infection with SARS-CoV-2 (NHP correlate analysis and van der Lubbe et al [35]).

Two SARS-CoV-2 challenge studies in Syrian hamsters and 1 NHP challenge study showed no indications of VAERD based on monitoring of clinical signs and viral load of Ad26.COV2.S vaccinated animals after SARS-CoV-2 challenge, and based on histopathologic assessment of lung tissue from these animals compared with challenged control animals. In conclusion, data from the preclinical studies performed by the Sponsor suggest that the theoretical risk of VAERD with Ad26.COV2.S is low, however, it is acknowledged that there is limited understanding of the value of nonclinical models in predicting the risk of VAERD in humans.

Biodistribution

Biodistribution studies in rabbits showed a pattern of limited distribution of the Ad26 vector. Clearance (i.e., reflected by a downward trend in number of positive tissues and vector copies over time, to levels close to, or below the respective detection limits) of the Ad26 vector was observed following IM injection, indicating that the vector does not replicate and/or persist in the tissues.

As biodistribution is considered dependent on the viral vector platform and not on the transgene insert, the biodistribution results obtained with Ad26.ENVA.01 and Ad26.RSV.preF are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same (i.e., IM) route.

Toxicology

Ad26.COV2.S administered on three occasions over 4 weeks (i.e., every 2 weeks) at $1x10^{11}$ vp/dose in the toxicity studies was well tolerated, and was not associated with any adverse vaccine-related effects. The vaccine-related effects noted were considered to reflect a normal, immunologic response consistent with vaccination. The nonclinical safety profile of Ad26.COV2.S is largely similar to the profile observed previously for other Ad26-based vaccines.

The EF-PPND toxicity study did not reveal any evidence of impaired female fertility and did not indicate harmful effects with respect to reproductive toxicity. In addition, the repeat-dose toxicity studies with Ad26.COV2.S or other Ad26-based vaccines have not revealed any effects on male sex organs that would impair male fertility.

Overall Conclusion

Ad26.COV2.S was immunogenic and protected from SARS-CoV-2 infection in nonclinical studies. The nonclinical data did not show any adverse vaccine-related effects and support the use of Ad26.COV2.S in humans. We have shown in Ad26.COV2.S immunized Syrian hamsters and NHP absence of enhanced lung pathology and clinical signs of disease compared with controls after SARS-CoV-2 inoculation, even under conditions of suboptimal immunity allowing breakthrough infection. Together with induction of neutralizing antibodies and a Th1 skewed immune response after Ad26.COV2.S dosing these data suggest that the theoretical risk of VAERD and VAED for Ad26.COV2.S is low.

6. LIST OF LITERATURE CITATIONS

Literation citations are located in Mod4.3.

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APPENDIX 1: JUSTIFICATION FOR ABSENCE OF DOCUMENTS IN MODULE 4

Module	Module Title	Justification
4.2.1.2	Secondary Pharmacodynamics	Not applicable for vaccines
4.2.1.3	Safety Pharmacology	No separate safety pharmacology studies were performed since data from the Ad26.COV2.S-specific and Ad26 platform toxicity studies (which include detailed clinical examinations) did not suggest that the vaccine has a significant impact on physiological functions (e.g., central nervous system, respiratory, an cardiovascular functions) other than those of the immune system.
4.2.1.4	Pharmacodynamic Drug Interactions	Not applicable for vaccines
4.2.2.1	Analytical Methods and Validation Reports	In the biodistribution studies, specific quantitative polymerase chain reaction (q-PCR) assays were used to detect and quantify Ad26-vector DNA expression in tissues and blood collected at specified time points following vector administration. A description of the assays is available in the different biodistribution reports (in Module 4.2.2.3).
4.2.2.2	Absorption	Not applicable for vaccines
4.2.2.4	Metabolism	Not applicable for vaccines
4.2.2.5	Excretion	Not applicable for vaccines
4.2.2.6	Pharmacokinetic Drug Interactions	Not applicable for vaccines
4.2.2.7	Other Pharmacokinetic Studies	Other pharmacokinetic studies were not performed
4.2.3.1	Singe-Dose Toxicity	Separate studies to determine single dose toxicity were not performed. Possible signs of acute toxicity effects were monitored following the first vaccination in the repeat-dose toxicity studies in Module 4.2.3.2.
4.2.3.3	Genotoxicity	In accordance with WHO Guidelines on Nonclinical Evaluation of Vaccines, no genotoxicity studies were performed for Ad26.COV2.S. In addition, adenoviral vectors are classified as non-integrating, so the risk of integration of genetic material from the Ad26 vector in the host genome, and possible associated insertional mutagenesis is unlikely.
4.2.3.4	Carcinogenicity	In accordance with WHO Guidelines on Nonclinical Evaluation of Vaccines, no carcinogenicity testing was performed for Ad26.COV2.S.
4.2.3.5.1	Fertility and Early Embryonic Development	No dedicated fertility studies have been conducted. The histopathology data from the Ad26.COV2.S-specific (in Module 4.2.3.2) and Ad26-platform repeat-dose toxicity studies in rabbits do not raise any concerns that the vaccine adversely affects male or female reproductive organs. Female fertility was evaluated as part of the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2), e.g., via the premating vaccine administration, and did not show any adverse effects on female fertility.
4.2.3.5.3	Prenatal and Postnatal Development	Assessment of prenatal and postnatal development was included in the combined embryo-fetal and pre- and postnatal development study which is located in Module 4.2.3.5.2.

Module	Module Title	Justification
4.2.3.5.4	Studies in Juvenile Animals	The repeat-dose toxicity and local tolerance study (in Module 4.2.3.2) and the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2) are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children. There were no findings in these nonclinical studies that would indicate a concern for the use of the vaccine regimen in the pediatric population from birth onwards (see Section 4.3 for discussion).
4.2.3.6	Local Tolerance	The local tolerance following IM injection of the vaccines was evaluated as part of the pivotal repeat-dose toxicity (in Module 4.2.3.2) and reproductive toxicity studies (Module 4.2.3.5.2). In these toxicology studies, Ad26.COV2.S was well tolerated when given at a dose above the maximum anticipated human dose and when receiving a number of injections that is higher than what is used in the clinical regimen.
4.2.3.7	Other Toxicity Studies	Other toxicity studies besides the repeat-dose toxicity and local tolerance study (in Module 4.2.3.2) and the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2) were not performed. Neurovirulence and/or neurotoxicity testing was not performed since (1) Ad26.COV2.S is replication-incompetent; and (2) nonclinical biodistribution studies conducted with the Ad26 vector did not show any distribution of the vectors to the brain, and (3) Ad26.COV2.S did not show any adverse effects on nervous tissues in the repeat-dose toxicity study TOX14382.

APPENDIX 2: OVERVIEW OF SUPPORTIVE GLP TOXICOLOGY STUDIES TESTING AD26-VECTORED VACCINES IN RABBITS OR RATS AFTER IM INJECTIONS

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
RABBIT STUDIES			
Repeated dose (IND016112/0000/Mod4.2.3.2/1006-001)*	HIV[d]	5 or 6 injections (Days 1, 22, 43, 64, 85, 106; 3-week interval between doses) • Saline on each dosing day • MVA.Natural ($1x10^8$ pfu) on Days 1, 22, 43, 64 and 85 • MVA.Mosaic ($1x10^8$ pfu) on Days 1, 22, 43, 64 and 85 • **Ad26.ENVA.01** ($5x10^{10}$ vp) on Days 1, 22 and 43; MVA-Natural ($1x10^8$ pfu) on Days 64, 85 and 106 • **Ad26.ENVA.01** ($5x10^{10}$ vp) on Days 1, 22 and 43; MVA-Mosaic ($1x10^8$ pfu) on Days 64, 85 and 106	*Clin obs/clin path:* ↑ BT (x1.02), ↑ CRP (x79), ↑ neutrophils (x2.21), ↑ fibrinogen (x1.96) *Necropsy/histology*: iliac LN (enlargement, ↑ weight, edema, erythrocytosis/erythrophagocytosis, hyperplasia), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016263/0000/Mod4.2.3.2/TOX10873)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on each dosing day + Clade C gp140/aluminum phosphate (250 µg/425 µg) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; Clade C gp140/aluminum phosphate (250 µg/425 µg) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; Clade C gp140/aluminum phosphate (250 µg/425 µg) + MVA-Mosaic ($1x10^8$ pfu) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; MVA-Mosaic ($1x10^8$ pfu) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on each dosing day	*Clin obs/clin path*: transient ↓ FC (x0.63), ↑ CRP (x222), ↑ fibrinogen (x1.97), ↑ globulin (x1.18), ↑ BT (x1.10) *Necropsy/histology*: iliac LN and spleen (↑ weight, ↑ germinal centers, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016263/0115/Mod4.2.3.2/1645-06077)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.ENVA.01** ($1x10^{10}$ vp) on each dosing day • **Ad26.ENVA.01** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* ↑ monocytes (x2.20), ↑ globulin (x1.23), ↑ total protein (x1.02), ↑ albumin (x1.02), ↓ albumin/globulin[b] (x0.85) *Necropsy/histology*: injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND016263/0043/Mod4.2.3.2/TOX12014)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.Mos4.HIV** ($5x10^{10}$ vp) on Days 1 and 22; **Ad26.Mos4.HIV** ($5x10^{10}$ vp) + Clade C gp140/Mosaic gp140/aluminum phosphate (125 µg/125 µg/425 µg) on Days 43 and 64	*Clin obs/clin path:* transient ↓ FC (x0.92), ↑ CRP (x26), ↑ fibrinogen (x2.10), ↑ total protein (x1.10), ↑ globulin (x1.30), ↓ prothrombin time (x0.90), ↓ albumin/globulin (x0.80) *Necropsy/histology*: iliac LN and spleen (↑ germinal centers, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (1854-09764)	Malaria[d]	5 injections (Days 1, 22, 43, 64, 85; 3-week interval between doses) • Saline on each dosing day • Ad35.CS.01 ($1x10^{11}$ vp) on Days 1, 22 and 43; **Ad26.CS.01** ($0.77x10^{11}$ vp) on Days 64 and 85	*Clin obs/clin path:* transient ↓ BW (x0.99), ↓ FC (x0.62), ↑ BT (x1.01), ↑ globulin (x1.16), ↑ CRP (x22), ↑ fibrinogen (x1.06), ↑ APTT (x1.38), ↑ monocytes (x2.78), minimal changes in RBC/platelet parameters *Necropsy/histology*: iliac LN and spleen (↑ weight, hyperplasia), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016401/0000/Mod4.2.3.2/TOX10931)*	RSV[d]	4 injections (Days 1, 15, 29, 43/44; 2-week interval between doses) • Formulation Buffer 5 on each dosing day • **Ad26.RSV.FA2** ($0.65x10^{11}$ vp) on each dosing day • Ad35.RSV.FA2 ($1.3x10^{11}$ vp) on each dosing day • **Ad26.RSV.FA2** ($0.65x10^{11}$ vp) on Days 1 and 15; Ad35.RSV.FA2 ($1.3x10^{11}$ vp) on Days 29 and 43/44	*Clin obs/clin path:* transient ↓ FC (x0.50), ↑ CRP (x33), ↑ fibrinogen (x2.50), ↑ total protein (x1.10), ↑ globulin (x1.30), ↓ prothrombin time (x0.90), ↓ APTT (x0.90), ↓ albumin/globulin (x0.80), ↓ RBC parameters, minor disturbances in differential WBC numbers (neutrophils [x0.40], monocytes [x3.50]) *Necropsy/histology*: iliac LN and spleen (↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 22-day treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND017148/0002/Mod4.2.3.2/TOX11592)*	RSV[d]	5 injections (Days 1, 15, 29, 43, 57/58; 2-week interval between doses) • Formulation Buffer 5 on each dosing day • **Ad26.RSV.preF** ($1x10^{11}$ vp) on each dosing day • Ad35.RSV.FA2 ($0.65x10^{11}$ vp) on Days 43 and 57/58	*Clin obs/clin path:* transient BW loss (1-2%) and ↓ FC (x0.64), ↑ CRP (x33), ↑ fibrinogen (x2.10), ↑ globulin (x1.21), ↓ albumin/globulin (x0.74) *Necropsy/histology*: iliac LN and spleen (enlargement and/or ↑ weight, ↑ cellularity, ↑ germinal center development), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND017148/0017/Mod4.2.3.2/TOX12288)*	RSV	3 injections (Days 1, 15, 29; 2-week interval between doses) • Saline on each dosing day • RSV-F Protein (250 µg) on each dosing day • **Ad26.RSV.preF** ($1x10^{11}$ vp) on Day 1; RSV-F Protein (250 µg) on Days 15 and 29 • **Ad26.RSV.preF**/RSV-F Protein mix[c] ($1x10^{11}$ vp/250 µg) on Days 1,15 and 29	*Clin obs/clin path*: transient BW loss (2%) and ↓ FC (x0.75), resulting in overall ↓ BWG (the latter only in group 4 receiving mix Ad26.RSV.PreF with RSV-F protein) (x0.70), ↑ fibrinogen (x2.00), ↑ globulin (x1.20), ↑ CRP (x19) ↓ albumin/globulin (x0.80); no findings in RSV-F Protein only group *Necropsy/histology*: iliac LN and spleen (enlargement and/or ↑ cellularity), injection sites (dark discoloration, inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (TOX14253)	RSV	2 injections (Days 1, 15; 2-week interval between doses) • Saline on each dosing day • **Ad26.RSV.preF**/RSV preF protein mix[b] ($2×10^{11}$ vp /240 µg) on each dosing day • **Ad26.RSV.preF** ($4x10^{11}$ vp) on each dosing day	*Clin obs/clin path*: ↑ BT (x1.03), ↑ fibrinogen (x2.15), ↑ CRP (x36.7), ↑ WBCs (lymphocytes x1.34, monocytes x3.11, neutrophils x1.86 [Ad26.RSV.preF group]), ↑ creatine kinase (x2.72; Ad26.RSV.preF group), ↑ globulin (x1.23; Ad26.RSV.preF group), ↓ albumin (x0.96), ↓ albumin/globulin (x0.80; Ad26.RSV.preF group), ↓ RBC parameters, ↓ ALP (x0.58) *Necropsy/histology*: iliac and popliteal LN and spleen (↑ cellularity germinal centers), injection sites (mononuclear cell infiltration, inflammation) *Recovery*: ↑ neutrophils (x1.24; Ad26.RSV.preF group), ↑ monocytes (x1.91). Full or partial recovery of microscopic findings after 17-day treatment-free period. **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016280/0004/Mod4.2.3.2/TOX11059)* (EMEA/H/C/005337/0000/Mod4.2.3.2/ TOX11059)**	Ebola[d]	2 injections (Days 1 and 15/16; 14-day interval between doses) • Saline on each dosing day • **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 1; MVA-BN-Filo ($4.4x10^{8}$ $TCID_{50}$) on Day 15/16 • MVA-BN-Filo ($4.4x10^{8}$ $TCID_{50}$) on Day 1; **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 15/16 • **Ad26.ZEBOV** ($5x10^{10}$ vp) + MVA-BN-Filo ($1x10^{8}$ $TCID_{50}$) on Day 1; Ad26.ZEBOV ($5x10^{10}$ vp) + MVA-BN-Filo ($1x10^{8}$ $TCID_{50}$) on Day 15/16 • **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 1; Ad26.ZEBOV ($5x10^{10}$ vp) on Day 15/16	*Clin obs/clin path:* transient ↓ FC (x0.46), ↑ CRP (x95), ↑ fibrinogen (x2.32), ↑ total protein (x1.10), ↑ globulin (x1.31), ↓ RBC parameters, ↓ neutrophils (x0.38), ↓ albumin/globulin (x0.73) *Necropsy/histology*: iliac LN and spleen (enlargement, ↑ weight, and/or ↑ cellularity, ↑ germinal centers), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND017088/0000/Mod4.2.3.2/TOX11260)*	Filo[d]	3 injections (Days 1, 15, 29/30; 14-day interval between doses)[e] • Saline on each dosing day • **Ad26.Filo** ($1.2x10^{11}$ vp) on Day 1; MVA-BN-Filo ($5x10^{8}$ Inf.U) on Days 15 and 29/30 • **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 1 and Day 29/30; MVA-BN-Filo ($5x10^{8}$ Inf.U) on Day 15 • MVA-BN-Filo ($5x10^{8}$ Inf.U) on Day 1; **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 15 and 29/30 • **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 1 and 15; Ad35.ZEBOV ($1.2x10^{11}$ vp) on Day 29/30	*Clin obs/clin path:* transient BW loss, ↓ FC (x0.52), ↑ BT (x1.02) (individual animals), ↑ CRP (x81), ↑ fibrinogen (x2.4), ↓ prothrombin time (x0.9), ↑ globulin (x1.38), ↓ albumin/globulin (x0.76) *Necropsy/histology*: iliac LN and spleen (enlargement, ↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND017749/0002/Mod4.2.3.2/TOX13018)*	Zika	3 injections (Days 1, 15, 29; 2-week interval between doses) • Saline on each dosing day • **Ad26.ZIKV.001** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* transient ↓ FC (day after 1st and 2nd injection) (x0.80), ↑ monocytes (x3.00), ↑ CRP (x50), ↑ fibrinogen (x2.00), ↑ globulin (x1.30), ↓ albumin/globulin (x0.78) *Necropsy/histology*: iliac LN and spleen (enlargement LN, ↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (TOX13736)	Uniflu[f]	3 injections (Days 1, 22, 43/44; 3-week interval between doses) • Saline on each dosing day • $AlOH_3$ (750 µg) on each dosing day • G1 mini-HA protein (135 µg) on each dosing day • G1 mini-HA protein + $AlOH_3$ (135 µg + 750 µg) on each dosing day • **Ad26.FLU.003** ($5x10^{10}$ vp) on each dosing day • G1 mini-HA protein + $AlOH_3$/**Ad26.FLU.003** mix[c] (135 µg + 750 µg/$5x10^{10}$ vp) on each dosing day • G1 mini-HA protein + **Ad26.FLU.003** (135 µg/$5x10^{10}$ vp) on each dosing day	*Clin obs/clin path:* ↑ monocytes (x4.65), ↑ fibrinogen (x2.26), ↑ globulin (x1.40), ↑ total protein (x1.10), ↑ CRP (x55), ↓ RBC parameters (x0.91), ↓ albumin/globulin (x0.69) *Necropsy/histology*: iliac/poplitheal LN and spleen (↑ cellularity; trend for spleen), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Embryo-fetal/Pre- and Postnatal Development (IND016280/0014/Mod4.2.3.5.2/ TOX11212)* (EMEA/H/C/005337/0000/Mod4.2.3.5.2/ TOX11212)**	Ebola[d]	2 injections (Day -8 and GD6; 14-day interval between doses)[g] • Saline on each dosing day • **Ad26.ZEBOV** ($1x10^{11}$ vp) on Day -8; MVA-BN-Filo ($3.61 x10^8$ Inf U)[d] on GD6 • MVA-BN-Filo ($3.61 x10^8$ Inf.U) on Day -8; **Ad26.ZEBOV** ($1x10^{11}$ vp) on GD6 • **Ad26.ZEBOV** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* ↑ CRP (x97), ↑ fibrinogen (x1.88), ↑ globulin (x1.14), ↑ monocytes (x2.84), ↑ lymphocytes (x1.36), ↓ albumin/globulin (x0.83) **Above findings were considered to be a non-adverse, normal response to vaccine administration** *Embryo-fetal/pre-postnatal development*: no effects on reproductive performance, fertility, and litter data (corpora lutea count, number of implantation sites, viable fetuses, litter size, pre- and post-implantation loss, and number of resorptions), parturition, or macroscopic evaluations in parental females. Similarly, no adverse effect of vaccination was seen on fetal body weights, external, visceral, and skeletal evaluations or F1 pup evaluations from LD0-28 (sex ratios, survival, body weights, clinical findings, developmental evaluations, and macroscopic evaluations)
RAT STUDIES			
Repeated dose (IND017959/0001/Mod4.2.3.2/TOX12276)*	HPV[d]	4 injections (Days 1, 15, 29, 43; 2-week interval between doses) • Saline on each dosing day • **Ad26.HPV16–HPV18 mix** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^9$ Inf U) on Days 29 and 43 • **Ad26.HPV16** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^9$ Inf U) on Days 29 and 43 • **Ad26.HPV18** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^9$ Inf U) on Days 29 and 43 • MVA.HPV16-18 ($1x10^9$ Inf U) on Days 1 and 15; **Ad26.HPV16-18 mix** ($5x10^{10}$ vp) on Days 29 and 43	*Clin obs/clin path:* ↑ neutrophils (x1.85), ↑ fibrinogen (x1.56), ↑ globulin (x1.15), ↑ α2-macroglobulin[h] (x2.57), ↓ albumin (x0.89), ↓ albumin/globulin (x0.78) *Necropsy/histology*: iliac and popliteal LN (enlargement, ↑ weight, and/or ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 18-day treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

* Reference to the vaccine-specific INDs
** Reference to the Ebola MAA file/reports
[a] The indicated fold changes are the maximum mean fold changes (irrespective of sampling time or sex) measured during the study, versus controls
[b] CRP and fibrinogen were not measured, and iliac (draining) lymph node was not sampled in this study.
[c] Pharmacy mix as a single injection.
[d] No relevant differences between study results (previous versus new buffer) were observed.
[e] The Ad26.Filo vaccine is a trivalent mix (in a 1:1:1 ratio) of Ad26 vectors encoding the GP of EBOV Mayinga (Ad26.ZEBOV), MARV Angola (Ad26.MARVA), and SUDV Gulu (Ad26.SUDV).
[f] A tetracycline operon (TetO) was inserted in the CMV promotor sequence of the transgene expression cassette (Uniflu; TOX13736).
[g] A first vaccination was administered 8 days prior to mating, followed by a second vaccination 6 days after mating (ie, on gestation day 6).
[h] CRP was not measured; α2-macroglobulin was measured instead as it is a more relevant acute phase protein in rats.
APTT: activated partial thromboplastin time; BT: body temperature; BW: body weight; BWG: body weight gain; clin obs: clinical observations; clin path: clinical pathology CRP: C-reactive protein; FC: food consumption; GD: gestation day; HPV: human papilloma virus; Inf U: infectious unit; LD: lactation day; LN: lymph nodes; RBC: red blood cell; pfu: plaque-forming units; RSV: respiratory syncytial virus; $TCID_{50}$: 50% tissue culture infective dose; vp: virus particles; WBC: white blood cell

2.4. Nonclinical Overview

Drug Substance	AZD1222
ANGEL ID	Doc ID-004365565
Date	21 December 2020

2.4 Nonclinical Overview AZD1222

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1 OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

AstraZeneca (the Sponsor) is developing AZD1222 for the prevention of coronavirus disease-2019 (COVID-19). AZD1222 is a recombinant chimpanzee adenovirus (ChAd) expressing the severe respiratory syndrome-coronavirus-2 (SARS CoV-2) spike (S) surface glycoprotein. Development of AZD1222, previously referred to as ChAdOx1 nCoV-19, was initiated by the University of Oxford with subsequent transfer of development activities to the Sponsor.

AZD1222 is a recombinant replication-defective ChAd vector expressing the SARS CoV-2 S surface glycoprotein, driven by the human cytomegalovirus major immediate early promoter that includes intron A with a human tissue plasminogen activator (tPA) leader sequence at the N terminus. Spike (S) is a type I, trimeric, transmembrane protein located at the surface of the viral envelope, giving rise to spike shaped protrusions from the SARS-CoV-2 virion. The S protein's subunits are responsible for cellular receptor angiotensin-converting enzyme 2 (ACE-2) binding via the receptor-binding domain and fusion of virus and cell membranes, thereby mediating the entry of SARS-CoV-2 into the target cells. The S protein has an essential role in virus entry and determines tissue and cell tropism, as well as host range. The roles of the S in receptor binding and membrane fusion make it a desirable target for vaccine and antiviral development. AZD1222 expresses a codon-optimised coding sequence for S protein from the SARS-CoV-2 genome sequence accession MN908947.

The ChAdOx1 platform technology was used to support the first-in-human (FIH) and other early clinical AZD1222 studies. This approach of using platform data to support a FIH clinical study is consistent with the views expressed by global regulators at the International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 (ICMRA 2020).

To date, immunology and biological activity studies (including prime boost vaccination) of AZD1222, have been conducted in mice, non-human primates, ferrets and pigs (Table 1).

For the FIH study, biodistribution studies with AZD1222 were not performed based upon previously generated biodistribution data with similar replication-defective ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice that showed no evidence of replication of the virus or presence of disseminated infection after intramuscular (IM) injections (Table 2). A recent biodistribution study (Table 2) of IM ChAdOx1 HBV in mice detected, based on interim data using a qPCR method, low levels of disseminated ChAdOx1 HBV. Low copy numbers were found in a range of organs (spleen, brain, heart, kidney, liver, lung, lymph node, testes, ovary) at levels 1,000 to 100,000 fold less than at the injection site (skeletal muscle). Toxicology studies on a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein, as well as other ChAd vaccines (AdCh63 MSP-1, ChAd OX1 NP+M1) were also used to support the FIH study and are shown as a reference

(Table 2). Toxicology studies with AZD1222 have either been recently completed or are ongoing (Table 1 and Table 3). Ongoing or planned nonclinical studies are listed in Table 3.

All pivotal nonclinical safety studies were conducted in OECD member countries and in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP), and according to relevant International Conference on Harmonisation guidelines.

Table 1 List of Nonclinical Pharmacology Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Source	GLP Y/N
Primary Pharmacology				
Effect of D614G Mutation in SARS-CoV-2 Spike Protein on AZD1222 (20-01700)	In vitro	NA	CSIRO Health and Biosecurity, Australia	N
Murine Immunogenicity (van Doremalen et al 2020)	Balb/C and CD-1 mice	Single dose, IM 6×10^9 vp AZD1222	Jenner Institute - Oxford University, UK / NIH, USA	N
Murine Immunogenicity (Graham et al 2020)	Balb/C and CD-1 mice	Day 0 and 28 IM, 6.02 × 109 vp/animal AZD1222	Jenner Institute - Oxford University / Pirbright Institute, UK	N
Non-human Primate Efficacy and Immunogenicity (van Doremalen et al, 2020)	Rhesus macaques	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University, UK / NIH, USA	N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Rhesus Macaques (6284)	Rhesus macaques	Single dose, IM 2.5×10^{10} vp AZD1222	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N
Assessment of Efficacy of SARS-CoV-2Vaccine Candidates in the Ferret Mode (20-01125)	Ferret	Single dose, IM, IN 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	CSIRO Health and Biosecurity, Australia	N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Ferrets (6285)	Ferret	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N

Table 1 List of Nonclinical Pharmacology Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Source	GLP Y/N
Porcine Immunogenicity (Graham et al 2020)	White-Landrace-Hampshire cross-bred pigs	Day 0 and 28, IM 5.12×10^{10} vp AZD1222	Jenner Institute - Oxford University / Pirbright Institute, UK	N
ChAdOxl-nCoV19 immunopotency assay (INT-ChadOx1 nCov19-POT004)	Balb/C and CD-1 mice	5×10^{9} vp AZD1222	Jenner Institute - Oxford University, UK	N
Safety Pharmacology				
Cardiovascular and Respiratory Assessment Following Intramuscular Administration to Male Mice (617078)	CD-1 mice	Day 4, IM 2.59×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y
Repeat Dose Toxicology				
AZD1222 (ChAdOx1-nCovd-19): A 6 Week Intermittent Dosing Intramuscular Vaccine Toxicity Study in the Mouse with a 4 Week Recovery (513351)	CD-1 mice	Days 1, 22 and 43, IM 3.7×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y
Developmental and Reproductive Toxicology				
ChAdOx1-nCovd19: A Preliminary Intramuscular Injection Vaccine Development and Reproductive Study in Female CD-1 Mice (490838)	CD-1 mice	Day 1 (13 days prior to pairing for mating) and GD 6 to EFD phase animals and on GD 6 and GD 15 to littering phase animals, IM 2.59×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y

CSIRO = Commonwealth Scientific and Industrial Research Organisation, Geelong, Australia; EFD = embryo-fetal development; GD = gestation day; IM = intramuscular; IN = intranasal; NIH = National Institute of Health

Table 2 List of Nonclinical Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
AdCh63 MSP-1 and MVA MSP-1 Tissue Distribution Study By Intra-Muscular Administration To Mice (Report UNO0014/RMBIODIST-001)	Balb/C mice	Day 1, IM 1.11×10^{10} vp AdCh63 MSP-1 1.04×10^{8} pfu MVA MSP-1	Huntingdon Life Sciences,[a] UK	Y[b]
AdCh63ME-TRAP Tissue Distribution Study By Intra-Dermal Administration To Mice (UNO0009/MAB-001)	Balb/C mice	Day 1, ID 3.3×10^{9} vp	Huntingdon Life Sciences,[a] UK	Y[b]
ChAdOx-1 HBV and MVA-HBV Biodistribution Study in BALB/c Mice with Shedding Assessment (0841MV38.001)	Balb/C mice	Days 1 and 28, IM 2.4×10^{10} vp ChAdOx-1-HBV 6.1×10^{7} pfu MVA-HBV	Calvert Laboratories, USA	Y
ChAdOx1 Chik Vaccine or ChAdOx1 MERS: Toxicity Study by Intramuscular Administration to Mice (QS18DL)	Balb/C mice	Day 1 and 15, IM 1×10^{10} vp	Envigo CRS Limited UK	Y
ChAd OX1 NP+M1 and MVA NP+M1: Toxicity Study by Intramuscular Administration to Mice (XMM0003)	Balb/C mice	Day 1, IM ChAd OX1 NP+M1 1×10^{10} vp and Day 15, IM MVA NP+M1 1.5×10^{7} pfu	Huntingdon Life Sciences,[a] UK	Y
Mouse Toxicity AdCh63 MSP-1 and MVA MSP-1 or a Combination of AdCh63 ME-TRAP and MVA ME-TRAP (UNO0013)	Balb/C mice	Day 1, IM AdCh63 MSP-1 1.11×10^{10} vp Day 15, IM MVA MSP -1 10.4×10^{7} pfu Day 1 and 15, IM AdCh63ME-TRAP/ MVA ME TRAP 0.78×10^{10} vp / 6.85×10^{7} pfu	Huntingdon Life Sciences,[a] UK	Y

[a] Currently Covance CRS Ltd.

[b] In-life phase conducted to GLP; biodistribution phase (RBIODIST-001 or MAB-001) not conducted to GLP

Table 3 List of Ongoing and Planned Nonclinical Studies with AZD1222

Study (Report Number)	Species	Status	GLP Y/N
AZD1222 (ChAdOx1-nCovd-19): A Single Dose Intramuscular Vaccine Biodistribution Study in the Mouse (514559)	CD-1 mice	Ongoing Audited draft February 2021	Y
AZD1222 (ChAdOx1 -nCovd19): An Intramuscular Vaccine Development and Reproductive Study in Female CD-1 Mice (490843)	CD-1 mice	Ongoing Audited draft February 2021	Y

2 PHARMACOLOGY

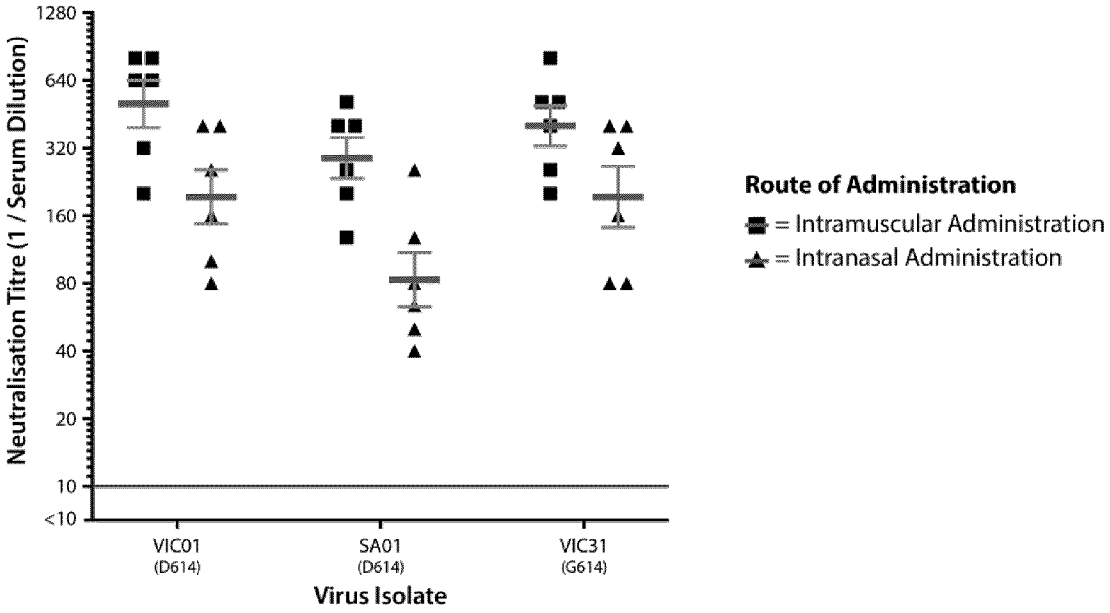
2.1 Primary Pharmacodynamics

Immunogenicity studies in animal models responsive to AZD1222 were conducted to evaluate the immunologic properties of this COVID-19 vaccine candidate to support FIH clinical trials. AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. These studies included evaluation of humoral, cellular and functional immune responses. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated enhanced respiratory disease (ERD). A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

Mutations are occurring naturally within the SARS-CoV-2 genome. Most vaccines in development rely upon inducing immune responses towards Spike protein (S), the main virus surface protein. A D614G mutation in S is increasing in prevalence amongst sequenced viruses worldwide. The mutation is thought to increase infectivity of the virus by reducing S1 shedding, increasing infection (Zhang et al 2020). The effect of the D614G mutation on the efficacy of virus neutralisation following vaccination of ferrets with AZD1222 was assessed in study 20-01700 (Figure 1). A new Australian isolate containing the D614G mutation (VIC31) was obtained from VIDRL. Three isolates were used for virus neutralisation assays: SA01: has identical amino acid sequence in S to Wuhan-Hu-1. VIC01: S differs from SA01 by an Ser247Arg mutation. VIC31: S differs from SA01 by the Asp614Gly mutation.

Overall, there were no significant effects of the D614G mutation in the SARS-CoV-2 Spike protein on relative neutralisation of D614 and G614 variants with serum samples collected from ferrets that had received prime-boost administrations of AZD1222. Therefore, animal challenge studies presented are relevant to strains circulating in the human population.

Figure 1 Effect of D614G mutation on vaccine-induced antibody-mediated neutralisation.



Mean neutralising titres (calculated from log2-values) to three circulating Australian SARS-CoV-2 isolates. Neutralisation titres of serum samples collected following prime-boost vaccination with AZD1222 in ferrets, administered by two routes (intramuscular and intranasal). Bold horizontal lines represent overall mean titre of the vaccination route/isolate combination with uncertainty bars representing Standard Error of the Mean (SEM). Square and triangle marks represent mean titres of the triplicate titres for each serum sample/isolate combination.

Viral RNA in Gastrointestinal Tract

In the NHP pharmacology study (van Doremalen et al 2020), there was an unexpected finding of viral RNA in tissues of the gastrointestinal (GI) tract at 7 days post-challenge in immunised, but not control, animals. Viral gRNA load in intestinal tissues of prime-boost-vaccinated animals was higher than the levels measured in control and prime-only-vaccinated animals at 7 days post-challenge and was associated with the detection of sgRNA. However, no infection of intestinal tissue was observed by immunohistochemistry, nor were we able to detect infectious virus in intestinal tissue. Given that spike-specific antibodies were significantly increased after the second immunization (two-tailed signed-rank Wilcoxon test) higher viral gRNA load intestinal in prime-boost animals may correlate with

greater intestinal clearance and retention of opsonised virus following challenge. FcRn allows the entry and retrieval of IgG from the intestinal lumen throughout health and disease. This bidirectional transport allows the secretion of IgG into the lumen, the subsequent uptake of opsonized bacteria and viruses (Castro and Clatworthy 2020). As previously reported, SARS-CoV-2 antigen can be detected in lymphocytes and macrophages in the lamina propria of the intestinal tract of control animals (Munster et al 2020). This may indicate a higher proportion of plasma cells secreting IgA2 in the gut lamina propria of prime-boost-vaccinated animals and trapping of SARS-CoV-2 virus. Whilst SARS-CoV-2 virus may make its way to the gastric lumen, it would be subjected to the adverse effect of the acidic environment of the stomach that would significantly affects viability.

Nevertheless, SARS-CoV-2 can cause gastrointestinal symptoms, such as loss of appetite, vomiting, diarrhoea, or abdominal pain during the early phases of the disease (Villapol 2020). It has been reported in some patients that although SARS-CoV-2 has been cleared in the respiratory tract, the virus continues to replicate in the gastrointestinal tract and could be shed in faeces (Yang et al 2020). Currently, the exact mechanism of SARS-CoV-2 interaction with the gastrointestinal tract is still not fully understood. However, SARS-CoV-2 shows a high affinity to ACE2 receptors, making sites of high ACE2 receptor expression such as lungs and GI tract prime targets for infection (Dahiya et al 2020). It is therefore possible that gastrointestinal symptoms in COVID-19 are somehow caused by the direct attack of SARS-CoV-2 to gastrointestinal tract (Zhong et al 2020). If higher viral gRNA loads in intestinal tissues of prime-boost vaccinated animals is associated with continued replication then it was not associated with any signs of lesions or infection.

Lung Histopathology

In rhesus macaques 3 out of 6 control animals developed some degree of viral interstitial pneumonia following SARS-CoV-2 challenge. Lesions were widely separated and characterised by thickening of alveolar septum. Alveoli contained small numbers of pulmonary macrophages and rarely oedema. Type-II pneumocyte hyperplasia was observed. No histological lesions were observed in the lungs of vaccinees.

In comparison, the majority of histopathological findings made in the lungs of ferrets following SARS-CoV-2 challenge were modest at most. In control group 3a that received a prime with ChAdOx1 vector expressing green fluorescent protein (GFP) one ferret showed mild lesions compatible with acute bronchiolitis and the other animals were similar to group 1 primed with AZD1222. Only mild inflammatory cell foci and no lesions were observed in group 1. In group 2 that received a prime and boost with AZD1222, inflammatory cell were also detected in lungs. These changes are likely associated with an immune response to challenge as they were also observed in controls. In group 4 immunised with inactivated SARS CoV-2, mild to moderate lesions were observed in the lungs with inflammatory cells and perivascular cuffing at day 7 post challenge potentially indicative of enhanced respiratory

disease. In a second ferret study, no significant histological lung changes were present in any of the animals examined.

Enhanced respiratory disease (ERD) can result from immunization with antigen that is not processed in the cytoplasm, resulting in a nonprotective antibody response and CD4+ T helper priming in the absence of anti-viral cytotoxic T lymphocytes. This type of vaccine response can lead to a pathogenic Th2 memory response with eosinophil and immune complex deposition in the lungs after respiratory infection. For example, infants and toddlers immunized with a formalin-inactivated virus vaccine against respiratory syncytial virus (RSV) experienced an enhanced form of RSV disease characterized by high fever, wheezing and bronchopneumonia when they became infected with wild-type virus in the community (Acosta et al 2015). AZD1222 not expected to cause ERD because antigens are expressed intracellularly, generating anti-viral cytotoxic T cell and protective antibody responses.

In the van Doremalen et al study, significantly reduced viral load in the bronchoalveolar lavage fluid and lower respiratory tract tissue of vaccinated rhesus macaques challenged with SARS-CoV-2 with no pneumonia was observed compared to control animals. No evidence of immune-enhanced disease after viral challenge in vaccinated SARS-CoV-2-infected animals was found in terms of increased severity of viral infection. At present, there are no known clinical findings, immunological assays or biomarkers that can differentiate any severe viral infection from immune-enhanced disease, whether by measuring antibodies, T cells or intrinsic host responses (Arvin et al 2020). Carefully controlled human studies of sufficient size to enable the detection of increased frequency of severe cases in vaccinated cohorts compared to control group are required to determine if antiviral host responses may become harmful in humans.

In conclusion, the rhesus macaque is more predictive than ferret of histological lung changes and the ability of immunisation with AZD1222 to mitigate these following challenge with SAR-CoV-2. No enhanced respiratory disease was observed post challenge in AZD1222 immunised animals.

2.2 Secondary Pharmacodynamics

Secondary pharmacodynamic studies have not been conducted with AZD1222.

2.3 Safety Pharmacology

In a mouse cardiovascular and respiratory safety pharmacology study, a group of 8 male CD-1 mice were dosed by IM injection with the control item for AZD1222 (A438 buffer) on Day 1 and AZD1222 (2.59 x 10^{10} vp dose) on Day 4 (617078).

There were no changes in arterial blood pressure, heart rate, body temperature or respiratory parameters considered to be AZD1222-related. The No Observed Effect Level (NOEL) for cardiovascular and respiratory assessment was an AZD1222 dose of 2.59 x 10^{10} vp .

Irwin Screen observations (autonomic, neuromuscular, sensorimotor, behavioural parameters) and effects on body temperature and pupil size were made in the repeat-dose IM toxicity study (513351) in male and female CD-1 mice on Days 8 and 29 following administration of AZD1222 at 3.7 x 10^{10} vp on Days 1, 22. There were no effects on body temperature, pupil size or Irwin Screen observations considered to be AZD1222-related.The NOEL for the Irwin Screen phase was 3.7 x 10^{10}.

2.4 Pharmacodynamic Drug Interactions

Pharmacodynamic drug interaction studies have not been conducted with AZD1222.

3 PHARMACOKINETICS

3.1 Absorption

Absorption studies evaluations are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional absorption, distribution, metabolism, and excretion (ADME) evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune responses.

