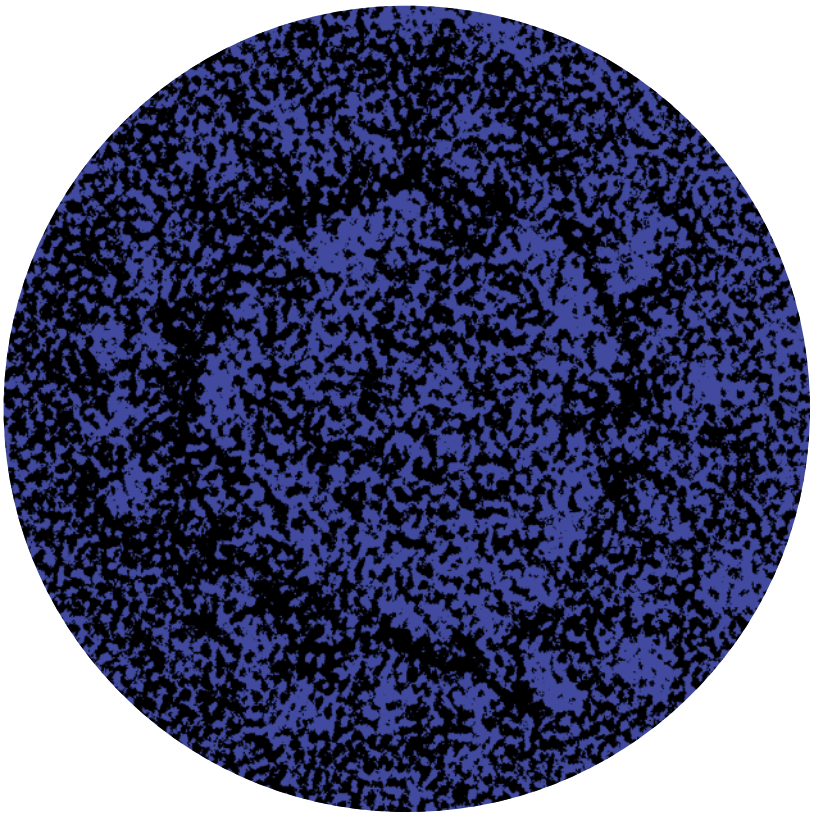

ADVANCES IN CLINICAL DEVELOPMENT FOR VACCINES AND THERAPEUTICS AGAINST RESPIRATORY VIRUS INFECTIONS

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IMAGE: Electron microscopy image framed in blue

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CHAPTER 1

INTRODUCTION

The evolutionary origin of viruses is unclear, although there appears to be consensus that they originate from DNA or RNA of living organisms. In the beginning of the 20th century the Dutch scientist Beijerinck demonstrated the existence of viruses and the first images of viruses appeared with the availability of electron microscopy in the 1930s.[1,2] Since the second half of 20th century many (thousands) viruses have been identified and are commonly named by the name of the organ/system that is affected most. This thesis focusses on respiratory viruses, which have probably been around since time immemorial. Respiratory viruses can be transmitted through excreted droplets, exhaled aerosols or contact with contaminated surfaces.[3] These viruses may infect the respiratory epithelial cells of the nose, throat and sometimes also the lower respiratory tract.[3,4] Respiratory viruses can cause various respiratory and systemic symptoms such as: sneezing, coughing, rhinitis, throat ache, nasal congestions, fever, malaise, myalgia. It is however not possible to discriminate causative pathogens based on clinical presentation alone because symptoms are overlapping and non-specific. Molecular diagnostics, such as multiplex real-time polymerase chain reaction (PCR), are therefore needed to reliably detect the causative agent.[5] Respiratory viruses have a wide spectrum of clinical disease: from asymptomatic infection and upper respiratory complaints (common cold) to acute lower respiratory disease with respiratory insufficiency, systemic inflammatory response syndrome and may even lead to death in individuals at risk.[6-9] Respiratory virus infections occur in persons of all ages and re-infections with the same virus species occur throughout an individual's lifetime.[10] This is because natural respiratory virus infection does not confer lasting sterile immunity. Respiratory viruses have various mechanisms of host immune evasion and virus surface antigens can change relatively quickly through evolutionary pressure.[11,12]