3.2 Distribution

Distribution studies are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional ADME evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune response.

Biodistribution studies are more informative when a replication-competent virus is administered since the amount of virus present in the subject (experimental animal or human volunteer) will increase following injection, and some viruses have a known propensity to accumulate in particular organs. For example, Vaccinia virus may be found at high titres in the ovaries and adenovirus accumulates in the liver. However, replication-deficient viruses are known to infect cells at the injection site, and although some infectious viral particles may drain to local lymph nodes and travel through the blood to other sites in the body, concentrations of virus at these sites are so low after dilution in the blood and other tissues that they are not reliably detected. A biodistribution study would demonstrate if, unexpectedly, viral replication was taking place after injection. However, this is not an

appropriate assay to use to detect replication competent virus, which is tested for in an in vitro assay which has much greater sensitivity for detecting even small amounts of replication competent virus in the vaccine preparation.

AZD1222 is replication-incompetent in human cells due to a block in gene expression caused by the deletion of the E1 genes. Therefore, after the initial infection of the cells that the virus enters, there will be no further infection and no spread of the virus within the body. Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injection. A biodistribution and shedding study using the ChAdOx1 vector with an hepatitis B virus (HBV) insert after IM injection on Days 1 and 28 in mice was conducted (0841MV38.001). Distribution to some samples of all tissues was noted on day 2 and Day 29. The highest levels (copies/mg sample) were noted at the site of administration (skeletal muscle), ranging from 3×10^8 to 9.97×10^9 copies/mg sample. In the majority of samples of other tissues taken on Day 56, the levels were below the level of quantification, indicating elimination. Low levels were noted in 1 sample (of 6) for each of heart and liver, 1 of 3 for ovary and testes, and 3 of 6 lymph node samples at this timepoint. This study does not contain assessment of CNS, relevant peripheral nerves or bone marrow and it does not include analysis at shorter time points compared to the already available studies and no description of the validation of method analysis. This platform study will be superseded by a biodistribution study with AZD1222 (514559). This study includes additional early timepoints, an assessment of a full set of tissues including spinal cord and bone marrow. A draft report is due February 2021.

Intramuscular administration of AZD1222 is expected to minimise risk of systemic exposure. The biodistribution of AZD1222 following intramuscular administration is expected to be similar to that of AdCh63, confined to the site of injection and draining lymph nodes.

3.3 Metabolism

Metabolism studies have not been conducted with AZD1222. The expected consequence of metabolism of biotechnology-derived vaccines is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood.

3.4 Excretion

Excretion studies have not been conducted with AZD1222. No virus excretion is expected with AZD1222 as it is a non-replicating vaccine vector. Shedding of ChAdOx1 HBV in mice following IM administration of Days 1 and 28 have been assessed. DNA was extracted from mouse fecal and urine samples collected were all negative, suggesting that no shedding had occurred in these matrices at the times sampled.

3.5 Pharmacokinetic Drug Interactions

Pharmacokinetic drug interaction studies have not been conducted with AZD1222.

3.6 Other Pharmacokinetic Studies

Other pharmacokinetic studies have not been conducted with AZD1222.

4 TOXICOLOGY

4.1 SINGLE DOSE TOXICITY

No single dose toxicity studies have been performed with AZD1222.

4.2 REPEAT DOSE TOXICITY

A repeat-dose GLP toxicity study with AZD1222 in mice was initiated on the 9th September 2020, the audited draft results (excluding recovery pathology) are discussed below. A final report is due January 2021 (513351).

As the ChAdOx1 platform technology utilized for AZD1222 is well characterized, toxicology data with ChAdOx1 MERS-CoV vaccine expressing the full-length Spike protein in mice (Report QS18DL), was used to support first in human (FIH) clinical trials for AZD1222 (International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 [ICMRA 2020]). In addition, toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) are also discussed.

4.2.1 A 6 -Week Intermittent Dosing Intramuscular AZD1222 Toxicity Study in Mice with a 4 Week Recovery

At the time of toxicology species selection only ChAdOx1 immunogenicity data for mouse and rhesus macaques was available to the sponsor. Pig and ferret immunogenicity data were subsequently made available. Considering the need to expedite toxicity testing given the urgency of the ongoing pandemic, the longer lead time for NHP toxicity studies and longer reproductive toxicity study requirements, the CD-1 mouse strain was selected as the toxicology species due to its larger size compared to the Balb/c mouse strain.

The objective of this study was to determine the potential toxicity of AZD1222 (total viral particle dose of $3.7x10^{10}$) when given by intramuscular injection intermittently (on Days 1, 22 and 43) to mice, with a 28 day recovery period to evaluate the potential reversibility of any findings (513351). In addition, the immunogenicity was evaluated. Scheduled necropsies were conducted either at the end of the 6 week treatment period (Day 45) or at the end of the 28 day recovery period.

The following parameters and end points were evaluated in this study: clinical signs, body temperature, body weights, body weight gains, food consumption, dermal scoring, Irwin screen observations, clinical pathology parameters (hematology and plasma chemistry), immunogenicity, gross necropsy findings, organ weights, and histopathological examinations.

In comparison to controls and pre-study data, a slightly higher body temperature was observed in AZD1222 treated males, notably on Days 22, 4 hours post dose (range 36.2-39.5°C compared to 36.2-38.7°C in controls) but was comparable to controls by 24 hours post-dose. There was no AZD1222-related change in body temperature recorded in males, as part of the Irwin observations.

In animals administered AZD1222, there was a mild decrease in monocytes on Day 45, which was consistent with expected pharmacology following immunisation. Additionally, globulin was mildly higher, and albumin and albumin/globulin ratio were minimally to mildly lower, which was consistent with an acute phase response. Following the recovery period, globulin remained mildly higher and albumin/globulin ratio remained mildly lower in AZD1222-treated females, the other changes had reversed.

All samples collected from animals during the pre-treatment phase prior to immunisation were below the limit of quantification (BLQ) for the assay (BLQ; 0.250 AU/mL) and considered seronegative. Samples collected indicate that all animals mounted an antibody response to S-glycoprotein following a single administration of AZD1222 on Day 1 with most animals showing a marked increase in the level of antibody response following a second administration of AZD1222 on Day 22. On Day 74, a further increase in antibody response or maintenance of response was observed in all animals following a third administration of AZD1222.

At histopathological examination of the main study animals, mononuclear and/or mixed cell inflammation was observed in the subcutaneous tissue and underlying skeletal muscle at the control and AZD1222 administration sites. This finding was of a higher incidence in animals dosed with AZD1222. In some animals there was an extension of the inflammatory cells into the fascia and connective tissue below the skeletal muscle at the administration sites, that extended to surround the sciatic nerve. The inflammatory cells did not extend into the endoneurium of the sciatic nerve and no findings were present in the underlying axons, which appeared histologically normal. Inflammatory cells were not observed in the nerve roots contained within the lumbar spinal cord sections, confirming that the epineurial/perineurial inflammatory cells noted in the sciatic nerve samples resulted from an extension of the inflammation from the adjacent injection site.

In conclusion, administration of AZD1222 to CD-1 mice (total viral particle dose of 3.7 x 10^{10}) by intramuscular injection on 3 occasions (once every 3 weeks) over a 43 day period was well tolerated, with a transiently higher body temperature in males, decreases in

monocytes in males and females (consistent with the expected pharmacology of AZD1222) and increase in globulin and decrease in albumin and albumin/globulin ratio, consistent with an acute phase response, observed.

In all animals dosed with AZD1222, antibodies against the S-glycoprotein were raised and maintained throughout the dosing and recovery periods in all animals.

In AZD1222 animals, higher spleen weights were observed but with no correlating macroscopic or microscopic changes. Non adverse, mixed and/or mononuclear cell inflammation was observed in the subcutaneous tissues and skeletal muscle of the administration sites and adjacent sciatic nerve of animals dosed with AZD1222 which were consistent with the anticipated findings after intra-muscular injection of vaccines.

4.2.2 Repeat-dose Toxicology Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

A brief summary of the key findings from the ChAdOx1 MERS vaccine toxicology study in in mice is provided below.

- Changes at the intramuscular injection sites (inflammatory cell infiltrates) were observed in the majority of females and in several males.
- Histopathological changes in the spleen (increased germinal center development) correlated with an increased spleen weight in females. Increased germinal center development of the right lumbar lymph nodes (draining lymph node), correlated macroscopically with enlargement, was observed for the majority of treated animals. Slightly higher circulating white blood cell numbers were observed.
- At the end of the study treatment there was a slightly lower than control body weight gain for treated males and females. For males this was due mainly to slightly lower than control weight gains during Days 15 to 18 however for females this was due mainly to small weight losses during this period. Mice were dosed on Day 1 and 15, with necropsy on day 28.
- Slightly lower group mean liver weight for males and females (0.92X and 0.90X control), higher phosphorus concentration for females (1.2X control) or lower triglyceride concentration for males and females (0.56X and 0.64X) were observed. There was no correlation with histopathological changes.

The spectrum and severity of these changes were consistent with the administration of an antigenic substance such as ChAdOx1 MERS, and were considered to be non-adverse.

Results from the toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) were consistent with ChAdOx1 MERS and were well tolerated with no associated adverse effects. The toxicity data (and toxicity in the target organs) from the ChAdOx1 and ChAd63 based vaccines follows the same pattern, where findings were consistent with a predicted response to vaccine administration.

4.3 Toxicokinetics

Toxicokinetic studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Pharmacokinetic studies (eg, for determining serum or tissue concentrations of vaccine components) are normally not needed.

4.4 Genotoxicity (Mutagenicity)

Genotoxicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines, (WHO 2005), genotoxicity studies are normally not required for the final vaccine formulation and therefore have not been conducted.

4.5 Carcinogenicity

Carcinogenicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Carcinogenicity studies are not required for vaccine antigens. AZD1222 is a replication deficient, non-integrating adenovirus vector so there is no risk of carcinogenicity. To date there have been no clinical reports of chromosomal vector integration following adenovirus vector-mediated gene transfer.

4.6 Developmental and Reproductive Toxicity

An evaluation of the impact of AZD1222 on embryo-foetal development was completed in a dose-range study (490838). The main GLP embryo-foetal development study is ongoing with an audited draft due end January 2021.

Intramuscular administration of AZD1222 to groups of CD-1 female mice on Day 1 (13 days prior to pairing for mating) and again on Gestation Day (GD) 6 at 2.59 x 10^{10} per occasion (embryofetal development phase), or on GD 6 and GD 15 at 2.59 x 10^{10} per occasion (littering phase) was well tolerated (490838). Anti-S glycoprotein antibody responses were raised in dams following administration of AZD1222 and these were maintained through the gestational and lactation periods. Seropositivity of fetuses and pups was confirmed and was indicative of placental and lactational anti-S glycoprotein antibody transfer, respectively. There were no AZD1222-related effects seen for dams in-life including at the injection site, for female reproduction, fetal or pup survival and no abnormal gross pathology findings in pups or in dams in either phase. There were no AZD1222-related fetal visceral or skeletal findings.

4.7 Local Tolerance

Local tolerance of AZD1222 was evaluated as part of the repeat dose toxicity study in mice (513351). There was no erythema or oedema at the injection sites after administration of

AZD1222 on any dosing occasion. Histopathology showed minimal subcutaneous oedema was observed in the administration sites in male and female animals from both the control group and those administered AZD1222 and was considered to be related to the route of administration. Minimal mononuclear or mixed cell inflammation was observed in the subcutaneous tissue and underlying skeletal muscle at the administration sites in both male and female animals. This finding was of a higher incidence in animals administered with AZD1222. In some animals there was an extension of the inflammatory cells into the fascia and connective tissue below the skeletal muscle at the administration sites. This resulted in inflammatory cells being noted surrounding the epineurium/perineurium of the sciatic nerve samples. In the hind limb, inflammation around the sciatic nerve due to local extension from the administration site is a well-recognized effect (Sellers et al 2020). Local tolerance was also evaluated as part of a repeat dose GLP toxicology study in mice with the related ChAdOx1 MERS vaccine (QS18DL). Changes related to treatment with ChAdOx1 MERS vaccine were seen in the tissues of the intramuscular injection site, the right lumbar lymph node (draining lymph node) and the spleen of mice. The inflammatory cell infiltrate seen in the tissues of the intramuscular injection sites (infiltrates of lymphocytic/mononuclear inflammatory cells) were caused by the intramuscular injection of the vaccine with the increased germinal centre development of the right lumbar lymph node caused by immune stimulation of the lymphatic drainage from this area and are not considered adverse.

4.8 Other toxicity Studies

No other toxicity studies with AZD1222 were conducted.

5 INTEGRATED OVERVIEW AND CONCLUSIONS

AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated ERD. A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injections. WHO guidelines on nonclinical evaluation of vaccines states that pharmacokinetic studies (eg, for determining serum or tissue concentrations of vaccine components) are normally not needed and specific studies should be

considered on a case-by-case basis (eg, when using novel adjuvants or alternative routes of administration).

A biodistribution study using the ChAdOx1 vector with a hepatitis B virus (HBV) insert following IM injection on days 1 and 28 in mice has been conducted. This study shows distribution to some samples of all tissues on days 2 and 29. The highest levels (copies/mg sample) were noted at the site of administration (skeletal muscle), ranging from 3×10^8 to 9.97×10^9 copies/mg sample. In the majority of samples of other tissues taken on Day 56, the levels were below the level of quantification, indicating elimination. AZD1222 is made using a platform technology utilized for other previously studied investigational vaccines and is sufficiently characterized to use toxicology data with other vaccines that use the same platform (Development and Licensure of Vaccines to Prevent COVID-19, FDA Guidance for Industry, June 2020 FDA 2020). Administration of a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein was associated with treatment related changes in the right lumbar lymph node, spleen and intramuscular injection site. The spectrum and severity of the changes were consistent with the administration of an antigenic substance such as ChAdOx1 MERS which were considered to be non-adverse. This was also true for the similar replication-defective ChAd vaccines, ChAd OX1 NP+M1 and AdCh63 MSP-1.

In the mouse cardiovascular and respiratory safety pharmacology study there were no changes in arterial blood pressure, heart rate, body temperature or respiratory parameters considered to be AZD1222-related. Irwin Screen observations showed no effects considered to be AZD1222-related.

In the repeat dose (once every 3 weeks over a 43 day period) toxicity study in CD-1 mice, AZD122 was well tolerated, with a transiently higher body temperature in males, decreases in monocytes in males and females (consistent with the expected pharmacology of AZD1222) and increase in globulin and decrease in albumin and albumin/globulin ratio, consistent with an acute phase response, observed. In all animals dosed with AZD1222, antibodies against the S-glycoprotein were raised and maintained throughout the dosing and recovery periods in all animals. In AZD1222 animals, higher spleen weights were observed but with no correlating macroscopic or microscopic changes. Non adverse, mixed and/or mononuclear cell inflammation was observed in the subcutaneous tissues and skeletal muscle of the administration sites and adjacent sciatic nerve of animals dosed with AZD1222 which were consistent with the anticipated findings after intra-muscular injection of vaccines.

In the preliminary DART study in mice, there were no AZD1222-related effects seen for dams in-life including at the injection site, for female reproduction, fetal or pup survival and no abnormal gross pathology findings in pups or in dams in either phase. There were no AZD1222-related fetal visceral or skeletal findings.

In conclusion, AZD1222 and similar ChAd vaccines are well tolerated and are not associated with any adverse effects in mice. Further, similar ChAd vaccines show no evidence of replication or dissemination after IM injection in mice. AZD1222 is immunogenic in mice, ferrets, NHP and pig models inducing humoral and cellular immune responses. Vaccination with AZD1222 significantly reduced viral load following a SARS-CoV-2 challenge in rhesus macaques with no evidence of ERD.

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Janssen Vaccines & Prevention B.V. *

Nonclinical Overview

MODULE 2.4

VAC31518 (JNJ-78436735)

* Janssen Vaccines & Prevention B.V. is a Janssen pharmaceutical company of Johnson & Johnson and is hereafter referred to as the sponsor.

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LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

Ad26	adenovirus type 26
Ad26.COV2.S	Ad26 vector encoding a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike protein
Ad26.ENVA.01	Ad26 vector encoding the Clade A envelope protein of HIV type 1
Ad26.RSV.preF	Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain
Ad26.ZEBOV	recombinant, replication-incompetent, adenovirus type 26 (Ad26) vector encoding the GP of EBOV Mayinga variant
BAL	bronchoalveolar lavage
BALT	bronchial-associated lymphoid tissue
BIDMC	Beth Israel Deaconess Medical Center
CAR	coxsackievirus adenovirus receptor
CBER	Center for Biologics Evaluation and Research
CHMP	Committee for Medicinal Products for Human Use
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
DNA	deoxyribonucleic acid
E1, E3	early gene regions (of Ad26)
EF-PPND	embryo-fetal and pre- and postnatal development
EMA	European Medicines Agency
ELISA	enzyme-linked immunosorbent assay
EUA	Emergency Use Authorization
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
HIV	human immunodeficiency virus
HPV	human papillomavirus
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ICMRA	International Coalition of Medicines Regulatory Authorities
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IM	intramuscular
LLOD	lower limit of detection
LRT	lower respiratory tract
MAA	Marketing Authorization Application
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	messenger ribonucleic acid
MVA	Modified Vaccinia Virus
MVA-BN	Modified Vaccinia Ankara-Bavarian Nordic A/S vector backbone
MVA-BN-Filo	Recombinant, non-replicating in human cells, Modified Vaccinia Ankara - Bavarian Nordic (MVA-BN®) vector encoding the GP of EBOV Mayinga, SUDV Gulu, MARV Musoke, and the TAFV nucleoprotein
NHP	nonhuman primate(s)
NZW	New Zealand white
OECD	Organization for Economic Co-operation and Development
PBMCs	peripheral blood mononuclear cells

PCR	polymerase chain reaction
PDCO	Pediatric Committee
PIP	Pediatric Investigation Plan
PSP	Pediatric Study Plan
pre-F	pre-fusion conformation-stabilized F protein
PSP	Pediatric Study Plan
ppVNA (pVNA)	pseudotyped virus neutralization assay
q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-qPCR	reverse transcription followed by quantitative polymerase chain reaction
S	Spike
SARS-CoV-1	severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
sgRNA	subgenomic ribonucleic acid
$TCID_{50}$	50% tissue culture infective dose
TetO	tetracycline operon
TetR	tetracycline repressor
URT	upper respiratory tract
VAERD	vaccine-associated enhanced respiratory disease
vp	virus particles
(wt)VNA	(wild type) virus neutralization assay
VRBPAC	Vaccines and Related Biological Products Advisory Committee
WHO	World Health Organization

1. OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

1.1. Introduction

Ad26.COV2.S[1] (also known as Ad26COVS1, VAC31518, or JNJ-78436735) is a monovalent vaccine composed of a recombinant, replication-incompetent human adenovirus type 26 (Ad26) vector, constructed to encode the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike (S) protein, stabilized in its prefusion conformation. It is being developed for prophylactic immunization against coronavirus disease 2019 (COVID-19), which has spread rapidly and globally since its emergence.

Ad26.COV2.S encodes a full-length S protein, derived from a SARS-CoV-2 clinical isolate (Wuhan, 2019, whole genome sequence NC_045512), with 2 amino acid changes in the S1/S2 junction that knock out the furin cleavage site, and 2 proline substitutions in the hinge region. Prior to selection of Ad26.COV2.S for clinical development, the sponsor evaluated different vaccine constructs with design elements previously shown to be successful for other coronavirus S protein based vaccines, eg, prefusion-stabilizing substitutions and heterologous signal peptides [27,38]. In vitro characterization of these SARS-CoV-2 S protein constructs demonstrated that the introduction of stabilizing substitutions (ie, furin cleavage site mutations and two consecutive prolines in the hinge region of S2) increased the ratio of neutralizing versus non-neutralizing antibody binding, suggestive of a benefit of the prefusion conformation of the S protein for improved antigenicity. Furthermore, the wild-type signal peptide was best suited for the correct cleavage needed for a natively folded protein. The combination of the wild-type signal peptide, the furin cleavage site mutations and the proline substitutions translated into superior immunogenicity in mice [2]. In addition, several vaccine constructs were tested for immunogenicity and efficacy in NHP. The in vivo testing in NHP showed that the design of Ad26.COV2.S was optimal in inducing robust neutralizing antibody responses as well as protection following SARS-CoV-2 challenge [25]. The increase in neutralizing antibodies in mice, rabbits, hamsters and NHP, and the increase in protective efficacy in hamsters and NHP, induced by Ad26.COV2.S compared with an Ad26 vector encoding wild-type S protein (Ad26NCOV006) is shown in nonclinical studies in this document, and in the articles by Mercado et al [25], Bos et al [2], and van der Lubbe et al [35]. The selection of the S protein stabilized in its prefusion conformation was further substantiated in additional ongoing nonclinical studies [32,34].

The proposed clinical vaccine regimen consists of a single vaccination with Ad26.COV2.S at a dose level of $5x10^{10}$ virus particles (vp). The vaccine is a suspension for intramuscular (IM) injection provided in multi-dose vials. The dose volume is 0.5 mL. The vaccine composition is described in Table 1.

[1] In nonclinical studies performed prior to selection of the Ad26.COV2.S vaccine candidate, Ad26.COV2.S was named Ad26NCOV030. The name Ad26.S.PP is used in some publications.

Table 1: Ad26.COV2.S Vaccine Composition

Vaccine	Nature and Composition
Ad26.COV2.S	Adenovirus type 26 (Ad26) vectored vaccine encoding the SARS-CoV-2 S protein. Produced in the human PER.C6® TetR cell line. $5x10^{10}$ vp as a 0.5 mL single dose. Excipients: sodium chloride, citric acid monohydrate, polysorbate 80, 2 hydroxypropyl-β-cyclodextrin, ethanol, sodium hydroxide, water for injection.

This nonclinical overview covers a description of the nonclinical pharmacology testing strategy, a summary of immunogenicity and efficacy data, and an assessment of the theoretical risk of vaccine-associated enhanced respiratory disease (VAERD). In addition, this overview covers a description of the pharmacokinetic (biodistribution) and toxicology testing strategy. An integrated assessment of the biodistribution profile of the Ad26 vector, as well as the nonclinical safety profile of Ad26.COV2.S, based on an Ad26.COV2.S-specific repeat-dose and local tolerance study, and a developmental and reproductive toxicity study are presented. To further support the toxicological evaluation of Ad26.COV2.S, a high-level summary of toxicology data from other Ad26-based vaccines is provided.

Overall, Ad26.COV2.S was immunogenic and protected animals from SARS-CoV-2 infection in nonclinical studies. The available nonclinical data did not show any adverse vaccine-related effects and support the use of Ad26.COV2.S in humans. The data further suggest that the theoretical risk of VAERD with Ad26.COV2.S is low.

An overview of the nonclinical packages can be found in Table 2 (pharmacology), Table 4 (pharmacokinetics), and Table 5 (toxicology). An overview of nonclinical studies not included in the final submission dossier, with justification for their absence, is included in Appendix 1.

1.2. Pharmacology Testing Strategy

1.2.1. Study Overview and Characteristics

Primary pharmacology studies are listed in Table 2; detailed descriptions are provided in the Pharmacology Written Summary in Mod 2.6.2.

Table 2: Overview of Primary Pharmacology Studies

Test Species	Study Identifiers	Study Description	Regimen Dose Level	Study Report
mouse	9346-20004 (TV-TEC-171843)	Immunogenicity study to support vaccine candidate selection.	1-dose 10^8, 10^9, 10^{10} vp	Mod4.2.1.1/9346-20004
mouse	9346-20007 (TV-TEC-171881)	Th1/Th2 skewing study to support vaccine candidate selection.	1-dose 10^{10} vp	Mod4.2.1.1/9346-20007
NHP	NHP 20-09 (TV-TEC-175059)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose 10^{11} vp	Mod4.2.1.1/NHP 20-09
NZW rabbit	TOX14369 (TV-TEC-175060)	Immunogenicity study to support vaccine candidate selection.	2-dose $5x10^9$, $5x10^{10}$ vp	Mod4.2.1.1/TOX 14369
Syrian Hamster	TKO 707 (TV-TEC-175626)	Immunogenicity and efficacy study to support vaccine candidate selection. Lung histopathology assessment.	1-dose and 2-dose 10^9, 10^{10} vp	Mod4.2.1.1/TKO 707
Syrian Hamster	TKO 766 (TV-TEC-176250)	Immunogenicity and efficacy study (vaccine titration study) with histopathology assessment after breakthrough SARS-CoV-2 infection.	1-dose 10^7,10^8, 10^9, 10^{10} vp	Mod4.2.1.1/TKO 766
NHP	NHP correlate analysis	Across-study report, correlation of immunogenicity with protection in nonhuman primates		Mod4.2.1.1/NHP correlate analysis

NHP: nonhuman primate; NZW: New Zealand white; vp: virus particles.

The nonclinical pharmacology testing of Ad26.COV2.S was guided by the testing principles laid out by the World Health Organization (WHO) Guidelines on Nonclinical Evaluation of Vaccines [37], European Medicines Agency (EMA) Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7], Food and Drug Administration (FDA) Guidance on development and licensing of prophylactic Covid-19 vaccines [10] and FDA Guidance for Industry for emergency use authorization for vaccines to prevent COVID-19 [11]. In addition, the International Coalition of Medicines Regulatory Authorities [ICMRA] convened international regulatory workshops to discuss regulatory considerations related to the development of COVID-19 vaccine candidates, including the type and extent of nonclinical studies required to support proceeding to first-in-human clinical studies (18 March 2020 [17]), and Phase 3 clinical studies (22 June 2020 [18]). A Vaccines and Related Biological Products Advisory Committee (VRBPAC) meeting held on October 22, 2020 included a discussion of assessment and de-risking of VAERD. In these meetings it was acknowledged that there is limited understanding of the value of available nonclinical models in predicting the likelihood of VAERD in humans. However, studies in animal models were considered important to address the potential for VAERD.

The nonclinical pharmacology testing strategy has been presented in previous Scientific Advice interactions to EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Questions 5 and 9) and FDA/Center for Biologics Evaluation and Research (CBER) (PTS

5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Questions 1 and 5) in the context of the clinical development program. Data presented so far have been considered by multiple agencies as sufficient to support the clinical development including the currently ongoing phase 3 efficacy studies. The proposed nonclinical data package, including data to assess a theoretical VAERD risk, was considered overall acceptable in a meeting with EMA (Co)-Rapporteurs was held on 30 September 2020 to outline the nonclinical data package for conditional marketing authorization application (MAA). FDA feedback on 09 January 2021 stated that proposed nonclinical Emergency Use Authorization (EUA) data packages showing a low theoretical risk of VAERD are sufficient to support the review of the EUA application. An outline of procedural aspects of the nonclinical pharmacology studies is described below.

The primary pharmacology (immunogenicity and efficacy) studies were performed in countries that are part of the Organization for Economic Co-operation and Development (OECD. All of the non-GLP studies performed under Janssen Vaccines & Prevention supervision were conducted according to good research practices at facilities with suitable expertise. Janssen Preclinical QA conducted an audit of a selection of data for study NHP 20-09 conducted by the Beth Israel Deaconess Medical Center (BIDMC) and for Syrian hamster study TKO 707 conducted at [redacted]. The audits focused on data traceability (retrieval, reconstruction, full disclosure, safe storage and review) and data integrity (data consistency and risk for bias). Through Janssen's assessment of the available data and study reports, several data handling and source verification gaps were noted, however the audits did not reveal any findings that compromise the integrity or interpretation of the data. Information on the Good Laboratory Practice (GLP) status of the pharmacology studies and the location of the test facilities is provided in the Pharmacology Tabulated Summary (Mod2.6.3).

Ad26.COV2.S material used in nonclinical pharmacology studies was produced firstly in a small scale laboratory process (non-Good Manufacturing Practice [non-GMP]). This research-grade material (batch P758-028A) was used in mouse studies 9346-2004 and 9346-20007, rabbit study TOX14369, Syrian hamster study TKO 707, and study NHP 20-09). Later, development-grade material generated in a large scale process similar to the 50 L process used for Phase 1/2a clinical trial material was evaluated (batch P763-004#06: lot E006614 in Syrian hamster study TKO 766 and lot E006613 in study NHP 20-14 described as part of the NHP correlates analysis report).

Data from ongoing studies

Additional studies with Ad26.COV2.S are ongoing from which interim data have been published. The data from these ongoing studies support the results presented in this submission, and are cited where appropriate. In addition, interim study data from study NHP 20-14 are presented in an across-study report of immune correlates of protection (Mod4.2.1.1/NHP correlate analysis). These ongoing studies are summarized in Table 3.

Table 3: Ongoing Pharmacology Studies With Data Referenced in the Submission

Test Species	Study Identifiers	Study Description	Regimen Dose Level	Reference
Syrian Hamster	Hamster 20-09 (TV-TEC-180196)	Immunogenicity and efficacy study to support vaccine candidate selection (high inoculum study). Lung histopathology assessment.	1-dose and 2-dose 10^9, 10^{10} vp	Tostanoski et al [34]
NHP	VH808.681 (TV-TEC-182907)	Immunogenicity and efficacy in aged NHP. Lung histopathology assessment.	1-dose and 2-dose $5x10^{10}$, 10^{11} vp	Solforosi et al [32]
NHP	CRL 2020-3373 (TV-TEC-179493)	Durability of immune response and protection (6 and 12 months after first vaccination). Includes assessment of cellular immunogenicity. Lung histopathology assessment.	1-dose and 2-dose $5x10^{10}$ vp (2-dose), 10^{11} vp (1-dose)	Solforosi et al [32][a]
NHP	NHP 20-14 (TV-TEC-176763)	Immunogenicity and efficacy study (vaccine titration study). Lung histopathology assessment.	1-dose $2x10^9$, $1.125x10^{10}$, $5x10^{10}$, 10^{11} vp	Mod4.2.1.1/NHP correlate analysis

[a] At this time, immunogenicity data up to 14 weeks post immunization are published.
NHP: nonhuman primate; vp: virus particles.

1.2.2. Animal Model Rationale

Nonclinical pharmacology of Ad26.COV2.S was evaluated in murine, rabbit, Syrian hamster, and nonhuman primate (NHP) (rhesus monkey) animal models for immunogenicity, including assessment of immunological parameters relevant to the theoretical risk of VAERD (see Section 1.2.5). Vaccine efficacy including clinical signs and lung histopathology for assessment of VAERD was evaluated in Syrian hamsters and NHP. The list of all studies intended for evaluation is provided in Table 2. All studies were performed in animals of an age range corresponding to adult age in humans. Female animals were used in the murine and rabbit studies, male animals were used in the Syrian hamster studies, and both male and female animals were used in the NHP study.

The murine model was used to comprehensively examine immunogenicity and Th1/Th2 skewing of the immune response by assessing antibody and cellular immune responses after immunization. In addition, an alternative immunogenicity model was developed in rabbits as non-rodent species and to verify rabbits as an appropriate species for toxicology studies (Section 1.5).

A SARS-CoV-2 challenge model has been developed in Syrian hamsters, which are permissive to SARS-CoV-2 replication and display widespread lung pathology and body weight loss upon infection. A nonlethal challenge model was used, applying intranasal inoculation of 10^2 $TCID_{50}$ SARS-CoV-2 strain BetaCoV/Munich/BavPat1/2020, a European isolate with glycine at position 614 of the S protein (G614). Peak viral load in the lung measured by $TCID_{50}$ assay was around day 4 with clearance from lung tissue and throat swabs of the Syrian hamsters within 7 days [35] In addition, SARS-CoV-2-induced lung pathology was also evident 4 days post inoculation, and therefore a 4 day follow-up was selected for these studies. Body weight loss is transient in Syrian hamsters after SARS-CoV-2 infection, with peak body weight loss typically at Day 6 post infection, and is a clinical sign of disease.

A SARS-CoV-2 challenge model in rhesus monkeys has also been developed by the sponsor [4]. In this challenge model, sites of viral replication are predominantly the upper and lower respiratory tract, leading to lung histopathology consistent with mild human disease. This NHP species recapitulates asymptomatic to moderate disease observed in the majority of human cases [4,26,28], and other SARS-CoV-2 vaccines with proven efficacy in phase 3 clinical trials were previously assessed in comparable rhesus monkey challenge studies [5,36]. This is a nonlethal challenge model, applying intranasal and intratracheal inoculation of $1x10^5$ 50% tissue culture infective dose ($TCID_{50}$) SARS-CoV-2 strain USA-WA1/2020, a US isolate with aspartic acid at position 614 of the S protein (D614). In this SARS-CoV-2 challenge model, rhesus monkeys showed modestly decreased appetite and responsiveness suggestive of mild clinical signs of disease; fever, weight loss, and respiratory distress were not observed [4]. Lower levels of virus were found in the gastrointestinal tract, liver, and kidney. In nonvaccinated animals, peak viral load occurred approximately 2 days after inoculation, and viral clearance in the lower and upper respiratory tract was observed within approximately 2 weeks of inoculation [4 and study NHP 20-09]. This is very similar to the kinetics of SARS-CoV-2 clearance in humans [3]. A comparable SARS-CoV-2 challenge model has been developed by the sponsor in aged rhesus monkeys using a G614 virus variant that showed a significant rise in body temperature after virus inoculation and is reported in Solforosi et al [32].

In addition to the efficacy model, the high level of genetic homology between NHP and humans, and their comparative immunology have made NHP the animal model of choice for studies of vaccine immunogenicity [14,19].

1.2.3. Vaccine Immunogenicity

Humoral immune responses were measured in mice, rabbits, Syrian hamsters, and NHP. Based on experience with SARS-CoV, and in recent SARS-CoV-2 vaccine efficacy studies in NHP, binding and neutralizing antibody responses have been identified as the predominant correlates of protection [24,34,39], and the correlation has also been confirmed for Ad26.COV2.S (NHP correlate analysis, and van der Lubbe et al [35]). Neutralizing antibody titers were determined in a virus neutralization assay (wild-type [wt]VNA) with live SARS-CoV-2, or in a pseudotyped virus neutralization assay (ppVNA [pVNA]). In addition, binding antibody titers to the full length ectodomain of the S protein were determined by enzyme-linked immunosorbent assay (ELISA).

Cellular immune responses were measured in mice, rabbits, and NHP. SARS-CoV-2 specific cellular responses were determined by measuring cytokine production after ex vivo stimulation of splenocytes or peripheral blood mononuclear cells (PBMCs) with peptide pools covering the complete SARS-CoV-2 S wild type protein sequence. Cytokine production was determined by interferon gamma (IFN-γ) ELISpot.

While innate responses may contribute to protection early after vaccination, they are not considered to be a major aspect of the protective effect of the vaccine and were therefore not characterized.

1.2.4. Vaccine Efficacy

In the Syrian hamster challenge studies, the main efficacy readouts were SARS-CoV-2 viral load in throat swab samples taken daily after inoculation and in lung and nasal turbinate tissue samples collected at day 4 after inoculation. Infectious viral load, ie, the presence of replication competent virus, was measured by $TCID_{50}$ assay, in which samples were titrated on confluent monolayers of Vero E6 cells and the viral titers were calculated. Additionally, body weight loss was monitored daily during a 4 day follow up period.

In the NHP challenge study, the main efficacy readouts were viral load in bronchoalveolar lavage (BAL) and nasal swab samples. The BAL and nasal swab viral loads were determined at 0, 1, 2, 4, 7, 10 and 14 days post inoculation for each NHP, measured by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) of SARS-CoV-2 E gene subgenomic ribonucleic acid (sgRNA).

1.2.5. Assessment of VAERD

There is a theoretical risk of VAERD for SARS-CoV-2 vaccines, based on nonclinical experience with SARS-CoV and MERS-CoV based vaccines. It is acknowledged that there is limited understanding of the value of available nonclinical models in predicting the risk of VAERD in humans. However, studies in animal models are considered important to address the potential for VAERD, in combination with clinical assessments. To the sponsor's knowledge, VAERD for SARS-CoV-2 vaccines has not been observed in nonclinical models or in human efficacy studies. Actions taken to assess the potential risk of VAERD included lung histopathology assessment in Ad26.COV2.S immunized Syrian hamsters and NHP after SARS-CoV-2 challenge in comparison with unvaccinated and SARS-CoV-2 infected control animals. In 1 Syrian hamster study (TKO 766) lower dose levels of Ad26.COV2.S were included, which induced a suboptimal humoral immune response allowing breakthrough viral replication in the lungs after SARS-CoV-2 challenge, conditions which are hypothesized to contribute to a risk of VAERD. Increased clinical signs of disease and viral load are thought to be evidence for an increased risk of VAERD, therefore these factors have been assessed. In addition, immunogenicity was assessed to show induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to minimize the potential risk of VAERD.

The induction of neutralizing antibodies was assessed in each nonclinical pharmacology study. A study to assess the Th skewing of the immune response induced by Ad26.COV2.S was performed

in BALB/c mice (study 9346-20007). For this purpose, a comparison group received recombinant S protein in aluminum phosphate ($AlPO_4$) adjuvant, which is associated with a Th2 skewed immune response. Immunologic readouts for Th1/Th2 skewing included IgG subclass binding antibody titers, and cytokine production measured by ELISpot, Multiplex ELISA, and intracellular cytokine staining (ICS).

To assess signs for general vaccine associated enhanced disease (VAED), viral load and signs of clinical disease have been monitored in Syrian hamsters and in NHP during the post inoculation follow-up. At the end of follow-up, a histopathology evaluation was conducted in all efficacy studies, and for Syrian hamsters, a gross pathology examination and immunohistochemistry analysis of selected respiratory tissues for the presence of SARS-CoV-2 nucleoprotein, was performed. Selected respiratory tract tissues were evaluated and assessed (semi-quantitatively graded/scored) for disease-associated pathology findings, such as the severity and extent of alveolar, bronchial, bronchiolar, or tracheal inflammation and/or inflammatory infiltrates, evidence of alveolar edema, or any other degenerative inflammatory changes.

1.3. Safety Pharmacology Testing Strategy

Safety pharmacology studies have not been performed with Ad26.COV2.S, since data (e.g., detailed clinical observations) from repeat-dose toxicity studies with Ad26.COV2.S (see Section 4.1.1) and other Ad26-based vaccines (see Section 4.1.2) did not suggest that these vaccines have a significant impact on physiological functions (e.g., central nervous system, respiratory, and cardiovascular functions) other than those of the immune system. Therefore, and in line with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], stand-alone safety pharmacology studies are not deemed necessary.

1.4. Pharmacokinetic Testing Strategy

In accordance with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], pharmacokinetic studies are usually not needed for vaccines. However, in line with the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7] and the FDA Guidance on considerations for plasmid DNA vaccines for infectious disease indications [9], biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 viral vector platform following IM injection. The biodistribution profile of the Ad26 vaccine platform has been evaluated in rabbits using an Ad26-based human immunodeficiency virus (HIV) vaccine, i.e., Ad26.ENVA.01 (Ad26 vector encoding the Clade A envelope protein of HIV type 1), and an Ad26-based respiratory syncytial virus (RSV) vaccine, i.e., Ad26.RSV.preF (Ad26 vector encoding the pre-fusion conformation-stabilized F protein [pre-F] of RSV A2 strain) (see Table 4).

As explained in Section 3, these vector platform data are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S, for which the same Ad26 vector backbone is used. This position has been confirmed and agreed by the Agency in a Scientific Advice by EMA (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, EMA Response to Sponsor Question 6) and CBER (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Question 2). It is further noted that the same platform biodistribution data were part of the MAA

file for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001), which was approved in 2020. Hence, no pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S specifically.

Table 4: Overview of Biodistribution Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine, Route, Interval, and Dose Level	GLP	Study Report
NZW rabbit	Single dose biodistribution study with a 91-day observation period	Ad26.ENVA.01, IM, single dose, 5×10^{10} vp	Yes	Mod4.2.2.3/ 1645-06074
NZW rabbit	Single dose biodistribution study with a 180-day observation period	Ad26.RSV.preF, IM, single dose, 1×10^{11} vp	Yes	Mod4.2.2.3/ TOX13342

GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

The biodistribution studies with Ad26.ENVA.01 and Ad26.RSV.preF were conducted using the IM route, which is also the intended route for use of Ad26.COV2.S in humans.

The studies were done in rabbits as this is a widely accepted species to assess the nonclinical safety of vaccines. In available nonclinical (safety) studies, Ad26-based vaccines (including Ad26.COV2.S, Ad26.ENVA.01 and Ad26.RSV.preF) were shown to elicit immune responses against the vaccine antigen in the animals, indicating the rabbit as a relevant nonclinical species [33]. In addition, the rabbits have sufficient muscle mass to receive a full human vaccine dose via the IM route with a single injection. The use of one species is generally considered sufficient to assess the nonclinical safety/biodistribution profile of vaccines [7,37].

The dose levels applied in the biodistribution studies for Ad26.ENVA.01 and Ad26.RSV.preF were similar to, or above the human dose level for Ad26.COV2.S ($5x10^{10}$ vp), hence full human dose levels were tested. Specific quantitative polymerase chain reaction (q-PCR) assays were used to detect and quantify Ad26-vector DNA in various tissues, including the gonads, brain and blood collected at specified time points following vector administration.

The sampling timepoints used in the biodistribution studies with the Ad26 vectors (i.e., Days 11, 61, 91 post-vaccination for Ad26.ENVA.01, and Days 11, 90, 120 or 180 post-vaccination for Ad26.RSV.preF) are in line with biodistribution studies conducted with adenovirus type 5 (Ad5) and type 35 (Ad35) based vaccines as reported by Sheets et al. 2008 [30]. The sampling timepoints for the biodistribution studies were selected to allow sufficient time to confirm clearance (i.e., confirming a downward trend over time) of the Ad26 vector.

The biodistribution studies (with Ad26.ENVA.01 and Ad26.RSV.preF) were conducted as standalone studies (i.e., not integrated in a pivotal nonclinical safety study). For such pharmacokinetics studies, GLP compliance is not generally required. Nevertheless, the biodistribution studies for the Ad26 vector platform were conducted in compliance with GLP.

Nonclinical shedding studies were not conducted for the Ad26 vaccine platform. The risk for shedding of Ad26.COV2.S will be based on clinical shedding data for the Ad26 vaccine platform and is discussed briefly in Section 3.

1.5. Toxicology Testing Strategy

The nonclinical safety profile of the Ad26.COV2.S vaccine has been assessed in two pivotal toxicology studies in New Zealand white (NZW) rabbits, i.e., a combined repeat-dose toxicity and local tolerance study, and a combined embryo-fetal and pre- and postnatal development (EF-PPND) toxicity study. The testing is consistent with applicable guidelines, including the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], the EMA Guideline on quality, nonclinical and clinical aspects of live recombinant viral vectored vaccines [7], the ICH-S5 Guideline on Detection of Reproductive and Developmental Toxicity for Human Pharmaceuticals [16], and the FDA Guidance for Industry: Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications [8]. An overview of these toxicology studies is provided in Table 5.

In addition, the Sponsor has significant nonclinical experience with Ad26-based vaccines using various gene inserts encoding for HIV, malaria, RSV, Zika virus, Filovirus (Ebolavirus, Marburgvirus), influenza and human papillomavirus (HPV) antigens. More than 10 GLP combined repeat-dose toxicity and local tolerance studies as well as one EF-PPND toxicity study (with the Ebola vaccine component Zabdeno [Ad26.ZEBOV-GP [recombinant]]) have been performed in rabbits (or rats), testing the nonclinical safety of these various Ad26-based vaccines. A high-level summary of these supportive data is given in Sections 4.1.2 and 4.2.2 and in Appendix 2.

In the two pivotal GLP toxicology studies (TOX14382 and TOX14389), 3 doses of Ad26.COV2.S at $1x10^{11}$ vp/dose were administered IM with a 14-day interval period between the injections. The vaccine dose, the applied dose volume (1 mL) and the number of doses (3) administered in the toxicology studies cover the proposed clinical regimen of the vaccine ($5x10^{10}$ vp, administered as a single dose in 0.5 mL). The IM route is the intended route for use in humans.

The use of one species is considered sufficient to assess the nonclinical safety profile of a vaccine [37]. A relevant animal is defined as a species capable of mounting an immune response to the vaccine antigen [33]. The rabbit was considered a relevant toxicological species to assess the nonclinical safety profile of the Ad26.COV2.S vaccine since it was shown to elicit specific immune responses against the SARS-CoV-2 S protein (see Sections 2.3, 4.1.1 and 4.2.1). The use of the rabbit is further supported by the fact that it is a species that is widely used to assess preclinical toxicity and local tolerance of vaccine candidates, and for which sufficient historical control data exist. In addition, in contrast to rodents (e.g., rats, mice), rabbits have sufficient muscle mass to receive a full human dose via the IM route, which increases the relevance of this species to assess local tolerance of vaccines.

Table 5: Overview of Toxicology Studies in Support of the Development of the Ad26.COV2.S Vaccine

Test Species	Study Description	Vaccine, Route, Interval, and Dose Level	GLP	Study Report
NZW rabbit	Combined repeat-dose toxicity and local tolerance study	3 injections (Days 1, 15, 29; 2-week interval between doses), IM • Saline • $1x10^{11}$ vp	Yes	Mod4.2.3.2/ TOX14382
NZW rabbit	Combined embryo-fetal and pre- and postnatal development study	3 injections (Day 1[a], GD6 and GD20; 2-week interval between doses), IM • Saline[b] • $1x10^{11}$ vp[b]	Yes	Mod4.2.3.5.2/ TOX14389

[a] Day 1 = 7 days prior to pairing
[b] The study includes 2 subgroups: 1 group consisting of females that are necropsied on GD29 and have a uterine and fetal examination (external, visceral, and skeletal exams), and 1 group consisting of females that are allowed to give birth and in which the survival and development of the kits is evaluated through lactation day 28
GD: gestation day; GLP: Good Laboratory Practice; IM: intramuscular; NZW: New Zealand white; vp: virus particles

The repeat-dose toxicity (including local tolerance) and EF-PPND toxicity studies were conducted with the Phase 1/2a clinical Ad26.COV2.S material which is representative for the material that is used in the (Phase 3) clinical studies, as well as for commercial batches (refer to respective quality sections 3.2.S.2.6-Manufacturing Process Development-Comparability and 3.2.P.2.3–Manufacturing Process Development-Comparability). Actual and potential (process-related) impurities present in the Ad26.COV2.S drug product have been toxicologically assessed and do not raise a safety concern at the maximum anticipated levels per vaccine dose (refer to respective quality sections 3.2.S.2.6 - Impurity Criticality Evaluation).

The toxicology studies were conducted in conformance with GLP, 21 CFR, Part 58 and/or OECD Principles of GLP in a country that is part of the OECD Mutual Acceptance of Data Process and include the appropriate documentation. The immunogenicity assessment which was included in the toxicology studies was not conducted in compliance with GLP but was conducted and documented by qualified staff in accordance with good scientific practices and standard operating procedures. Since these data are not generally considered a pivotal safety endpoint, but are mainly included as a pharmacodynamic endpoint to confirm responsiveness of the test species to the vaccine administration, this GLP exception does not affect the quality and integrity of the study (conclusions), and overall GLP status of the study.

The local tolerance of the IM administered Ad26.COV2.S vaccine was evaluated as part of the pivotal toxicity studies (i.e., reactions at the injection site were assessed by Draize scoring in the repeat-dose and EF-PPND toxicity studies and by histopathological evaluation in the repeat-dose toxicity study). Therefore, no standalone local tolerance study was conducted. Also, a separate study to determine single dose toxicity was not performed. Possible signs of acute toxicity were monitored following the first vaccination in the repeat-dose toxicity study.

In accordance with the WHO Guidelines on Nonclinical Evaluation of Vaccines [37], genotoxicity and carcinogenicity studies have not been performed. Also, the risk for genotoxicity and carcinogenicity is deemed low since adenoviral vectors are classified as non-integrating because they lack the machinery to integrate their genome into the host chromosomes (eg., *"EMA Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors,*

2006" [6]; *"Long Term Follow-Up After Administration of Human Gene Therapy Products, 2020" [12]*). As such, upon transduction of a cell, the adenoviral DNA does not integrate into the host genome, but rather resides episomally in the host nucleus. Such episomal transduction reduces the risk of insertional mutagenesis [13,21].

Dedicated fertility studies are not routinely required for vaccines and have not been performed because histopathology data from the (repeat-dose) toxicity study are considered to provide sufficient relevant information concerning a possible impact of the vaccine regimen on the integrity of the reproductive organs [33]. Histopathology data from the Ad26.COV2.S repeat-dose toxicity study (see Section 4.1.1) did not reveal any effects on male or female sex organs that would impair male or female fertility. Also, the histopathology data from the available repeat-dose GLP toxicity studies for the Ad26 platform in rabbits (see Section 4.1.2) do not raise any concerns that Ad26-based vaccines adversely affect male or female reproductive organs. This is further supported by results from the available biodistribution studies (see Section 3 and Mod2.6.4), showing that the Ad26 vector does not distribute to the gonads (testes, ovaries) following IM injection. An assessment of female fertility was included in the EF-PPND toxicity study with first vaccination in the premating period (study TOX14389). Based on the above, further dedicated male or female fertility studies are not deemed necessary. This position was confirmed by CHMP (EMA/CHMP/SAWP/369281/2020, dated 9 July 2020, CHMP Response to Sponsor Question 8) and CBER (PTS 5513.9, CBER feedback dated 24 June 2020, CBER Response to Sponsor Question 4).

Studies in juvenile animals were not performed because the repeat-dose toxicity and local tolerance study and the combined EF-PPND toxicity study are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children from birth onwards (see Section 4.3 for discussion). During the assessment procedure of the Pediatric Investigation Plan (PIP) and the Pediatric Study Plan (PSP), the Pediatric Committee (PDCO) and CBER, respectively, confirmed that no further nonclinical studies were deemed necessary to support the pediatric development. Final approval of both documents is pending.

Neurovirulence testing was not done since (1) Ad26.COV2.S is replication-incompetent, (2) distribution into the brain was not seen for the Ad26 vector following IM injection as explained in Section 3, and (3) Ad26.COV2.S, as well as other Ad26-based vaccines, did not show any adverse effects on nervous tissues in the available repeat-dose toxicity studies (see Mod2.6.6/Sec3 and 5.2).

The nonclinical safety package presented for Ad26.COV2.S (i.e., Ad26.COV2.S specific repeat-dose toxicity, and developmental and reproductive toxicity studies, as well as biodistribution studies covering the Ad26 vector) is similar to the package that was submitted and approved (in 2020) under the MAA for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001).

2. PHARMACOLOGY

2.1. Mechanism of Action

Induction of protective immunity by Ad26.COV2.S is thought to occur through antibody responses, predominantly a neutralizing antibody response, against the S protein. The role of cellular immune responses, particularly during a severe course of infection and for the clearance of virus-infected cells, is currently unclear.

The S protein on the surface of SARS-CoV-2 binds to the ACE2 receptor of a host cell, allowing the virus to infect the cell. Vaccination with Ad26.COV2.S leads to humoral and cellular immune responses directed against the S protein. The production of neutralizing and other functional S-specific antibodies may block ACE2 receptor binding to the S protein, thereby inhibiting viral entry into host cells, and mediate cellular effector mechanisms via Fc function, leading to clearance of SARS-CoV-2 virus particles and infected cells. Cellular immune responses may further contribute to protection by clearing SARS-CoV-2 infected cells via cytotoxic mechanisms.

Although unable to replicate in normal cells, Ad26.COV2.S can be produced in the laboratory on genetically engineered cell lines (eg, human PER.C6® and PER.C6 tetracycline repressor [TetR] cell lines) that complement for the missing E1 region. Since the genes needed for replication are lacking in normal human cells and these genes do not naturally occur in the human genome, this precludes replication of Ad26.COV2.S following IM administration to humans. Upon IM administration of Ad26.COV2.S, binding of the Ad26 vector to cellular receptors is mediated by fibers on the capsid. Currently suggested receptors on the host cell are cluster of differentiation (CD)46, the coxsackievirus adenovirus receptor (CAR), desmoglein 2, and cell surface sialic acid bearing glycans [1,22]. After transduction of the cell, vector DNA enters the nucleus without integrating into the genome, and is driving cellular production of the S protein. The S protein is then presented on the cell membrane to the host immune system and stimulates an immune response in local lymphoid organs by presentation on antigen presenting cells.

2.2. Study Rationale

The nonclinical pharmacology studies are listed in Table 2. All studies with the exception of Syrian hamster study TKO 766 were performed to inform on vaccine candidate selection for clinical development. The murine studies and rabbit study tested the immunogenicity of the vaccine, and murine study 9346-20007 was specifically designed to determine the Th1/Th2 balance induced by a single dose of vaccine. The Th1/Th2 balance has been linked with the theoretical risk of VAERD. Study NHP 20-09 was performed to assess the immunogenicity and protective efficacy of a single vaccine dose, testing different vaccine candidates including Ad26.COV2.S. Study TKO 707 in Syrian hamsters was performed to assess immunogenicity and protective efficacy of different vaccine candidates, in both 1-dose and 2-dose regimens.

After selection of Ad26.COV2.S for clinical development, Syrian hamster study TKO 766 was performed with lower dose levels of Ad26.COV2.S, which intentionally induced a suboptimal humoral response allowing breakthrough viral replication in the lungs after SARS-CoV-2 inoculation, conditions which are hypothesized to contribute to a risk of VAERD.

Additional studies are ongoing where interim data has been or will be published in support of Ad26.COV2.S development, or interim data is presented. These studies are listed in Table 3: Ongoing Pharmacology Studies With Data Referenced in the Submission.

2.3. Immunogenicity and Protective Efficacy of Ad26.COV2.S

Murine study 9346-20004 (Mod2.6.2/Sec2.3.1.1) demonstrated the humoral (binding and neutralizing antibody) and cellular immunogenicity of a single dose of Ad26.COV2.S, and also showed the benefit of the stabilizing mutations in increasing humoral immunogenicity compared with an Ad26 vector encoding the wild type S protein.

Murine study 9346-20007 (Mod2.6.2/Sec2.3.1.2) was designed to determine the Th1/Th2 balance induced by a single dose of Ad26.COV2.S. In this study, Ad26.COV2.S was shown to skew towards a Th1 skewed response. This was measured by the ratio of the Th1 associated cytokine IFN-γ, over the Th2 associated cytokines interleukin-4 (IL-4), IL-5 and IL-10, as well as by the ratio of anti-S protein immunoglobulin G (IgG) antibody subclasses IgG2a and IgG1.

Study TOX14369 in New Zealand White rabbits (Mod2.6.2/Sec2.3.2) demonstrated humoral (binding and neutralizing antibody) and cellular immunogenicity of a single dose of Ad26.COV2.S, and showed that binding and neutralizing antibody titers and cellular immune responses were further increased after a second dose of Ad26.COV2.S. This study also showed the benefit of the stabilizing mutations in increasing humoral immunogenicity compared with an Ad26 vector encoding the wild type S protein.

Syrian hamster study TKO 707 (Mod2.6.2/Sec2.3.3.1) assessed the immunogenicity, efficacy, and histopathology of 2 dose levels (10^9 and 10^{10} vp) of Ad26.COV2.S in a 1-dose regimen, and in a 2-dose regimen with a 4-week interval. A single dose of Ad26.COV2.S was immunogenic, and a second dose of Ad26.COV2.S further increased binding and neutralizing antibody titers. Ad26.COV2.S given in 1-dose and 2-dose regimens resulted in a significantly lower infectious viral load in lung tissue samples after intranasal SARS-CoV-2 challenge compared with Ad26.Empty mock-immunized animals. Overall, the majority of animals immunized with Ad26.COV2.S showed undetectable infectious viral load in the lungs. Ad26.COV2.S induced significantly higher antibody titers than the Ad26 vector encoding the wild type S protein, and a significant difference in infectious viral load in the lung was observed after challenge in the single dose regimen.

Syrian hamster study TKO 766 (Mod2.6.2/Sec2.3.3.2) assessed a single dose immunization regimen and included also lower dose levels of Ad26.COV2.S (dose level range 10^7 to 10^{10} vp). Ad26.COV2.S induced dose level-dependent S protein binding and SARS-CoV-2 neutralizing antibody titers. The study confirmed robust protective efficacy of Ad26.COV2.S against SARS-CoV-2 challenge at high vaccine doses, which is consistent with data from study TKO 707, while lower vaccine dose levels resulted in dose-dependent reduction of efficacy and partial breakthrough infection.

Study NHP 20-09 in rhesus monkeys (Mod2.6.2/Sec2.3.4) was designed to assess the immunogenicity and efficacy of a single dose of $1x10^{11}$ vp Ad26.COV2.S, one of the regimens in clinical studies VAC31518COV1001 and VAC31518COV2001. A single dose of Ad26.COV2.S induced binding and neutralizing antibodies. The NHP were inoculated with SARS-CoV-2 6 weeks after vaccination. In the group immunized with Ad26.COV2.S, viral load in the lower respiratory tract (ie, BAL) was below the lower limit of detection (LLOD) for all NHP (N=6) at all timepoints. Viral load in the upper respiratory tract (ie, nasal swabs) was below the LLOD in 5 out of 6 NHP immunized with Ad26.COV2.S; 1 animal had low viral load detectable at days 1 and 2 post inoculation. In contrast, all sham immunized NHP had high viral loads in the lower respiratory tract (LRT) and upper respiratory tract (URT) that persisted up to 14 days post inoculation .

In these studies, immune responses including neutralizing antibody responses were induced as early as 2 weeks post immunization. In addition, a favorable immunogenicity profile was observed in the nonclinical studies, ie, the induction of neutralizing antibodies and a Th1 skewed immune response. In both Syrian hamsters and NHP SARS-CoV-2 specific binding and neutralizing antibodies significantly correlate with protection from infection with SARS-CoV-2 (NHP correlate analysis and van der Lubbe et al [35]).

Similar results have been observed in ongoing studies. The increase in neutralizing antibody titers after a second dose of Ad26.COV2.S and the Th1 skewing of the immune response has been confirmed in NHP [32]. Antibody titers persisted for at least 14 weeks after a single dose of $5x10^{10}$ vp or $1x10^{11}$ vp [32]. The protective efficacy of Ad26.COV2.S in Syrian hamsters has been demonstrated in an additional, more stringent SARS-CoV-2 challenge model using a D614 variant of the virus [34], complementing current data with a G614 SARS-CoV-2 variant (studies TKO707 and TKO766). Interim data from an ongoing study in aged NHP showed that a single dose of $1x10^{11}$ vp Ad26.COV2.S afforded protection from challenge with $1x10^{5}$ $TCID_{50}$ of the G614 variant strain SARS-CoV-2/human/NLD/Leiden-0008/2020 three months post single dose immunization [32]. In the ongoing dose level titration study NHP 20-14, dose levels of $1x10^{11}$ vp and $5x10^{10}$ vp Ad26.COV2.S afforded complete protection from viral load in the lung, and lower dose levels of $1.125x10^{10}$ vp and $2x10^{9}$ vp afforded protection in 4 out of 5 animals (Mod2.6.2/Sec2.4).

2.4. VAERD Risk Monitoring

Nonclinical data have shown the induction of neutralizing antibodies and a Th1 skewed immune response, factors that are thought to minimize potential risk of VAERD (Section 2.3). VAERD was also monitored in nonclinical efficacy studies by assessment of histopathology in respiratory tract tissues taken after SARS-CoV-2 inoculation and by clinical monitoring of infected animals.

In Syrian hamster studies TKO 707 (Mod2.6.2/Sec2.3.3.1) and TKO 766 (Mod2.6.2/Sec2.3.3.2) the following histopathological parameters were assessed: alveolitis, extent of alveolitis/alveolar damage, alveolar edema, alveolar hemorrhage, type II pneumocyte hyperplasia, bronchitis, bronchiolitis, peribronchial and perivascular cuffing, tracheitis and rhinitis. Study TKO 766 included lower dose levels of Ad26.COV2.S (dose level range 10^{7} to 10^{10} vp), which resulted in

dose-dependent reduction in protective efficacy against SARS-CoV-2 challenge and breakthrough infection in the lung. In these studies, there was no evidence of VAERD, including Syrian hamsters with breakthrough infection in the lung (total 28 out of 56 immunized hamsters).

In study NHP 20-09 (Mod2.6.2/Sec2.3.4) the following LRT histopathological parameters were assessed in Ad26.COV2.S immunized and sham-treated animals after SARS-CoV-2 challenge: alveolar edema, inflammation interstitial/septal thickening, mononuclear cell infiltrates in perivascular/ peribronchiolar space, macrophage infiltrates in alveolar space, macrophage infiltrates in bronchiolar space, neutrophil infiltrates in alveoli, bronchioloalveolar hyperplasia, bronchial-associated lymphoid tissue (BALT) hyperplasia. No signs of VAERD were observed in this NHP challenge study, either when assessing the cumulative histopathology score or any individual score compared with the unvaccinated control group. The histopathology scores were reduced in Ad26.COV2.S immunized animals, when compared with the mock immunized animals. Conclusions on viral load are included in Section 2.3. In this study, breakthrough infection in the lung was not detected in Ad26.COV2.S immunized NHP.

The Th1 skewing of the immune response has been confirmed in interim data from an ongoing study in NHP [32]. Moreover, interim data from an ongoing studies in Syrian hamsters and in (aged) NHP showed that Ad26.COV2.S afforded protection from challenge with SARS-CoV-2, with no histopathologic evidence of VAERD [32,34].

We have shown in Ad26.COV2.S immunized Syrian hamsters and NHP absence of enhanced lung pathology and clinical signs of disease compared with controls after SARS-CoV-2 inoculation, even under suboptimal immunity allowing breakthrough infection. Together with induction of neutralizing antibodies and a Th1 skewed immune response after Ad26.COV2.S dosing these data suggest that the theoretical risk of VAERD and VAED for Ad26.COV2.S is low.

3. PHARMACOKINETICS

Biodistribution studies have been conducted to assess the distribution, persistence, and clearance of the Ad26 vaccine platform following IM injection in NZW rabbits using Ad26.ENVA.01 and Ad26.RSV.preF. No pharmacokinetic or biodistribution studies have been conducted with Ad26.COV2.S.

The Ad26 vector contains deletions in the early region (E1) of the Ad26 genome, rendering it replication-incompetent. Ad26-based vaccines, including Ad26.COV2.S, require recombinant E1 complementing cell lines, like the PER.C6 (TetR) cells, for virus replication. Outside of these specific cellular environments, Ad26-based vaccines, including Ad26.COV2.S, cannot replicate or reproduce and are therefore expected to show a limited distribution and persistence following administration. This is confirmed by the biodistribution studies with Ad26.ENVA.01 (study 1645-06074; Mod2.6.4/Sec4.1) and Ad26.RSV.preF (study TOX13342; Mod2.6.4/Sec4.2). In these studies, animals were sacrificed on Days 11, 61, or 91 (Ad26.ENVA.01), and on Days 11, 90, 120 or 180 (Ad26.RSV.preF) following single IM injection at a dose level of 5×10^{10} vp (Ad26.ENVA.01) or 1×10^{11} vp (Ad26.RSV.preF). Tissues from these animals were harvested for analysis of Ad26 vector DNA using q-PCR. As a general pattern, both Ad26 vectors showed a

limited biodistribution profile following IM administration, as they were primarily detected at the site of injection, regional (iliac) lymph nodes and (to a lesser extent) the spleen. No Ad26 vector DNA was detected in the gonads or in the brain. Comparing the various necropsy timepoints following IM administration (i.e., Days 11, 61, and 91 for Ad26.ENVA.01; Days 11, 90, 120 and 180 for Ad26.RSV.preF), a clear downward trend in the number of positive tissues, and/or vector copy number present in those positive tissues was observed, to levels close to, or below the respective limits of detection towards the end of the observation period, indicating clearance of the Ad26 vector. The data further indicate that the Ad26 vector does not replicate and/or persist in the tissues following IM injection. Despite differences in the expressed transgene insert, both Ad26 vectors showed a similar pattern of biodistribution and clearance when delivered via the IM route at full human dose levels in the rabbit. This pattern of clearance and (limited) distribution as observed for the Ad26 vector is in line with results from other (replication-incompetent) adenoviral vectors, including Ad5 and Ad35 [30].

The Ad26 vector backbone used for Ad26.COV2.S is identical to the vector backbone of the Ad26-based vaccines that were tested in the available biodistribution studies (i.e., Ad26.ENVA.01 and Ad26.RSV.preF). The only difference between the vectors, apart from the encoded antigen transgene, is the insertion of a tetracycline operon (TetO) motif in the cytomegalovirus promoter sequence of the transgene expression cassette of Ad26.COV2.S. The TetO motif was included in the Ad26 vector to improve vector bioreactor yields, as described in Mod3.2.S.2.3 Control of Materials – Source, History, and Generation of the Pre-Master Virus Seed.

The insertion of the TetO sequence in the transgene expression cassette is not considered to impact the biodistribution profile of the Ad26 vector. Adenoviruses are non-enveloped viruses whose cell entry, and therefore tropism, is dictated via interactions of structural capsid proteins (mainly the fiber and penton base) with specific cellular receptors [29]. The adenoviral capsid is a highly complex and organized structure [23] which does not easily allow for the introduction or exchange of other proteins. The transgene expression cassette itself, which is inserted into the site where the early E1 gene was previously located, is thus not considered to impact on the formation or the composition of the Ad26 vector capsid, and hence tropism of the vector. As a consequence, the biodistribution profile of the Ad26 vector is considered independent of the transgene/expression cassette, which is supported by the comparable biodistribution profile observed for Ad26.ENVA.01 and Ad26.RSV.preF. This is in line with a study from Sheets et al, 2008 [30], which concluded that the biodistribution profile for Ad5 and Ad35 vectors was consistent, regardless of differences in transgene inserts.

As described above (see Section 1.5), adenoviral vectors are classified as non-integrating because they lack the machinery to integrate their genome into the host chromosomes (e.g., EMA Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors [6]; FDA Guidance for Industry, Long Term Follow-Up After Administration of Human Gene Therapy Products [12]). As such, upon transduction of a cell, the adenoviral DNA does not integrate into the host genome, but rather resides episomally in the host nucleus [13,21]. Therefore, and also supported by the results from the biodistribution studies where clearance of the vector was shown, the risk of integration of genetic material from an Ad26 vector in the human host genome and

potentially associated delayed/long-term adverse events is unlikely. In fact, clinical data from long-term follow-up observations of subjects who have received adenovirus (gene-therapy) products in trials conducted over the last years, further support the assessment of low risk for delayed/long term side effects of these products [12].

Ad26 vectors show limited shedding following intramuscular injection in clinical trials. Shedding studies with Ad26.RSV.preF, Ad26.ZEBOV and Ad26.Mos4.HIV showed that Ad26 vector DNA is rarely found and disappears quickly over time in secreted body fluids after vaccination. No replication-competent virus could be detected in samples positive for vector DNA (Environmental Risk Assessment).

In a human shedding study performed with Ad26.RSV.preF (study VAC18193RSV1005), vector DNA was observed in a single semen specimen at a single timepoint (Day 8 post vaccination), but all subsequent samples up to 183 days post vaccination (i.e., covering 2 sperm cycles) from this subject were negative for vector DNA (Environmental Risk Assessment). This, together with the results from the nonclinical biodistribution studies described above, in which no Ad26 vector DNA was detected in the gonads (i.e., testes and ovary), indicates that it is unlikely that the Day 8 positive semen sample represents association of the Ad26.RSV.preF vector with the sperm cells, and that the Ad26 vector does not persist in the semen. In addition, no infectious virus was detected in the culture from this sample, indicating that no replication of vaccine vector or adenovirus was found in this single semen sample. Overall, considering the non-integrating characteristic of adenoviral vectors, together with the fact that the nonclinical biodistribution studies did not show any dissemination/persistence of the Ad26 vector in the male and female gonads, there is no need to conduct dedicated nonclinical germline transmission studies.

Dissemination of the Ad26 vector to breast milk or to/across the placenta has not been specifically assessed in the available nonclinical biodistribution studies. However, considering the pattern of limited biodistribution (as summarized above), and in line with the limited shedding as observed in clinical studies (Environmental Risk Assessment), Ad26.COV2.S is unlikely to be excreted in breastmilk. Even if a small quantity of the vector would be (transiently) excreted via the milk, it would not be considered a risk to lactating newborns, specifically with regard to infections, as Ad26.COV2.S is replication-incompetent and does not encode a complete SARS-CoV-2 virus. In addition, Ad26.COV2.S is unlikely to be disseminated to the placenta considering its non-replicating characteristic, and consistent with the limited distribution as observed following IM injection. In line with the above, even if a small quantity of the (non-replicating) vector would be (transiently) disseminated to/across the placenta, it would not be considered a risk for the growing fetus. It is further noted that the pivotal combined EF-PPND toxicity study in rabbits (study TOX14389, see Section 4.2.1) did not indicate any signs of reproductive toxicity following maternal immunization with Ad26.COV2.S during pregnancy.

In conclusion, the biodistribution data obtained with Ad26.ENVA.01 and Ad26.RSV.preF are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same route of administration (IM) and at a comparable dose level ($5x10^{10}$ vp to $1x10^{11}$ vp). The results from these studies show a pattern of limited distribution and indicate clearance over

time of the Ad26 vector following IM injection. The data do not indicate any dissemination/persistence of the Ad26 vector to or in the male and female gonads of the rabbits.

4. TOXICOLOGY

The nonclinical safety profile of the Ad26.COV2.S vaccine was assessed in a pivotal repeat-dose toxicity study, including local tolerance, as well as in a combined EF-PPND toxicity study. In addition, a high-level summary of supportive toxicology data with other Ad26-based vaccines using various antigen transgenes is described.

4.1. General Toxicity and Local Tolerance

4.1.1. Ad26.COV2.S

In the pivotal repeat-dose toxicity and local tolerance study in rabbits (study TOX14382; Mod2.6.6/Sec 3), Ad26.COV2.S was well tolerated when administered on three occasions over 4 weeks (i.e., every 2 weeks) at 1 x 10^{11} vp/dose. The observed changes were related to a normal, anticipated (local and systemic) immunologic response to vaccination, and consisted clinically of (rare) transient local injection site dermal reactions, with transient minimal hyperthermia and minimal body weight loss or lower body weight gain after injection. This was associated with a transient (acute phase/immune) response in clinical pathology parameters, characterized by increases in plasma proteins (C-reactive protein [CRP], fibrinogen and globulins), and white blood cell counts (monocytes and lymphocytes). Microscopic pathology findings of minimal to slight inflammation and hemorrhage were observed at the injection sites, along with increased lymphoid cellularity of germinal centers in popliteal and iliac lymph nodes and the spleen, which is consistent with an immune response to the vaccine administration. Overall, the findings were considered non-adverse and were partially or completely reversible after a 3-week treatment-free period. All vaccinated animals developed an antibody response against SARS-CoV-2 S protein, confirming responsiveness of the rabbits to the vaccine.

The target organs or tissues identified in this study (i.e., injection site, [draining] lymph nodes and spleen) were consistent with the tissues that tested mostly positive for Ad26 DNA in the biodistribution studies for the Ad26 platform (Section 3).

Immunogenicity of Ad26.COV2.S was tested in mice, rabbits, Syrian hamsters, and NHPs, and vaccine efficacy was tested in Syrian hamsters and NHPs (see Section 2). Ad26.COV2.S was tested up to a dose level of 1×10^{10} vp in mice and Syrian hamsters, 5×10^{10} vp in rabbits, and 1×10^{11} vp in NHP. No vaccine-related adverse effects were noted in these non-GLP immunogenicity/efficacy studies.

4.1.2. Ad26-Platform: Supportive Toxicity Data

The Sponsor has significant nonclinical experience with Ad26-vectored vaccines using various transgenes encoding for HIV, malaria, RSV, Zikavirus, Filovirus (Ebolavirus, Marburgvirus), influenza (Universal influenza) and HPV antigens. More than 10 GLP combined repeat-dose toxicity and local tolerance studies as well as one EF-PPND toxicity study (with Ad26.ZEBOV; see Section 4.2.2) have been performed in rabbits (or rats) testing the nonclinical safety of these

various Ad26-based vaccines either alone or in combination with various other vaccine modalities, including Ad35-based vaccines, Modified Vaccinia Virus Ankara (MVA)-based vaccines and/or (glyco)proteins with or without an aluminum phosphate adjuvant. In these studies, up to 5 sequential IM dose administrations have been tested at levels up to 4×10^{11} vp. The tested vaccine regimens in these supportive Ad26-platform studies were well tolerated. Irrespective of the antigen transgene used, the vaccine-related effects observed in these supportive studies were largely similar across studies and considered to be reflective of a normal, immunologic response to the administered vaccines, similar to the responses noted with Ad26.COV2.S in TOX14382. No vaccine-related adverse effects were observed. Therefore, these studies with other Ad26-based vaccines are considered to provide supportive repeat-dose toxicity (including local tolerance) data for Ad26.COV2.S, which is based on the same Ad26 vector backbone.

An overview of the completed GLP toxicology studies testing various Ad26-based vaccines and/or vaccine regimens is provided in Appendix 2.

4.2. Reproductive Toxicity

4.2.1. Ad26.COV2.S

Female reproductive toxicity and fertility were assessed in a combined EF-PPND toxicity study in the rabbit using a 3-dose vaccine regimen (study TOX14389; Mod2.6.6/Sec5.2). In this study, a first vaccine dose of Ad26.COV2.S at $1x10^{11}$ vp was administered 7 days prior to mating (i.e., Day 1) with untreated male rabbits to ensure induction of a maternal immune response during mating and early gestation.

In order to evaluate potential direct embryotoxic effects of the components of the vaccine formulation and to ensure high antibody titers during early gestation, a second vaccine dose was administered 6 days after mating (i.e., on Gestation Day [GD] 6), corresponding to the start of organogenesis, around implantation in rabbits. This was followed by a third vaccination on GD20, in order to assess the (direct) effects of the vaccine during late gestation and to ensure a sustained maternal immune response during late gestation as well as during lactation.

This dose scheme allows an assessment of potential effects of the vaccine and/or the maternal immune response induced by the vaccine on the various stages of pregnancy. The design of this study has been discussed with CHMP and CBER and was found acceptable (EMA/CHMP/SAWP/221031/2020, dated 24 April 2020, CHMP response to Sponsor Question 10; PTS 5513, CRMTS 12536, CBER feedback dated 5 June 2020, CBER Response to Sponsor Question 10).

There was no adverse effect of Ad26.COV2.S on reproductive performance, fertility, ovarian and uterine examinations, parturition, or macroscopic evaluations in parental females. In addition, there was no adverse effect of vaccination on fetal body weights, external, visceral and skeletal evaluations, or on postnatal development of the offspring (sex ratios, survival, body weights, clinical findings, developmental evaluations, and macroscopic evaluations). The parental females as well as their fetuses and offspring (kits) exhibited SARS-CoV-2 S protein-specific antibody titers, indicating that maternal antibodies were transferred to the fetuses during gestation.

Overall, the EF-PPND toxicity study with Ad26.COV2.S did not reveal any evidence of impaired female fertility and did not indicate harmful effects with respect to reproductive toxicity. In addition, the combined repeat-dose toxicity and local tolerance study with Ad26.COV2.S as well as the general (supportive) toxicity studies with other Ad26-based vaccines have not revealed any effects on male sex organs that would impair male fertility.

4.2.2. Ad26-Platform: Supportive Reproductive Toxicity Data

As part of the Ad26 platform, a supportive EF-PPND toxicity study (TOX11212) has been conducted in rabbits using an Ad26-based Ebola vaccine, i.e., Ad26.ZEBOV (Ad26 vector encoding the glycoprotein of Zaïre ebolavirus Mayinga variant; 1×10^{11} vp) in a 2-dose homologous regimen, or in a 2-dose heterologous regimen with MVA-BN-Filo (3.61×10^{8} Inf U). This study is considered to provide supportive reproductive and developmental toxicity data for Ad26.COV2.S, as Ad26.ZEBOV is produced from the same Ad26 vector backbone as Ad26.COV2.S. Similar to the EF-PPND toxicity study with Ad26.COV2.S, the vaccine regimens in this study with Ad26.ZEBOV did not induce maternal or developmental toxicity in rabbits following maternal exposure during the premating and gestation period. This study (TOX11212) was part of the MAA file for the Ebola vaccine component Zabdeno (Ad26.ZEBOV-GP [recombinant]) (EU/1/20/1444/001).

4.3. Juvenile Toxicity

Studies in juvenile animals were not performed. During the assessment procedure of the PIP and the PSP, the PDCO and CBER, respectively, confirmed that no further nonclinical studies were deemed necessary to support the pediatric development. Final approval of both documents is pending.

All vaccine-related effects noted in the dedicated Ad26.COV2.S studies, as well as in the supportive studies with other Ad26-based vaccines (see Appendix 2) in (young) adult rabbits were considered to reflect a non-adverse, immunologic response to the injection of a vaccine. These studies did not indicate any other target organs than those anticipated based on the pharmacological mode of action, i.e., the immune system. Hence, the main consideration when immunizing infants or children would be any untoward effect associated with the immune system.

It is noted that immune system development is significantly more advanced in humans at birth when comparing with routine preclinical species [15,31]. Therefore, from a preclinical perspective, vaccine studies in juvenile animals (i.e., very young or newborn animals) would not yield any additional relevant information and have therefore not been conducted. Interpretation of any findings derived from such models would pose challenges, especially since it may not be possible to extrapolate immune system developmental stages from the animal model to humans [33].

In the repeat-dose toxicity and local tolerance studies with Ad26.COVS2.S and other Ad26-based vaccines, the lower age range of the NZW rabbits used was approximately 12 weeks at start of the study, which is an age range equivalent to young adolescent age in humans [20]. These animals were confirmed to be immunologically responsive to the vaccine, i.e., in all toxicology studies a

specific antibody response was detected. This immune response was not associated with any adverse effects in the animals.

In the EF-PPND toxicity study with Ad26.COV2.S, fetuses and offspring (kits) from vaccinated parental females exhibited SARS-CoV-2 S protein-specific antibody titers, indicating that maternal antibodies were transferred to the fetuses during gestation. No adverse effects from exposure to these (maternal) antibodies were observed on fetal or postnatal development in the rabbit.

Overall, the available (general) repeat-dose toxicology studies with Ad26.COV2.S and Ad26-based vaccines are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children/adolescents. There were no findings in these nonclinical studies that would indicate a concern for the use of the vaccine in infants/children/adolescents.

5. INTEGRATED OVERVIEW AND CONCLUSIONS

Pharmacology

Immunogenicity data from studies in mice, rabbits, Syrian hamsters, and NHP show that a single dose of Ad26.COV2.S induces humoral and cellular immune responses as early as 2 weeks post immunization. Ad26.COV2.S induces neutralizing antibodies and a Th1 skewed immune response, factors that are thought to be beneficial to minimize potential risk of VAERD.

In a Syrian hamster SARS-CoV-2 challenge model, Ad26.COV2.S provided dose-level dependent protection from infectious viral load in the lung. In a SARS-CoV-2 NHP challenge model, immunization with Ad26.COV2.S at dose levels of 1×10^{11} vp and 5×10^{10} vp fully protected 16 out of 16 NHP from viral replication in the lung and protected 14 out of 16 NHP from viral replication in the nose (studies NHP 20-09 and NHP 20-14). In both Syrian hamsters and NHP SARS-CoV-2 specific binding and neutralizing antibodies significantly correlate with protection from infection with SARS-CoV-2 (NHP correlate analysis and van der Lubbe et al [35]).

Two SARS-CoV-2 challenge studies in Syrian hamsters and 1 NHP challenge study showed no indications of VAERD based on monitoring of clinical signs and viral load of Ad26.COV2.S vaccinated animals after SARS-CoV-2 challenge, and based on histopathologic assessment of lung tissue from these animals compared with challenged control animals. In conclusion, data from the preclinical studies performed by the Sponsor suggest that the theoretical risk of VAERD with Ad26.COV2.S is low, however, it is acknowledged that there is limited understanding of the value of nonclinical models in predicting the risk of VAERD in humans.

Biodistribution

Biodistribution studies in rabbits showed a pattern of limited distribution of the Ad26 vector. Clearance (i.e., reflected by a downward trend in number of positive tissues and vector copies over time, to levels close to, or below the respective detection limits) of the Ad26 vector was observed following IM injection, indicating that the vector does not replicate and/or persist in the tissues.

As biodistribution is considered dependent on the viral vector platform and not on the transgene insert, the biodistribution results obtained with Ad26.ENVA.01 and Ad26.RSV.preF are considered sufficient to inform on the biodistribution profile of Ad26.COV2.S when administered via the same (i.e., IM) route.

Toxicology

Ad26.COV2.S administered on three occasions over 4 weeks (i.e., every 2 weeks) at $1x10^{11}$ vp/dose in the toxicity studies was well tolerated, and was not associated with any adverse vaccine-related effects. The vaccine-related effects noted were considered to reflect a normal, immunologic response consistent with vaccination. The nonclinical safety profile of Ad26.COV2.S is largely similar to the profile observed previously for other Ad26-based vaccines.

The EF-PPND toxicity study did not reveal any evidence of impaired female fertility and did not indicate harmful effects with respect to reproductive toxicity. In addition, the repeat-dose toxicity studies with Ad26.COV2.S or other Ad26-based vaccines have not revealed any effects on male sex organs that would impair male fertility.

Overall Conclusion

Ad26.COV2.S was immunogenic and protected from SARS-CoV-2 infection in nonclinical studies. The nonclinical data did not show any adverse vaccine-related effects and support the use of Ad26.COV2.S in humans. We have shown in Ad26.COV2.S immunized Syrian hamsters and NHP absence of enhanced lung pathology and clinical signs of disease compared with controls after SARS-CoV-2 inoculation, even under conditions of suboptimal immunity allowing breakthrough infection. Together with induction of neutralizing antibodies and a Th1 skewed immune response after Ad26.COV2.S dosing these data suggest that the theoretical risk of VAERD and VAED for Ad26.COV2.S is low.

6. LIST OF LITERATURE CITATIONS

Literation citations are located in Mod4.3.

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APPENDIX 1: JUSTIFICATION FOR ABSENCE OF DOCUMENTS IN MODULE 4

Module	Module Title	Justification
4.2.1.2	Secondary Pharmacodynamics	Not applicable for vaccines
4.2.1.3	Safety Pharmacology	No separate safety pharmacology studies were performed since data from the Ad26.COV2.S-specific and Ad26 platform toxicity studies (which include detailed clinical examinations) did not suggest that the vaccine has a significant impact on physiological functions (e.g., central nervous system, respiratory, an cardiovascular functions) other than those of the immune system.
4.2.1.4	Pharmacodynamic Drug Interactions	Not applicable for vaccines
4.2.2.1	Analytical Methods and Validation Reports	In the biodistribution studies, specific quantitative polymerase chain reaction (q-PCR) assays were used to detect and quantify Ad26-vector DNA expression in tissues and blood collected at specified time points following vector administration. A description of the assays is available in the different biodistribution reports (in Module 4.2.2.3).
4.2.2.2	Absorption	Not applicable for vaccines
4.2.2.4	Metabolism	Not applicable for vaccines
4.2.2.5	Excretion	Not applicable for vaccines
4.2.2.6	Pharmacokinetic Drug Interactions	Not applicable for vaccines
4.2.2.7	Other Pharmacokinetic Studies	Other pharmacokinetic studies were not performed
4.2.3.1	Singe-Dose Toxicity	Separate studies to determine single dose toxicity were not performed. Possible signs of acute toxicity effects were monitored following the first vaccination in the repeat-dose toxicity studies in Module 4.2.3.2.
4.2.3.3	Genotoxicity	In accordance with WHO Guidelines on Nonclinical Evaluation of Vaccines, no genotoxicity studies were performed for Ad26.COV2.S. In addition, adenoviral vectors are classified as non-integrating, so the risk of integration of genetic material from the Ad26 vector in the host genome, and possible associated insertional mutagenesis is unlikely.
4.2.3.4	Carcinogenicity	In accordance with WHO Guidelines on Nonclinical Evaluation of Vaccines, no carcinogenicity testing was performed for Ad26.COV2.S.
4.2.3.5.1	Fertility and Early Embryonic Development	No dedicated fertility studies have been conducted. The histopathology data from the Ad26.COV2.S-specific (in Module 4.2.3.2) and Ad26-platform repeat-dose toxicity studies in rabbits do not raise any concerns that the vaccine adversely affects male or female reproductive organs. Female fertility was evaluated as part of the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2), e.g., via the premating vaccine administration, and did not show any adverse effects on female fertility.
4.2.3.5.3	Prenatal and Postnatal Development	Assessment of prenatal and postnatal development was included in the combined embryo-fetal and pre- and postnatal development study which is located in Module 4.2.3.5.2.