Most respiratory virus infections are self-limiting with only mild symptoms in healthy immunocompetent adults, however, some viruses can cause severe disease in specific subpopulations. Respiratory syncytial virus (RSV) and influenza virus are two RNA viruses that have been historically associated with substantial mortality and hospitalization rates.[9,13] In developed countries the mortality in adults aged 65 years or older has been estimated around 21 and 15 per 100,000 individuals for seasonal influenza and RSV, respectively.[9] Mortality rates in low-to-middle income countries are expected to be even higher.[14] Both viruses are associated with excess mortality in elderly individuals (>65 years), chronically ill and children, but RSV especially poses a great risk to young infants.[9] RSV can cause severe lower respiratory tract infections (bronchiolitis, bronchospasms, pneumonia and respiratory failure) and is a leading cause of hospitalization and death of infants, worldwide.[15,16] Influenza can cause serious complications such as secondary pneumonia and exacerbations of chronic lung diseases.[17,18] Children (younger than 5 years), elderly and chronically ill are at increased of developing such complications.[19]

Severe disease can also occur in seemingly healthy individuals if a novel antigenic virus variant occurs for which there is no pre-existing immunity. An example is the devastating 1918 influenza pandemic caused by the H1N1 virus that contained genes of avian origin for which there was pre-existing immunity in the human population.[20] The estimated death-toll of this pandemic is estimated to exceed 20 million people.[21] In the last decades novel highly pathogenic corona viruses emerged from zoonotic spillover, such as Severe Acute Respiratory Syndrome (SARS, 2003) and Middle East Respiratory Syndrome (MERS, 2012).[22,23] At the end of 2019, the fear of a new pandemic suddenly became reality when the novel human coronavirus SARS-COV-2 was first identified following a cluster of pneumonia casus of unknown etiology in the Wuhan region of China.[24] Infection rates grew exponentially and Corona Virus Diseases 2019 (COVID-19) was declared a pandemic on March 11th 2020 by the World Health Organization (WHO) and resulted in the largest public health crisis of this century.[25] Infection with SARS-COV-2 leads to a wide spectrum of disease: from asymptomatic and mild flu-like illness to serious complications such as septic shock, pneumonia, acute respiratory distress syndrome and cardiovascular events. The WHO currently estimates that there have been more than 600 million confirmed cumulative cases and over 6 million deaths.[26] In addition, COVID-19 has generated a substantial financial burden on health care systems and the general population.[27] During the writing of this thesis, COVID-19 still has a large impact on health care and society as a whole.

Historically, respiratory syncytial RSV and influenza have received the most research interest of all respiratory viruses due to their high global disease burden. Research into therapies and vaccines against these viruses have been ongoing for almost a century, ever since the first isolation of influenza in 1933.[28] One of the largest scientific breakthroughs was the development of the influenza vaccine in the 1940s.[28] However, shortly after its

discovery it became apparent that vaccines against influenza needed to be updated annually. This became painfully clear in the influenza epidemic of 1947. The vaccine failed almost completely due to marked (intrasubtypic) antigenic variation in the prevalent influenza strain (H1N1) of 1947.[29] Since then, there has been an ongoing endeavor to adapt, re-formulate and re-administer influenza vaccines annually to keep up with the evolution of the influenza virus. Modern seasonal influenza vaccines are tri- or quadrivalent, they contain antigens derived from multiple virus strains (2 influenza A subtypes and 1 or 2 B lineages). Unfortunately, current vaccines are far from perfect; their effectiveness is variable and partly depends on the match of the vaccine strain with the most prevalent circulating strain of that particular year.[30] The overall effectivity of influenza vaccines in adults is modest: 59% for inactivated parental vaccines and 53% for live-attenuated mucosal vaccines).[31] In addition, current influenza vaccines do not sufficiently prevent virus transmission.[32,33] Preventing transmission of influenza virus throughout the population would be highly desirable from a public health perspective.

Current seasonal influenza vaccines are designed to elicit serum antibodies to the highly antigenically variable and immunodominant heads of the hemagglutinin (HA) protein. Immunity induced by these vaccines is specific for influenza strains that match the vaccine antigen and generally lack efficacy against other strains.[34] The development of a broadly protective 'universal' influenza vaccine has been on the research agenda for decades. A universal influenza vaccine would also serve as the best defense against an emerging pandemic influenza strain. Such a vaccine might target more conserved influenza virus epitopes to induce immunity against multiple strains.[35,36] Recently, new universal flu candidates have entered clinical development with some promising preliminary results.[37] However, until a universal vaccine is available, efforts should also be made to improve immunogenicity and cross-reactivity of currently available seasonal influenza vaccines, especially in populations at risk for serious complication. New adjuvants could increase the immunogenicity of current and investigational vaccine technologies while development of improved mucosal vaccine platforms could elicit local immunity (next to a sufficient systemic antibody response).