Module	Module Title	Justification
4.2.3.5.4	Studies in Juvenile Animals	The repeat-dose toxicity and local tolerance study (in Module 4.2.3.2) and the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2) are considered to provide sufficient assurance of safety regarding possible effects associated with an immune response to the vaccine in infants/children. There were no findings in these nonclinical studies that would indicate a concern for the use of the vaccine regimen in the pediatric population from birth onwards (see Section 4.3 for discussion).
4.2.3.6	Local Tolerance	The local tolerance following IM injection of the vaccines was evaluated as part of the pivotal repeat-dose toxicity (in Module 4.2.3.2) and reproductive toxicity studies (Module 4.2.3.5.2). In these toxicology studies, Ad26.COV2.S was well tolerated when given at a dose above the maximum anticipated human dose and when receiving a number of injections that is higher than what is used in the clinical regimen.
4.2.3.7	Other Toxicity Studies	Other toxicity studies besides the repeat-dose toxicity and local tolerance study (in Module 4.2.3.2) and the combined embryo-fetal and pre- and postnatal development study (in Module 4.2.3.5.2) were not performed. Neurovirulence and/or neurotoxicity testing was not performed since (1) Ad26.COV2.S is replication-incompetent; and (2) nonclinical biodistribution studies conducted with the Ad26 vector did not show any distribution of the vectors to the brain, and (3) Ad26.COV2.S did not show any adverse effects on nervous tissues in the repeat-dose toxicity study TOX14382.

APPENDIX 2: OVERVIEW OF SUPPORTIVE GLP TOXICOLOGY STUDIES TESTING AD26-VECTORED VACCINES IN RABBITS OR RATS AFTER IM INJECTIONS

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
RABBIT STUDIES			
Repeated dose (IND016112/0000/Mod4.2.3.2/1006-001)*	HIV[d]	5 or 6 injections (Days 1, 22, 43, 64, 85, 106; 3-week interval between doses) • Saline on each dosing day • MVA.Natural ($1x10^8$ pfu) on Days 1, 22, 43, 64 and 85 • MVA.Mosaic ($1x10^8$ pfu) on Days 1, 22, 43, 64 and 85 • **Ad26.ENVA.01** ($5x10^{10}$ vp) on Days 1, 22 and 43; MVA-Natural ($1x10^8$ pfu) on Days 64, 85 and 106 • **Ad26.ENVA.01** ($5x10^{10}$ vp) on Days 1, 22 and 43; MVA-Mosaic ($1x10^8$ pfu) on Days 64, 85 and 106	*Clin obs/clin path:* ↑ BT (x1.02), ↑ CRP (x79), ↑ neutrophils (x2.21), ↑ fibrinogen (x1.96) *Necropsy/histology*: iliac LN (enlargement, ↑ weight, edema, erythrocytosis/erythrophagocytosis, hyperplasia), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016263/0000/Mod4.2.3.2/TOX10873)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on each dosing day + Clade C gp140/aluminum phosphate (250 µg/425 µg) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; Clade C gp140/aluminum phosphate (250 µg/425 µg) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; Clade C gp140/aluminum phosphate (250 µg/425 µg) + MVA-Mosaic ($1x10^8$ pfu) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on Days 1 and 22; MVA-Mosaic ($1x10^8$ pfu) on Days 43 and 64 • **Ad26.Mos.HIV** ($5x10^{10}$ vp) on each dosing day	*Clin obs/clin path*: transient ↓ FC (x0.63), ↑ CRP (x222), ↑ fibrinogen (x1.97), ↑ globulin (x1.18), ↑ BT (x1.10) *Necropsy/histology*: iliac LN and spleen (↑ weight, ↑ germinal centers, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016263/0115/Mod4.2.3.2/1645-06077)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.ENVA.01** ($1x10^{10}$ vp) on each dosing day • **Ad26.ENVA.01** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* ↑ monocytes (x2.20), ↑ globulin (x1.23), ↑ total protein (x1.02), ↑ albumin (x1.02), ↓ albumin/globulin[b] (x0.85) *Necropsy/histology*: injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND016263/0043/Mod4.2.3.2/TOX12014)*	HIV[d]	4 injections (Days 1, 22, 43, 64; 3-week interval between doses) • Saline on each dosing day • **Ad26.Mos4.HIV** ($5x10^{10}$ vp) on Days 1 and 22; **Ad26.Mos4.HIV** ($5x10^{10}$ vp) + Clade C gp140/Mosaic gp140/aluminum phosphate (125 µg/125 µg/425 µg) on Days 43 and 64	*Clin obs/clin path:* transient ↓ FC (x0.92), ↑ CRP (x26), ↑ fibrinogen (x2.10), ↑ total protein (x1.10), ↑ globulin (x1.30), ↓ prothrombin time (x0.90), ↓ albumin/globulin (x0.80) *Necropsy/histology*: iliac LN and spleen (↑ germinal centers, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (1854-09764)	Malaria[d]	5 injections (Days 1, 22, 43, 64, 85; 3-week interval between doses) • Saline on each dosing day • Ad35.CS.01 ($1x10^{11}$ vp) on Days 1, 22 and 43; **Ad26.CS.01** ($0.77x10^{11}$ vp) on Days 64 and 85	*Clin obs/clin path:* transient ↓ BW (x0.99), ↓ FC (x0.62), ↑ BT (x1.01), ↑ globulin (x1.16), ↑ CRP (x22), ↑ fibrinogen (x1.06), ↑ APTT (x1.38), ↑ monocytes (x2.78), minimal changes in RBC/platelet parameters *Necropsy/histology*: iliac LN and spleen (↑ weight, hyperplasia), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016401/0000/Mod4.2.3.2/TOX10931)*	RSV[d]	4 injections (Days 1, 15, 29, 43/44; 2-week interval between doses) • Formulation Buffer 5 on each dosing day • **Ad26.RSV.FA2** ($0.65x10^{11}$ vp) on each dosing day • Ad35.RSV.FA2 ($1.3x10^{11}$ vp) on each dosing day • **Ad26.RSV.FA2** ($0.65x10^{11}$ vp) on Days 1 and 15; Ad35.RSV.FA2 ($1.3x10^{11}$ vp) on Days 29 and 43/44	*Clin obs/clin path:* transient ↓ FC (x0.50), ↑ CRP (x33), ↑ fibrinogen (x2.50), ↑ total protein (x1.10), ↑ globulin (x1.30), ↓ prothrombin time (x0.90), ↓ APTT (x0.90), ↓ albumin/globulin (x0.80), ↓ RBC parameters, minor disturbances in differential WBC numbers (neutrophils [x0.40], monocytes [x3.50]) *Necropsy/histology*: iliac LN and spleen (↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 22-day treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND017148/0002/Mod4.2.3.2/TOX11592)*	RSV[d]	5 injections (Days 1, 15, 29, 43, 57/58; 2-week interval between doses) • Formulation Buffer 5 on each dosing day • **Ad26.RSV.preF** ($1x10^{11}$ vp) on each dosing day • Ad35.RSV.FA2 ($0.65x10^{11}$ vp) on Days 43 and 57/58	*Clin obs/clin path:* transient BW loss (1-2%) and ↓ FC (x0.64), ↑ CRP (x33), ↑ fibrinogen (x2.10), ↑ globulin (x1.21), ↓ albumin/globulin (x0.74) *Necropsy/histology*: iliac LN and spleen (enlargement and/or ↑ weight, ↑ cellularity, ↑ germinal center development), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND017148/0017/Mod4.2.3.2/TOX12288)*	RSV	3 injections (Days 1, 15, 29; 2-week interval between doses) • Saline on each dosing day • RSV-F Protein (250 µg) on each dosing day • **Ad26.RSV.preF** ($1x10^{11}$ vp) on Day 1; RSV-F Protein (250 µg) on Days 15 and 29 • **Ad26.RSV.preF**/RSV-F Protein mix[c] ($1x10^{11}$ vp/250 µg) on Days 1,15 and 29	*Clin obs/clin path*: transient BW loss (2%) and ↓ FC (x0.75), resulting in overall ↓ BWG (the latter only in group 4 receiving mix Ad26.RSV.PreF with RSV-F protein) (x0.70), ↑ fibrinogen (x2.00), ↑ globulin (x1.20), ↑ CRP (x19) ↓ albumin/globulin (x0.80); no findings in RSV-F Protein only group *Necropsy/histology*: iliac LN and spleen (enlargement and/or ↑ cellularity), injection sites (dark discoloration, inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (TOX14253)	RSV	2 injections (Days 1, 15; 2-week interval between doses) • Saline on each dosing day • **Ad26.RSV.preF**/RSV preF protein mix[b] ($2×10^{11}$ vp /240 µg) on each dosing day • **Ad26.RSV.preF** ($4x10^{11}$ vp) on each dosing day	*Clin obs/clin path*: ↑ BT (x1.03), ↑ fibrinogen (x2.15), ↑ CRP (x36.7), ↑ WBCs (lymphocytes x1.34, monocytes x3.11, neutrophils x1.86 [Ad26.RSV.preF group]), ↑ creatine kinase (x2.72; Ad26.RSV.preF group), ↑ globulin (x1.23; Ad26.RSV.preF group), ↓ albumin (x0.96), ↓ albumin/globulin (x0.80; Ad26.RSV.preF group), ↓ RBC parameters, ↓ ALP (x0.58) *Necropsy/histology*: iliac and popliteal LN and spleen (↑ cellularity germinal centers), injection sites (mononuclear cell infiltration, inflammation) *Recovery*: ↑ neutrophils (x1.24; Ad26.RSV.preF group), ↑ monocytes (x1.91). Full or partial recovery of microscopic findings after 17-day treatment-free period. **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND016280/0004/Mod4.2.3.2/TOX11059)* (EMEA/H/C/005337/0000/Mod4.2.3.2/ TOX11059)**	Ebola[d]	2 injections (Days 1 and 15/16; 14-day interval between doses) • Saline on each dosing day • **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 1; MVA-BN-Filo ($4.4x10^{8}$ $TCID_{50}$) on Day 15/16 • MVA-BN-Filo ($4.4x10^{8}$ $TCID_{50}$) on Day 1; **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 15/16 • **Ad26.ZEBOV** ($5x10^{10}$ vp) + MVA-BN-Filo ($1x10^{8}$ $TCID_{50}$) on Day 1; Ad26.ZEBOV ($5x10^{10}$ vp) + MVA-BN-Filo ($1x10^{8}$ $TCID_{50}$) on Day 15/16 • **Ad26.ZEBOV** ($5x10^{10}$ vp) on Day 1; Ad26.ZEBOV ($5x10^{10}$ vp) on Day 15/16	*Clin obs/clin path:* transient ↓ FC (x0.46), ↑ CRP (x95), ↑ fibrinogen (x2.32), ↑ total protein (x1.10), ↑ globulin (x1.31), ↓ RBC parameters, ↓ neutrophils (x0.38), ↓ albumin/globulin (x0.73) *Necropsy/histology*: iliac LN and spleen (enlargement, ↑ weight, and/or ↑ cellularity, ↑ germinal centers), injection sites (inflammation) *Recovery*: (ongoing) recovery after 2-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Repeated dose (IND017088/0000/Mod4.2.3.2/TOX11260)*	Filo[d]	3 injections (Days 1, 15, 29/30; 14-day interval between doses)[e] • Saline on each dosing day • **Ad26.Filo** ($1.2x10^{11}$ vp) on Day 1; MVA-BN-Filo ($5x10^{8}$ Inf.U) on Days 15 and 29/30 • **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 1 and Day 29/30; MVA-BN-Filo ($5x10^{8}$ Inf.U) on Day 15 • MVA-BN-Filo ($5x10^{8}$ Inf.U) on Day 1; **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 15 and 29/30 • **Ad26.Filo** ($1.2x10^{11}$ vp) on Days 1 and 15; Ad35.ZEBOV ($1.2x10^{11}$ vp) on Day 29/30	*Clin obs/clin path:* transient BW loss, ↓ FC (x0.52), ↑ BT (x1.02) (individual animals), ↑ CRP (x81), ↑ fibrinogen (x2.4), ↓ prothrombin time (x0.9), ↑ globulin (x1.38), ↓ albumin/globulin (x0.76) *Necropsy/histology*: iliac LN and spleen (enlargement, ↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (IND017749/0002/Mod4.2.3.2/TOX13018)*	Zika	3 injections (Days 1, 15, 29; 2-week interval between doses) • Saline on each dosing day • **Ad26.ZIKV.001** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* transient ↓ FC (day after 1st and 2nd injection) (x0.80), ↑ monocytes (x3.00), ↑ CRP (x50), ↑ fibrinogen (x2.00), ↑ globulin (x1.30), ↓ albumin/globulin (x0.78) *Necropsy/histology*: iliac LN and spleen (enlargement LN, ↑ weight, ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**
Repeated dose (TOX13736)	Uniflu[f]	3 injections (Days 1, 22, 43/44; 3-week interval between doses) • Saline on each dosing day • $AlOH_3$ (750 µg) on each dosing day • G1 mini-HA protein (135 µg) on each dosing day • G1 mini-HA protein + $AlOH_3$ (135 µg + 750 µg) on each dosing day • **Ad26.FLU.003** ($5x10^{10}$ vp) on each dosing day • G1 mini-HA protein + $AlOH_3$/**Ad26.FLU.003** mix[c] (135 µg + 750 µg/$5x10^{10}$ vp) on each dosing day • G1 mini-HA protein + **Ad26.FLU.003** (135 µg/$5x10^{10}$ vp) on each dosing day	*Clin obs/clin path:* ↑ monocytes (x4.65), ↑ fibrinogen (x2.26), ↑ globulin (x1.40), ↑ total protein (x1.10), ↑ CRP (x55), ↓ RBC parameters (x0.91), ↓ albumin/globulin (x0.69) *Necropsy/histology*: iliac/poplitheal LN and spleen (↑ cellularity; trend for spleen), injection sites (inflammation) *Recovery*: (ongoing) recovery after 3-week treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

Study Type (Study Number)	Target	Vaccine Regimen, Interval, and Dose Level	Main Findings[a]
Embryo-fetal/Pre- and Postnatal Development (IND016280/0014/Mod4.2.3.5.2/ TOX11212)* (EMEA/H/C/005337/0000/Mod4.2.3.5.2/ TOX11212)**	Ebola[d]	2 injections (Day -8 and GD6; 14-day interval between doses)[g] • Saline on each dosing day • **Ad26.ZEBOV** ($1x10^{11}$ vp) on Day -8; MVA-BN-Filo ($3.61 x10^{8}$ Inf U)[d] on GD6 • MVA-BN-Filo ($3.61 x10^{8}$ Inf.U) on Day -8; **Ad26.ZEBOV** ($1x10^{11}$ vp) on GD6 • **Ad26.ZEBOV** ($1x10^{11}$ vp) on each dosing day	*Clin obs/clin path:* ↑ CRP (x97), ↑ fibrinogen (x1.88), ↑ globulin (x1.14), ↑ monocytes (x2.84), ↑ lymphocytes (x1.36), ↓ albumin/globulin (x0.83) **Above findings were considered to be a non-adverse, normal response to vaccine administration** *Embryo-fetal/pre-postnatal development*: no effects on reproductive performance, fertility, and litter data (corpora lutea count, number of implantation sites, viable fetuses, litter size, pre- and post-implantation loss, and number of resorptions), parturition, or macroscopic evaluations in parental females. Similarly, no adverse effect of vaccination was seen on fetal body weights, external, visceral, and skeletal evaluations or F1 pup evaluations from LD0-28 (sex ratios, survival, body weights, clinical findings, developmental evaluations, and macroscopic evaluations)
RAT STUDIES			
Repeated dose (IND017959/0001/Mod4.2.3.2/TOX12276)*	HPV[d]	4 injections (Days 1, 15, 29, 43; 2-week interval between doses) • Saline on each dosing day • **Ad26.HPV16–HPV18 mix** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^{9}$ Inf U) on Days 29 and 43 • **Ad26.HPV16** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^{9}$ Inf U) on Days 29 and 43 • **Ad26.HPV18** ($5x10^{10}$ vp) on Days 1 and 15; MVA.HPV16-18 ($1x10^{9}$ Inf U) on Days 29 and 43 • MVA.HPV16-18 ($1x10^{9}$ Inf U) on Days 1 and 15; **Ad26.HPV16-18 mix** ($5x10^{10}$ vp) on Days 29 and 43	*Clin obs/clin path:* ↑ neutrophils (x1.85), ↑ fibrinogen (x1.56), ↑ globulin (x1.15), ↑ α2-macroglobulin[h] (x2.57), ↓ albumin (x0.89), ↓ albumin/globulin (x0.78) *Necropsy/histology*: iliac and popliteal LN (enlargement, ↑ weight, and/or ↑ cellularity), injection sites (inflammation) *Recovery*: (ongoing) recovery after 18-day treatment-free period **Above findings were considered to be a non-adverse, normal response to vaccine administration**

* Reference to the vaccine-specific INDs
** Reference to the Ebola MAA file/reports
[a] The indicated fold changes are the maximum mean fold changes (irrespective of sampling time or sex) measured during the study, versus controls
[b] CRP and fibrinogen were not measured, and iliac (draining) lymph node was not sampled in this study.
[c] Pharmacy mix as a single injection.
[d] No relevant differences between study results (previous versus new buffer) were observed.
[e] The Ad26.Filo vaccine is a trivalent mix (in a 1:1:1 ratio) of Ad26 vectors encoding the GP of EBOV Mayinga (Ad26.ZEBOV), MARV Angola (Ad26.MARVA), and SUDV Gulu (Ad26.SUDV).
[f] A tetracycline operon (TetO) was inserted in the CMV promotor sequence of the transgene expression cassette (Uniflu; TOX13736).
[g] A first vaccination was administered 8 days prior to mating, followed by a second vaccination 6 days after mating (ie, on gestation day 6).
[h] CRP was not measured; α2-macroglobulin was measured instead as it is a more relevant acute phase protein in rats.
APTT: activated partial thromboplastin time; BT: body temperature; BW: body weight; BWG: body weight gain; clin obs: clinical observations; clin path: clinical pathology CRP: C-reactive protein; FC: food consumption; GD: gestation day; HPV: human papilloma virus; Inf U: infectious unit; LD: lactation day; LN: lymph nodes; RBC: red blood cell; pfu: plaque-forming units; RSV: respiratory syncytial virus; $TCID_{50}$: 50% tissue culture infective dose; vp: virus particles; WBC: white blood cell

2.4. Nonclinical Overview

Drug Substance	AZD1222
ANGEL ID	Doc ID-004493554
Date	26 April 2021

2.4 Nonclinical Overview AZD1222

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1 OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

AstraZeneca (the Sponsor) is developing AZD1222 for the prevention of coronavirus disease-2019 (COVID-19). AZD1222 is a recombinant chimpanzee adenovirus (ChAd) expressing the severe respiratory syndrome-coronavirus-2 (SARS CoV-2) spike (S) surface glycoprotein. Development of AZD1222, previously referred to as ChAdOx1 nCoV-19, was initiated by the University of Oxford with subsequent transfer of development activities to the Sponsor.

AZD1222 is a recombinant replication-defective ChAd vector expressing the SARS CoV-2 S surface glycoprotein, driven by the human cytomegalovirus major immediate early promoter that includes intron A with a human tissue plasminogen activator (tPA) leader sequence at the N terminus. Spike (S) is a type I, trimeric, transmembrane protein located at the surface of the viral envelope, giving rise to spike shaped protrusions from the SARS-CoV-2 virion. The S protein's subunits are responsible for cellular receptor angiotensin-converting enzyme 2 (ACE-2) binding via the receptor-binding domain and fusion of virus and cell membranes, thereby mediating the entry of SARS-CoV-2 into the target cells. The S protein has an essential role in virus entry and determines tissue and cell tropism, as well as host range. The roles of the S in receptor binding and membrane fusion make it a desirable target for vaccine and antiviral development. AZD1222 expresses a codon-optimised coding sequence for S protein from the SARS-CoV-2 genome sequence accession MN908947.

The ChAdOx1 platform technology, as well as other ChAd related vaccines, were used to support the first-in-human (FIH) and other early clinical AZD1222 studies. This approach of using platform data to support a FIH clinical study is consistent with the views expressed by global regulators at the International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 (ICMRA 2020). To support the FIH study, biodistribution studies with similar replication-defective ChAd vaccines (AdCh63 ME TRAP and AdCh63 MSP-1) and toxicology studies with a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein, as well as other ChAd related vaccines (AdCh63 MSP-1, ChAd OX1 NP+M1) in mice were used (Table 2).

To date, immunology and biological activity studies (including prime boost vaccination) of AZD1222, have been conducted in mice, non-human primates, ferrets and pigs (Table 1). A mouse cardiovascular and respiratory safety pharmacology study has also been conducted with AZD1222, along with Irwin assessment as part of the repeat-dose toxicity study.

To support licensure, biodistribution studies with AZD1222 IM (Table 1) or ChAdOx1 HBV IM (Table 2) and toxicology studies with AZD1222 were conducted, which included a Good Laboratory Practice (GLP) repeat-dose toxicity and 2 GLP embryo-fetal development (EFD) studies in CD-1 mice (Table 1).

All pivotal nonclinical safety studies were conducted in OECD member countries and in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP), and according to relevant International Conference on Harmonisation guidelines.

Table 1 List of Nonclinical Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Sponsor / Test Facility	GLP Y/N
Primary Pharmacology				
Effect of D614G Mutation in SARS-CoV-2 Spike Protein on AZD1222 (20-01700)	In vitro	NA	Jenner University of Oxford / CSIRO Health and Biosecurity, Australia	N
Murine Immunogenicity (van Doremalen et al 2020)	Balb/C and CD-1 mice	Single dose, IM 6×10^9 vp AZD1222 or Control - ChAdOx1 GFP	Jenner Institute - Oxford University, UK / NIH, MT, USA	N
Murine Immunogenicity (Graham et al 2020)	Balb/C and CD-1 mice	Day 0 and 28 or 28 only IM, 6.02×10^9 vp AZD1222	Jenner Institute – University of Oxford / Pirbright Institute, UK	N
Non-human Primate Efficacy and Immunogenicity (van Doremalen et al, 2020)	Rhesus macaques	Day -56 and -28 or -28 only before challenge, IM 2.5×10^{10} vp AZD1222 or Control - ChAdOx1 GFP	Jenner Institute - University of Oxford, UK / NIH, USA	N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Rhesus Macaques (6284)	Rhesus macaques	Single dose, Day -27 before challenge, IM 2.5×10^{10} vp AZD1222, or Control – PBS	Jenner Institute - University of Oxford / Public Health England, Porton Down, UK	N
Assessment of Efficacy of SARS-CoV-2Vaccine Candidates in the Ferret Model (20-01125)	Ferret	Day -56 and -28 or -28 only before challenge, IM, IN 2.5×10^{10} vp AZD1222, or Control – PBS	Jenner University of Oxford / CSIRO Health and Biosecurity, Australia	N

Table 1 List of Nonclinical Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Sponsor / Test Facility	GLP Y/N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Ferrets (6285)	Ferret	Day -56 and -28 or -28 only before challenge, IM 2.5 x 10^{10} vp AZD1222, or Control - ChAdOx1 GFP or Day -14, IM, formalin-inactivated SARS CoV-2	Jenner Institute - University of Oxford / Public Health England, Porton Down, UK	N
Porcine Immunogenicity (ar001111 / Graham et al 2020)	White-Landrace-Hampshire cross-bred pigs	Day 0 and 28, IM 5.12 × 10^{10} vp AZD1222	Jenner Institute - University of Oxford / Pirbright Institute, UK	N
ChAdOxl-nCoV19 immunopotency assay (INT-ChadOx1 nCov19-POT004)	Balb/C and CD-1 mice	5 × 10^{9} vp AZD1222	Jenner Institute – University of Oxford, UK	N
Safety Pharmacology				
Cardiovascular and Respiratory Assessment Following Intramuscular Administration to Male Mice (617078)	CD-1 mice	Day 4, IM 2.59 x 10^{10} vp AZD1222 or IP, 1 mg/kg, Salbutamol (0.9% w/v sodium chloride) and Day 1, IM, A438 buffer,	AstraZeneca / Charles River Laboratories Ltd, UK	Y
Distribution				
AZD1222 (ChAdOx1-nCovd-19): A Single Dose Intramuscular Vaccine Biodistribution Study in the Mouse (514559)	CD-1 mice	Single dose, IM 3.7 x 10^{10} vp AZD1222,	AstraZeneca / Charles River Laboratories Ltd, UK	Y
Repeat Dose Toxicology				
AZD1222 (ChAdOx1-nCovd-19): A 6 Week Intermittent Dosing Intramuscular Vaccine Toxicity Study in the Mouse with a 4 Week Recovery (513351)	CD-1 mice	Days 1, 22 and 43, IM 3.7 x 10^{10} vp AZD1222	AstraZeneca / Charles River Laboratories Ltd, UK	Y

Table 1 List of Nonclinical Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Sponsor / Test Facility	GLP Y/N
Developmental and Reproductive Toxicology				
ChAdOx1-nCovd19: A Preliminary Intramuscular Injection Vaccine Development and Reproductive Study in Female CD-1 Mice (490838)	CD-1 mice	Day 1 (13 days prior to pairing for mating) and GD 6 to EFD phase animals and on GD 6 and GD 15 to littering phase animals, IM 2.59 x 10^{10} vp AZD1222 or Control – A438 buffer	AstraZeneca / Charles River Laboratories Ltd, UK	Y
AZD1222 (ChAdOx1 - nCovd19): An Intramuscular Vaccine Development and Reproductive Study in Female CD-1 Mice (490843)	CD-1 mice	Day 1 (13 days prior to pairing for mating) and GD 6 to EFD phase animals and on GD 6 and GD 15 to littering phase animals, IM 3.71 x 10^{10} vp AZD1222 or Control[b]	AstraZeneca / Charles River Laboratories Ltd, UK	Y

CSIRO = Commonwealth Scientific and Industrial Research Organisation, Geelong, Australia; EFD = embryo-foetal development; GD = gestation day; IM = intramuscular; IN = intranasal; NIH = National Institute of Health

[a] AZD1222 Vehicle (10 mM histidine, 7.5% [v/w] sucrose, 35 mM sodium chloride, 1 mM magnesium chloride, 0.1% [v/w] Polysorbate-80, 0.1 mM EDTA and 0.5% [v/w] ethanol, pH 6.6)

Table 2 List of Nonclinical Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
AdCh63 MSP-1 and MVA MSP-1 Tissue Distribution Study By Intra-Muscular Administration To Mice (Report UNO0014/RMBIODIST-001)	Balb/C mice	Day 1, IM 1.11×10^{10} vp AdCh63 MSP-1 1.04×10^{8} pfu MVA MSP-1	Jenner Institute – University of Oxford / Huntingdon Life Sciences,[a] UK	Y[b]
AdCh63ME-TRAP Tissue Distribution Study By Intra-Dermal Administration To Mice (UNO0009/MAB-001)	Balb/C mice	Day 1, ID 3.3×10^{9} vp	Jenner Institute – University of Oxford / Huntingdon Life Sciences,[a] UK	Y[b]
ChAdOx-1 HBV and MVA-HBV Biodistribution Study in BALB/c Mice with Shedding Assessment (0841MV38.001)	Balb/C mice	Days 1 and 28, IM 2.4×10^{10} vp ChAdOx-1-HBV 6.1×10^{7} pfu MVA-HBV	Jenner Institute – University of Oxford / Calvert Laboratories, USA	Y
ChAdOx1 Chik Vaccine or ChAdOx1 MERS: Toxicity Study by Intramuscular Administration to Mice (QS18DL)	Balb/C mice	Day 1 and 15, IM 1×10^{10} vp	Jenner Institute – University of Oxford / Envigo CRS Limited UK	Y
ChAd OX1 NP+M1 and MVA NP+M1: Toxicity Study by Intramuscular Administration to Mice (XMM0003)	Balb/C mice	Day 1, IM ChAd OX1 NP+M1 1×10^{10} vp and Day 15, IM MVA NP+M1 1.5×10^{7} pfu	Jenner Institute – University of Oxford / Huntingdon Life Sciences,[a] UK	Y

Table 2 List of Nonclinical Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
Mouse Toxicity AdCh63 MSP-1 and MVA MSP-1 or a Combination of AdCh63 ME-TRAP and MVA ME-TRAP (UNO0013)	Balb/C mice	Day 1, IM AdCh63 MSP-1 1.11×10^{10} vp Day 15, IM MVA MSP -1 10.4×10^{7} pfu Day 1 and 15, IM AdCh63ME-TRAP/ MVA ME TRAP 0.78×10^{10} vp / 6.85×10^{7} pfu	Jenner Institute – University of Oxford / Huntingdon Life Sciences,[a] UK	Y

[a] Currently Covance CRS Ltd.

[b] In-life phase conducted to GLP; biodistribution phase (RBIODIST-001 or MAB-001) not conducted to GLP

2 PHARMACOLOGY

2.1 Primary Pharmacodynamics

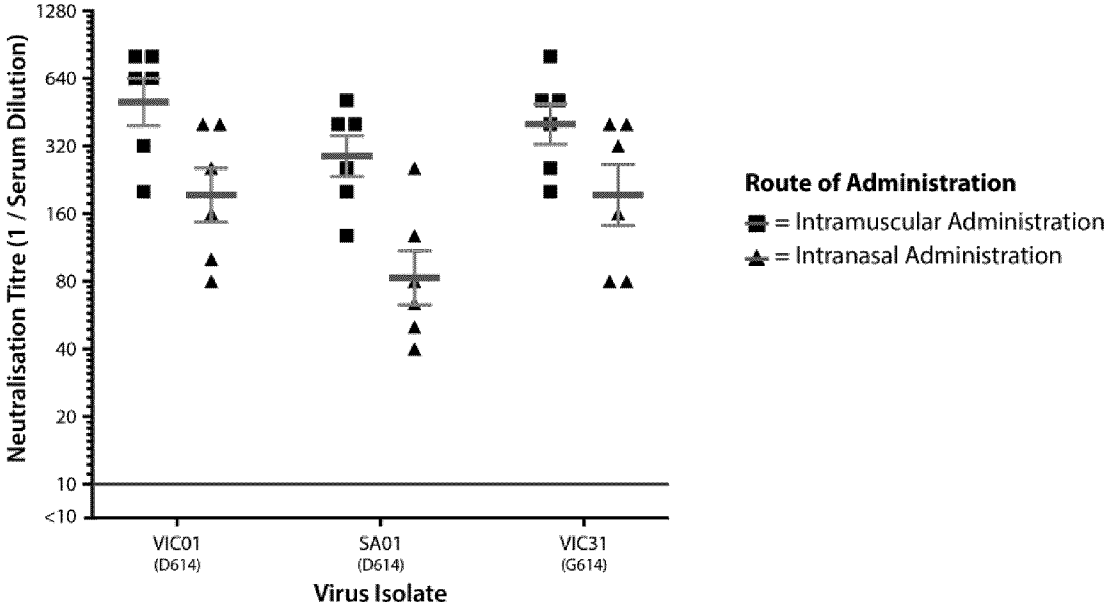
Immunogenicity studies in animal models responsive to AZD1222 were conducted to evaluate the immunologic properties of this COVID-19 vaccine candidate to support FIH clinical trials. AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. These studies included evaluation of humoral, cellular and functional immune responses. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated enhanced respiratory disease (ERD). A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

Mutations are occurring naturally within the SARS-CoV-2 genome. Most vaccines in development rely upon inducing immune responses towards Spike protein (S), the main virus surface protein. A D614G mutation in S is increasing in prevalence amongst sequenced

viruses worldwide. The mutation is thought to increase infectivity of the virus by reducing S1 shedding, increasing infection (Zhang et al 2020). The effect of the D614G mutation on the efficacy of virus neutralisation following vaccination of ferrets with AZD1222 was assessed in study 20-01700 (Figure 1). A new Australian isolate containing the D614G mutation (VIC31) was obtained from VIDRL. Three isolates were used for virus neutralisation assays: SA01: has identical amino acid sequence in S to Wuhan-Hu-1. VIC01: S differs from SA01 by an Ser247Arg mutation. VIC31: S differs from SA01 by the Asp614Gly mutation.

Overall, there were no significant effects of the D614G mutation in the SARS-CoV-2 Spike protein on relative neutralisation of D614 and G614 variants with serum samples collected from ferrets that had received prime-boost administrations of AZD1222. Therefore, animal challenge studies presented are relevant to strains circulating in the human population.

Figure 1 **Effect of D614G mutation on vaccine-induced antibody-mediated neutralisation.**



Mean neutralising titres (calculated from log2-values) to three circulating Australian SARS-CoV-2 isolates. Neutralisation titres of serum samples collected following prime-boost vaccination with AZD1222 in ferrets, administered by two routes (intramuscular and intranasal). Bold horizontal lines represent overall mean titre of the vaccination route/isolate combination with uncertainty bars representing Standard Error of the Mean (SEM). Square and triangle marks represent mean titres of the triplicate titres for each serum sample/isolate combination.

Viral RNA in Gastrointestinal Tract

In the NHP pharmacology study (van Doremalen et al 2020), there was an unexpected finding of viral RNA in tissues of the gastrointestinal (GI) tract at 7 days post-challenge in immunised, but not control, animals. Viral gRNA load in intestinal tissues of prime-boost-vaccinated animals was higher than the levels measured in control and prime-only-vaccinated animals at 7 days post-challenge and was associated with the detection of sgRNA. However, no infection of intestinal tissue was observed by immunohistochemistry, nor were we able to detect infectious virus in intestinal tissue. Given that spike-specific antibodies were significantly increased after the second immunization (two-tailed signed-rank Wilcoxon test) higher viral gRNA load intestinal in prime-boost animals may correlate with greater intestinal clearance and retention of opsonised virus following challenge. FcRn allows the entry and retrieval of IgG from the intestinal lumen throughout health and disease. This bidirectional transport allows the secretion of IgG into the lumen, the subsequent uptake of opsonized bacteria and viruses (Castro and Clatworthy 2020). As previously reported, SARS-CoV-2 antigen can be detected in lymphocytes and macrophages in the lamina propria of the intestinal tract of control animals (Munster et al 2020). This may indicate a higher proportion of plasma cells secreting IgA2 in the gut lamina propria of prime-boost-vaccinated animals and trapping of SARS-CoV-2 virus. Whilst SARS-CoV-2 virus may make its way to the gastric lumen, it would be subjected to the adverse effect of the acidic environment of the stomach that would significantly affects viability.

Nevertheless, SARS-CoV-2 can cause gastrointestinal symptoms, such as loss of appetite, vomiting, diarrhoea, or abdominal pain during the early phases of the disease (Villapol 2020). It has been reported in some patients that although SARS-CoV-2 has been cleared in the respiratory tract, the virus continues to replicate in the gastrointestinal tract and could be shed in faeces (Yang et al 2020). Currently, the exact mechanism of SARS-CoV-2 interaction with the gastrointestinal tract is still not fully understood. However, SARS-CoV-2 shows a high affinity to ACE2 receptors, making sites of high ACE2 receptor expression such as lungs and GI tract prime targets for infection (Dahiya et al 2020). It is therefore possible that gastrointestinal symptoms in COVID-19 are somehow caused by the direct attack of SARS-CoV-2 to gastrointestinal tract (Zhong et al 2020). If higher viral gRNA loads in intestinal tissues of prime-boost vaccinated animals is associated with continued replication then it was not associated with any signs of lesions or infection.

Lung Histopathology

In rhesus macaques 3 out of 6 control animals developed some degree of viral interstitial pneumonia following SARS-CoV-2 challenge. Lesions were widely separated and characterised by thickening of alveolar septum. Alveoli contained small numbers of pulmonary macrophages and rarely oedema. Type-II pneumocyte hyperplasia was observed. No histological lesions were observed in the lungs of vaccinees.

In comparison, the majority of histopathological findings made in the lungs of ferrets following SARS-CoV-2 challenge were modest at most. In control group 3a that received a prime with ChAdOx1 vector expressing green fluorescent protein (GFP) one ferret showed mild lesions compatible with acute bronchiolitis and the other animals were similar to group 1 primed with AZD1222. Only mild inflammatory cell foci and no lesions were observed in group 1. In group 2 that received a prime and boost with AZD1222, inflammatory cell were also detected in lungs. These changes are likely associated with an immune response to challenge as they were also observed in controls. In group 4 immunised with inactivated SARS CoV-2, mild to moderate lesions were observed in the lungs with inflammatory cells and perivascular cuffing at day 7 post challenge potentially indicative of enhanced respiratory disease. In a second ferret study, no significant histological lung changes were present in any of the animals examined.

Enhanced respiratory disease (ERD) can result from immunization with antigen that is not processed in the cytoplasm, resulting in a nonprotective antibody response and CD4+ T helper priming in the absence of anti-viral cytotoxic T lymphocytes. This type of vaccine response can lead to a pathogenic Th2 memory response with eosinophil and immune complex deposition in the lungs after respiratory infection. For example, infants and toddlers immunized with a formalin-inactivated virus vaccine against respiratory syncytial virus (RSV) experienced an enhanced form of RSV disease characterized by high fever, wheezing and bronchopneumonia when they became infected with wild-type virus in the community (Acosta et al 2015). AZD1222 not expected to cause ERD because antigens are expressed intracellularly, generating anti-viral cytotoxic T cell and protective antibody responses.

In the van Doremalen et al study, significantly reduced viral load in the bronchoalveolar lavage fluid and lower respiratory tract tissue of vaccinated rhesus macaques challenged with SARS-CoV-2 with no pneumonia was observed compared to control animals. No evidence of immune-enhanced disease after viral challenge in vaccinated SARS-CoV-2-infected animals was found in terms of increased severity of viral infection. At present, there are no known clinical findings, immunological assays or biomarkers that can differentiate any severe viral infection from immune-enhanced disease, whether by measuring antibodies, T cells or intrinsic host responses (Arvin et al 2020). Carefully controlled human studies of sufficient size to enable the detection of increased frequency of severe cases in vaccinated cohorts compared to control group are required to determine if antiviral host responses may become harmful in humans.

In conclusion, the rhesus macaque is more predictive than ferret of histological lung changes and the ability of immunisation with AZD1222 to mitigate these following challenge with SAR-CoV-2. No enhanced respiratory disease was observed post challenge in AZD1222 immunised animals.

2.2 Secondary Pharmacodynamics

Secondary pharmacodynamic studies have not been conducted with AZD1222.

2.3 Safety Pharmacology

In a mouse cardiovascular and respiratory safety pharmacology study, a group of 8 male CD-1 mice were dosed by IM injection with the control item for AZD1222 (A438 buffer) on Day 1 and AZD1222 (2.59 x 10^{10} vp dose) on Day 4 (617078).

There were no changes in arterial blood pressure, heart rate, body temperature or respiratory parameters considered to be AZD1222-related. The No Observed Effect Level (NOEL) for cardiovascular and respiratory assessment was an AZD1222 dose of 2.59 x 10^{10} vp .

Irwin Screen observations (autonomic, neuromuscular, sensorimotor, behavioural parameters) and effects on body temperature and pupil size were made in the repeat-dose IM toxicity study (513351) in male and female CD-1 mice on Days 8 and 29 following administration of AZD1222 at 3.7 x 10^{10} vp on Days 1, 22. There were no effects on body temperature, pupil size or Irwin Screen observations considered to be AZD1222-related.The NOEL for the Irwin Screen phase was 3.7 x 10^{10}.

2.4 Pharmacodynamic Drug Interactions

Pharmacodynamic drug interaction studies have not been conducted with AZD1222.

3 PHARMACOKINETICS

3.1 Absorption

Absorption studies evaluations are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional absorption, distribution, metabolism, and excretion (ADME) evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune responses.

3.2 Distribution

Distribution studies are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), states that traditional ADME evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune response.

In an AZD1222 biodistribution study in mice, there was no biodistribution to blood and faeces samples with the exception of low signal from 2 blood and 1 faeces samples on Day 2. Both blood samples had signals below the limit of quantification (<LLOQ) and the faeces sample returned a low signal of 1.30 x 10^3 copies/µg DNA (LLOQ was 50 copies/Q-PCR reaction). In tissues, AZD1222 vector DNA showed biodistribution to the intramuscular administration sites, sciatic nerve, bone marrow, liver, lung and spleen. The highest levels of AZD1222 vector DNA (10^3 to 10^7 copies/µg DNA) were observed in the intramuscular administration sites and sciatic nerve (close proximity to the administration sites) on Day 2. Lower levels of AZD1222 vector DNA (<LLOQ to 10^4 copies/µg DNA) were observed in bone marrow, liver, spleen and lung, on Day 2. The levels of AZD1222 and the number of tissues with detectable levels of AZD1222 vector DNA decreased from Day 2 to 29, indicating elimination.