Therapeutic and non-vaccine prophylactic compounds against influenza are scarce. For influenza there are a handful of antivirals authorized by European Medical Agency (EMA). The majority belong to the class of neuraminidase inhibitors (NAIs), such as oseltamivir and zanamivir. By inhibiting neuraminidase – a glycoprotein with enzymatic activity conserved within all influenza viruses – the release of virions from host cells is diminished.[38,39] Although the mechanism of action is appealing, the clinical effects are modest with a reduction of the time of symptom alleviation in adults by less than day.[40] Treatment initiation is recommended as soon as possible after illness onset, as clinical benefit has been shown to be highest within the first days after onset.[41,42] A second group of authorized antivirals consist of viral ion channel M2 inhibitors (such as amantadine and rimantadine). These antivirals are only effective against influenza A strains and widespread resistance has been reported.[42,43] The novel cap-dependent endonuclease inhibitor baloxavir marboxil did not improve time to symptom alleviation compared to oseltamivir in uncomplicated influenza.[44] Large scale use of antivirals has been debated due to their cost-effectiveness ratio's, associated adverse events and the development of antiviral drug resistance.[40,45,46] Advances in effective antivirals that reduce mortality and disease progression are highly needed, especially considering that antiviral therapies are the first-line of defense during a influenza pandemic, when vaccines are still in development or supply is still insufficient.

In contrast to influenza, there is no vaccine available yet for RSV and only very few authorized anti-infectious compounds. Palivuzimab, a humanized IgG_1 monoclonal antibody (MAB) targeting the surface fusion (F) protein, is the only compound authorized in the European Union for (passive) prophylaxis.[47] Its use is currently restricted to children <2 years with a high risk of severe RSV disease (such as preterm infants). Unfortunately, the high cost of passive immunization with palivizumab and repeated intramuscular administration limits widespread global use. This applies especially to low- and middle-income countries where disease burden and RSV-related mortality are highest.[48]

Attempts to develop a safe and effective vaccine for RSV have been ongoing for decades. A major setback was the unpredicted occurrence of vaccine-enhanced disease in an RSV trial with formalin-inactivated RSV (1967).[49,50] Children were not protected and subsequent RSV infection led to worsening of respiratory symptoms, hospitalization of many children and the dead of two.[50,51] To mitigate the risk of vaccine enhanced disease,

new vaccines-candidate now have to show compelling and robust pre-clinical safety and immunogenicity data before clinical testing.[52] In addition, safety and immunogenicity data must first be obtained from healthy adults, before exposure to non-naïve children and subsequently immune-naïve infants.[52] Due to an improved molecular understanding of RSV and innovative biotechnologies, the vaccine pipeline has been filled with various new platforms.[53] Hopefully, one of these candidates will succeed to bring forth the first RSV-vaccine soon.

The previous paragraphs illustrate that despite decades of research there are still substantial knowledge gaps that hinder the development of safe and (more) effective vaccines and therapeutics for influenza and RSV. Aware of the difficulties of the development of vaccines and therapeutics for respiratory viruses, the medical community was forced to tackle the COVID-19 pandemic. With no effective treatments or vaccines against coronaviruses, investigators and regulators were challenged with the enormous task to expedite development of vaccines, anti-infectious and disease modifying agents. This required *drug repurposing* of existing authorized compounds with potential antiviral or immunomodulatory properties (e.g. hydroxychloroquine and chloroquine) and investigating promising antiviral candidates in late stage clinical development for other diseases (e.g. remdesivir).[54-57] As more data became available about SARS-COV-2 genome, structural biology and pathophysiology, novel compounds were to be developed and brought from bench-to-bed at an unprecedented pace. Both pre-existing and experimental vaccine platform technologies were used as a base to develop COVID-19 vaccines.[58]