A biodistribution and shedding study using the ChAdOx1 vector with an hepatitis B virus (HBV) insert after IM injection on Days 1 and 28 in mice was conducted (0841MV38.001). Distribution to some samples of all tissues was noted on day 2 and Day 29. The highest levels (copies/mg sample) were noted at the site of administration (skeletal muscle), ranging from 3 x 10^8 to 9.97 x 10^9 copies/mg sample. In the majority of samples of other tissues taken on Day 56, the levels were below the level of quantification, indicating elimination. Low levels were noted in 1 sample (of 6) for each of heart and liver, 1 of 3 for ovary and testes, and 3 of 6 lymph node samples at this timepoint.

Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infectious virus after IM injections.

3.3 Metabolism

Metabolism studies have not been conducted with AZD1222. The expected consequence of metabolism of biotechnology-derived vaccines is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood.

3.4 Excretion

Excretion studies have not been conducted with AZD1222. No virus excretion is expected with AZD1222 as it is a non-replicating vaccine vector. Shedding of ChAdOx1 HBV in mice following IM administration of Days 1 and 28 have been assessed. DNA was extracted from mouse fecal and urine samples collected were all negative, suggesting that no shedding had occurred in these matrices at the times sampled.

3.5 Pharmacokinetic Drug Interactions

Pharmacokinetic drug interaction studies have not been conducted with AZD1222.

3.6 Other Pharmacokinetic Studies

Other pharmacokinetic studies have not been conducted with AZD1222.

4 TOXICOLOGY

4.1 SINGLE DOSE TOXICITY

No single dose toxicity studies have been performed with AZD1222.

4.2 REPEAT DOSE TOXICITY

A 6-week repeat-dose GLP toxicity study with AZD1222 in mice was conducted.

As the ChAdOx1 platform technology utilized for AZD1222 is well characterized, toxicology data with ChAdOx1 MERS-CoV vaccine expressing the full-length Spike protein in mice (Report QS18DL), was used to support first in human (FIH) clinical trials for AZD1222 (International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 [ICMRA 2020]). In addition, toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) are also discussed.

At the time of toxicology species selection only ChAdOx1 immunogenicity data for mouse and rhesus macaques was available to the sponsor. Pig and ferret immunogenicity data were subsequently made available. Given that both mouse and NHP elicit appropriate immune responses to AZD1222 and considering the need to expedite toxicity testing, the mouse was selected as the toxicology species given the urgency of the ongoing pandemic, the longer lead time for NHP toxicity studies and longer reproductive toxicity study requirements. For dosing considerations, the CD-1 mouse strain was selected due to its larger size compared to the Balb/c mouse strain.

4.2.1 A 6 -Week Intermittent Dosing Intramuscular AZD1222 Toxicity Study in Mice with a 4 Week Recovery

The objective of this study was to determine the potential toxicity of AZD1222 (total viral particle dose of $3.7x10^{10}$) when given by intramuscular injection intermittently (on Days 1, 22 and 43) to mice, with a 28 day recovery period to evaluate the potential reversibility of any findings (513351). In addition, the immunogenicity was evaluated. Scheduled necropsies were conducted either at the end of the 6 week treatment period (Day 45) or at the end of the 28 day recovery period.

The following parameters and end points were evaluated in this study: clinical signs, body temperature, body weights, body weight gains, food consumption, dermal scoring, Irwin

screen observations, clinical pathology parameters (hematology and plasma chemistry), immunogenicity, gross necropsy findings, organ weights, and histopathological examinations.

In comparison to controls and pre-study data, a slightly higher body temperature was observed in AZD1222 treated males, notably on Days 22, 4 hours post dose (range 36.2-39.5°C compared to 36.2-38.7°C in controls) but was comparable to controls by 24 hours post-dose. There was no AZD1222-related change in body temperature recorded in males, as part of the Irwin observations.

In animals administered AZD1222, there was a mild decrease in monocytes on Day 45, which was consistent with expected pharmacology following immunisation. Additionally, globulin was mildly higher, and albumin and albumin/globulin ratio were minimally to mildly lower, which was consistent with an acute phase response. Following the recovery period, globulin remained mildly higher and albumin/globulin ratio remained mildly lower in AZD1222-treated females, the other changes had reversed.

All samples collected from animals during the pre-treatment phase prior to immunisation were below the limit of quantification (BLQ) for the assay (BLQ; 0.250 AU/mL) and considered seronegative. Samples collected indicate that all animals mounted an antibody response to S-glycoprotein following a single administration of AZD1222 on Day 1 with most animals showing a marked increase in the level of antibody response following a second administration of AZD1222 on Day 22. On Day 74, a further increase in antibody response or maintenance of response was observed in all animals following a third administration of AZD1222.

At histopathological examination of the main study animals, mononuclear and/or mixed cell inflammation was observed in the subcutaneous tissue and underlying skeletal muscle at the control and AZD1222 administration sites. This finding was of a higher incidence in animals dosed with AZD1222. In some animals there was an extension of the inflammatory cells into the fascia and connective tissue below the skeletal muscle at the administration sites, that extended to surround the sciatic nerve. The inflammatory cells did not extend into the endoneurium of the sciatic nerve and no findings were present in the underlying axons, which appeared histologically normal. Inflammatory cells were not observed in the nerve roots contained within the lumbar spinal cord sections, confirming that the epineurial/perineurial inflammatory cells noted in the sciatic nerve samples resulted from an extension of the inflammation from the adjacent injection site. There were no findings in the administration sites or sciatic nerves at the end of the recovery period, indicating complete recovery of the AZD1222 related inflammation.

In conclusion, administration of AZD1222 to CD-1 mice (total viral particle dose of 3.7 x 10^{10}) by intramuscular injection on 3 occasions (once every 3 weeks) over a 43 day period was well tolerated, with a transiently higher body temperature in males, decreases in

monocytes in males and females (consistent with the expected pharmacology of AZD1222) and increase in globulin and decrease in albumin and albumin/globulin ratio, consistent with an acute phase response, observed.

In all animals dosed with AZD1222, antibodies against the S-glycoprotein were raised and maintained throughout the dosing and recovery periods in all animals.

In AZD1222 animals, higher spleen weights were observed but with no correlating macroscopic or microscopic changes. Non adverse, mixed and/or mononuclear cell inflammation was observed in the subcutaneous tissues and skeletal muscle of the administration sites and adjacent sciatic nerve of animals dosed with AZD1222 which were consistent with the anticipated findings after intra-muscular injection of vaccines.

4.2.2 Repeat-dose Toxicology Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

A brief summary of the key findings from the ChAdOx1 MERS vaccine toxicology study in in mice is provided below.

- Changes at the intramuscular injection sites (inflammatory cell infiltrates) were observed in the majority of females and in several males.
- Histopathological changes in the spleen (increased germinal center development) correlated with an increased spleen weight in females. Increased germinal center development of the right lumbar lymph nodes (draining lymph node), correlated macroscopically with enlargement, was observed for the majority of treated animals. Slightly higher circulating white blood cell numbers were observed.
- At the end of the study treatment there was a slightly lower than control body weight gain for treated males and females. For males this was due mainly to slightly lower than control weight gains during Days 15 to 18 however for females this was due mainly to small weight losses during this period. Mice were dosed on Day 1 and 15, with necropsy on day 28.
- Slightly lower group mean liver weight for males and females (0.92X and 0.90X control), higher phosphorus concentration for females (1.2X control) or lower triglyceride concentration for males and females (0.56X and 0.64X) were observed. There was no correlation with histopathological changes.

The spectrum and severity of these changes were consistent with the administration of an antigenic substance such as ChAdOx1 MERS, and were considered to be non-adverse.

Results from the toxicology studies on similar replication-defective ChAd vaccines (ChAd OX1 NP+M1 and AdCh63 MSP-1) were consistent with ChAdOx1 MERS and were well tolerated with no associated adverse effects. The toxicity data (and toxicity in the target organs) from the ChAdOx1 and ChAd63 based vaccines follows the same pattern, where findings were consistent with a predicted response to vaccine administration.

4.3 Toxicokinetics

Toxicokinetic studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Pharmacokinetic studies (eg, for determining serum or tissue concentrations of vaccine components) are normally not needed.

4.4 Genotoxicity (Mutagenicity)

Genotoxicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines, (WHO 2005), genotoxicity studies are normally not required for the final vaccine formulation and therefore have not been conducted.

4.5 Carcinogenicity

Carcinogenicity studies have not been performed with AZD1222. Consistent with WHO guidelines on the nonclinical evaluation of vaccines (WHO 2005), Carcinogenicity studies are not required for vaccine antigens. AZD1222 is a replication deficient, non-integrating adenovirus vector so there is no risk of carcinogenicity. To date there have been no clinical reports of chromosomal vector integration following adenovirus vector-mediated gene transfer.

4.6 Developmental and Reproductive Toxicity

An evaluation of the impact of AZD1222 on embryo-foetal development was completed in a dose-range study (490838). Intramuscular administration of AZD1222 to groups of CD-1 female mice on Day 1 (13 days prior to pairing for mating) and again on Gestation Day (GD) 6 at 2.59 x 10^{10} vp per occasion (embryofoetal development phase), or on GD 6 and GD 15 at 2.59 x 10^{10} per occasion (littering phase) was well tolerated (490838). Anti-S glycoprotein antibody responses were raised in dams following administration of AZD1222 and these were maintained through the gestational and lactation periods. Seropositivity of foetuses and pups was confirmed and was indicative of placental and lactational anti-S glycoprotein antibody transfer, respectively. There were no AZD1222-related effects seen for dams in-life including at the injection site, for female reproduction, foetal or pup survival and no abnormal gross pathology findings in pups or in dams in either phase. There were no AZD1222-related foetal visceral or skeletal findings.

In the main GLP embryo-foetal development study, IM administration of AZD1222 to groups of CD-1 female mice on Day 1 (13 days prior to pairing for mating) and again on GD 6 at 3.71 x 10^{10} vp per occasion (embryofoetal development phase), or on GD 6 and GD 15 at 3.71 x 10^{10} vp per occasion (littering phase) was well tolerated (490843). Anti-S glycoprotein antibody responses were raised in dams following administration of AZD1222 and these were maintained through the gestational and lactation periods. Seropositivity of fetuses and pups

was confirmed and was indicative of placental and lactational anti-S glycoprotein antibody transfer, respectively. There were no test item-related effects seen for dams in-life including at the injection site, for female reproduction, foetal or pup survival, pup physical development and no abnormal gross pathology findings in pups prior to or post weaning or in dams in either phase. There were no test item-related foetal external, visceral or skeletal findings.

4.7 Local Tolerance

Local tolerance of AZD1222 was evaluated as part of the repeat dose toxicity study in mice (513351). There was no erythema or oedema at the injection sites after administration of AZD1222 on any dosing occasion. Histopathology showed minimal subcutaneous oedema was observed in the administration sites in male and female animals from both the control group and those administered AZD1222 and was considered to be related to the route of administration. Minimal mononuclear or mixed cell inflammation was observed in the subcutaneous tissue and underlying skeletal muscle at the administration sites in both male and female animals. This finding was of a higher incidence in animals administered with AZD1222. In some animals there was an extension of the inflammatory cells into the fascia and connective tissue below the skeletal muscle at the administration sites. This resulted in inflammatory cells being noted surrounding the epineurium/perineurium of the sciatic nerve samples. In the hind limb, inflammation around the sciatic nerve due to local extension from the administration site is a well-recognized effect (Sellers et al 2020). Local tolerance was also evaluated as part of a repeat dose GLP toxicology study in mice with the related ChAdOx1 MERS vaccine (QS18DL). Changes related to treatment with ChAdOx1 MERS vaccine were seen in the tissues of the intramuscular injection site, the right lumbar lymph node (draining lymph node) and the spleen of mice. The inflammatory cell infiltrate seen in the tissues of the intramuscular injection sites (infiltrates of lymphocytic/mononuclear inflammatory cells) were caused by the intramuscular injection of the vaccine with the increased germinal centre development of the right lumbar lymph node caused by immune stimulation of the lymphatic drainage from this area and are not considered adverse.

4.8 Other toxicity Studies

No other toxicity studies with AZD1222 were conducted.

5 INTEGRATED OVERVIEW AND CONCLUSIONS

AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, NHP and pig models. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated ERD. A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of

vaccinated animals as compared to vector controls (van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

In the mouse cardiovascular and respiratory safety pharmacology study there were no changes in arterial blood pressure, heart rate, body temperature or respiratory parameters considered to be AZD1222-related. Irwin Screen observations showed no effects considered to be AZD1222-related.

In an AZD1222 biodistribution study in mice, there was no biodistribution to blood and faeces samples with the exception of low signal from 2 blood and 1 faeces samples on Day 2. Both blood samples had signals below the limit of quantification (<LLOQ) and the faeces sample returned a low signal of 1.30×10^3 copies/µg DNA (LLOQ was 50 copies/Q-PCR reaction). In tissues, AZD1222 showed biodistribution to the intramuscular (IM) administration sites, sciatic nerve, bone marrow, liver, lung and spleen. The highest levels of AZD1222 (10^3 to 10^7 copies/µg DNA) were observed in the IM administration sites and sciatic nerve (close proximity to the administration sites) on Day 2. Lower levels of AZD1222 (<lower limit of quantification [LLOQ] to 10^4 copies/µg DNA) were observed in bone marrow, liver, spleen and lung, on Day 2. The levels of AZD1222 and the number of tissues with detectable levels of AZD1222 decreased from Day 2 to 29, indicating elimination. Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injections. WHO guidelines on nonclinical evaluation of vaccines states that pharmacokinetic studies (eg, for determining serum or tissue concentrations of vaccine components) are normally not needed and specific studies should be considered on a case-by-case basis (eg, when using novel adjuvants or alternative routes of administration).

A biodistribution study using the ChAdOx1 vector with a hepatitis B virus (HBV) insert following IM injection on days 1 and 28 in mice has been conducted. This study shows distribution to some samples of all tissues on days 2 and 29. The highest levels (copies/mg sample) were noted at the site of administration (skeletal muscle), ranging from 3×10^8 to 9.97×10^9 copies/mg sample. In the majority of samples of other tissues taken on Day 56, the levels were below the level of quantification, indicating elimination. AZD1222 is made using a platform technology utilized for other previously studied investigational vaccines and is sufficiently characterized to use toxicology data with other vaccines that use the same platform (Development and Licensure of Vaccines to Prevent COVID-19, FDA Guidance for Industry, June 2020 FDA 2020). Administration of a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein was associated with treatment related changes in the right lumbar lymph node, spleen and intramuscular injection site. The spectrum and severity of the changes were consistent with the administration of an antigenic

substance such as ChAdOx1 MERS which were considered to be non-adverse. This was also true for the similar replication-defective ChAd vaccines, ChAd OX1 NP+M1 and AdCh63 MSP-1.

In the repeat dose (once every 3 weeks over a 43 day period) toxicity study in CD-1 mice, AZD122 was well tolerated, with a transiently higher body temperature in males, decreases in monocytes in males and females (consistent with the expected pharmacology of AZD1222) and increase in globulin and decrease in albumin and albumin/globulin ratio, consistent with an acute phase response, observed. In all animals dosed with AZD1222, antibodies against the S-glycoprotein were raised and maintained throughout the dosing and recovery periods in all animals. In AZD1222 animals, higher spleen weights were observed but with no correlating macroscopic or microscopic changes. Non adverse, mixed and/or mononuclear cell inflammation was observed in the subcutaneous tissues and skeletal muscle of the administration sites and adjacent sciatic nerve of animals dosed with AZD1222 which were consistent with the anticipated findings after intra-muscular injection of vaccines.

In the preliminary and main GLP DART studies in mice, there were no AZD1222-related effects seen for dams in-life including at the injection site, for female reproduction, foetal or pup survival and no abnormal gross pathology findings in pups or in dams in either phase. There were no AZD1222-related foetal visceral or skeletal findings.

In conclusion, AZD1222 and similar ChAd vaccines are well tolerated and are not associated with any adverse effects in mice. Further, similar ChAd vaccines show no evidence of replication or dissemination after IM injection in mice. AZD1222 is immunogenic in mice, ferrets, NHP and pig models inducing humoral and cellular immune responses. Vaccination with AZD1222 significantly reduced viral load following a SARS-CoV-2 challenge in rhesus macaques with no evidence of ERD.

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Janssen Vaccines & Prevention B.V.*

Nonclinical Overview Addendum

Final Study Results of Nonclinical Studies Proposed in the EU-RMP to Address the Safety Concerns TTS, Thrombocytopenia, Including ITP and/or VTE

VAC31518 (JNJ-78436735)

* Janssen Vaccines & Prevention B.V. is a Janssen pharmaceutical company of Johnson & Johnson and is referred to as the Company in this document.

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LIST OF ABBREVIATIONS

abbreviation	description of abbreviated term
Ad26.COV2.S	Ad26 vector encoding a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S protein
aa	amino acid
ACE2	angiotensin converting enzyme 2
ADAMTS-13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
APTT	activated partial thromboplastin time
ChAdOx1	viral vector, adapted from chimpanzee adenovirus Y25, used in ChAdOx1 nCoV-19 vaccine
ChAdOx1 nCoV-19	AstraZeneca's adenoviral vectored COVID-19 vaccine
CMV	cytomegalovirus
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
DP	drug product
DS	drug substance
dsDNA	double stranded deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EU-RMP	European Union Risk Management Plan
HCP	host cell protein
HIT	heparin-induced thrombocytopenia
HIV	human immunodeficiency virus
hPF4	Human PF4
Ig	immunoglobulin
IHC	immunohistochemistry
IM	intramuscular
ITP	immune thrombocytopenia
IV	intravenous
MMR	Measles/Mumps/Rubella
MS	mass spectrometry
MS-PP	mass spectrometry protein profiling
NCBI	National Center for Biotechnology Information
NCO	Nonclinical Overview
NHP	non-human primate
NZW	New Zealand White
PCR	polymerase chain reaction
PF4	platelet factor 4
PRAC	Pharmacovigilance Risk Assessment Committee
PT	prothrombin time
PV	pharmacovigilance
RNA	ribonucleic acid

RSV	respiratory syncytial virus
qPCR	quantitative polymerase chain reaction
RP-UPLC	reversed phase ultra performance liquid chromatography
S (protein)	Spike (protein) of SARS-CoV-2
SA	splice acceptor
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
$TCID_{50}$	50% tissue culture infectious dose
TetR	tetracycline repressor
TM	transmembrane domain
TTS	thrombosis with thrombocytopenia syndrome
UL	ultra-large
vp	viral particle
VITT	vaccine-induced immune thrombotic thrombocytopenia
VTE	venous thromboembolism
vWF	Von Willebrand factor
wt	wild-type

1. OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

1.1. Rationale for the Nonclinical Overview Addendum

This Nonclinical Overview (NCO) Addendum is a follow-up of Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan which included hypotheses for the potential mechanisms underlying (vaccine-associated) thrombosis with thrombocytopenia syndrome (TTS) and the proposal for new studies. The previous NCO Addendum was submitted on 3 August 2021 as part of procedure EMEA/H/C/005737/II/0018 to support the inclusion of proposed nonclinical studies, aimed at further characterization of vaccine-associated TTS and thrombocytopenia, as additional pharmacovigilance activities in the EU-RMP. In subsequent updates of the EU-RMP (procedures EMEA/H/C/005737/II/0018 and EMEA/H/C/005737/II/0029 respectively) the safety concern 'thrombocytopenia' was revised to 'thrombocytopenia, including immune thrombocytopenia (ITP)', and one of the nonclinical studies initially proposed to address TTS and thrombocytopenia was proposed in the updated RMP to also address venous thromboembolism (VTE) (see Section 1.3.1). The present NCO Addendum provides an overview of the current status and results of these nonclinical studies (see Section 2), and an integrated conclusion on how the results contribute to understanding of the potential mechanisms underlying vaccine-associated TTS (see Section 5.1). These studies may also provide insights relevant to thrombocytopenia including ITP, and/or VTE. The current status and results of studies using clinical samples aimed at further characterizing TTS (final report milestone date in the EU-RMP of 31 March 2022) are provided in the Clinical Overview Addendum ('Update to the European Union Risk Management Plan Regarding Thrombosis with Thrombocytopenia Syndrome'), which will be submitted to EMA in a separate package.

In summary, the data from nonclinical studies presented in this document do not allow to conclude on a final pathogenesis mechanism of vaccine-induced TTS, however they provide relevant data that can be used to put published data into perspective and/or deprioritize some aspects of the proposed hypotheses or underlying mechanisms. The results and conclusions from each study are presented in detail in the pharmacology Section 2. Overall conclusions and perspectives from the various nonclinical studies are further summarized in Table 11. A more detailed and integrated conclusion on the nonclinical and clinical results (as also presented in the Clinical Overview Addendum 'Update to the European Union Risk Management Plan Regarding Throbosis with Thrombocytopenia Syndrome') to be submitted to EMA in a separate package, is provided in Section 5.2 of this NCO Addendum.

1.2. Summary of Interactions Between EMA PRAC and the Company

A summary of the interactions between the European Medicines Agency (EMA) Pharmacovigilance Risk Assessment Committee (PRAC) and the Company which are relevant to the studies presented in this document is provided in Table 1.

Table 1: Interactions Between EMA PRAC and the Company Relevant for this Document

Interaction	Procedure	EMA Request	Company Reaction
PRAC assessment report dated 06 May 2021	Procedure: SDA 018.1	To present a plan, including proposals for new studies, aimed at further characterization of the important identified risk "thrombosis with thrombocytopenia syndrome".	On 3 August 2021, the Company submitted a Type II variation (II/0018), including proposed preclinical studies aimed at further characterization of TTS and of thrombocytopenia (EU-RMP version 2.2). Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan
Request for Supplementary Information received on 30 Sep 2021	Procedure EMEA/H/C/005737/II/0018	PRAC stated: *It is not accepted at this stage to replace the term thrombocytopenia with ITP since the safety concern has not been sufficiently characterized to allow such narrowing of the term*	On 12 October 2021, the Company submitted a response to the Request for Supplementary Information, including a revised EU-RMP version 2.3, in which the terminology of the safety concerns was updated and the proposed nonclinical studies were aligned accordingly. Terminology of the safety concerns was further updated in EU-RMP version 2.5, submitted on 1 December 2021, also as part of procedure II/0018. Mod1/Response to RSI related to Variation II/0018 - Update EU-RMP and Mod1.8.2/EU Risk-Management Plan Version 2.5 - Clean - HA Approved (02Dec2021)
PRAC assessment report of MEA 032, dated 30 September 2021	Procedure EMEA/H/C/005737/MEA/032	To update the EU-RMP with upgrading of 'venous thromboembolism (VTE)' from important potential risk to important identified risk and to present a proposal for further characterization of VTE.	On 29 October 2021, the Company submitted a Type II variation (II/0029), proposing Study TOX15252 to address VTE (EU-RMP version 2.4). Mod2.4/ Nonclinical Overview Addendum - Update of the EU-RMP to version 2.4 (VTE Reclassification as Important Identified Risk)

EMA: European Medicines Agency; EU-RMP: European Risk Management Plan; ITP: Immune thrombocytopenia; PRAC: Pharmacovigilance Risk Assessment Committee; TTS: thrombosis with thrombocytopenia syndrome; VTE: venous thromboembolism

1.3. Nonclinical Testing Strategy

1.3.1. Studies Listed in the EU-RMP

This document provides study results and conclusions of nonclinical studies proposed in the EU-RMP to address the following safety concerns: TTS; thrombocytopenia, including ITP; and/or VTE.

Thrombosis with thrombocytopenia syndrome

TTS after vaccination with Ad26.COV2.S is very rare, which complicates the identification of a causal pathway leading to this serious clinical outcome. The very low incidence of TTS may imply that the clinical outcome is associated with Ad26.COV2.S related factors in combination with other factors, potentially including a predisposition of the host. In literature, this phenomenon is also referred to as vaccine-induced immune thrombotic thrombocytopenia (VITT). Different hypotheses to explain the pathogenesis of TTS following vaccination with Ad26.COV2.S were proposed as part of procedure II/0018 in Mod2.4/Nonclinical Overview Addendum – Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2 submitted with the EU-RMP version 2.2. Several nonclinical studies were listed as additional research / pharmacovigilance activities to gain insights into potential mechanisms of TTS, though it is recognized that a number of human host factors (not defined) may not be present in nonclinical models (see Table 2).

Thrombocytopenia, including immune thrombocytopenia

As part of procedure II/0018 the safety concern 'thrombocytopenia' in the EU-RMP was revised to 'thrombocytopenia, including ITP'. Several nonclinical studies initially proposed to address TTS were then proposed to also gain insights into potential mechanisms of vaccine related ITP, or thrombocytopenia including ITP (see Table 2).

The studies that may provide conclusions related to the pathogenesis of vaccine associated TTS, may also have relevance for the understanding of the pathogenesis of vaccine associated thrombocytopenia, including ITP. Therefore, in this document, thrombocytopenia (including ITP) is not mentioned separately from TTS.

Venous thromboembolism

In Mod2.4/ Nonclinical Overview Addendum - Update of the EU-RMP to version 2.4 (VTE Reclassification as Important Identified Risk) submitted with the EU-RMP version 2.4, one nonclinical study initially proposed to address TTS (TOX15252) was proposed to also gain insight into VTE.

The above information is reflected in the Mod1.8.2/EU Risk Management Plan Version 4.1.

An overview of the nonclinical studies as described in the EU-RMP Version 4.1 is presented in Table 2. In this table, for each study a (hyper)link is included to the associated hypotheses for the potential mechanisms underlying vaccine-associated TTS, as described in the previous Mod2.4/Nonclinical Overview Addendum – Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2 submitted in August 2021 with the EU-RMP version 2.2. The study designs and results are summarized in Section 2.1. Because of the cross-disciplinary nature of the studies, all study results (Pharmacology and Toxicology) are reported together under Section 2 Pharmacology.

Table 2: Overview of Nonclinical Studies Included as Additional PV Activities in the EU-RMP, to Investigate Potential Causes of TTS, Thrombocytopenia Including ITP and/or VTE

Study Reference	Study Title As Used in the EU-RMP (Study Report Title, if Different Than the EU-RMP Study Title)	Study Objective	Related Safety Concern in EU-RMP	Research Question*/ Potential Mechanism Addressed	Link to Description of Associated Hypothesis
Mod4.2.1.1/ TV-TEC-207316	Molecular mimicry of PF4 (Assessment of Ad26.COV2.S protein sequence homologies with PF4, vWF, thrombopoietin, and ADAMTS-13, as well as with the human proteome)	To compare linear amino acid sequences of the SARS-CoV-2 S protein, TetR, and adenovirus proteins present on the outside of the virion with the known human proteome sequences, and PF4, vWF, thrombopoietin, and ADAMTS-13 -see Section 2.1.1-	TTS; thrombocytopenia including ITP (this study addressed only ITP)	Potential role of autoimmune responses induced by proteins encoded by the Ad26.COV2.S vector	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.1 and Sec1.1.2.2
Mod4.2.1.1/ TV-TEC-207437	RNA sequencing of Ad26.COV2.S transduced cells in vitro (RNA secquencing of cell lines transduced with Ad26.COV2.S in vitro)	To analyze cells transduced with Ad26.COV2.S by RNA sequencing for possible alternative splicing events -see Section 2.1.2-	TTS; thrombocytopenia including ITP (this study addressed only ITP)	Potential role of soluble S protein (fragments) / alternative splicing variants	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.2
Mod4.2.1.1/ TOX15155	Study in NZW rabbits to determine S protein expression after Ad26.COV2.S immunization (VAC31518 SARS-COV-2 Vaccine (Covid-19): Immunogenicity and biodistribution/protein expression study in New Zealand White rabbits)	To evaluate the expression/distribution of the S protein and compare to non-stabilized spike transgenes that are known to shed the S1 portion following a single IM injection with Ad26.COV2.S New Zealand White (NZW) rabbits -see Section 2.1.3-	TTS; thrombocytopenia including ITP (this study addressed only ITP)	Potential role of soluble S protein (fragments)	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.2

Study Reference	Study Title As Used in the EU-RMP (Study Report Title, if Different Than the EU-RMP Study Title)	Study Objective	Related Safety Concern in EU-RMP	Research Question*/ Potential Mechanism Addressed	Link to Description of Associated Hypothesis
Mod4.2.1.1/ TV-TEC-215524	Anti-PF4 antibody levels in immune sera of Ad26.COV2.S immunized animals (Screening for cross-reactive antibodies to human Platelet Factor-4 protein in serum of animals immunized with different SARS-CoV-2 spike based vaccines)	To assess anti-PF4 antibody induction by different Ad26-based vaccine candidates encoding SARS-CoV-2 S protein antigens or non-SARS-CoV-2 antigens compared with control treatment in animals -see Section 2.1.4-	TTS; thrombocytopenia including ITP (this study addressed only ITP)	Assess potential animal models of hPF4 binding antibody induction	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.1 and Sec1.1.2.2
Mod4.2.1.1/ TOX15252	Systemic exposure to Ad26.COV2.S (Ad26.COV2.S: single dose investigative intravenous toxicity study with Ad26.COV2.S vaccine in New-Zealand white rabbits)	To assess effects of Ad26.COV2.S on platelets/coagulation and immunogenicity parameters following intravenous (IV) dosing. -see Section 2.1.5-	TTS; thrombocytopenia including ITP; VTE	Potential role of systemic exposure to Ad26.COV2.S (e.g., following accidental IV administration)	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.3 and Sec1.1.2.5
TOX15258 **	RNA transcriptome analysis after dosing with Ad26.COV2.S in Cynomolgus monkey	RNA transcriptome analysis after single and 2-dose immunization with Ad26.COV2.S compared to control vaccines.	TTS; thrombocytopenia including ITP	To assess host responses that may potentially be linked to TTS, by comparing clinical chemistry, hematology, coagulation and molecular (transcriptomics, in whole blood) endpoints across vaccines	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.3

*See also Table 11. ** TOX15258 is ongoing; the final report is expected by 30 Sep 2022
All studies listed in this table are non-GLP studies.
ADAMTS-13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; IM: intramuscular ITP: immune thrombocytopenia; IV: intravenous; PF4: platelet factor 4 (CXCL4); PV: pharmacovigilance; RNA: ribonucleic acid; S: Spike protein; TTS: thrombosis with thrombocytopenia syndrome; VTE: venous thromboembolism; vWF: Von Willebrand factor

1.3.2. Supplementary Studies (Not Listed in the EU-RMP)

In addition to the studies listed in Table 2, the Company listed a few other studies in the Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan, submitted in August 2021 (II/0018), which were labelled as 'Under Discussion to Investigate Potential Causes of TTS'. The Company has updated the status of these studies in Table 3. The Company has been able to analyse the study results of 'Re-evaluation of analytical data of all vaccine drug product (DP) batches', and has conducted a further characterization of the host cell protein (HCP) fraction of the vaccine. In addition, preliminary data are available of studies that examine whether there is an interaction of Ad26.COV2.S with platelet factor 4 (PF4), a study which was not listed in the Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan. The Company considers the findings of these studies of relevance to the overall conclusion of this NCO Addendum. Therefore, a summary of these results is provided in Section 2.2.

Table 3: The Current Status of the Studies Listed as the "Studies Under Discussion to Investigate Potential Causes of TTS" in Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS Including Updated PV Plan Submitted in August 2021 (II/0018)

Study Title and Research Question	Current Status	Link to Description of Associated Hypothesis
Re-evaluation of analytical data of all vaccine drug product batches (including further characterization of the host cell protein fraction) Research Question*: Potential role of CMC/product quality attributes (including potential role of HCP) -see Sections 2.2.1 and 2.2.2-	Completed	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.4
Ad26.COV2.S and S protein interaction studies with PF4 (in house) ** Research Question*: Potential role of interaction between Ad26.COV2.S and PF4 -see Section 2.2.3-	Study ongoing	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.1 and Sec1.1.2.2
In-silico prediction of T cell immunogenicity of host cell proteins and Ad26.COV2.S proteins	Study ongoing	
Confirm, and if feasible further explore the binding epitope on PF4 of antibodies from patients with TTS***	MTA signed	
Ad26.COV2.S and S protein interaction studies with platelets and PF4	Contract set up ongoing	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.1, Sec1.1.2.2 and Sec1.1.2.5
Examine molecular profile of Ad26.COV2.S transduced cells***	MTA signed	Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.3
HIT mouse model for mechanistic studies	Discussion with SME	NA

* See also Table 11.

** This refers to an in house study which was not listed in the Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan. The Company considers the findings of this study relevant, therefore it is included in this document. For this study only results from interaction between Ad26.COV2.S and PF4 are currently shown, and not between S protein and PF4.

*** Studies under Material Transfer Agreement (MTA) at external academic partner, the Company is providing material but is not involved in experimental set up and reporting. These studies will not be reported to the EMA by the Company.

CMC: Chemistry Manufacturing and Controls; EMA: Europen Medicine Agency; EU-RMP: European Union Risk Management Plan; HCP: host cell proteins; HIT: Heparin-induced thrombocytopenia; MTA: material transfer agreement; NA: not applicable; TTS: thrombosis with thrombocytopenia syndrome; PF4: platelet factor 4 (CXCL4); PV: pharmacovilgilance; S: Spike; SME: subject matter expert

1.4. Description of Ad26.COV2.S and Other Ad26-based COVID-19 Vaccine Candidates and Soluble S Proteins Presented in This Document

The Ad26.COV2.S (previously named Ad26NCOV030) vaccine is conditionally authorized for use in the prevention of coronavirus disease 2019 (COVID-19). It is a monovalent vaccine composed of a recombinant, replication-incompetent human Ad26 vector. The Ad26 vector encodes a full-length severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike (S) protein that is stabilized in its prefusion conformation, with point mutations in the S1/S2 junction that disable the furin cleavage site, and 2 newly introduced prolines in the hinge region in S2 (Figure 1). Ad26.COV2.S is produced in the PER.C6 TetR cell line. The transgene encoding the SARS-CoV-2 S protein was human gene optimized and cloned into the Ad26 vector using recombinant DNA technology. Modification of the furin cleavage site has been shown to inhibit shedding of the S1 subunit of the S protein (Bos 2020).

Ad26.COV2.S and other Ad26 based COVID-19 vaccine candidates, as well as soluble S proteins, were evaluated in studies TOX15155 (Section 2.1.3) and TV-TEC-215524 (Section 2.1.4). The constructs differ in the presence or absence of stabilizing mutations, a functional furin cleavage site, a transmembrane domain (TM), and/or a trimerization domain. By utilizing these different constructs, the aim was to characterize potential differences in biodistribution of the encoded S proteins or their capacity to induce human PF4 (hPF4)-reactive antibodies in animal models. For example, transgenes encoding soluble S protein or non-stabilized S protein with the capacity to shed the S1 protein subunit may distribute more broadly or induce hPF4-reactive antibodies more easily compared with membrane-bound spike transgenes that are stabilized, and cannot shed the S1 subunit of S protein (eg, Ad26.COV2.S). The different transgene constructs that were assessed in these studies are listed below and presented in Figure 1.

- Ad26NCOV006 encodes the wild-type (wt) S protein without any modifications (ie, based on the Wuhan strain); this vector was included in the studies to investigate the impact of the stabilizing mutations in the Ad26.COV2.S encoded S protein on biodistribution of the S protein and the ability to induce anti-PF4 antibodies. In addition, due to the presence of a functional furin cleavage site, the Ad26NCOV006 encoded S protein can shed its S1 subunit into the extracellular space of transduced host cells. S1 includes the receptor binding domain capable of interacting with angiotensin converting enzyme 2 (ACE2) of the host (Bos 2020).

- Ad26NCOV034 encodes an S protein stabilized in its prefusion conformation, however with an intact furin cleavage site allowing S1 subunit shedding (used in study TOX15155 only).

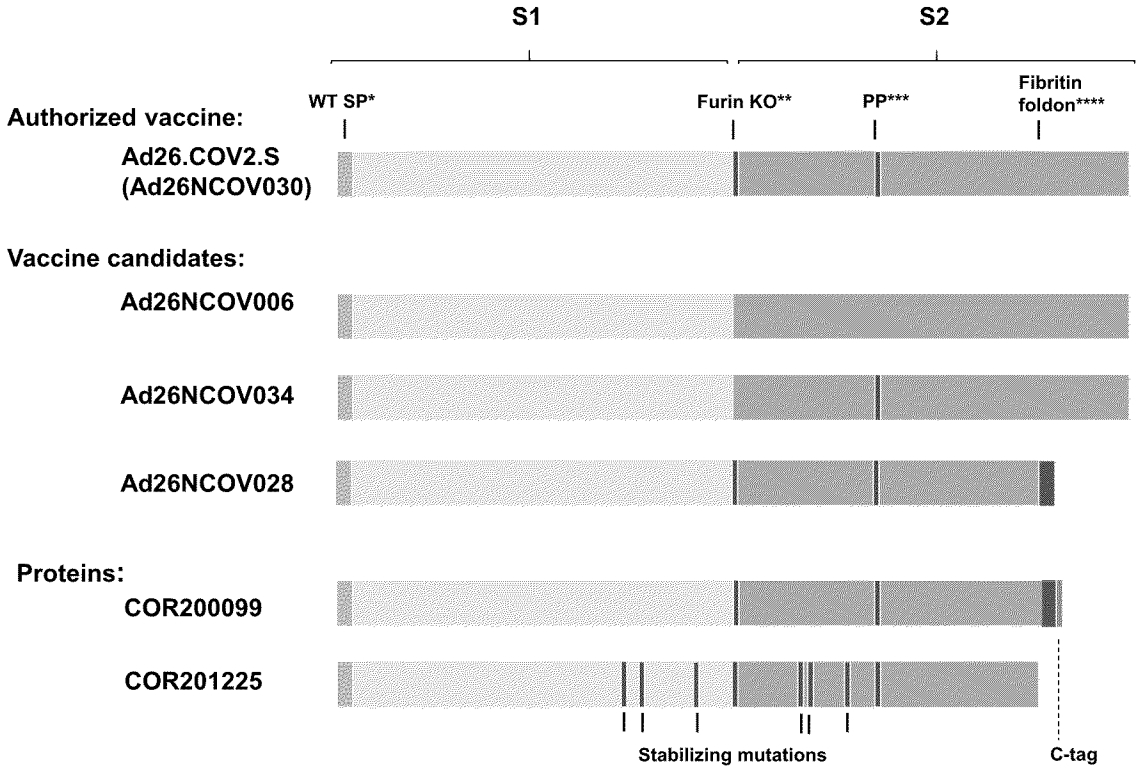
- Ad26NCOV028 encodes a soluble version of the S protein encoded by Ad26.COV2.S (ie, stabilized in its prefusion conformation and with disabled furin cleavage site), in which the transmembrane and cytoplasmic regions of the S protein were replaced by a foldon domain for trimerization (used in study TV-TEC-215524 only).

- Protein COR200099, used in TV-TEC-215524, is almost identical to the S protein encoded by the transgene in Ad26NCOV028, but the protein has a C-tag at the C-terminus and the

furin cleavage site is modified using different mutations than those in the Ad26NCOV028 insert. Protein COR200099 has a foldon trimetization domain.

- Protein COR201225, used in study TOX15155, is a soluble S protein with additional mutations compared with COR200099, that stabilize the protein in a trimeric prefusion conformation in the absence of a foldon trimerization domain.

Figure 1: Schematic Visualization of the Alterations Made to the S Protein Inserts of the Various SARS-CoV-2 Ad26 Vaccine Candidates and to the Proteins Tested in Studies TOX15155 and TV-TEC-215524



Visualization of the alterations made to the genetic sequence of the SARS-CoV-2 S protein insert of Ad26.COV2.S (Ad26NCOV030) vaccine and Ad26NCOV006, Ad26NCOV034, Ad26NCOV028 vaccine candidates; and COR201225 and COR200099 proteins. Light grey indicates the S1 region of the S protein; dark grey indicates the S2 region of the S protein; c-tag is indicated in green; stabilizing mutations are indicated in purple.*WT SP = wild type signal peptide (in yellow); **Furin KO = position of point mutation to knock out furin cleavage site (in red); ***PP = position of the addition of two prolines in hinge region (in red); ****fibritin foldon (in blue).

2. PHARMACOLOGY

Study rationale, design, data summaries and conclusions of the studies presented in this document are included in Section 2.1 and Section 2.2. An integrated overview and conclusion of the studies is provided in Section 5.

2.1. Studies Listed in the EU-RMP

2.1.1. Assessment of Ad26.COV2.S Protein Sequence Homologies with the Human Proteome, PF4, vWF, Thrombopoietin, and ADAMTS-13 – TV-TEC-207316

Study Rationale and Design

It was hypothesized that molecular mimicry between Ad26.COV2.S DP and human proteins may be linked to TTS (referred to in Table 2). This means there is a theoretical possibility that sequence similarities between Ad26.COV2.S DP proteins and human proteins can result in the cross-activation of autoreactive B cells that could trigger the coagulation cascade. It was published that molecular mimicry between S protein and PF4 epitopes is theoretically possible, but in an updated publication antibody cross-reactivity was not demonstrated (Greinacher 2021a). In addition, a 5-amino acid (aa) peptide sequence homology between S protein and thrombopoietin was described (Nunez-Castilla 2021).

Study Mod4.2.1.1/TV-TEC-207316 was designed to evaluate the potential risk of an autoimmune response induced by the Ad26.COV2.S DP that may be linked to TTS. The aim of this study was to compare linear amino acid (aa) sequences of Ad26.COV2.S DP proteins (the Ad26.COV2.S transgene encoded S protein, the tetracycline repressor [TetR] protein, and the Ad26 capsid proteins) with human proteins.

The rationale for the selection of the Ad26.COV2.S DP proteins is described below.