To allow for rapid development and large-scale availability of vaccines and therapeutics, a paradigm shift in drug and vaccine development was needed.[59,60] Developing novel anti-infectious agents and vaccines from discovery to widespread public availability takes up to 10 years on average.[61] Traditionally, drug development is an iterative process characterized by different sequential phases, starting at early discovery of compounds through pre-clinical testing, clinical development (sequential phase I, II and III testing) leading to application of authorization, registration and finally marked introduction. To expedite development during a pandemic, the developmental phases needed to overlap to reach the finish-line earlier (*Figure 1*). To accelerate development time, early clinical studies may be performed in parallel to pre-clinical studies, provided that there is robust toxicology and clinical safety data from similar pharmaceutical products derived from the same platform technology.[62] Phase I and II clinical trials may be combined in larger study protocols as long as there are staggered dosing approaches and rigorous safety monitoring.[63-65] Timely availability also depends on regulators prioritizing review procedures by giving compounds for COVID-19 'emergency fast-tracks' designations.[66] Close collaboration and early discussions between investigators and regulators is needed to provide pivotal clinical data in the most efficient manner. The COVID-19 pandemic has revealed that innovation is not only needed on a level of basic science and drug development but also clinical trial conduct and regulations.

Necessity became the catalyst of innovation throughout the COVID-19 pandemic. The development of vaccines and therapeutics during the pandemic crisis required a collective effort from the medical and life science community. Currently, over 4000 interventional clinical studies have been registered for COVID-19 (ClinicalTrials.gov). Before the COVID-19 pandemic, data on attrition rates of vaccines and anti-infectious therapies showed that the vast majority of these compounds failed to reach market authorization (probability of market entry of vaccines was estimated to be 1.8% in 2014).[67] Nonetheless, vaccines and therapies were successfully developed for COVID-19 and they mostly relied on innovative technologies that were already in development years prior to the onset of the pandemic. The first vaccines became available approximately a year after the discovery of SARS-COV-2 and without compromising on safety. The first vaccines were based on novel delivery platforms such as mRNA and viral vectors. The therapeutic arsenal for COVID-19 has expanded significantly and currently includes immunomodulatory compounds, small molecule antivirals and monoclonal antibodies. However, due to the emergence of novel variants of concern and ongoing transmission, vaccines need to be adapted and the threat of resistance against therapies remain. Innovation is therefore still highly needed and ongoing.

AIM AND OUTLINE OF THIS THESIS

This thesis aims to assess several innovative novel compounds in clinical development for three of the most impactful respiratory viruses: RSV, Influenza and SARS-COV-2. A summary of biological and clinical characteristics of RSV, Influenza and SARS-COV-2 is provided in *Table 1*. Next to pharmacological

innovations in clinical development, this thesis also explores novel approaches for clinical trial conduct during a pandemic and provides means for regulators and investigators to accelerate early clinical development in pandemic situations. The studies described in this thesis took place before the COVID-19 pandemic (*Section 1 and 2*) and partly during the pandemic (*Section 3*).

In *Section 1 Respiratory Syncytial Virus* a novel live-attenuated RSV vaccine candidate lacking the surface G-protein is assessed for the first time in humans. The safety profile of this genetically modified intranasal vaccine should first be investigated in healthy adult volunteers who have been previously exposed to RSV before testing in the target population (naïve infants). To better assess viral shedding he immunogenicity (functional effect) we performed an observational study to examine the distribution of neutralizing RSV antibodies in the envisioned phase I adult study population (*Chapter 2*). It was hypothesized that a lower titer of antibodies could potentiate immune effects and allow for viral replication. Based on this study an eligibility criterion was defined for the randomized controlled clinical trial investigating the safety, immunogenicity and viral shedding of intranasal administration of the RSV vaccine candidate (*Chapter 3*).

Section 2 Influenza Virus described the use of a novel bacteria-like particle (BPL) as adjuvant to increase the immunogenicity of intranasally administered seasonal inactivated trivalent influenza vaccine (*Chapter 4*). This randomized controlled clinical trial explored three increasing dose levels of the adjuvant in healthy adults. The elderly population is known to be at risk of developing influenza related complications but tend to have generally lower vaccine-induced immune responses. The trial concluded with the testing of the most immunogenic dose of the adjuvant in individuals aged 65 years and older (target population).

Section 3 SARS-COV-2 and clinical development during pandemics starts with the development of a novel therapeutic for COVID-19 (*Chapter 5*). Ensovibep – a tri-specific DARPin molecule that binds to the SARS-COV-2 spike protein – was administered for the first time in patients with mild-to-moderate COVID-19 in an outpatient settings. This study served as a feasibility study in the clinical development trajectory of ensovibep, but the study was designed to also gain early clinical insight of the patient safety profile, pharmacokinetics and immunogenicity of two envisioned dose levels of ensovibep.

Chapter 6 investigates the immunomodulatory effect of hydroxychloroquine, a drug that was repurposed for COVID-19 and widely used during the first months of the pandemic. Hydroxychloroquine showed *in vitro* antiviral activity against SARS-COV-2 and is a known immunomodulatory drug. It was hypothesized that the immunosuppressive action of hydroxychloroquine could prevent the adverse immune reaction in severe COVID-19. Large randomized controlled efficacy trials later showed no clinical benefit of hydroxychloroquine for COVID-19. The reversed translational study (*from bed-to-bench*) presented in *Chapter 6* assessed and quantified the immunomodulatory effects of hydroxychloroquine on primary human immune cells, both *in vitro* and *ex vivo,* in a randomized clinical trial.

The last two chapters describe innovative approaches to clinical trial conduct and regulations during a pandemic. A novel approach to conducting vaccine field trials is introduced in *Chapter 7*. Through epidemic modelling and clinical trial stimulations a *hot spot* identification and recruitment strategy is compared to the traditional *wait-and-see* approach commonly used in phase III vaccine field trials. *Section 3* concludes with a pragmatic overview of recommendations that may facilitate accelerated development of early phase clinical trial in a pandemic crisis (*Chapter 8*).

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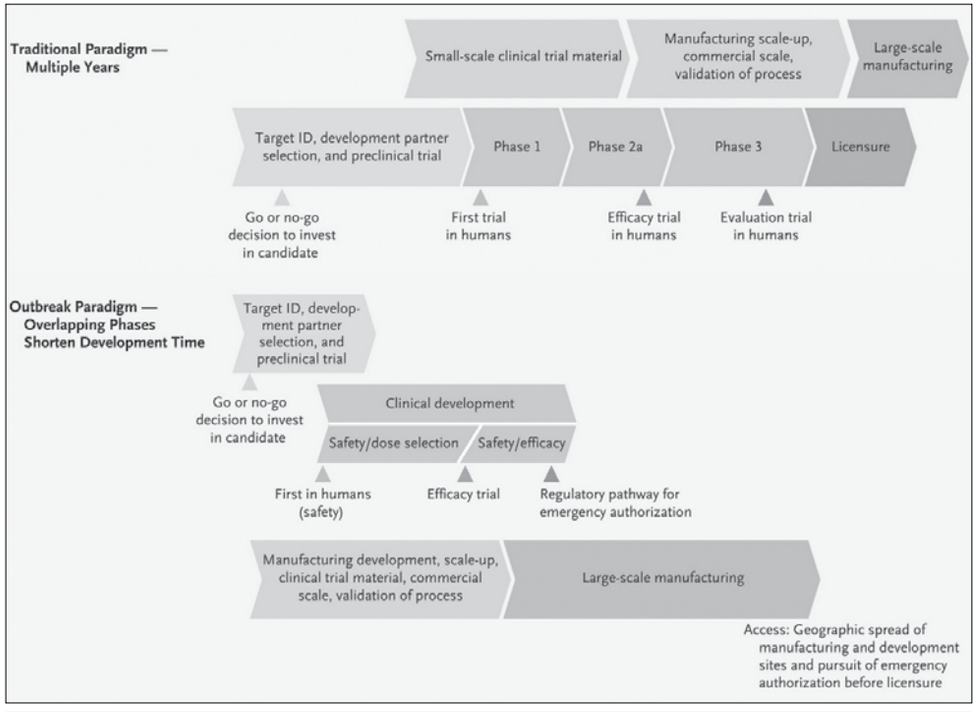
TABLE 1 SUMMARY OF VIRUS CHARACTERISTICS.