- The SARS-CoV-2 S protein is encoded by Ad26.COV2.S and the sequence for the homology analyses was based on the protein as encoded by Ad26.COV2.S.

- Tetracycline repressor protein was included in this analysis because the Ad26.COV2.S vaccine is generated on suspension PER.C6 cells expressing TetR. Trace amounts of residual TetR protein are detected in the vaccine (up to 0.05% of total protein intensity [measured by mass spectrometry] which translates to a maximum ~0.4 ng TetR/dose based on the highest determined HCP concentration in drug substance [DS]). In contrast to HCP derived from the human cell line PER.C6 that might be present in the vaccine, TetR protein is not a human protein. Non-human antigens are more likely to be immunogenic in humans. Therefore, TetR protein, although being present at very low levels, was included in this analysis. The sequence used in this study is identical to the sequence of the TetR protein as expressed in PER.C6 TetR cells.

- The selection of the adenoviral capsid proteins for this study focused on proteins of the outer capsid shell of the Ad26.COV2.S virus particle. The outer capsid shell of adenovirus particles contains the Hexon, Fiber and Penton proteins as well as the proteins pIX, pIIIa, and proteins VI

and VIII (Vujadinovic 2018). Protein sequences of the above mentioned Ad26 proteins were derived from a fully annotated wt Ad26 sequence (EF153474) which are identical to proteins in the Ad26.COV2.S vector.

In the first part of this study, these Ad26.COV2.S DP proteins were compared with the human proteome in the human protein database of the National Center for Biotechnology Information (NCBI) (non-redundant proteins: All non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF excluding environmental samples from WGS projects; organism: Homo sapiens, taxid: 9606) using the NCBI blast algorithm (analysis #1). For this analysis, hits with an Expect I-value ≤10 were investigated. The results were described in Mod2.4/Nonclinical Overview Addendum -Update EU-RMP Regarding TTS including Updated PV Plan/Sec 1.2.1.2.

Since large databases increase the chance of false positive hits, the study was extended with additional analyses using protein-protein BLAST to compare Ad26.COV2.S DP proteins specifically with 4 human proteins from the human proteome, which have or may have a link with vaccine-induced TTS (ie, PF4, von Willebrand factor [vWF], thrombopoietin, and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 [ADAMTS-13]) (analysis #2). For this analysis, hits with an Expect I(E)-value ≤1 were investigated.

For analyses #1 and #2, an NCBI protein BLAST search was performed. This is the most commonly used tool to detect similarities between sequences. These analyses report an EIct (E)-value (lower than the pre-set cut-off value) for each of the homologies found. The E-value is a parameter that describes the number of hits one can expect to see by chance and adjusted to the sequence database size. Since large databases increase the chance of false positive hits, the E-value corrects for the higher chance.

The rationale for the selection of the proteins from the human proteome which have or may have a link with vaccine-induced TTS for analysis #2 is described below:

- PF4 was linked to TTS because anti-PF4 antibodies were detected in vaccine-induced TTS patients (Greinacher 2021a, Greinacher 2021b, Greinacher 2021c) and similar to anti-PF4 antibodies in heparin-induced thrombocytopenia (HIT) may be implicated in TTS.

- Thrombopoietin and the S protein of SARS-CoV-2 share a 5-aa peptide sequence (Nunez-Castilla 2021). Therefore, a S protein specific antibody induced by Ad26.COV2.S may also bind to thrombopoietin and cause coagulation.

- vWF is a large multimeric glycoprotein present in the blood plasma and involved in platelet adhesion by forming ultra-large protein complexes (UL-vWF). vWF is involved in many diseases including thrombotic thrombocytopenic purpura and was therefore included in the analysis.

- ADAMTS-13 is involved in the reversion of the multimerization by cleavage of the UL-vWF complex. In COVID-19 patients, elevated levels of vWF have been observed, as well as the presence of antibodies against ADAMTS-13 which is linked to decreased ADAMTS-13 activity (Ward 2021). Chang et al. proposed that upon vaccination with

COVID-19 vaccines, mild endotheliopathy is induced that activates the UL-vWF path of hemostasis. If ADAMTS-13 is insufficiently able to breakdown the UL-vWF complexes, these complexes may be anchored to damaged endothelial cells, thereby recruiting platelets and triggering microthrombogenesis (Chang 2021). Furthermore, Claireaux et al. identified SARS-CoV-2 S-reactive naïve and unswitched memory B cells with potential cross-reactivity to ADAMTS-13, suggesting a potential role of these pre-existing S-reactive B cells in clotting events during SARS-CoV-2 infection (Claireaux 2021).

Greinacher et al. described linear homologies between SARS-CoV-2 S protein and PF4 using the SIM Alignment Tool. To confirm these findings, a third analysis was performed using the SIM Alignment Tool (https://web.expasy.org/sim/) with the same parameters as described previously (Greinacher 2021a). In addition, linear homology between PF4 and the other Ad26.COV2.S DP proteins was analyzed. To identify peptides with the potential for being a genuine hit, only peptides with a score >30 were further analyzed.

Results

Analysis #1 of linear peptide sequences of the Ad26 Capsid proteins, TetR, and the SARS-CoV-2 S protein that are present in Ad26.COV2.S DP against the human proteome did not show any homologies that are considered relevant for the risk of vaccine associated TTS (as described in Mod2.4/Nonclinical Overview Addendum -Update EU-RMP Regarding TTS including Updated PV Plan/Sec 1.2.1.2). Two peptides with linear homology to human proteins were found with a E-score >1 (low probability to be a genuine hit). In addition to the high E-score, both these human proteins are intracellular proteins and literature review did not reveal any clear link with platelet activation, PF4 expression, or blood clotting events.

Analysis #2 compared the Ad26.COV2.S DP proteins with PF4, vWF, thrombopoietin, and ADAMTS-13. This analysis revealed 4 potential linear peptides that share some homology to the 4 human proteins (Table 4) and are possibly exposed (located on the outside of the structure). The first peptide is an Ad26 Penton-derived peptide with linear homology to thrombopoietin (31% homology in 45 residues). The second peptide is an Ad26 Hexon-derived peptide with homology to ADAMTS-13 (25% homology in 71 residues). When the same analysis was performed using the sequences of the chimpanzee adenovirus Y25 (the vector used for AstraZeneca's adenoviral vectored COVID-19 vaccine ChAdOx1 nCoV-19) these homologies were not identified. There are no crystal structures known for thrombopoietin and ADAMTS-13, it can therefore not be assessed whether potential epitopes would be accessible for binding of cross-reactive antibodies. However, since most likely similar mechanisms are related to induction of TTS after vaccination with ChAdOx1 nCoV-19 and Ad26.COV2.S, these 2 potential homologies are unlikely to be related to TTS. The third peptide is a TetR-protein specific peptide with homology to PF4 (45% homology in 11 residues). Of note, when the PF4 homolog of the PF4-TetR overlapping peptide is mapped to the crystal structure of tetrameric PF4, the peptide is mostly buried inside PF4 which implies that antibodies (if formed) are unlikely to bind to PF4. Only a single amino acid residue of the overlapping peptide has been linked to binding of anti-PF4 antibodies as found in the serum of TTS patients (Huynh 2021). The fourth peptide is a peptide located in the SARS-CoV-2 S S2 domain with homology to thrombopoietin (31% homology in 26 residues). S protein is present

in/expressed by all COVID-19 vaccines, including mRNA based vaccines which are not linked to TTS and therefore linear homologies associated with the S protein are unlikely to be the root cause of TTS. This is in line with the findings of Greinacher et al., describing linear homology between S protein and PF4, but no cross-reactivity of the affinity-purified anti-PF4 antibodies from 14 VITT patients with SARS-CoV-2 S protein (Greinacher 2021a). However, there are many differences between the vaccines and S protein that might still play a role in a multifactorial mechanism. For example, biodistribution, expression level, duration of expression and/or innate immune activation can influence the potential effect of S protein. Sequence homology between S protein and thrombopoietin has also been previously described by others, but the peptide identified by the Company is not in the region of the 5-aa overlapping sequence as reported by Nunez-Castilla et al. (Nunez-Castilla 2021).

Analysis #3 did not identify any additional homologies that are considered relevant. Four peptides with linear homology to PF4 were found with a score value of >30. The first peptide is an Ad26 Hexon peptide that is partially hidden in the Hexon protein. The second peptide is an Ad26 protein VI peptide that is buried inside the virus particle. The third peptide is an SARS-CoV-2 S peptide that is part of the S signal peptide and cleaved off during intracellular protein processing. The fourth peptide is also a SARS-CoV-2 S peptide and as described above not considered relevant because affinity-purified anti-PF4 antibodies from VITT patients do not cross-react with SARS-CoV-2 S protein (Greinacher 2021a).

Table 4: Linear Sequence Homologies Identified Using Protein-Protein BLAST

Human protein (Query)	Target protein (Subject)	% homology (# of amino acid)	Overlapping sequence
Thrombopoietin	Ad26 Penton	31% (45aa)	Q 206 NFTASARTTGSGLLK---WQQGFRAKIPGLLNQTSRSLDQIPGYL 247 +FT S + G+ K +Q+GFR L +L +P YL S 242 DFTESRLSNLLGIRKKQPFQEGFRIMYEDLEGGNIPALLDVPKYL 286
ADAMTS-13	Ad26 Hexon	25% (71aa)	Q 772 PCPARWEVSEPSSCTS----------AGGAGLALENETCVP P P++WE E T A G+ + N+ + S 140 PNPSQWETKEKQGTTGGVQQEKDVTKTFGVAATGGINITNQGLLL GADGLEAPVTEGPGSVDEKLPAPEPCVG 830 G D E +K PEP VG GTD--ETAENGKKDIYADKTFQPEPQVG 210
PF4	TetR	45% (11aa)	Q 68 PTAQLIATLKN 78 PT + TL+N S 105 PTEKQYETLEN 115
Thrombopoietin	Spike	31% (26aa)	Q 306 LFPLPPTLPTPVVQLHPLLPDPSAPT 331 ++ PP +LPDPS P+ S 788 IYKTPPIKDFGGFNFSQILPDPSKPS 813

Q: query = human protein; S: subject = target protein

Discussion and Conclusion

Based on these data, the identified linear homologies are unlikely associated with the onset of TTS. However, conformational homology cannot be excluded at this point. The Company will closely monitor work ongoing in the field by organizations that have access to TTS case samples to further understand the specificity of antibodies identified in TTS cases. Any important new findings will be reported.

2.1.2. RNA Sequencing of Ad26.COV2.S Transduced Cells to Assess Alternative Splicing Events Leading to C-Terminal Truncated, Soluble S Protein Variants – TV-TEC-207437

Study Rationale and Design

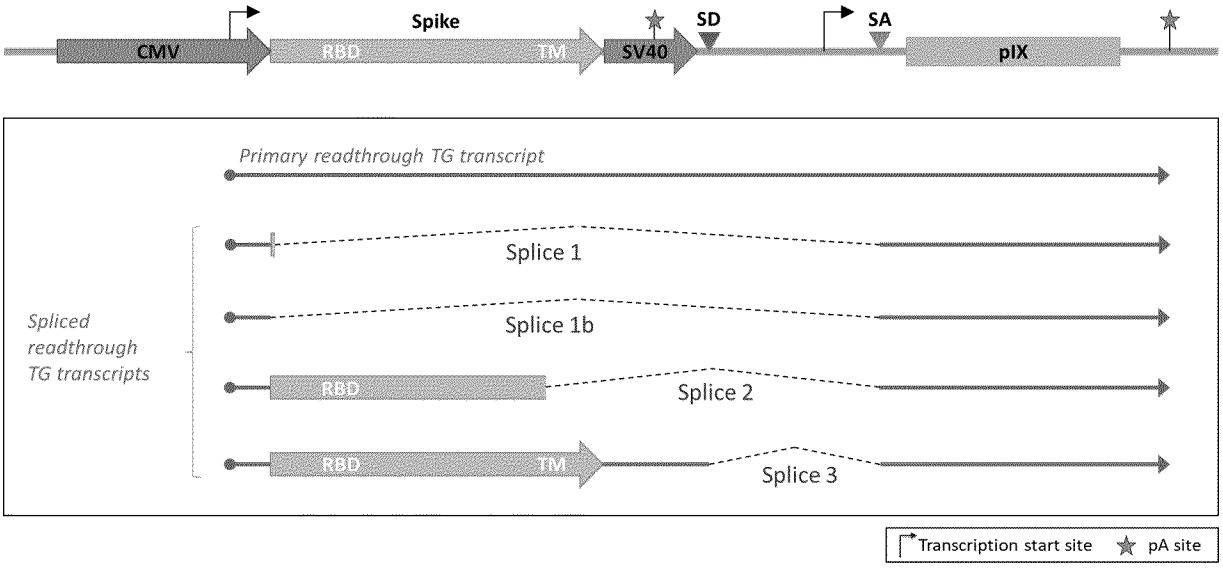
Kowarz et al., hypothesized that soluble S protein fragments, resulting from unintended splice events, may bind to ACE2-expressing endothelial cells and cause TTS in rare cases (Kowarz 2021, hypothesis referred to in Table 2). The Company considers the relevance of splice variants negligible and not applicable to Ad26.COV2.S because there are no strong splice donor sites left in the spike transgene sequence used in Ad26.COV2.S (as described in Mod2.4/Nonclinical Overview Addendum -Update EU-RMP Regarding TTS including Updated PV Plan/Sec 1.2.1.3). Nevertheless, the Company has further investigated to what extent alternative splicing of the spike insert in Ad26.COV2.S occurs. In study Mod4.2.1.1/TV-TEC-207437 the potential of alternative splicing events leading to S protein variants was investigated. The objective of the study was to use RNA sequencing to analyse Ad26.COV2.S transduced cells for the presence of splice variants of spike. To this end, the transcriptomes of the vector-transduced cell lines were sequenced using Illumina's massively parallel sequencing technology, which delivers short-read sequence data that can be used for splice (junction) discovery and quantification.

The cell lines that were used in this study are the human lung diploid fibroblast cell line MRC-5 and the adenocarcinomic human alveolar aneuploid epithelial cell line A549. These cell lines lack the adenovirus E1 gene and cannot support replication of the E1-deleted Ad26 vector, similar to human cells that are transduced in vivo upon vaccination. MRC-5 cells are considered the most relevant, as being a diploid cell line from a noncancerous origin. The short-read RNA sequencing data sets obtained for the different Ad26.COV2.S transduced cell samples were analyzed with the goal to identify and quantify possible splice events in the transgene transcript, particularly those that may be expected to result in transgene transcripts encoding truncated, soluble forms of the S protein. To this end, reads were mapped to the Ad26.COV2.S genome using a read mapping algorithm that allows to identify reads with splice events. The frequency of an identified splice event was calculated by dividing the number of reads supporting that splice event by the local total read coverage seen at that splice site.

Results

Four splice events in the Ad26.COV2.S transgene transcript, referred to as splices 1, 1b, 2, and 3, were identified. The transgene expression cassette consists of a cytomegalovirus (CMV) promoter, an spike coding sequence, and an SV40 polyadenylation sequence, and is located in the ΔE1A/E1B region between the left inverted terminal repeat and the pIX gene. All four splice events utilize the splice acceptor (SA) located outside the transgene expression cassette (ie, in the vector backbone), just before the pIX gene. The use of this SA that is located downstream of the transgene expression cassette implies that these splice events occur in readthrough transcripts that continue beyond the normal termination site of that cassette, as schematically illustrated in Figure 2. Splices 1, 1b, and 2 affect the transgene coding sequence while splice 3 does not. Table 5 describes the expected effects of the three identified splice evenst that affect the transgene coding sequence.

Figure 2. Predicted Readthrough Transgene Transcripts With Splices 1, 1b, 2, and 3



Predicted readthrough transgene transcripts with splices 1, 1b, 2, and 3. On top is a schematic visualisation of the Ad26.COV2.S transgene cassette region, including the adenoviral gene for protein IX (pIX). Shown below are identified RNA transcripts with splices that would affect the transmembrane domain (TM).
CMV: cytomegalovirus promoter; RBD: receptor binding domain; TM: transmembrane domain; SV40: simian virus 40 = SV40 polyadenylation sequence; SD: splice donor site; SA: splice acceptor site; pA site: polyadenylation site = transcription termination site. Figure adapted from Mod4.2.1.1/TV-TEC-207437

Table 5. Expected Effect of Identified Transgene Coding Sequence-Affecting Splice Events on the Transgene Product

Splice #	Splice Description	Effect on Spike Transgene	Splice Frequencies*	
			A549	MRC5
1	Splice from cryptic splice donor located in the signal peptide-encoding sequence (in spike transgene) to known SA just before pIX gene (in vector backbone).	C-terminal truncation leading to7 aa-long spike truncation/fusion product. The last 2 aa are not spike-specific.	0.03%	0%
1b		Complete deletion, no expression of S	0.01%	0%
2	Splice from cryptic splice donor located just before the transmembrane domain-encoding sequence (in spike transgene) to known SA just before pIX gene (in vector backbone)	C-terminal truncation exactly before the transmembrane domain leading to 1213 aa-long spike truncation product that theoretically represents a soluble protein	0.02%	0%

*data combined per cell type
SA: splice acceptor.

The transgene coding sequence-affecting splice events (ie, splice 1, 1b, and 2) were found in A549 cells, but not in MRC-5 cells.

Of the identified transgene coding sequence affecting splice events, only splice event 2 would result in a transgene transcript that potentially encodes a soluble form of S protein (Table 5). Splice event 1 would result in almost a complete deletion of spike, only a 7 aa-long spike truncation

product would be formed (which would not contain any functional domains of S protein anymore). Splice event 1b results in complete deletion of spike. Splice event 2, which spans from just upstream of the TM of S protein to the known SA just upstream of pIX, is expected to encode an S protein that is C-terminally truncated exactly before the transmembrane domain. Due to a stop codon formed at the splice junction, this truncation product would not carry any additional (non-spike) sequences. The frequencies of this splice event in the 2 data sets obtained with MOI 4000 and MOI 2000 in A549 cells were 0.03% and 0%, respectively. Combined, these 2 data sets show a frequency of this splice event of 0.02%.

Considering the low absolute number of reads supporting the identified transgene coding sequence-affecting splice events (ie, splices 1, 1b, and 2), the calculated frequencies for these splice events in the different data sets should be seen as (very rough) estimations. For example, splice event 2 was found in (only) 4 out of 17464 reads in A549 cells (0.02%), and 0 out of 12129 reads in MRC5 cells (0%). This analysis did not test if these low splice events lead to protein expression and secretion. However, even if these splice variants were translated and protein was expressed, the level of S protein in the circulation would be very low. In addition to these analyses, the Company has set up an external collaboration to perform a similar analysis of the Ad26.COV2.S transcriptome as has been described for ChAdOx1 nCoV-19 (Almuqrin 2021). The data on Ad26.COV2.S from this collaboration confirm that the dominant transcripts were coding for the S protein and evidence for low level novel splice events within the full length S protein was obtained (< 0.05% in MRC-5 cells, <0.1% in A549 cells) (confidential communication, data will be published by collaborator).

Discussion and Conclusion

Based on the findings by the Company and the collaborators, it is unlikely that splice events would contribute to TTS. In addition, published data suggest that soluble S protein alone is likely not sufficient for the induction of TTS-like events, since mRNA vaccines induce detectable soluble S protein in the circulation without causing increased TTS rates in human vaccinees (Röltgen 2022, Ogata 2021). However, the duration of mRNA induced S protein expression could be shorter compared with Ad26.COV2.S induced S protein expression. In addition, the magnitude and exact location of S protein expression as well as its conformation or glycosylation could be different. Therefore, soluble S protein may not be ruled out as a potential contributing factor in a multifactorial scenario of TTS induction following vaccination with Ad26.COV2.S.

2.1.3. S Protein Expression After Ad26.COV2.S Immunization – TOX15155

Study Rationale and Design

The biodistribution profile of the Ad26 vector was previously evaluated by quantitative polymerase chain reaction (qPCR) in rabbits using two Ad26-based vaccines encoding proteins of the respiratory syncytial virus (RSV) or the human immunodeficiency virus (HIV) (ie, other than the SARS-CoV-2 S protein). Following intramuscular (IM) injection, Ad26 vector DNA was primarily detected at the site of injection, in regional (iliac) lymph nodes and (to a lesser extent) the spleen, hence indicating a limited distribution profile (in Mod4.2.2.3/TOX13342 and 1645-06074 in EMEA/H/C/005737).

Considering the potential role of the SARS-CoV-2 S protein in TTS (see hypothesis referred to in Table 2), a study was conducted to evaluate the expression/distribution of the S protein following a single IM injection with Ad26.COV2.S, Ad26NCOV006 or Ad26NCOV034 in male New Zealand White (NZW) rabbits. Intramuscular injection with an aluminum hydroxide ($Al(OH)_3$) adjuvanted soluble S protein (COR201225) was included as a positive control and IM injection of an empty Ad26 vector (Ad26.Empty) as a negative control. The design of the study is provided in Table 6 (Mod4.2.1.1/TOX15155). Descriptions and schematic visualization of the adjuvanted soluble COR201225 protein, as well as the different S protein constructs encoded by Ad26.COV2.S, Ad26NCOV006, Ad26NCOV034 are presented in Section 1.4 and Figure 1.

Clinical observations and body weights were recorded during the study. For detection of the S protein, immunohistochemistry (IHC) was performed with two different SARS-CoV-2 S protein binding antibodies on tissue sections from the administration site, draining lymph nodes (iliac, popliteal), spleen (ie, on tissues that showed a qPCR signal for Ad26 vector DNA in the previously conducted biodistribution studies, see above), as well as on 2 veins (lateral saphenous/cava caudalis). These veins (along with vessels at the injection site tissue) were included in the IHC analysis considering the hypothesized role of S protein presence and/or expression in vascular endothelium in the onset of TTS (as summarized in Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan, Section 1.1.2.2). The 2 monoclonal anti-S protein antibodies were directed either towards the n-terminal (S1 antibody) or the c-terminal (S2 antibody) subunit of the membrane external portion of the S protein (see Figure 1). IHC was conducted following necropsies on Day 1 and Day 11. In addition, S protein levels were measured on Day 1 and Day 11 in blood (both in plasma and in blood derived cell fraction) with a commercial S-PLEX SARS-CoV-2 S protein detection assay, based on an electrochemiluminescence readout that detects presence of the S protein receptor binding domain (direct communication from the manufacturer). Day 11 was selected in this study to overlap with the first sampling timepoint used in the available biodistribution studies detecting the Ad26 vector using qPCR (see above). The study further included a measurement of anti-S protein antibodies, as well as neutralizing antibody titers against the Ad26 vector.

Table 6: Study Design of Biodistribution/Protein Expression Study in Rabbits (TOX15155)

Group	Dosage groups	Dosage** (per animal)	Concentration	Day of Dosing	Necropsy***
A	Ad26.COV2.S*	$5x10^{10}$ vp	$1x10^{11}$ vp/mL	0	Day 1
B	Ad26.COV2.S*			0	Day 11
C	Ad26NCOV006	$5x10^{10}$ vp	$1x10^{11}$ vp/mL	0	Day 1
D	Ad26NCOV006			0	Day 11
E	Ad26NCOV034	$5x10^{10}$ vp	$1x10^{11}$ vp/mL	0	Day 1
F	Ad26NCOV034			0	Day 11
G	COR201225+$Al(OH)_3$	50 µg protein + 250 µg $Al(OH)_3$ adjuvant	100 µg protein + 500 µg $Al(OH)_3$ adjuvant/mL	0	Day 1
H	COR201225+$Al(OH)_3$			0	Day 11
I	Ad26.Empty	$5x10^{10}$ vp	$1x10^{11}$ vp/mL	0	Day 1
J	Ad26.Empty			0	Day 11

*Also annotated as Ad26NCOV030 in the report
**Administration site: Left biceps femoris (left hind limb); Injection volume: 0.5 mL. Ad26 dosage represents a full human dose level.
***4 animals / subgroup/timepoint

Results - Toxicology and Immunohistochemistry Endpoints

The IM administration of the vaccines was well tolerated in all groups, and no adverse vaccine-related effects were noted. Local reactions were limited to transient erythema or slight swelling at the administration site in some of the animals/groups. A minimal, transient body weight loss was noted on the day after dosing in all vaccinated groups except groups G and H (COR201225+ $Al(OH)_3$). This was followed by a quick recovery in the following days, and no difference in body weight was observed during the remainder of the study.

One day after an IM injection in the left biceps femoris of male rabbits with Ad26-based COVID-19 vaccines (Ad26.COV2.S, Ad26NCOV006, Ad26NCOV034), IHC evaluation showed the presence of S protein in the administration site and draining lymph nodes (iliac and/or popliteal) with similar distribution for S1 and S2 subunits of the S protein, as detected with the two different antibodies directed towards the n-terminal (S1 antibody) and c-terminal (S2 antibody) subunit of the S protein, respectively. At the injection site, membranous/cytoplasmic staining of round to elongated cells (considered macrophages and/or fibroblasts) was mainly observed in connective tissue, while myocytes at the administration site were negative. The S protein was not detected in spleen and arteries/veins (ie, blood vessels at the injection site, or lateral saphenous vein and vena cava caudalis). Eleven days after the IM injection with Ad26-based vaccines, the S protein was no longer detected in any of the tissues examined. There was no difference in the distribution of the S protein between the Ad26-based vaccines.

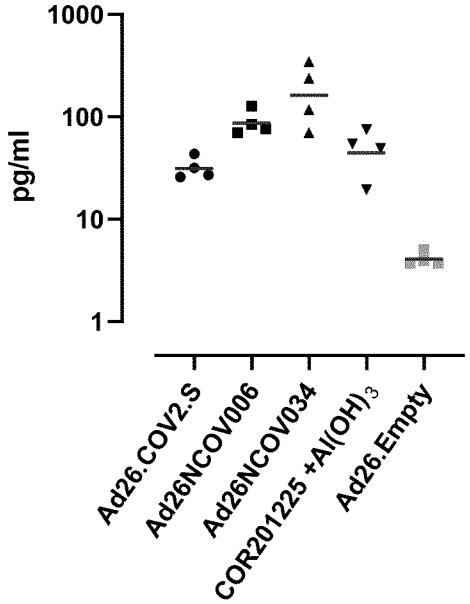
For animals dosed with the recombinant soluble S protein COR201225 (+$Al(OH)_3$), IHC staining showed that the S protein was only detected in the administration site (mainly without cellular association) one day after an IM injection, with similar distribution of anti S1 and S2 staining. The S protein was no longer detected in any of the tissues examined on Day 11.

No S protein was detected in administration site, draining lymph nodes, spleen or veins after dosing with Ad26.Empty (negative control).

Results - S protein in Blood (Plasma) and Immunogenicity

S protein levels were increased above background levels in plasma sampled 1 day post dosing with any of the S protein encoding Ad26 vectors, or with the COR201225 protein. There were statistically significantly lower S protein levels in Ad26.COV2.S dosed animals (group geomean 31.3 pg/mL ± 8.0) compared with animals receiving Ad26NCOV006 (group geomean 86.9 pg/mL ± 25.7; $p=0.0006$, Tobit model with Bonferroni adjustment) or Ad26NCOV034 (group geomean 161.8 pg/ml ± 124.7; $p<0.0001$ Tobit model with Bonferroni adjustment) (Figure 3). In the blood-derived cell fraction sampled 1 day post dosing, the S protein levels were in the range of the background measured in the control animals dosed with Ad26.empty. Similarly, the S protein levels in the plasma sampled at Day 11 post dosing with any of the S protein encoding Ad26 vectors, or with the COR201225 protein, were comparable to the background of the assay as measured for the Ad26.Empty control (data not shown).

Figure 3: SARS-COV-2 S Protein in Plasma Day 1 Post Dosing With Ad26.COV2.S in Rabbits



Plasma from New Zealand white rabbits (N=4 per group) dosed with Ad26.COV2.S, Ad26NCOV006, Ad26NCOV034, COR201225 + $Al(OH)_3$, or Ad26.Empty was analyzed in a commercial S protein detection S-PLEX SARS-CoV-2 S protein detection assay. Since the SARS-CoV-2 S protein detection assay is developed but not qualified to test rabbit plasma samples, no lower limit of detection is available for this assay. The assay background is defined by responses measured after immunization with Ad26.Empty, which does not include or encode SARS-CoV-2 S. Plasma was sampled Day 1 post dosing. Symbols represent the mean response per animal of 2 runs. The geometric mean response per group is indicated with a red horizontal line (Mod4.2.1.1/TOX15155/Annex Non-clinical Contributing Scientist Immunogenicity Report (TV-TEC-199150).

Induction of S protein-specific binding antibody titers was detected in all animals at Day 11 post dosing with an S protein encoding Ad26 vaccine, or with adjuvanted COR201225 protein. Furthermore, Ad26 neutralizing antibody titers were present at Day 11 in all animals dosed with Ad26 vectors, confirming exposure of the animals to the Ad26 based vaccines (data not shown).

Discussion and Conclusion

Using IHC, on Day 1 following immunization, expression of the S protein was observed in the IM administration site and draining lymph nodes, with all examined tissues being negative for S protein expression on Day 11 post dosing. This profile differs from the previously reported biodistribution studies for the Ad26 platform, using qPCR. In these studies, Ad26 vector DNA was still detectable in the injection site, draining lymph nodes and to a lesser extent the spleen on Day 11. This difference may be explained by the lower sensitivity of IHC versus qPCR. Although it is not clear whether the qPCR method used in these previous biodistribution studies detects intact (transcriptionally active) vectors / vector particles or only fragments thereof, some more prolonged expression (ie, beyond Day 1 and/or Day 11) of the S protein in these tissues may not be ruled out based on these IHC data.

Using IHC, no difference in the distribution of the S protein was observed between the various S protein encoding Ad26 vectors (ie, Ad26.COV2.S, Ad26NCOV006, Ad26NCOV034; see Figure 1) on Day 1, whereas in plasma the S protein levels in animals dosed with Ad26.COV2.S were lower than in animals that received Ad26NCOV006 or Ad26NCOV034. This difference between the two methodologies may be explained by the lower sensitivity and less quantifiable (with

variability in sampling and sectioning of tissues) method of IHC versus the S-PLEX SARS-CoV-2 S assay. The plasma data indicate that immunization with the stabilized S protein construct with mutations in the furin cleavage site (as used in Ad26.COV2.S, see Figure 1) leads to lower levels of the S protein in the circulation compared with similarly manufactured Ad26-based vaccines encoding unstabilized spike or stabilized spike with intact furin cleavage site.

Overall, following administration of Ad26.COV2.S in rabbits a transient expression of the S protein was observed, both locally as well as in the circulation. It is yet unknown whether soluble S protein can be found in the circulation of human subjects following vaccination with Ad26.COV2.S, but these rabbit data, in line with the discussion above (Section 2.1.2), indicate that soluble S protein may not be ruled out as a potential contributing factor in a multifactorial scenario of TTS induction following vaccination with Ad26.COV2.S.

2.1.4. PF4 Binding Antibody Levels in Immune Sera of Ad26.COV2.S Immunized Animals – TV-TEC-215524

Study Rationale and Design

Due to clinical similarities between TTS and HIT, anti-PF4 antibodies are part of some TTS case definitions and are hypothesized to play a central role in the pathogenesis of TTS (hypothesis refered to in Table 2). To study whether animal models can be used to assess the mechanism of anti-PF4 antibody induction in humans, the levels of hPF4 binding antibodies were determined in animal sera from historically performed nonclinical immunogenicity studies. If hPF4 binding antibody responses were induced in humans by epitope similarities between vaccine components and hPF4, animal studies might represent a sensitive model of hPF4 binding antibody induction, since self-tolerance mechanisms would limit hPF4 directed antibody responses to a lesser extent in animal models than in humans. Presence of hPF4 binding antibodies in sera of Ad26.COV2.S immunized mice, rabbits and non-human primate (NHP) was determined and compared to levels in sera from animals receiving either Ad26-based S protein vaccines, or Ad26-based vaccines encoding SARS-CoV-2 unrelated antigens, or used recombinant SARS-CoV-2 S protein as immunogen (refer to Section 1.4 for descriptions of S protein vaccines), or were entirely unrelated to Ad26.COV2.S (study Mod4.2.1.1/TV-TEC-215524, see Table 7).

Three Ad26-based SARS-CoV-2 vaccine candidates were evaluated to determine potential different capacities of the encoded antigens to induce hPF4 cross reactive antibodies in animals: Ad26.COV2.S, Ad26NCOV006 and Ad26NCOV028 (depending on the specific study, see Table 7). Descriptions and schematic visualization of Ad26.COV2.S, Ad26NCOV006 and Ad26NCOV028 and COR200099 are presented in Section 1.4 and in Figure 1. In some studies, additional groups immunized with recombinant S protein (COR200099) adjuvanted with aluminum salts, or an Ad26.empty, or Ad26 encoding other antigens, or other vaccines were also included (eg, a marketed influenza vaccine, Fluzone). Heparin dependency of hPF4 binding was tested since anti-hPF4 antibodies are known to depend on heparin binding in HIT, while in TTS the majority of evaluated serum immunoglobulin (Ig) G reactivity with PF4 was heparin independent.

Sera from immunized NHP (rhesus macaques), mice and rabbits were analysed. The original animal studies were conducted as part of the COVID-19 vaccine development program or other programs.

An overview of all studies included in this analysis is presented in Table 7.

Table 7: An Overview of the Origin of Serum Samples Analyzed in Study TV-TEC-215524, Including Original Animal Study Identifiers , Study Groups and Sample Time Points

Species (Number of Animals[N])	Study Number	Treatment	Regimen (dose interval) Dose Level, Immunization Route*,**,***	Time points (Days Relative to Dose 1)
NHP (Rhesus Macaque) (N=56	NHP 2020_3373	Ad26.COV2.S	1-dose, 5×10^{10} vp, 10^{11} vp 2-dose (4wk and 8wk), 5×10^{10} vp	pre-dose, 11, 57, 67
NHP (Rhesus Macaque) (N=14	VH808.681	Ad26.COV2.S	2-dose (8wk), 5×10^{10} vp	pre-dose, 14, 28
		S protein (COR200099) + alum (aluminum hydroxide)	2-dose (8wk), 100 mcg protein + 500 mcg aluminum hydroxide	
		Ad26RSV-F_{A2}-2A-Gluc****	2-dose (8wk), 10^{11} vp	
Mice (Balb/C) (N=100)	AEP9346_20004	Ad26NCOV006	1-dose, 10^7, 10^8, 10^9, 10^{10} vp	pre-dose, 14, 28
		Ad26NCOV028	1-dose, 10^8, 10^9, 10^{10} vp	
		Ad26.COV2.S	1-dose, 10^8, 10^9, 10^{10} vp	
		Ad26.Empty	1-dose, 10^{10} vp	
Mice (Balb/C) (N=21)	AEP9346_20007	Ad26NCOV028	1-dose, 10^{10} vp	pre-dose, 13
		Ad26.COV2.S	1-dose, 10^{10} vp	
		S protein (COR200099) + aluminum phosphate	50 mcg protein+100 mcg aluminum phosphate	
		Aluminum phosphate	100 mcg	
Mice (Balb/C) (N=20)	AEP9346_20013	Ad26.COV2.S	1-dose, 10^9 vp	pre-dose , 84
			2-dose (8wk), 10^9 , $2x10^8$, $4x10^7$ vp	-14 to -1***, 27/28, 56, 84
Mice (Balb/C) (N=18)	AEP203_16008	Ad26-RSV-F_{A2}-2A-Gluc****	1-dose, 10^8, 10^9, 10^{10} vp	pre-dose, 28
		Ad26.Fluc (Ad26.dE3.5orf.Luc+)	1-dose, 10^{10}	
Mice (Balb/C) (N=23)	AEP425-18002	UFV180251 (recombinant influenza protein) +2% adjuplex	3-dose (3wk), 3 mcg	pre-dose, 18, 41
		UFV180284 (recombinant influenza protein) +2% adjuplex	3-dose (3wk), 3 mcg	
		UFV170088 wt HA (B/Brisbane/60/08) +2% adjuplex	3-dose (3wk), 3 mcg	
		PBS	-	
Mice (Balb/C) (N=23)	V20331/39	PBS +2% adjuplex	3-dose (3wk),	pre-dose, 20, 41
		H3 HK68 FL HA +2% adjuplex	3-dose (3wk), 10 mcg	
		Fluzone HD® 17/18	3-dose (3wk), 1/5th human dose	
New Zealand white Rabbits (N=50)	TOX15252 (see Section 2.1.5)	AP1 buffer	IV, IM	pre-dose, 14, 28
		Ad26.COV2.S	1-dose, $2x10^8$ vp IV, 10^9 vp IV, $5x10^9$ vp IV/IM	
		Ad26.ZIKV.001	1-dose, $5x10^9$ vp IV/IM	
		Ad5.eGFP	1-dose, $5x10^9$ vp IV	
		MMR	Human dose: $1x10^2$ $TCID_{50}$ measles, $12.5x10^2$ $TCID_{50}$ mumps, $1x10^2$ $TCID_{50}$ rubella, IV	

*Immunization was given intramuscularly (IM) in all studies, with exception of TOX15252 where the route is indicated.
** The dose indicated in study TOX15252 is per kg, whereas the doses indicated for all other studies are per animal
*** Study VH808.681 no post Dose 2 samples were analyzed; study V20331/39 no post Dose 3 samples were analyzed.
**** Ad26-RSV-F_{A2}-2A-GLuc is a research vector encoding RSV-FA2-2A-GLuc where the RSV-FA2 protein is bicistronically joined with Gaussia Luciferase (GLuc) by a short linker sequence based on the 2A protein of the foot-and-mouth-disease virus, with substantial differences to any clinically developed RSV vaccine. In the studies also referred to as Ad26.RSV-F2A-Gluc, Ad26.ApoA1.RSV.F(2A)Gaussia Luc, Ad26.RSV.Luc.

HA: Influenza haemagglutinin glycoprotein, H3 HK68 FL HA = Recombinant full length HA antigen from Influenza A virus Hong Kong/1/68 (H3N2) isolate; IM: intramuscular; IV: intravenous; mcg: microgram; MMR: measles, mumps, rubella (vaccine) N: number of animals from each study contributing to the analysis; NHP: non-human primate; $TCID_{50}$: tissue culture infective dose; vp: virus particles; wk: weeks.

Results

Rhesus Macaque Studies VH808.681 and NHP2020_3373

Most animals showed detectable hPF4 binding antibody titers at baseline. No increase in serum hPF4 binding IgG levels in animals immunized with Ad26.COV2.S compared with baseline serum samples was observed. This was independent of the dose levels or vaccine regimens (1- or 2- doses at different intervals) tested. This observation also held true for animals immunized with recombinant S protein adjuvanted with aluminum hydroxide (alum). The type of enzyme-linked immunosorbent assay (ELISA) used ie, whether binding to un-complexed hPF4 was determined or binding to hPF4/heparin complexes, did not change the outcome. Taken together, the data indicate that Ad26.COV2.S does not generally induce hPF4 cross reactive antibodies in NHP.

Mouse studies AEPs 9346-20004, -20007, -20013, 203-16008, 425-18002 and V20331/39

In mice, a statistically significant induction of hPF4 binding antibodies was observed after a single dose of Ad26.COV2.S compared to pre-immunization levels. The hPF4 binding antibody levels were further increased by a second immunization with Ad26.COV2.S. An increase in hPF4 binding antibodies above baseline was also evident in mice that received a single dose of recombinant S protein adjuvanted with alum. Significant induction of hPF4 binding antibodies was also observed upon immunization with Ad26 encoding other antigens, including SARS-COV-2 unrelated antigens. Furthermore, also animals immunized with a recombinant Influenza protein adjuvanted with Adjuplex, or with Fluzone®, a licensed influenza vaccine, showed significantly higher hPF4 antibody titers compared with baseline responses.

Collectively, the data suggest that induction of hPF4 reactive antibodies in mice immunized with Ad26.COV2.S, or other Ad26 and non-Ad26 based vaccines is likely a general phenomenon associated with vaccination.

Rabbit study TOX15252

All animals showed detectable anti-hPF4 ELISA titers at baseline. No increase in hPF4 binding IgG above baseline levels was observed in rabbits immunized with Ad26.COV2.S. This observation was independent of the route of administration (IM versus intravenous [IV]), or the dose levels of the vaccine used. The data indicate that Ad26.COV2.S did not induce hPF4 cross-reactive antibodies in rabbits at the dose levels tested.

Discussion and Conclusion

In samples derived from rabbits and NHP, no general induction of hPF4 binding antibodies above baseline responses was observed, indicating no causal link between Ad26.COV2.S administration and induction of hPF4 binding antibodies in these species.

In contrast, sample analysis from historic nonclinical immunogenicity studies in mice showed a significant induction of hPF4 binding IgG after Ad26.COV2.S immunization, compared with baseline responses. Induction of hPF4 binding antibodies in mice but not in other animal species could be explained by higher vaccine dose levels used in mice in relation to body weight compared with rabbits and NHP. However, in mice, hPF4-reactive IgG was also detected after immunization with Ad26 vectors encoding other transgenes than S protein, with recombinant S protein adjuvanted with aluminum salts, and with recombinant influenza protein vaccine in adjuvant or a marketed influenza vaccine. Since there is no common antigen present in all vaccines tested in mice, these data suggest that unspecific immune activation led to the induction of hPF4 reactive IgG.

2.1.5. Systemic Exposure to Ad26.COV2.S – TOX15252

Study Rationale and Design

A direct interaction between the vaccine and blood components (eg, due to systemic exposure either following accidental IV administration, or by draining systemically from the IM injection site) has been hypothesized as a possible contributing factor to the onset of TTS (referred to in Table 2).