	Respiratory Syncytial Virus	Seasonal Influenza	SARS-CoV-2
Genome size (kilobases)	~ 15.2	~ 13.5	~ 29.9
Genetic material	Negative–sense RNA, non–segmented	Negative–sense RNA, segmented	Positive–sense RNA, non–segmented
Incubation time	4–8 days	1–4 days	4–5 days
Patients at risk of severe disease or complications*	**Children**: < 5 years (especially infants < 6 months), born < 35 weeks gestation, congenital heart and lung diseases, immunocompromised **Adults**: chronic cardiopul–monary disease, function–al disability, nursing home residents	Children < 5 years, adults ≥ 65 years, pregnant or 3 weeks postpartum, nursing home residents, diabetes mellites and various chronic co–morbidities	Age ≥ 65 years, chronic long, cancer, kidney and cerebrovascular diseases, immunocompromised, body mass index ≥ 30, physical inactivity, smoking
Major antigens	Fusion (F) protein, attachment (G) protein	Hemagglutinin (HA) and neuraminidase (NA)	Spike (S) protein
Vaccine availability and platform technology	No vaccine currently available	Multivalent inactivated and live–attenuated vaccines	RNA, viral vector, inactivated, protein subunit
Available therapies	Passive immune prophylaxis (palivizumab) for high risk infants	Antivirals: neuraminidase inhibitors, adamantanes, baloxavir marboxil	Various: monoclonal antibodies, small molecule antivirals, immunomodulators, dexamethasone, convalescent plasma

**Clinically relevant risk factors, however, not intended as an exhaustive list of all known risk factors for severe disease or complications.*

FIGURE 1 DIFFERENCE BETWEEN TRADITIONAL VACCINE DEVELOPMENT AND DEVELOPMENT USING A PANDEMIC PARADIGM.



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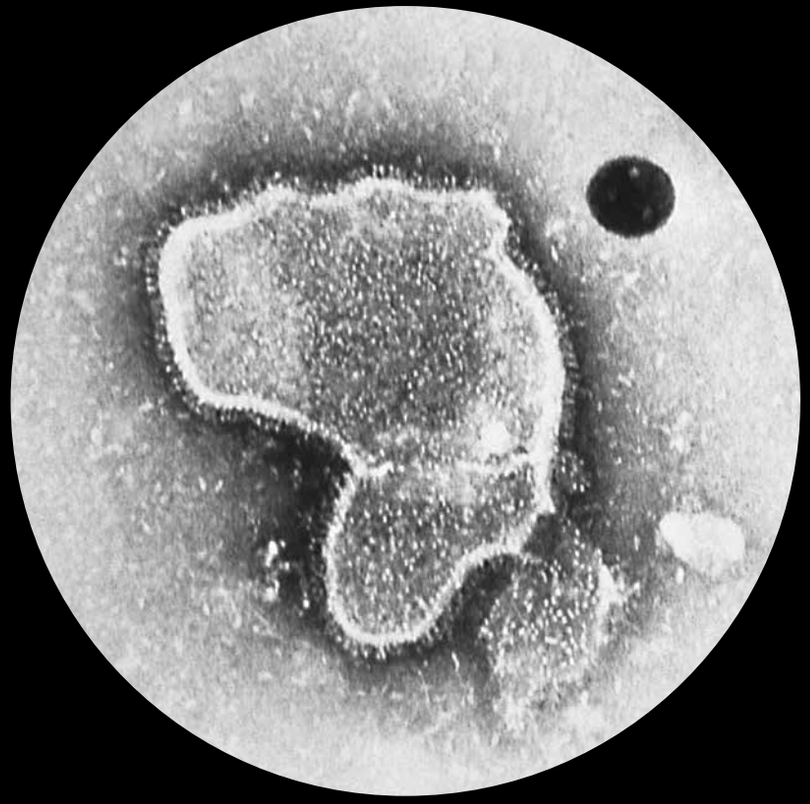

Section 1

RESPIRATORY SYNCYTIAL VIRUS

CHAPTER 2

PREVALENT LEVELS OF RSV SERUM NEUTRALIZING ANTIBODIES IN HEALTHY ADULTS OUTSIDE THE RSV-SEASON

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Abstract

One of the main challenges in early clinical research with respiratory syncytial virus (RSV) live-attenuated vaccines (LAVs) is to assess immunogenicity in healthy adults. Healthy adults will have pre-existing levels of serum neutralizing antibodies that could prematurely neutralize the LAV and underestimate the potential effect of the vaccine on the immune system. Data on prevalence and distribution of virus neutralizing titers (VNTs) in healthy adults is limited and there is no absolute threshold for protection against RSV-infection that can serve as eligibility criterion in early phase trials. We assessed the RSV-specific serum VNT in healthy adults outside the Dutch RSV-Season in two clinical studies performed in 2017 (exploratory study, n=100) and 2018 (first-in-human LAV-study, n=190) using the same neutralizing assay. Our findings show that the prevalence and distribution of serum VNT was overall consistent in the two clinical studies. Log_2 VNTs were normally distributed, distributions of VNTs were similar and there was no statistical difference in mean log_2 VNT for both studies (p=0.3). Serum VNTs were comparable during the six-months of screening in the first-in-human LAV-study. Our findings will help to determine a cut-off serum VNT to be used as an eligibility criterion in future early phase clinical trials.