The purpose of the current study was to evaluate potential effects associated with IV dosing of Ad26.COV2.S (Mod4.2.1.1/TOX15252). To this end, the effects of IV dosing of Ad26.COV2.S in female NZW rabbits were compared with IM dosing of Ad26.COV2.S, and with IV and IM dosing of Ad26.ZIKV.001 (which is based on the same Ad26 vector but encoding a different antigen), or a vehicle control. A further comparison was made with an Ad5 vector (Ad5.eGFP) and a Measles/Mumps/Rubella (MMR) vaccine, which was included as an established, widely used vaccine control. A 4-week follow-up period was included, and animals were necropsied on Day 28. The design of the study is outlined in Table 8. As indicated in Table 2, results from this study were considered of potential relevance for an initial understanding of the pathogenesis of vaccine associated TTS, as well as VTE.

Rather than injecting full human vaccine doses, which is typically done in toxicology studies when evaluating the nonclinical safety profile of IM administered vaccines, the IV vaccine dose levels used in the study were scaled to body weight in order to obtain a more realistic presentation of an accidental systemic (IV) administration. The clinical dose of Ad26.COV2.S for adults is $5x10^{10}$ viral particles (vp). This translates to $1x10^{9}$ vp/kg for a 50 kg adult, which was used as the mid-dose for IV dosing of Ad26.COV2.S (group A2). The high IV dose was set at $5x10^{9}$ vp/kg (group A3), which translates to a potential $2.5x10^{10}$ vp dose administered to a 5-kg child (still to be confirmed in clinical studies). To further assess a possible dose relationship of any findings, a low dose of $2x10^{8}$ vp/kg was included (group A1). To compare the effects of IV dosing with the intended IM route of administration, the high IV dose of $5x10^{9}$ vp/kg was also given IM (group A4). The comparator adenoviral vector-based vaccines (Ad26.ZIKV.001, Ad5.eGFP) were also administered at the high dose of $5x10^{9}$ vp/kg. The dose level for the MMR vaccine was based on a 1-year-old child (with estimated body weight of 10 kg) receiving a dose of $1x10^{3}$ $TCID_{50}$ Measles virus, $12.5x10^{3}$ $TCID_{50}$ Mumps virus and $1x10^{3}$ $TCID_{50}$ Rubella virus and scaled to body weight.

Table 8: Study Design of Investigative IV Toxicity Study With Ad26.COV2.S in Rabbits (TOX15252)

Group	Dosage groups	Dose (vp/kg)*	Concentration (vp/mL)	Route
V1	Vehicle	0	0	IV
V2	Vehicle	0	0	IM
A1	Ad26.COV2.S	$2x10^8$	$2x10^9$	IV
A2	Ad26.COV2.S	$1x10^9$	$1x10^{10}$	IV
A3	Ad26.COV2.S	$5x10^9$	$5x10^{10}$	IV
A4	Ad26.COV2.S	$5x10^9$	$5x10^{10}$	IM
B1	Ad26.ZIKV.001	$5x10^9$	$5x10^{10}$	IV
B2	Ad26.ZIKV.001	$5x10^9$	$5x10^{10}$	IM
C	Ad5.eGFP	$5x10^9$	$5x10^{10}$	IV
Group	**Dosage groups**	**Dose ($TCID_{50}$/kg)**	**Concentration ($TCID_{50}$/mL)**	**Route**
D	MMR	$1x10^2$ Measles $12.5x10^2$ Mumps $1x10^2$ Rubella	$2x10^3$ Measles $25x10^3$ Mumps $2x10^3$ Rubella	IV

*Dose volume: 0.1 mL/kg body weight, except for Group D: 0.05 mL/kg body weight
N=5/group
IM: intramuscular; IV: intravenous; MMR: Measles/Mumps/Rubella; $TCID_{50}$: 50% tissue culture infectious dose; vp: viral particle

The following parameters and endpoints were evaluated in this study: mortality, clinical observations, body weight, body temperature, clinical pathology parameters (hematology, coagulation, clinical chemistry and C-reactive protein [CRP]), immunogenicity and measurement of S protein in blood (reported in Mod4.2.1.1/TOX15252/Annex Immunogenicity Report TV-TEC-215523), measurement of adenovirus DNA copies in blood (reported in Mod4.2.1.1/TOX15252/Annex Measurement Report TV-TEC-215927), gross necropsy findings, and histopathologic examination (the latter only for animals from the IV dosed groups V1, A3, B1, C and D; on Day 28). The following tissues were examined microcopically: administration site, adrenal glands, heart, aorta, brain, kidney, stomach, duodenum, colon, liver, lung, mesenteric and mandibular lymph nodes, mesentery, and spleen.

Results - Toxicology Endpoints

All IV and IM vaccine administrations were well tolerated by the animals. There were no unscheduled deaths and no vaccine-related systemic clinical signs and effects on body temperature. Minimal to slight erythema at the administration site was noted in the groups (including vehicle groups) receiving an IM injection and was considered related to the IM injection procedure. A minimal, transient body weight loss was measured for group A4 (IM Ad26.COV2.S, $5x10^9$ vp/kg) on the day after immunization, returning to values comparable to other groups one day later.

Clinical pathology changes were consistent with an (expected) acute phase response. Mild to moderate, transient CRP increases (4.4- up to 20.3-fold) were seen 24 hours post-dosing in groups A3 (IV, Ad26.COV2.S, $5x10^9$ vp/kg), A4 (IM, Ad26.COV2.S, $5x10^9$ vp/kg) and B2 (IM, Ad26.ZIKV.001, $5x10^9$ vp/kg), with the highest increase observed for group A4. These changes occurred in parallel with mild fibrinogen increases (up to 1.9-fold) observed 24 to 72 hours post-

dosing. Acute phase protein increases were generally more pronounced after IM compared to IV administration of the same respective dose level, for both of these vaccines. Furthermore, a minimal, transient CRP increase (1.9-fold) was present 24 hours post-dosing in group D (MMR, IV). No test-article related changes in platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT), or other clinical pathology parameters were observed compared to vehicle.

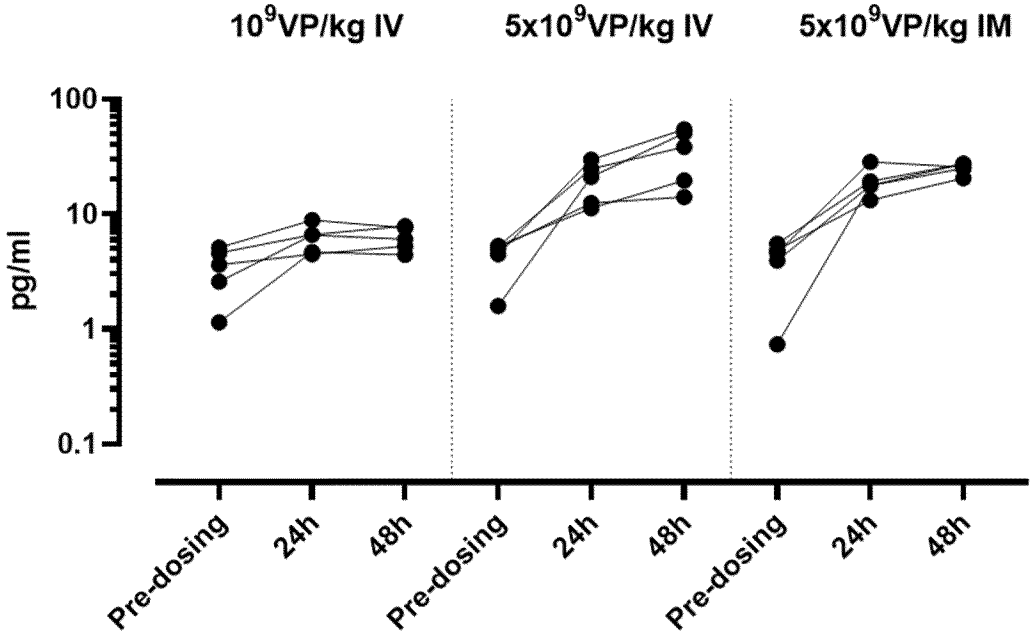
Intravenous dosing of Ad26.COV2.S (group A3, $5x10^9$ vp/kg), Ad26.ZIKV.001 (group B1, $5x10^9$ vp/kg), Ad5.eGFP (group C, $5x10^9$ vp/kg), and the MMR vaccine (group D) (see Table 8) was not associated with any gross or histopathological evidence of thrombosis, thromboembolic disease, or their sequelae, as assessed following necropsy of the animals on Day 28 post dosing. A comparable minimally or mildly increased cellularity of germinal centers in the spleen was observed in animals dosed IV with Ad26.COV2.S, Ad26.ZIKV.001 and Ad5.eGFP, but not MMR. Other than this expected and non-adverse immune response to vaccine administration observed in the spleen, there were no systemic pathological findings associated with IV administration of Ad26.COV2.S, or any of the other vaccines or vector (Ad5.eGFP). At the administration site (ear vein), minimal or mild, procedure-related perivenous hemorrhage, inflammatory infiltrates/inflammation or fibrosis were observed in all IV-dosed groups, at comparable incidences to controls. Intramuscular dosing did not result in gross pathology findings (no histopathology was done in animals dosed IM).

In summary, a single dose IV administration of Ad26.COV2.S (as well as Ad26.ZIKV.001, Ad5.eGFP or MMR vaccines) or IM dosing of Ad26.COV2.S or Ad26.ZIKV.001 to rabbits was well tolerated, not showing any adverse vaccine related effects. Dosing was not associated with any relevant changes in platelets, PT or APTT clotting times, or gross or histopathological changes related to thrombosis, thromboembolic disease, or their sequelae. For Ad26.COV2.S, the only vaccine-related findings noted following IV administration were transiently increased acute phase protein levels (CRP and fibrinogen), and minimally to mildly increased cellularity of germinal centers in the spleen. These changes were deemed a normal response to the injection of a vaccine and were not considered adverse. Overall, IV administration of either vaccine did not induce any additional (clinical pathology) changes nor changes which were more pronounced to what was observed in the corresponding IM group.

Results - Measurement of S Protein Levels and Adenovirus Vector DNA in Blood

S protein was measured in serum of the animals immunized with Ad26.COV2.S using an S-PLEX SARS-CoV-2 S protein assay at Day -8 or -7, Day 1 (24h) and Day 2 (48h). S protein was increased above the baseline at 24h and 48h post IV (18.4 pg/mL and 31.1 pg/mL, respectively) and post IM (18.6 and 25 pg/ml, respectively) dosing with $5x10^9$ vp/kg of Ad26.COV2.S, with no statistically significant difference observed between the IV and the IM route of vaccine administration (Figure 4).

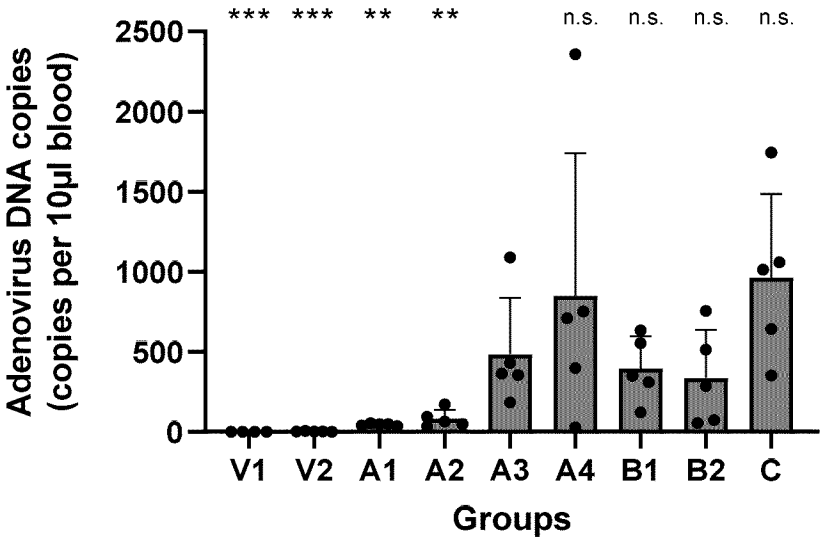
Figure 4: SARS-CoV-2 S Protein in Serum Samples Pre and Post Dosing with Ad26.COV2.S in Rabbits



Serum from New Zealand white rabbits (N=5 per group) dosed with Ad26.COV2.S, $1x10^9$ vp/kg IV, $5x10^9$ vp/kg IV, or $5x10^9$ vp/kg IM, were analyzed in a commercial S-PLEX SARS-CoV-2 Spike detection assay. Serum was sampled on day -8 (groups A3 and A4) or -7 (group A2) (pre-dosing), and day 1 (24h) and day 2 (48h) post dosing. Mod4.2.1.1/TOX15252/Annex Immunogenicity Report TV-TEC-215523
IM = intramuscular immunization; IV = intravenous immunization; vp = virus particles.

A dose level-dependent increase in Ad26 vector DNA copies in blood was observed when analyzed 30 minutes following IV administration with increasing doses of Ad26.COV2.S (ie, $2x10^8$, $1x10^9$ and $5x10^9$ vp/kg for groups A1, A2 and A3, respectively). Following IV or IM administration of Ad26.COV2.S, Ad26.ZIKV.001 or Ad5.eGFP at a dose level of $5x10^9$ vp/kg, a similar number of adenovirus vector DNA copies was detected in the blood using droplet digital PCR, irrespective of the route of administration used (Mod4.2.1.1/TOX15252/Annex Measurement Report (TV-TEC-215927)) (Figure 5). As noted in Section 2.1.3, it is not clear whether PCR detected vector DNA copies represent intact vectors / vector particles, or only fragments thereof, hence the presence of vector DNA does not preclude nor confirm the presence of intact Ad26 vectors in the blood.

Figure 5: Adenovirus DNA Copies in the Rabbit Blood upon Immunization (Expressed as Adenovirus DNA Copies per 10 µl Blood)



Each dot represents DNA copies per 10 µl blood from 1 rabbit, of which the vector DNA is measured in 3 technical replicates (N=3 per rabbit). All three adenoviral constructs used in the study (i.e., Ad26.COV2.S, Ad26.ZIKV.001, and Ad5.eGFP) contained a transgene cassette with the same Cytomegalovirus (CMV) promoter. The primer-probe set used in the droplet digital PCR assay was designed to amplify a sequence from this CMV promoter. For each group (N=5 rabbits per group, except group V1 N=4), the difference to group A3 (high dose Ad26.COV2.S IV administration) has been assessed (ANOVA on log10 [average per rabbit] with separate variance per group). Statistical significance is indicated with asterisks: *** $p<0.001$, ** $p<0.01$; n.s: not significant. Arithmetic means are shown; error bars indicate standard deviation. Mod4.2.1.1/TOX15252/Annex Measurement Report TV-TEC-215927

ANOVA: Analysis of variance; DNA: deoxyribonucleic acid, IV: intravenous; N: number of animals per group;
V1 = Vehicle (IV); V2 = Vehicle (IM); A1 = Ad26.COV2.S $2x10^8$ vp/kg (IV); A2 = Ad26.COV2.S $1x10^9$ vp/kg (IV); A3 = Ad26.COV2.S $5x10^9$ vp/kg (IV), A4 = Ad26.COV2.S $5x10^9$ vp/kg (IM); B1 = Ad26.ZIKV.001 $5x10^9$ vp/kg (IV); B2 = Ad26.ZIKV.001 $5x10^9$ vp/kg IM; C = Ad5.eGFP $5x10^9$ vp/kg (IV)

Results - Immunogenicity

A dose level-dependent induction of S protein-specific binding antibody titers was observed in serum taken on Day 28 from animals dosed IV with Ad26.COV2.S. Intramuscular dosing with Ad26.COV2.S induced comparable S protein-specific antibody titers as IV dosing, when administering the same dose level of $5x10^9$ vp/kg for each route. Low, but detectable S protein-specific binding antibody titers were observed in animals dosed with $5x10^9$ vp/kg Ad26.ZIKV.001 IV (group B1) or IM (group B2), which is likely related to antigenic homology between the SARS-CoV-2 S antigen and the Zika Envelope antigen encoded by Ad26.ZIKV.001, leading to cross-reactive binding antibodies (Lustig 2021). S protein-specific antibodies were not detected in serum of animals dosed with the commercial MMR vaccine (group D) (Mod4.2.1.1/TOX15252/Annex Immunogenicity Report (TV-TEC-215523)).

All animals dosed IV or IM with Ad26 vectors showed Ad26 neutralizing antibodies, and all animals dosed IV with Ad5.eGFP had Ad5 neutralizing antibodies at Day 28 post dosing, confirming exposure of the animals to the Ad26 or Ad5 based vaccines (Mod4.2.1.1/TOX15252/Annex Immunogenicity Report (TV-TEC-215523)).

Discussion and Conclusion

As summarized in Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan/Sec1.1.2.5, IV administration of (high doses of) adenoviral (Ad5) vectors has been associated with acute/transient thrombocytopenia in mice, rabbits, NHP and humans (Cichon 1999, Othman 2007, Raper 2003, Wolins 2003). Such studies have triggered the hypothesis that TTS may be linked to accidental IV administration of adenoviral vector-based COVID-19 vaccines. This was supported by more recent data from Nicolai et al, which showed a similar acute/transient thrombocytopenia following IV administration of ChAdOx1 nCoV-19 in mice (Nicolai 2021). In contrast, in TOX15252, IV dosing of Ad26.COV2.S (or Ad26.ZIKV.001 and Ad5.eGFP) did not cause any treatment-related effect on platelet counts.

The main difference between TOX15252 and the studies referenced above is the dose level of the adenoviral vectors that were tested. As indicated, to obtain a realistic mimic of an accidental IV dose administration, the vaccine dose levels used in TOX15252 were scaled to body weight, with the highest dose being $5x10^9$ vp/kg, which translates to a 5-fold margin versus a clinical Ad26.COV2.S dose of $5x10^{10}$ vp administered to a 50 kg subject ($1x10^9$ vp/kg). The acute thrombocytopenia (and associated platelet-adenovirus aggregate formation and platelet activation) as described by Nicolai et al, was observed following IV administration of 50 µL ChAdOx1 nCoV-19 to mice, with only a minimal effect on platelet counts noted at a lower dose of 5 µL (Nicolai 2021). Considering a ChAdOx1 nCoV-19 drug product of $1x10^{11}$ vp/mL (COVID-19 vaccine Astrazeneca, EPAR), a 50 µL dose volume would translate to a dose of $2x10^{11}$ vp/kg for a 25 g mouse[a], which is 40-fold higher than the highest Ad26.COV2.S dose tested in TOX15252. Similarly, in the IV dosing studies with Ad5 gene therapy vectors referenced above, animals received even higher dose levels in the ~$1x10^{12}$ vp/kg range (Cichon 1999, Othman 2007, Wolins 2003). This suggests that the acute effects on platelets as observed following IV dosing in these published studies should be interpreted with caution, as they likely represent high dose effects which may not be relevant for an Ad26.COV2.S vaccine dose that is accidentally administered IV.

In TOX15252 no difference in Ad26 vector DNA copies was observed in blood 30 minutes following either IV or IM administration of Ad26.COV2.S or Ad26.ZIKV.001 at a dose level of $5x10^9$ vp/kg. In addition, IV administration of Ad26.COV2.S did not yield higher levels of circulating S protein versus IM dosing. In line with this observation, IV administration of either Ad26-based vaccine did not induce any additional changes in safety parameters, nor changes which were more pronounced to what was observed in the corresponding IM group. The main difference observed between the two dosing routes was a generally more pronounced increase in acute phase proteins (CRP, fibrinogen) following IM administration of both Ad26-based vaccines. Overall, a single dose IV administration of Ad26.COV2.S to rabbits was well tolerated and did not induce any relevant changes in platelets, PT or APTT clotting times, or gross or histopathological changes related to thrombosis, thromboembolic disease, or their sequelae. Although based on a limited number of animals, these data indicate that an accidental IV injection of Ad26.COV2.S may not represent an increased risk of TTS and/or VTE.

[a] ($1x10^{11}$ vp/mL x 0.050 mL)/0.025 kg = $2x10^{11}$ vp/kg

2.2. Supplementary Studies (Not Listed in the EU-RMP)

As stated in Section 1.3, the studies described in this section were listed in the Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan, submitted in August 2021 (II/0018), as "Studies under discussion to Investigate Potential causes of TTS". These studies are not included in the EU-RMP. The Company was able to analyze results from "Re-evaluation of Analytical Data of all Vaccine Drug Product (DP) Batches", "Characterization of the HCP Content" and "Study to Explore Whether PF4 and Ad26.COV2.S Can Form a Complex". The findings from these studies are considered relevant for the overall conclusion of this NCO Addendum and are therefore summarized below in Sections 2.2.1, 2.2.2 and 2.2.3 respectively.

2.2.1. Re-Evaluation of Analytical Data of All Vaccine Drug Product Batches

Study Rationale and Design

The aim of the study was to compare release and product characterization data available from batches (that have been dispatched) with or without identified TTS cases (based on Brighton case definition, which was used as it is most conservative TTS case definition). Of note, TTS case identification is heavily dependent on the integrity regarding registration of possible symptoms by the individual health agencies. This analysis was conducted to determine whether there is any difference regarding product quality attributes (see Table 9) in batches with identified TTS cases versus batches with no identified TTS cases (see hypothesis referred to in Table 3). The total number of DS and DP batches analyzed is presented in Table 10. The study report (TV-TEC-221958) is available upon request.

Table 9: Product Quality Attributes Assessed For Batches with identified TTS Cases Versus Batches Without Identified TTS Cases

Product quality attribute	Method	DS and/or DP	Release or characterization
Virus particle titer	PCR	DS and DP	Release
Infectious unit titer	QPA	DS and DP	Release
Relative transgene expression	ELISA	DS and DP	Characterization
VP/IU	Calculated from virus particle and infectious unit titers	DS and DP	Release
HCP concentration	ELISA	DS	Release
HC-DNA concentration	PCR	DS	Release
Free hexon concentration	RP-UPLC	DS and DP	Characterization
Empty/incomplete virus particles	SV-AUC	DS and DP	Characterization
Average hydrodynamic radius	DLS	DS and DP	Characterization[a]
Polydispersity index	DLS	DS and DP	Characterization[a]
Level of particle larger than monomeric adenovirus	AF-MALS-UV	DS and DP	Characterization
Subvisible particles	MFI	DP	Characterization
Polysorbate-80 concentration	colorimetric assay	DS and DP	Release
pH	pH measurement	DS and DP	Release
Extractable volume		DP	Release

[a] This product quality attribute is part of the DP release package for European Medicines Agency and Rest of the World.
AF4-MALS-UV: asymmetric flow field-flow fractionation with multi angle light scattering and ultraviolet; DLS: Dynamic light scattering; DP: drug product; DS: drug substance; ELISA: enzyme linked immunosorbent assay; HC-DNA: host cell DNA; MFI: Micro-flow imaging; PCR: polymerase chain reaction; QPA: quantitative potency assay; RP-UPLC: Reversed phase ultra performance liquid chromatography; SV-AUC: Sedimentation velocity analytical ultracentrifuguation; TTS: thrombocytopenia with thrombosis syndrome; VP/IU: viraus particle/infectious units;

Table 10: Numbers of DS and DP Batches Analyzed

	Total number of DS batches	Total number of DP batches	Number of DS batches with identified TTS cases	Number of DP batches with identified TTS cases
Release data available	66	477	15	33
Characterization data available	28	19	15	6

DS: drug substance; DP: drug product; TTS: thrombocytopenia with thrombosis syndrome

The methods and specifications for DS batches are described in Mod3.2.S.4.1 Specification and those for the DP batches Mod3.2.P.5.1 Specifications. For the product characterization data refer to Mod3.2.S.3.1 Elucidation of Structure, Mod3.2.S.3.2 Impurities and Mod3.2.P.5.5 Characterization of Impurities.

Results

All DS and DP release and characterization test results were consistent between batches with and without identified TTS cases (up to November 15th 2021) and did not exceed release criteria, where applicable.

Discussion and Conclusion

No discrepancy regarding release and characterization data was found for product quality attributes assessed (see Table 9) of batches that have been associated with reported case(s) of TTS (Brighton definition) to batches currently on the market that have no reported TTS cases.

2.2.2. Characterization of Host Cell Protein Content

Study Rationale and Design

As described in Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan, Greinacher et al and Krutzke et al analyzed the composition of different lots of ChAdOx1 nCoV-19 by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS) (Quadrupole-Orbitrap), and observed that approximately 2/3 (45-71%) of the protein fraction in the vaccine lots consisted of HCP impurities (with more than 1,200 individual HCP entities) (Greinacher 2021b, Krutzke 2021). The total amount of human cell derived HCP and viral proteins in the ChAdOx1 nCoV-19 vaccine ranged from 32-40 µg per dose between vaccine lots, overall resulting in an HCP level of approximately 20 µg per dose of the ChAdOx1 nCoV-19 vaccine, and it was postulated that the residual HCP could potentially be associated with TTS (see hypothesis referred to Table 3). Based on these findings, the Company performed a study to further characterize the HCP content and provide an overall safety assessment of the residual HCP identified in Ad26.COV2.S when produced from PER.C6 TetR cells. The study report (TV-TEC-221745) is available upon request.

Results

The ELISA assay used to measure HCP levels in Ad26.COV2.S DS has a lower limit of quantification of 20 ng/mL. Most of the Ad26.COV2.S DS batches analyzed had HCP levels below this limit of quantification, with only few batches showing quantifiable levels up to 119 ng/mL. Irrespective of the HCP concentration there was no difference in batches associated with TTS versus batches not associated with TTS. Based on these results, the estimated HCP level per vaccine dose, based on the DP being manufactured to target a titer of 1.2×10^{11} vp/mL (i.e., a dilution dependent on the vp titer, where the DS target VP titer is 4.5×10^{11} VP/mL) with a vaccine dose volume of 0.5 mL, ranged from <2.0 to 14.3 ng/dose.

To have a more comprehensive understanding of the HCP profile, a subset of Ad26.COV2.S DS batches, irrespective of HCP concentration, was further characterized qualitatively by SDS-PAGE, and semi-quantitatively by Reversed phase ultra-performance liquid chromatography (RP-UPLC) and mass spectrometry protein profiling (MS-PP), using electrospray ionization-quadrupole-time of flight-mass spectrometry (see Mod3.2.S.3.2 Impurities for more details regarding the methods and results). These additional analyses demonstrated a consistent HCP profile (ie, same set of HCP with similar ratio's) across Ad26.COV2.S DS batches.

Additional exploratory profiling of the HCP proteome from one DS batch was conducted using Q-Orbitrap MS. Combining the results from the MS-PP and the Q-Orbitrap MS profiling, a total of 106 HCP were identified. These HCP were categorized into the following classes: Ribosomal

proteins, Histone proteins, Cell cycle regulation proteins, Heat shock proteins, Proteins potentially associated with thrombosis / platelet activation (in view of a potential association with TTS), Proteins involved in metabolism, and Structural proteins. The HCP category with the highest HCP level per vaccine dose were ribosomal proteins with a calculated highest total level of 8.1 ng/dose (extrapolated from the DS batch representing the highest total HCP level of 14.3 ng/dose). The remaining categories ranged from 3.3 ng/dose (histone proteins) to <0.1 ng/dose (proteins involved in metabolism) when extrapolated from the DS batch representing the highest total HCP level (14.3 ng/dose). As mentioned in Section 2.1.1 residual TetR protein was detected at a calculated maximum level of 0.4 ng/dose.

These HCP were assessed from a safety perspective, taking into account amongst others the (total) level of HCP, available generic HCP default limits used in the industry (Sharnez 2012; Wang 2018), potential (intrinsic) biological activity of the HCP (e.g., is it an enzyme, hormone, or cytokine), as well as endogenous (circulating) levels (if known). Overall, the currently available Ad26.COV2.S vaccine batches conform to generic HCP default limits used in the industry and do not contain HCP that raise a significant safety concern.

Discussion and Conclusion

Based on publications from Greinacher et al. and Krutzke et al., residual HCP have been suggested to be potentially linked with TTS (Greinacher 2021d, Krutzke 2021). For Ad26.COV.S, based on the release data across the available DS batches manufactured up to November 15th 2021, a maximum HCP level of approximately 14.3 ng/dose has been determined. These levels are significantly below residual HCP reported for other vaccines produced on human cell lines such as Havrix or Twinrix which are both produced on the human cell line MRC-5 and which contain up to 2.5-5.0 µg/dose residual MRC-5 proteins. In addition, the HCP levels are well below the 10 ppm limit (equivalent to 5 µg per 0.5 mL vaccine dose) that is proposed to classify biologics with the lowest (HCP-related) risk (Wang 2018). Although a potential risk of (auto)immune reactions associated with exposure to (human cell-derived) HCP is very difficult to predict from nonclinical data, Ad26.COV2.S does not contain HCP that raise a significant safety concern. Together these data suggest that trace levels of HCP are not likely a contributing factor in the pathogenesis of TTS.

2.2.3. Study to Explore Whether PF4 and Ad26.COV2.S Can Form a Complex

Study Rationale and Design

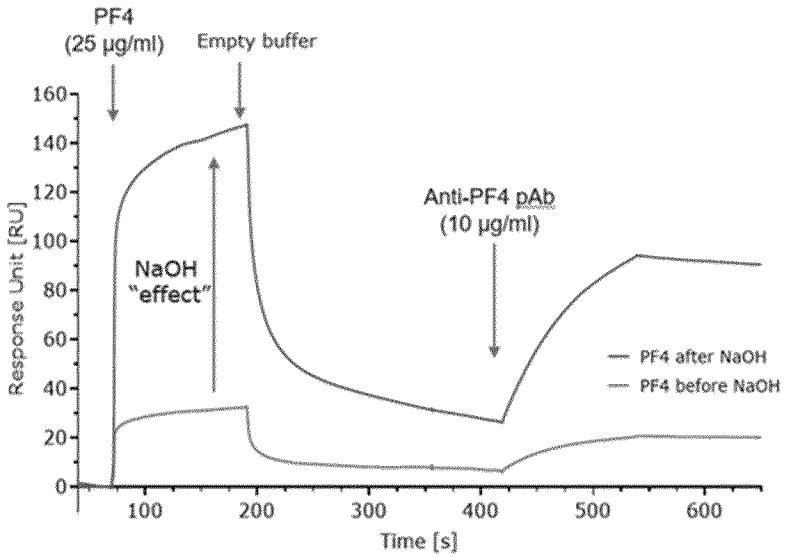
Heparin-induced thrombocytopenia is mediated by antibodies against complexes of PF4 and heparin. However, the induction of anti-PF4 antibodies in TTS is independent of heparin use (Li 2021). It has been hypothesized that Ad26.COV2.S or another component in the vaccine can mimic the role of heparin in binding to PF4 and as such trigger the induction of anti-PF4 antibodies (see hypothesis referred to in Table 3). It has to be noted that in silico and in vitro studies do not exactly represent what is happening in the human body and that these studies likely show a worst case scenario unless other factors in blood would promote binding. Baker et al used computational simulations to demonstrate an electrostatic interaction between ChAdOx1 and PF4 (Baker 2021). The electronegative surface potential of ChAdOx1 was compared to that of wt Ad26 and it was

observed that the electronegative surface potential of wt species D adenovirus type 26 (HAdV-D26) is less strong than ChAdOx1. To investigate the interaction of PF4 (with a strong electropositive surface potential) with adenovectors, a Biacore T200 instrument was used, which makes use of surface plasmon resonance technology. PF4 binding was observed to ChAdOx1 nCoV-19 and to Cesium Chloride purified adenoviruses in the ChAdOx1 vaccine, Ad5 and wt Ad26 (Baker 2021). To assess the interaction between PF4 and Ad26.COV2.S, the Company used the exact same experimental set up as described by Baker et al. The Company initiated a collaboration with Astra Zeneca and a detailed protocol was discussed and provided. Adenovectors were immobilized on a C1 chip by amine coupling using the same pre-dilution in acetate buffer (pH4.5). To be able to measure several test conditions (different concentrations or compounds) the chip containing amine-coupled Ad26.COV2.S needs to be regenerated after each run in order to remove the compound that was tested for its ability to bind Ad26.COV2.S (in this case PF4). Baker et al used 25mM NaOH treatment for regeneration. In addition to performing the experiments with the same protocol using 25mM NaOH for regeneration, the Company also assessed binding of PF4 to Ad26.COV2.S on a new chip prior to first regeneration with NaOH treatment.

Results

Preliminary findings showed that only low level binding of PF4 to Ad26.COV2.S was detected before first regeneration of the chip. However, binding of PF4 to Ad26.COV2.S was strongly increased (in same range as reported for wt Ad26 and ChAdOx1 by Baker 2021) after 25mM NaOH treatment (Figure 6).

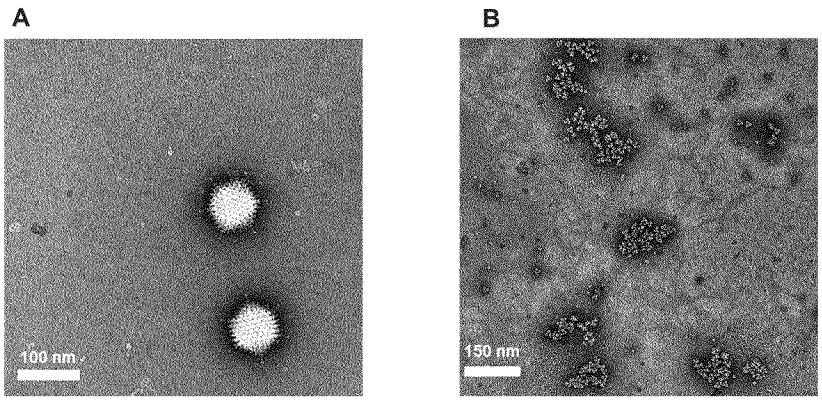
Figure 6: Effect of 25mM NaOH Regeneration on Binding of PF4 to Immobilized Ad26.COV2.S



PF4 (25 µg/mL) was injected to assess the binding to immobilized Ad26.COV2.S before 25mM NaOH regeneration (green curve) followed by injection of anti-PF4 IgG to confirm binding of PF4. After regeneration with 25mM NaOH PF4 was injected again (red curve), followed by anti-PF4 IgG. Electronic lab notebook E047091.
IgG: immunoglobulin gamma; PF4: platelet factor 4; RU: Relative Unit

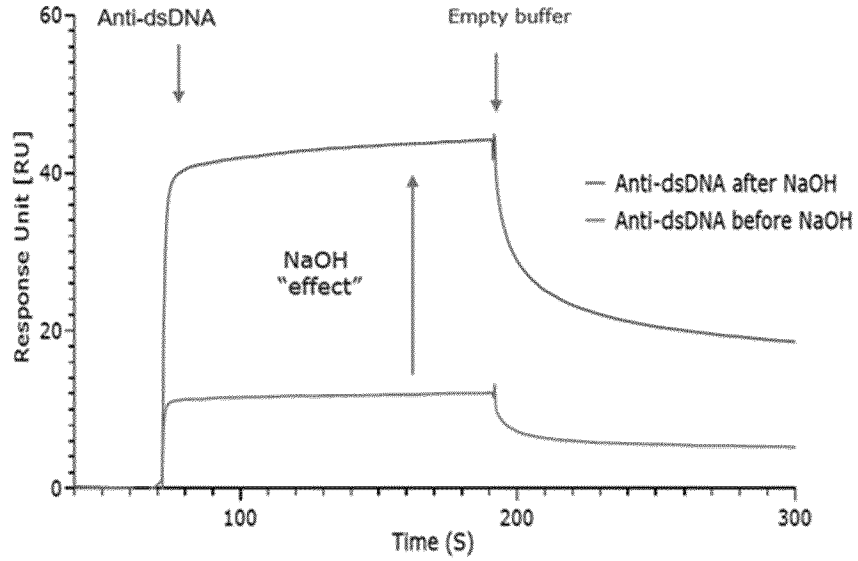
Further investigations into the effect of NaOH on Ad26.COV2.S by electron microscopy revealed that exposure to 25mM NaOH for 30 seconds (using the same buffer and duration as in the regeneration protocol) severely damaged (i.e., disintegrated) the Ad26 viral particles (Figure 7), leading to a drastic loss in potency (data not shown). This also complements the results of a forced degradation by means of pH (low and high) study (unpublished) where it was observed that a low pH significantly affected viral particle integrity, and thus also potency. To examine whether such a disruption of Ad26 viral particles can expose negatively charged genomic adenoviral DNA that can then bind to PF4, Biacore experiments were performed to test binding of an anti-double stranded DNA anti-(dsDNA) antibody to Ad26.COV2.S before and after regeneration with 25mM NaOH. These data indicated that indeed the binding of anti-dsDNA antibodies was also low before, but increased ~5-10-fold after regeneration with 25mM NaOH (similar as observed for PF4 binding), suggesting that PF4 is binding to genomic adenovirus DNA or newly exposed sites on viral particles after 25mM NaOH treatment (Figure 8). In addition, these data suggest that PF4 binds to DNA, which might also occur naturally in plasma as it contains cell free (genomic) DNA (e.g., Pös 2018).

Figure 7: Electron Microscopy of Ad26.COV2.S Before and After NaOH Treatment



Ad26.COV2.S vaccine was incubated on the grid for 30 seconds with either PBS (panel A, negative control) or 25 mM NaOH (panel B). After incubation grids were washed with PBS and stained with phosphotungstic acid pH7.4 followed by TEM analyses. Images are representative for a total of 15 per treatment and 2 independent experiments. Bars indicate size in nm.
PBS: phosphate buffered saline; TEM: transmission electron microscopy. Electronic lab notebook E051361.

Figure 8: Binding of Anti-dsDNA Monoclonal Antibody (mAb) to Immobilized Ad26.COV2.S Before and after 25mM NaOH Treatment



Anti-dsDNA mAb (10 µg/mL) was injected to assess the binding to Ad26.COV2.S before NaOH regeneration (green curve). After regeneration with NaOH the mAb anti-dsDNA was injected again (purple curve). Electronic lab notebook E040701.
dsDNA: double stranded deoxyribonucleic acid; mAb: monoclonal antibody; RU: relative unit

Discussion and Conclusion

PF4 binding to ChAdOx1 nCoV-19 and Ad26.COV2.S was also examined by Michalik et al. using dynamic light scattering (Michalik 2022). These data indicate that PF4 induced clustering of the ChAdOx1 nCoV-19 vaccine, but only marginally increased particle size when incubated with purified virions from ChAdOx1 nCoV-19 or Ad26.COV2.S. The Company's preliminary data demonstrate that the induced strong binding of PF4 to Ad26.COV2.S in Baker 2021 is an experimental artefact. Based on the above, we do not have conclusive evidence for binding of Ad26.COV2.S to PF4 in vitro. Therefore, PF4 binding to Ad26 DNA is unlikely to increase the risk of TTS.

Additional studies are needed to examine the interaction between Ad26.COV2.S and PF4 using different assays and alternative protocols.

3. PHARMACOKINETICS

The expression/distribution of the S protein after Ad26.COV2.S immunization of rabbits (TOX15155) is discussed in Section 2.1.3.

4. TOXICOLOGY

The nonclinical safety profile of IV dosing of Ad26.COV2.S in rabbits (TOX15252) is discussed in Section 2.1.5.

5. INTEGRATED OVERVIEW AND CONCLUSIONS

5.1. Overall Conclusion From Nonclinical Studies

This NCO Addendum provides an overview of the current status and results of the nonclinical studies that were conducted to characterize the potential mechanisms underlying vaccine associated TTS, thrombocytopenia including ITP and/or VTE. The studies were designed to evaluate several hypotheses that have been proposed as possible contributing factors to TTS pathogenesis (as summarized in Mod2.4/ Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan, submitted in August 2021 (II/0018)).

Although the available nonclinical studies provided relevant data that can be used to put published data into perspective and/or possibly deprioritize some aspects of the proposed hypotheses or underlying mechanisms, the data do not allow to conclude on a final pathogenesis mechanism of vaccine-induced TTS. It is noted in this context that likely not all human (host) factors may be replicated in the nonclinical studies (eg, rare host factors). The overall conclusions and perspectives from the various nonclinical studies are summarized in Table 11.

Table 11: Outcome and Conclusions Derived From Nonclinical Studies Conducted to Address TTS and/or VTE

Research Question/Potential Mechanism Addressed	Contributing Studies (Section)	Outcome / Overall conclusions
Potential role of autoimmune responses induced by proteins encoded by the Ad26.COV2.S vector	Mod4.2.1.1/TV-TEC-207316 (Section 2.1.1)	Analysis of sequence homologies of the Ad26.COV2.S antigens against the human proteome revealed only limited linear homologies. Linear epitope homology is unlikely to be the root cause to TTS. However, conformational homology cannot be excluded at this point. The Company will closely monitor work ongoing in the field by organizations that have access to TTS case samples to further understand specificity of antibodies identified in TTS cases. Any important new findings will be reported.
Potential role of soluble S protein (fragments) / alternative splicing variants	Mod4.2.1.1/TV-TEC-207437 (Section 2.1.2), Mod4.2.1.1/TOX15155 (Section 2.1.3), Mod4.2.1.1/TOX15252 (Section 2.1.5)	No or very low frequency of aberrant splicing events were found that would affect the spike transgene encoded by Ad26.COV2.S, making it unlikely to contribute to TTS. Low levels of S protein were detected in the circulation from rabbits following (IM or IV) administration of Ad26.COV2.S. It is yet unknown whether soluble S protein can be found in the circulation from human subjects following vaccination with Ad26.COV2.S. Nevertheless, systemic (soluble) S protein has been shown in the rabbit studies to be bioavailable for putative interactions with blood components after IM injection, and thus may be a contributing factor in a multifactorial scenario of TTS induction.
Assess potential animal models of hPF4 binding antibody induction	Mod4.2.1.1/ TV-TEC-215524 (Section 2.1.4)	Vaccination with Ad26.COV2.S did not induce hPF4 binding antibodies in NHP and rabbits. In mice, hPF4 binding antibody responses were induced, however responses were also seen with non-adeno based vaccines not including/encoding the SARS-CoV-2 S antigen (eg, Fluzone), hence indicative of an unspecific immune activation.

Research Question/Potential Mechanism Addressed	Contributing Studies (Section)	Outcome / Overall conclusions
Potential role of systemic exposure to Ad26.COV2.S (e.g., following accidental IV administration)	Mod4.2.1.1/TOX15252 (Section 2.1.5)	IV administration of Ad26.COV2.S did not induce any additional changes in safety parameters, nor changes which were more pronounced to what was observed in the corresponding IM dosing group (including S protein and Ad26 vector distribution in the blood). Although based on a limited number of animals, these data indicate that an accidental IV injection of Ad26.COV2.S may not represent an increased risk of TTS, and/or VTE.
Potential role of CMC/product quality attributes (including potential role of HCP)	Sections 2.2.1 and 2.2.2	There is no discrepancy regarding release and characterization data for product quality attributes of batches with or without reported TTS cases. Hence, this analysis did not identify a factor likely to contribute to TTS. Ad26.COV2.S contains only trace amount of HCP, well below generic HCP default limits used in the industry, and significantly lower than residual HCP levels that have been reported for other widely used vaccines produced on human cells (e.g. commercially available Havrix or Twinrix), making it unlikely a contributing factor to the pathogenesis of TTS.
Potential role of interaction between Ad26.COV2.S and PF4	Section 2.2.3	Study is ongoing; Currently available results using surface plasmon resonance show no conclusive evidence for strong binding of Ad26.COV2.S to PF4 in vitro. These findings are in contrast with the findings of Baker et. al. (Baker 2021), but in line with findings of Michalik et al. (Michalik 2022) using dynamic light scattering, showing no complex formation of PF4 with Ad26.COV2.S.

ADAMTS-13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; CMC: chemistry manufacturing control; HCP: host cell proteins; (h)PF4: (human) platelet factor 4 (CXCL4); IM: intramuscular ITP: immune thrombocytopenia; IV: intravenous;); PV: pharmacovigilance; RMP: risk management plan; RNA: ribonucleic acid; S: Spike protein; TTS: thrombosis with thrombocytopenia syndrome; VTE: venous thromboembolism; vWF: Von Willebrand factor

5.2. Overall Conclusion From Nonclinical and Clinical Studies (Benefits and Risks Conclusions)

This Section is a copy of Section 6 of the Clinical Overview Addendum 'Update to the European Union Risk Management Plan Regarding Thrombosis with Thrombocytopenia Syndrome', which will be submitted to EMA in a separate package.

TTS, also called VITT, has been reported following vaccination with Ad26.COV2.S. The pathogenesis of vaccine-associated TTS is not fully understood and the Clinical Overview Addendum describes the results of studies using clinical samples, to address TTS that are included in the EU-RMP (with a due date in the EU-RMP of 31 March 2022). More specifically, it describes the analyses of clinical study samples by the Company to evaluate levels of anti-PF4 antibodies after vaccination with Ad26.COV2.S or with non-COVID-19 Ad26-based Company vaccines. In general, the very low incidence of TTS may imply that the clinical outcome is related to a combination of Ad26.COV2.S and other factors, potentially including a predisposition of the host. To understand the pathogenesis of vaccine-associated TTS it is crucial to have TTS samples, however due to the very rare incidence of TTS the access to samples is limited.

Initially Proposed Hypotheses for Pathogenesis of Vaccine-associated TTS

Early after the first reports of TTS cases following administration of ChAdOx1 nCoV-19 or Ad26.COV2.S vaccine, several hypotheses to explain the mechanism of TTS after vaccination have been formulated and described in scientific publications. The Company described several of these in Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan submitted to the EMA with the EU-RMP version 2.2 in August 2021.

Vaccine-associated TTS resembles HIT with regards to the rare clinical combination of thrombosis with thrombocytopenia, though the incidence of TTS after vaccination is lower than the incidence of HIT after heparin treatment and the sites of thrombosis are different. Heparin is negatively charged and can bind to positively-charged PF4, leading to the formation of heparin/PF4 complexes that trigger a conformational change in PF4 and the creation of a neo epitope. This HIT epitope is the main target for pathogenic HIT antibodies. These antibodies, elicited by the HIT epitope, can induce Fc receptor mediated activation of platelets in the presence of heparin/PF4 complexes leading to a hypercoagulation state (Greinacher 2021c; Schultz 2021). In addition, so called autoimmune or spontaneous HIT has been described in rare HIT cases that were not associated with heparin treatment (Greinacher 2017). Platelet activating anti-PF4 antibodies have also been detected in vaccine associated TTS cases, which has led to the hypothesis that anti-PF4 antibodies play a central role in the pathogenesis of vaccine-associated TTS (Greinacher 2021c; Schultz 2021).

The similarities led to a strong focus in the field on the current main hypothesis where a negatively charged vaccine component would take the role that heparin has in HIT. This vaccine component, for instance the expressed S protein, adenoviral vector, or excipients, would directly bind to PF4 and thereby induce the conformational change exposing the neo-epitope. This would result in the induction of anti-PF4 antibodies against this "HIT epitope," either by de-novo activation of PF4-HIT-epitope-specific B cells, or activation of pre-existing PF4 HIT epitope specific B cells, finally

leading to the production of anti-PF4 antibodies, the activation of platelets, and the subsequent cascade of events leading to TTS (Greinacher 2021b).

Additional research has identified other factors potentially contributing to the pathogenesis of vaccine-associated TTS. These factors could play a role in the hypothesis described above or point towards a separate mechanism (Mod2.4/Nonclinical Overview Addendum - Update EU-RMP Regarding TTS including Updated PV Plan).

For instance, it was hypothesized that impurities in adenoviral vector-based vaccines (eg, HCP, host-cell DNA, formulation components) could interact with platelets and/or PF4 (Greinacher 2021b), or play a potential role in the modulation of innate and adaptive immune responses (Krutzke 2021). Soluble S protein generated by alternative splicing was hypothesized to be associated with vaccine-associated TTS by potentially damaging endothelial cells or triggering inflammation and platelet activation (Kowarz 2022). Another hypothesis postulated that systemic exposure to the vaccine (eg, by inadvertent IV administration of the vaccine) could lead to the formation of platelet/adenoviral vector complexes inducing platelet activation (Nicolai 2021).

Hypotheses Considered Unlikely Based on Currently Available Results

Over the last months, experiments have been performed to investigate some of those reported findings and hypotheses. Based on the experiments conducted by the Company (described in this NCO Addendum) it is considered that the following observations are no longer being pursued:

- ***Direct binding of the Ad26 vector to PF4***: Results of the investigations performed by the Company do not confirm this binding. As mentioned above, the main hypothesis in the TTS field focuses on the direct binding of negatively-charged Adenovectors to positively-charged PF4, leading to complex formation and subsequent triggering of anti-PF4 antibodies that can elicit the coagulation cascade via platelet aggregation and activation. Recently, Baker et al (Baker 2021) observed that the electronegative surface potential of wt species D Adenovirus type 26 (HAdV-D26) is less strong than that of ChAdOx1. They demonstrated binding of the ChAdOx1 nCoV-19 vaccine and of wt Ad26 virus to PF4. However, using the same experimental set up under multiple conditions, the Company could not confirm strong binding of Ad26.COV2.S to PF4. On the contrary, data generated by the Company suggest that the strong binding observed by Baker et al is induced by the applied experimental conditions (25 mM NaOH) that lead to degradation of the Adenovirus particles resulting in exposure of eg, the DNA to which PF4 could bind (Section 2.2.3). Furthermore, these Company data are in line with findings of Michalik et al (Michalik 2022) using dynamic light scattering, showing no complex formation of PF4 with Ad26.COV2.S.
- ***Product quality attributes and impurities including HCP:*** The results of investigations by the Company in this area support that product quality attributes and/or impurities are not considered a contributing factor to TTS. For Ad26.COV2.S, a maximum HCP level of approximately 14 ng/dose has been determined across the various Ad26.COV2.S batches. These trace amounts are well below generic HCP default limits used in the industry (Sharnez 2012; Wang 2018), and significantly lower than residual HCP levels that have been reported for other widely used vaccines produced on human cells (eg, Havrix or Twinrix which contain up to 2.5 to 5 µg/dose residual MRC-5 proteins). Other product quality attributes were also assessed, test results were within specifications and consistent between

batches that have been associated with TTS cases and batches that were not associated with TTS cases. Based on these results, vaccine product quality attributes, impurities and excipients are not considered a contributing factor to TTS (Section 2.2.1 and Section 2.2.2).

- ***Splice variants of S protein:*** By design, the S protein transgene in Ad26.COV2.S does not contain major splice donor-acceptor sites. RNA sequencing of in vitro transduced cells with Ad26.COV2.S identified no or very low frequencies of aberrant splicing events that would affect the S protein, making it unlikely that splice variants are a contributing factor in TTS (Section 2.1.2).
- ***Intravenous administration:*** Results of the investigations by the Company indicate that accidental IV injection is unlikely to contribute to TTS. An IV administration study with Ad26.COV2.S was conducted in rabbits. In this study, IV administration of Ad26.COV2.S did not induce any additional changes in safety parameters, nor changes which were more pronounced to what was observed in the corresponding intramuscular dose group (based on eg, clinical chemistry, coagulation and hematology parameters including platelet counts, as well as S protein and Ad26 vector distribution in the blood). Dosing was not associated with any relevant changes in platelets, prothrombin time, or activated partial thromboplastin time clotting times, or gross or histopathological changes related to thrombosis, thromboembolic disease, or their sequelae. Although based on a limited number of animals, these data indicate that an accidental IV injection of Ad26.COV2.S is unlikely to represent an increased risk of TTS (Section 2.1.5).
- ***TTS is speculated to be the most severe clinical presentation of a spectrum of milder to severe TE events with a similar underlying vaccine-associated pathogenic mechanism:*** Results obtained by the Company do not support this hypothesis. A current hallmark of TTS appears to be the presence of antibody responses against PF4. To address the possibility that TTS is the most severe manifestation of a continuum of TE events with the same underlying mechanism, the Company explored whether there is general induction of anti-PF4 antibodies after Ad26.COV2.S vaccination in humans. Study 1 (Clinical Overview Addendum 'Update to the European Union Risk Management Plan Regarding Thrombosis with Thrombocytopenia Syndrome', Section 5.1.1, which will be submitted to EMA separately), showed no evidence of increased rates of anti-PF4 antibody positivity associated with Ad26.COV2.S vaccination of humans for TE cases versus non-TE controls. There was a nonsignificant, numerically higher prevalence of anti-PF4 antibodies pre-vaccination observed in the Ad26.COV2.S group, which was maintained post-vaccination. Overall, the prevalence of TEs potentially attributable to Ad26.COV2.S vaccination remains very low. Furthermore, Study 3 (Clinical Overview Addendum 'Update to the European Union Risk Management Plan Regarding Thrombosis with Thrombocytopenia Syndrome', Section 5.1.3) indicated that Ad26.COV2.S does not induce anti-PF4 antibodies after vaccination in human participants across different ages, gender and regions (n=913) who do not develop TE events. These data suggest that the underlying mechanism leading to TE events and TTS are different ie, there is no evidence suggesting a general mechanism for coagulation disorders caused by Ad26.COV2.S leading to a spectrum of different TE events with TTS being the most severe manifestation.

Development of, and Supportive Work on New Hypotheses for the Pathogenesis of Vaccine-associated TTS

The data generated by the Company as described above, and new insights published by others, indicate that alternative hypotheses for the pathogenesis of vaccine-associated TTS need to be developed. These hypotheses assume involvement of a HIT-like coagulation pathway stimulated by other triggering events or could be independent of such a pathway.

For instance, it has been shown recently that the amino acid residues in the epitope of the anti-PF4 antibodies present in sera from patients who developed TTS after vaccination with ChAdOx1 nCoV-19 partially overlap with amino acid residues involved in the heparin binding site on PF4 (Huynh 2021). These so-called "VITT" antibodies can bind to free PF4 and are not dependent on the neo-epitope that is exposed as a consequence of the conformational change after heparin binding to PF4. This indicates that a polyanionic component that takes over the role of heparin in HIT is not required to elicit anti-PF4 antibodies. Furthermore, it was shown that VITT antibodies are monoclonal or at most oligoclonal, while anti-PF4 antibodies in HIT patients are polyclonal (Singh 2021) and that these VITT antibodies trigger platelet activation in the presence of PF4 (Kanack 2022), independent of the presence of heparin. It has been proposed that such a mechanism of anion-independent PF4 complexes can be present in spontaneous HIT and is also mediated by PF4 antibodies binding to the heparin binding site (Greinacher 2017).

It remains unclear how those anti-PF4 antibodies directed against the VITT epitope that overlaps with the heparin binding site on PF4 are triggered and further investigations are needed. In this context, molecular or conformational mimicry of the Spike protein or any component of the Ad26.COV2.S or an unspecific activation in the context of a strong pro-inflammatory immune response post-vaccination (induced by Spike protein and/or the Adenovirus) needs to be considered.

Other reports (Grobbelaar 2021; Letarov 2020) suggest a direct impact of the Spike protein on the pathogenesis of TE events in COVID-19. This has led to the hypothesis that the Spike protein is a potential risk factor for TE events in COVID-19 patients. Accordingly, the Spike protein alone has been shown to initiate an inflammatory phenotype in endothelial cells, to induce leukocyte adhesion, and to promote pro-inflammatory cytokine secretion after intravenous Spike protein administration in mice (Robles 2022).

It has also been suggested that SARS-CoV-2 infection can trigger TTS-like TE events at a higher rate than reported for the Adenovirus-based COVID-19 vaccines (Hippisley-Cox 2021). Additionally, the incidence of suspected "HIT" cases with high anti-PF4 antibody positivity in severe COVID-19 cases appeared to be 10-fold greater than in non-COVID cases in the ICU (8% versus 0.89%) (Daviet 2020). This suggests a similarity between vaccine-associated TTS and COVID-19 pathology. In severe cases requiring ICU care there is an increased frequency of cerebral venous sinus thrombosis (Bikdeli 2021; Zicarelli 2021).

While the Spike protein is also encoded by mRNA vaccines the duration of Spike expression could be shorter. In addition, the magnitude and exact location of Spike protein expression as well as its

conformation or glycosylation could be different. Therefore, the Spike protein could contribute to the pathogenesis of TTS, in combination with other factors.

To further develop and understand the value of alternative hypotheses, the Company is considering the following investigations:

- Explore cross-reactivity of anti-PF4 antibodies obtained from vaccine-associated TTS cases with components in Ad26.COV2.S or with the Spike protein. Access to TTS patient material will be a critical step in these follow-up investigations and collaborations with external partners are being discussed.
- Explore the epitope specificity of anti-PF4 antibodies in severe COVID-19 patients who developed cerebral venous sinus thrombosis or other severe TE events to identify if there are similarities with anti-PF4 antibodies in vaccine-associated TTS cases. Sera from these patients will also be tested in the heparin-independent platelet activation assay that can distinguish between vaccine-associated TTS antibodies and spontaneous HIT antibodies. For this, the Company is examining the feasibility of collaborations with external partners.
- Analyze inflammatory responses induced by Ad26 vaccination in humans including different doses and ideally compare with inflammatory responses induced by COVID-19 vaccines that are based on other platforms (mRNA, protein) and currently associated with much lower or no TTS risk. As the S protein was shown to be present in blood and can influence inflammation and/or coagulation, Ad26.COV2.S will be compared with the Company's Ad26 platform-based vaccines that are expressing non-SARS-CoV-2 antigens (Section 2.1.5). Gene expression profiles, and levels of serum inflammatory cytokines and coagulation factors, at early and later timepoints after vaccination with Ad26.COV2.S will be investigated to capture kinetics of expression of these factors. The Company is also planning to conduct a similar transcriptomics analysis in whole blood obtained from Ad26.COV2.S vaccinated nonhuman primate in ongoing study TOX15258 (Table 2).

Further insights into the pathogenesis of vaccine-associated TTS may be obtained from the aforementioned planned analysis of the potential cross-reactivity of the VITT antibodies with a component in the Ad26.COV2.S vaccine or S protein, and from the analysis of the transcriptome as well as of serum inflammatory cytokine and coagulation factors. Together with the results reported here, these additional data will act as a guide in defining next steps to elucidate underlying mechanisms which cause vaccine-associated TTS. Prospective evaluation of coagulation parameters in clinical studies will also be performed. The Company will continue to monitor the developments in the field and interact with experts in the field, as well as groups with access to material from TTS patients. Pharmacovigilance activities and monitoring of TTS post primary and booster vaccination (in clinical studies/post-marketing studies) as described in the Risk Management Plan will continue.

Data from clinical trials have shown that Ad26 vector vaccines have the capacity to elicit robust and durable humoral responses (with a unique combination of antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, antibody-dependent complement deposition, plus neutralizing antibodies) and cellular immune responses (strong CD8 and CD4 T cell responses with a Th1 signature). This expedited new vaccine development allows for rapid

at-scale manufacturing thanks to the platform approach and cumulative knowledge. Several hundred thousand people have received the Company Ad26-vector-based Ebola vaccine, and millions of people have been vaccinated with the Company Ad26-vector-based COVID-19 vaccine. Given the broad immunoprofile, Ad26 vaccines have the potential to be highly effective prophylactic vaccines with significant favorable impact on health globally.

Taking the new information presented in this NCO Addendum and in the Clinical Overview Addendum (which will be submitted to EMA in a separate package) into account, the overall benefit-risk assessment remains favorable for Ad26.COV2.S when used as recommended in the currently approved indication(s). Based on the data presented in this document no updates to the product information are considered warranted. The EU-RMP has been updated to reflect the completion of the studies presented in this document.

6. LIST OF LITERATURE CITATIONS

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2.4. Nonclinical Overview

Drug Substance	AZD1222
ANGEL ID	Doc ID-004365565
Date	21 December 2020

2.4 Nonclinical Overview AZD1222

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1 OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

AstraZeneca (the Sponsor) is developing AZD1222 for the prevention of coronavirus disease-2019 (COVID-19). AZD1222 is a recombinant chimpanzee adenovirus (ChAd) expressing the severe respiratory syndrome-coronavirus-2 (SARS CoV-2) spike (S) surface glycoprotein. Development of AZD1222, previously referred to as ChAdOx1 nCoV-19, was initiated by the University of Oxford with subsequent transfer of development activities to the Sponsor.

AZD1222 is a recombinant replication-defective ChAd vector expressing the SARS CoV-2 S surface glycoprotein, driven by the human cytomegalovirus major immediate early promoter that includes intron A with a human tissue plasminogen activator (tPA) leader sequence at the N terminus. Spike (S) is a type I, trimeric, transmembrane protein located at the surface of the viral envelope, giving rise to spike shaped protrusions from the SARS-CoV-2 virion. The S protein's subunits are responsible for cellular receptor angiotensin-converting enzyme 2 (ACE-2) binding via the receptor-binding domain and fusion of virus and cell membranes, thereby mediating the entry of SARS-CoV-2 into the target cells. The S protein has an essential role in virus entry and determines tissue and cell tropism, as well as host range. The roles of the S in receptor binding and membrane fusion make it a desirable target for vaccine and antiviral development. AZD1222 expresses a codon-optimised coding sequence for S protein from the SARS-CoV-2 genome sequence accession MN908947.

The ChAdOx1 platform technology was used to support the first-in-human (FIH) and other early clinical AZD1222 studies. This approach of using platform data to support a FIH clinical study is consistent with the views expressed by global regulators at the International Coalition of Medicines Regulatory Authorities – Global Regulatory Workshop on COVID-19 vaccine development, 18 March 2020 (ICMRA 2020).

To date, immunology and biological activity studies (including prime boost vaccination) of AZD1222, have been conducted in mice, non-human primates, ferrets and pigs (Table 1).

For the FIH study, biodistribution studies with AZD1222 were not performed based upon previously generated biodistribution data with similar replication-defective ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice that showed no evidence of replication of the virus or presence of disseminated infection after intramuscular (IM) injections (Table 2). A recent biodistribution study (Table 2) of IM ChAdOx1 HBV in mice detected, based on interim data using a qPCR method, low levels of disseminated ChAdOx1 HBV. Low copy numbers were found in a range of organs (spleen, brain, heart, kidney, liver, lung, lymph node, testes, ovary) at levels 1,000 to 100,000 fold less than at the injection site (skeletal muscle). Toxicology studies on a related betacoronavirus (MERS-CoV) ChAdOx1 vectored vaccine expressing full-length S protein, as well as other ChAd vaccines (AdCh63 MSP-1, ChAd OX1 NP+M1) were also used to support the FIH study and are shown as a reference

(Table 2). Toxicology studies with AZD1222 have either been recently completed or are ongoing (Table 1 and Table 3). Ongoing or planned nonclinical studies are listed in Table 3.

All pivotal nonclinical safety studies were conducted in OECD member countries and in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP), and according to relevant International Conference on Harmonisation guidelines.

Table 1 List of Nonclinical Pharmacology Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Source	GLP Y/N
Primary Pharmacology				
Effect of D614G Mutation in SARS-CoV-2 Spike Protein on AZD1222 (20-01700)	In vitro	NA	CSIRO Health and Biosecurity, Australia	N
Murine Immunogenicity (van Doremalen et al 2020)	Balb/C and CD-1 mice	Single dose, IM 6×10^9 vp AZD1222	Jenner Institute - Oxford University, UK / NIH, USA	N
Murine Immunogenicity (Graham et al 2020)	Balb/C and CD-1 mice	Day 0 and 28 IM, 6.02 × 109 vp/animal AZD1222	Jenner Institute - Oxford University / Pirbright Institute, UK	N
Non-human Primate Efficacy and Immunogenicity (van Doremalen et al, 2020)	Rhesus macaques	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University, UK / NIH, USA	N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Rhesus Macaques (6284)	Rhesus macaques	Single dose, IM 2.5×10^{10} vp AZD1222	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N
Assessment of Efficacy of SARS-CoV-2Vaccine Candidates in the Ferret Mode (20-01125)	Ferret	Single dose, IM, IN 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	CSIRO Health and Biosecurity, Australia	N
Efficacy of ChAdOx1 nCoV-19 Against Coronavirus Infection in Ferrets (6285)	Ferret	Day 0 and 28, IM 2.5×10^{10} vp AZD1222 or ChAdOx1 GFP	Jenner Institute - Oxford University / Public Health England, Porton Down, UK	N

Table 1 List of Nonclinical Pharmacology Studies with AZD1222

Study (Report Number or publication)	Species	Dose and route of administration	Source	GLP Y/N
Porcine Immunogenicity (Graham et al 2020)	White-Landrace-Hampshire cross-bred pigs	Day 0 and 28, IM 5.12×10^{10} vp AZD1222	Jenner Institute - Oxford University / Pirbright Institute, UK	N
ChAdOxl-nCoV19 immunopotency assay (INT-ChadOx1 nCov19-POT004)	Balb/C and CD-1 mice	5×10^{9} vp AZD1222	Jenner Institute - Oxford University, UK	N
Safety Pharmacology				
Cardiovascular and Respiratory Assessment Following Intramuscular Administration to Male Mice (617078)	CD-1 mice	Day 4, IM 2.59×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y
Repeat Dose Toxicology				
AZD1222 (ChAdOx1-nCovd-19): A 6 Week Intermittent Dosing Intramuscular Vaccine Toxicity Study in the Mouse with a 4 Week Recovery (513351)	CD-1 mice	Days 1, 22 and 43, IM 3.7×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y
Developmental and Reproductive Toxicology				
ChAdOx1-nCovd19: A Preliminary Intramuscular Injection Vaccine Development and Reproductive Study in Female CD-1 Mice (490838)	CD-1 mice	Day 1 (13 days prior to pairing for mating) and GD 6 to EFD phase animals and on GD 6 and GD 15 to littering phase animals, IM 2.59×10^{10} vp AZD1222	Charles River Laboratories Ltd, UK	Y

CSIRO = Commonwealth Scientific and Industrial Research Organisation, Geelong, Australia; EFD = embryo-fetal development; GD = gestation day; IM = intramuscular; IN = intranasal; NIH = National Institute of Health

Table 2 List of Nonclinical Studies with Similar Replication-defective ChAd Vaccines (AdCh63 and ChAdOx1)

Study (Report Number)	Species	Dose and route of administration	Source	GLP Y/N
AdCh63 MSP-1 and MVA MSP-1 Tissue Distribution Study By Intra-Muscular Administration To Mice (Report UNO0014/RMBIODIST-001)	Balb/C mice	Day 1, IM 1.11×10^{10} vp AdCh63 MSP-1 1.04×10^{8} pfu MVA MSP-1	Huntingdon Life Sciences,[a] UK	Y[b]
AdCh63ME-TRAP Tissue Distribution Study By Intra-Dermal Administration To Mice (UNO0009/MAB-001)	Balb/C mice	Day 1, ID 3.3×10^{9} vp	Huntingdon Life Sciences,[a] UK	Y[b]
ChAdOx-1 HBV and MVA-HBV Biodistribution Study in BALB/c Mice with Shedding Assessment (0841MV38.001)	Balb/C mice	Days 1 and 28, IM 2.4×10^{10} vp ChAdOx-1-HBV 6.1×10^{7} pfu MVA-HBV	Calvert Laboratories, USA	Y
ChAdOx1 Chik Vaccine or ChAdOx1 MERS: Toxicity Study by Intramuscular Administration to Mice (QS18DL)	Balb/C mice	Day 1 and 15, IM 1×10^{10} vp	Envigo CRS Limited UK	Y
ChAd OX1 NP+M1 and MVA NP+M1: Toxicity Study by Intramuscular Administration to Mice (XMM0003)	Balb/C mice	Day 1, IM ChAd OX1 NP+M1 1×10^{10} vp and Day 15, IM MVA NP+M1 1.5×10^{7} pfu	Huntingdon Life Sciences,[a] UK	Y
Mouse Toxicity AdCh63 MSP-1 and MVA MSP-1 or a Combination of AdCh63 ME-TRAP and MVA ME-TRAP (UNO0013)	Balb/C mice	Day 1, IM AdCh63 MSP-1 1.11×10^{10} vp Day 15, IM MVA MSP -1 10.4×10^{7} pfu Day 1 and 15, IM AdCh63ME-TRAP/ MVA ME TRAP 0.78×10^{10} vp / 6.85×10^{7} pfu	Huntingdon Life Sciences,[a] UK	Y

[a] Currently Covance CRS Ltd.

[b] In-life phase conducted to GLP; biodistribution phase (RBIODIST-001 or MAB-001) not conducted to GLP

Table 3 List of Ongoing and Planned Nonclinical Studies with AZD1222

Study (Report Number)	Species	Status	GLP Y/N
AZD1222 (ChAdOx1-nCovd-19): A Single Dose Intramuscular Vaccine Biodistribution Study in the Mouse (514559)	CD-1 mice	Ongoing Audited draft February 2021	Y
AZD1222 (ChAdOx1 -nCovd19): An Intramuscular Vaccine Development and Reproductive Study in Female CD-1 Mice (490843)	CD-1 mice	Ongoing Audited draft February 2021	Y

2 PHARMACOLOGY

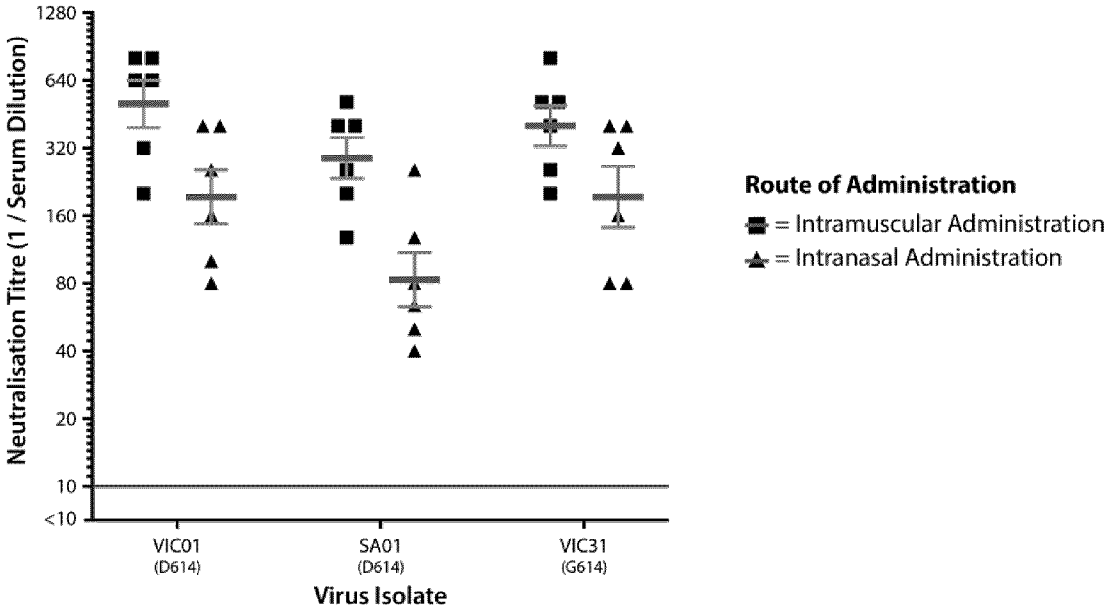
2.1 Primary Pharmacodynamics

Immunogenicity studies in animal models responsive to AZD1222 were conducted to evaluate the immunologic properties of this COVID-19 vaccine candidate to support FIH clinical trials. AZD1222 has been shown to be immunogenic in BALB/c, CD-1 mice, ferrets, non-human primate (NHP) and pig models. These studies included evaluation of humoral, cellular and functional immune responses. Whilst a single dose of AZD1222 induced antigen-specific antibody and T cell responses, a booster immunisation enhanced antibody responses, particularly in pigs, with significant increases in SARS-CoV-2 neutralising antibody titres (Graham et al 2020). A post-vaccination SARS-CoV-2 challenge in rhesus macaques was conducted to evaluate protection and the potential for vaccine-associated enhanced respiratory disease (ERD). A single administration of AZD1222 significantly reduced viral load in bronchoalveolar lavage fluid and respiratory tract tissue of vaccinated animals as compared to vector controls (van Doremalen et al 2020). Importantly, no evidence of ERD following SARS-CoV-2 challenge in vaccinated rhesus macaques was observed (van Doremalen et al 2020).

Mutations are occurring naturally within the SARS-CoV-2 genome. Most vaccines in development rely upon inducing immune responses towards Spike protein (S), the main virus surface protein. A D614G mutation in S is increasing in prevalence amongst sequenced viruses worldwide. The mutation is thought to increase infectivity of the virus by reducing S1 shedding, increasing infection (Zhang et al 2020). The effect of the D614G mutation on the efficacy of virus neutralisation following vaccination of ferrets with AZD1222 was assessed in study 20-01700 (Figure 1). A new Australian isolate containing the D614G mutation (VIC31) was obtained from VIDRL. Three isolates were used for virus neutralisation assays: SA01: has identical amino acid sequence in S to Wuhan-Hu-1. VIC01: S differs from SA01 by an Ser247Arg mutation. VIC31: S differs from SA01 by the Asp614Gly mutation.

Overall, there were no significant effects of the D614G mutation in the SARS-CoV-2 Spike protein on relative neutralisation of D614 and G614 variants with serum samples collected from ferrets that had received prime-boost administrations of AZD1222. Therefore, animal challenge studies presented are relevant to strains circulating in the human population.

Figure 1 Effect of D614G mutation on vaccine-induced antibody-mediated neutralisation.



Mean neutralising titres (calculated from log2-values) to three circulating Australian SARS-CoV-2 isolates. Neutralisation titres of serum samples collected following prime-boost vaccination with AZD1222 in ferrets, administered by two routes (intramuscular and intranasal). Bold horizontal lines represent overall mean titre of the vaccination route/isolate combination with uncertainty bars representing Standard Error of the Mean (SEM). Square and triangle marks represent mean titres of the triplicate titres for each serum sample/isolate combination.

Viral RNA in Gastrointestinal Tract

In the NHP pharmacology study (van Doremalen et al 2020), there was an unexpected finding of viral RNA in tissues of the gastrointestinal (GI) tract at 7 days post-challenge in immunised, but not control, animals. Viral gRNA load in intestinal tissues of prime-boost-vaccinated animals was higher than the levels measured in control and prime-only-vaccinated animals at 7 days post-challenge and was associated with the detection of sgRNA. However, no infection of intestinal tissue was observed by immunohistochemistry, nor were we able to detect infectious virus in intestinal tissue. Given that spike-specific antibodies were significantly increased after the second immunization (two-tailed signed-rank Wilcoxon test) higher viral gRNA load intestinal in prime-boost animals may correlate with

greater intestinal clearance and retention of opsonised virus following challenge. FcRn allows the entry and retrieval of IgG from the intestinal lumen throughout health and disease. This bidirectional transport allows the secretion of IgG into the lumen, the subsequent uptake of opsonized bacteria and viruses (Castro and Clatworthy 2020). As previously reported, SARS-CoV-2 antigen can be detected in lymphocytes and macrophages in the lamina propria of the intestinal tract of control animals (Munster et al 2020). This may indicate a higher proportion of plasma cells secreting IgA2 in the gut lamina propria of prime-boost-vaccinated animals and trapping of SARS-CoV-2 virus. Whilst SARS-CoV-2 virus may make its way to the gastric lumen, it would be subjected to the adverse effect of the acidic environment of the stomach that would significantly affects viability.

Nevertheless, SARS-CoV-2 can cause gastrointestinal symptoms, such as loss of appetite, vomiting, diarrhoea, or abdominal pain during the early phases of the disease (Villapol 2020). It has been reported in some patients that although SARS-CoV-2 has been cleared in the respiratory tract, the virus continues to replicate in the gastrointestinal tract and could be shed in faeces (Yang et al 2020). Currently, the exact mechanism of SARS-CoV-2 interaction with the gastrointestinal tract is still not fully understood. However, SARS-CoV-2 shows a high affinity to ACE2 receptors, making sites of high ACE2 receptor expression such as lungs and GI tract prime targets for infection (Dahiya et al 2020). It is therefore possible that gastrointestinal symptoms in COVID-19 are somehow caused by the direct attack of SARS-CoV-2 to gastrointestinal tract (Zhong et al 2020). If higher viral gRNA loads in intestinal tissues of prime-boost vaccinated animals is associated with continued replication then it was not associated with any signs of lesions or infection.

Lung Histopathology

In rhesus macaques 3 out of 6 control animals developed some degree of viral interstitial pneumonia following SARS-CoV-2 challenge. Lesions were widely separated and characterised by thickening of alveolar septum. Alveoli contained small numbers of pulmonary macrophages and rarely oedema. Type-II pneumocyte hyperplasia was observed. No histological lesions were observed in the lungs of vaccinees.

In comparison, the majority of histopathological findings made in the lungs of ferrets following SARS-CoV-2 challenge were modest at most. In control group 3a that received a prime with ChAdOx1 vector expressing green fluorescent protein (GFP) one ferret showed mild lesions compatible with acute bronchiolitis and the other animals were similar to group 1 primed with AZD1222. Only mild inflammatory cell foci and no lesions were observed in group 1. In group 2 that received a prime and boost with AZD1222, inflammatory cell were also detected in lungs. These changes are likely associated with an immune response to challenge as they were also observed in controls. In group 4 immunised with inactivated SARS CoV-2, mild to moderate lesions were observed in the lungs with inflammatory cells and perivascular cuffing at day 7 post challenge potentially indicative of enhanced respiratory

disease. In a second ferret study, no significant histological lung changes were present in any of the animals examined.

Enhanced respiratory disease (ERD) can result from immunization with antigen that is not processed in the cytoplasm, resulting in a nonprotective antibody response and CD4+ T helper priming in the absence of anti-viral cytotoxic T lymphocytes. This type of vaccine response can lead to a pathogenic Th2 memory response with eosinophil and immune complex deposition in the lungs after respiratory infection. For example, infants and toddlers immunized with a formalin-inactivated virus vaccine against respiratory syncytial virus (RSV) experienced an enhanced form of RSV disease characterized by high fever, wheezing and bronchopneumonia when they became infected with wild-type virus in the community (Acosta et al 2015). AZD1222 not expected to cause ERD because antigens are expressed intracellularly, generating anti-viral cytotoxic T cell and protective antibody responses.

In the van Doremalen et al study, significantly reduced viral load in the bronchoalveolar lavage fluid and lower respiratory tract tissue of vaccinated rhesus macaques challenged with SARS-CoV-2 with no pneumonia was observed compared to control animals. No evidence of immune-enhanced disease after viral challenge in vaccinated SARS-CoV-2-infected animals was found in terms of increased severity of viral infection. At present, there are no known clinical findings, immunological assays or biomarkers that can differentiate any severe viral infection from immune-enhanced disease, whether by measuring antibodies, T cells or intrinsic host responses (Arvin et al 2020). Carefully controlled human studies of sufficient size to enable the detection of increased frequency of severe cases in vaccinated cohorts compared to control group are required to determine if antiviral host responses may become harmful in humans.

In conclusion, the rhesus macaque is more predictive than ferret of histological lung changes and the ability of immunisation with AZD1222 to mitigate these following challenge with SAR-CoV-2. No enhanced respiratory disease was observed post challenge in AZD1222 immunised animals.

2.2 Secondary Pharmacodynamics

Secondary pharmacodynamic studies have not been conducted with AZD1222.

2.3 Safety Pharmacology

In a mouse cardiovascular and respiratory safety pharmacology study, a group of 8 male CD-1 mice were dosed by IM injection with the control item for AZD1222 (A438 buffer) on Day 1 and AZD1222 (2.59 x 10^{10} vp dose) on Day 4 (617078).

There were no changes in arterial blood pressure, heart rate, body temperature or respiratory parameters considered to be AZD1222-related. The No Observed Effect Level (NOEL) for cardiovascular and respiratory assessment was an AZD1222 dose of 2.59 x 10^{10} vp .

Irwin Screen observations (autonomic, neuromuscular, sensorimotor, behavioural parameters) and effects on body temperature and pupil size were made in the repeat-dose IM toxicity study (513351) in male and female CD-1 mice on Days 8 and 29 following administration of AZD1222 at 3.7 x 10^{10} vp on Days 1, 22. There were no effects on body temperature, pupil size or Irwin Screen observations considered to be AZD1222-related.The NOEL for the Irwin Screen phase was 3.7 x 10^{10}.

2.4 Pharmacodynamic Drug Interactions

Pharmacodynamic drug interaction studies have not been conducted with AZD1222.

3 PHARMACOKINETICS

3.1 Absorption

Absorption studies evaluations are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional absorption, distribution, metabolism, and excretion (ADME) evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune responses.

3.2 Distribution

Distribution studies are not generally needed for vaccines. WHO guidelines on nonclinical evaluation of vaccines (WHO 2005) and vaccine adjuvants and adjuvanted vaccines (WHO 2013), traditional ADME evaluations are not generally needed for vaccines. The safety concerns associated with vaccines are generally not related to the pharmacokinetics but are related to the potential induction of immune response.

Biodistribution studies are more informative when a replication-competent virus is administered since the amount of virus present in the subject (experimental animal or human volunteer) will increase following injection, and some viruses have a known propensity to accumulate in particular organs. For example, Vaccinia virus may be found at high titres in the ovaries and adenovirus accumulates in the liver. However, replication-deficient viruses are known to infect cells at the injection site, and although some infectious viral particles may drain to local lymph nodes and travel through the blood to other sites in the body, concentrations of virus at these sites are so low after dilution in the blood and other tissues that they are not reliably detected. A biodistribution study would demonstrate if, unexpectedly, viral replication was taking place after injection. However, this is not an

appropriate assay to use to detect replication competent virus, which is tested for in an in vitro assay which has much greater sensitivity for detecting even small amounts of replication competent virus in the vaccine preparation.

AZD1222 is replication-incompetent in human cells due to a block in gene expression caused by the deletion of the E1 genes. Therefore, after the initial infection of the cells that the virus enters, there will be no further infection and no spread of the virus within the body. Biodistribution studies with similar ChAd vaccines (AdCh63 ME-TRAP and AdCh63 MSP-1) in mice have previously been performed and showed no evidence of replication of the virus or presence of disseminated infection after IM injection. A biodistribution and shedding study using the ChAdOx1 vector with an hepatitis B virus (HBV) insert after IM injection on Days 1 and 28 in mice was conducted (0841MV38.001). Distribution to some samples of all tissues was noted on day 2 and Day 29. The highest levels (copies/mg sample) were noted at the site of administration (skeletal muscle), ranging from 3×10^8 to 9.97×10^9 copies/mg sample. In the majority of samples of other tissues taken on Day 56, the levels were below the level of quantification, indicating elimination. Low levels were noted in 1 sample (of 6) for each of heart and liver, 1 of 3 for ovary and testes, and 3 of 6 lymph node samples at this timepoint. This study does not contain assessment of CNS, relevant peripheral nerves or bone marrow and it does not include analysis at shorter time points compared to the already available studies and no description of the validation of method analysis. This platform study will be superseded by a biodistribution study with AZD1222 (514559). This study includes additional early timepoints, an assessment of a full set of tissues including spinal cord and bone marrow. A draft report is due February 2021.

Intramuscular administration of AZD1222 is expected to minimise risk of systemic exposure. The biodistribution of AZD1222 following intramuscular administration is expected to be similar to that of AdCh63, confined to the site of injection and draining lymph nodes.

3.3 Metabolism

Metabolism studies have not been conducted with AZD1222. The expected consequence of metabolism of biotechnology-derived vaccines is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood.

3.4 Excretion

Excretion studies have not been conducted with AZD1222. No virus excretion is expected with AZD1222 as it is a non-replicating vaccine vector. Shedding of ChAdOx1 HBV in mice following IM administration of Days 1 and 28 have been assessed. DNA was extracted from mouse fecal and urine samples collected were all negative, suggesting that no shedding had occurred in these matrices at the times sampled.