Introduction

Respiratory Syncytial Virus (RSV) usually causes mild upper respiratory tract infections in healthy adults. However, it can cause severe acute lower respiratory infections (ALRIs) in infants, elderly subjects and immunocompromised adults.[1-3] RSV-associated ALRI is a major cause of pediatric mortality worldwide.[4] Immunoprophylaxis with neutralizing monoclonal antibodies (Palivizumab) is used in high risk infants, however, treatment is relatively expensive and thus its use is limited to high-income countries. Consequently, there is a high need for an active immunization strategy to reduce mortality and the high disease burden of RSV infections.[4,5] However, an effective RSV vaccine is yet to be licensed despite considerable research and development efforts. Fortunately, new promising candidate RSV vaccines are currently in development.[6] A large proportion of these vaccines use a live-attenuated vaccine (LAV) concept.[7] RSV LAVs have several benefits: LAVs have the potential to induce a broad and durable humoral and cellular immune response, can be administered intranasally and are considered to be safe because they do not seem to cause vaccine-enhanced RSV disease in naïve recipients.[8,9]

One of the main challenges in early clinical research with RSV LAVs is to assess immunogenicity in first-in-human (FIH) trials. A commonly used immunogenicity endpoint in these trials is the neutralizing activity of serum expressed as the fold change in virus neutralizing titer (VNT) determined by RSV neutralization assays.[6] For obvious safety reasons FIH vaccine studies are performed in healthy (non-naïve) adult volunteers. Because all healthy adults have been previously exposed to RSV, they will have acquired serum neutralizing antibodies. The potential effects of the LAV on the immune system could be underestimated when the LAV is prematurely neutralized by high levels of circulating neutralizing antibodies. Eligibility criteria based on serum VNTs in healthy adults are frequently used in RSV-controlled human infection model (CHIM) studies to increase the chance of successful infection after inoculation with a wild-type RSV strain.[10-15] Likewise, using low pre-existing serum VNTs as an eligibility criterion in LAV clinical trials would be a rational approach to improve the chance of observing an immune response in healthy adults. However, the use of a VNT cut-off value will impact inclusion rates because healthy adults will have varying pre-existing serum VNTs.[14]

There is currently insufficient data available on the prevalence and distribution of the RSV-specific serum VNTs in the healthy adult population. For this reason we assessed RSV-specific serum VNTs in a healthy adult population in NL outside the RSV-season in two different clinical studies. Here, we present the collective findings that will aid investigators to determine a cut-off value for RSV-specific serum VNT in future LAV and CHIM studies.

Methods

We determined RSV-specific serum VNTs in two studies: an exploratory study and a FIH vaccine trial investigating A RSV LAV. For the exploratory study, a single blood sample was drawn from 100 healthy male and female adults at the Centre for Human Drug Research (CHDR; Leiden, NL). Blood samples were drawn between 20-29 June 2017. Subjects were included if they were 18-45 years of age. Subjects were excluded if they were immunocompromised, had chronic airway diseases, signs of airway infection/common cold within two weeks prior to blood sampling or had (active) hay fever or other allergies that involve the airway. Subjects were not allowed to use medication that may affect the immune system within 30 days before blood sample collection. As part of the screening procedure in the FIH vaccine trial, pre-existing serum VNTs were determined in 190 volunteers between the end of April and mid-September 2018. Subjects were aged 18-50 years and had to comply with similar in- and exclusion criteria as in the exploratory study. Blood sampling in both studies was performed outside the RSV-season in NL, which typically occurs annually from November until early April.[16,17] The exploratory study was approved by the Medical Review and Ethics Committee Foundation BEBO (Assen, NL). The FIH vaccine trial (EudraCT number: 2016-002437-30) was approved by the Central Committee on Research Involving Human Subjects (CCMO; The Hague, NL). All subjects provided written informed consent prior to participation in the study. All study procedures were performed in accordance with the Dutch Act regarding Medical Research involving Human Subjects.

Blood sample handling was similar between both studies. Blood was collected in 3.0 mL Clot Activating Tubes. The tubes were centrifuged within 30 minutes to 2 hours of collection at approximately 2000*g* for 10 minutes and serum was collected and stored at -20°C or lower.

All samples were analyzed by Viroclinics B.V. (Rotterdam, NL). In the VNT assays a constant amount of RSV-A2 (ATCC® VR1540™, aimed at 100 plaque forming units/well) was mixed with serial 2 fold dilutions of the subject serum. The serum/virus mixtures were transferred to 96-well plates with Hep-2 cells. Following a 24 hours incubation period, cells were fixed and immunostained with a murine monoclonal antibody directed against RSV F protein (Millipore, MAB858), followed by HRP-conjugated goat-anti-mouse antibody (Life technologies, A16072) and TrueBlue (KPL, 50-78-02). The plates were then scanned with a SX UV Analyzer (CTL). Spot counts per well at each serum/antibody concentration were quantified by using the ImmunoSpot/BioSpot software (CTL) and values were used in the inhibitory concentration formula to determine the dilution of serum/antibody that showed the selected 50% reduction point.[18] Titers were reported as reciprocal of the dilution.

Analyses were performed using SPSS Statistics for Mac, version 25.0 (IBM Corp) and GraphPad Prism version 6.05 for Windows (GraphPad Software). All titers were $\log_2$ transformed prior to analysis. All values above the upper limit of quantification (ULOQ) were set equal to the value of the ULOQ (12.0 $\log_2$). Values below the lower limit of quantification (LLOQ) were set to half of the LLOQ (3.9 $\log_2$). Normal distribution of $\log_2$ serum VNTs in both studies was tested by Shapiro-Wilk test. An independent samples t-test was conducted to compare means of $\log_2$ serum VNT between the exploratory and the FIH vaccine trial. Relative frequency histograms and relative cumulative frequency distribution curves were used to visualize the distribution of VNTs in the two studies. Means, standard deviations (SD) and ranges (minimum, maximum) of $\log_2$ VNTs were determined per month for the FIH vaccine trial.

Results

For the exploratory study serum samples were collected from 100 healthy volunteers; the mean age of the subjects was 23 years (range 18-40), 78% were female. For the FIH vaccine trial serum samples from 190 healthy volunteers were collected. The mean age of the subjects was 26 years (range 18-50), the percentage of females in this study was also 78%.

Shapiro-Wilk test was used to examine the normality of $\log_2$ serum VNTs in both studies and was not significant (p>0.05) for both studies, indicating normal distribution. The normal distribution of $\log_2$ serum VNT is also visually apparent in *Figure 1*, illustrating the prevalence and distribution of $\log_2$ serum VNT. Virus neutralizing titers were most frequently observed

in the range of 9.0 to 10.5 log_2. Values above the ULOQ were observed in a subset of samples (n=5 [5.0%] in the exploratory study and n=11 [5.8%] in the subsequent FIH vaccine trial), this contributed to the small peak in relative frequency observed at 12.0 log_2 (*Figure 1*).

The relative cumulative distribution curves of the exploratory and FIH vaccine trial overlap until a VNT of approximately 9.9 log_2 (*Figure 2*). *Figure 2* also shows that approximately 54% of the healthy adult volunteers in both studies had a VNT below 9.9 log_2 (dotted line). There is a slight difference between the cumulative distribution curves for titers above 9.9 log_2 due to relatively more values above 9.9 log_2 in the FIH vaccine trial compared to the exploratory study (*Figure 1* and *Figure 2*). There was no statistical difference in mean log_2 VNT for the exploratory study (mean=9.7, standard deviation [SD] = 1.3) and the FIH vaccine trial (mean=9.9, SD=1.3); p=0.3.

The mean (SD) log_2 serum VNT of the six-month screening period of the FIH vaccine trials was summarized per month (*Table 1*). The lowest mean log_2 VNT values were observed in August and were the highest in May and June. The total range of serum VNTs observed in this period ranged from 6.4 to 12.0 log_2.

Discussion

To our knowledge, this is the first publication that reports in this detail on the levels and distribution of RSV neutralizing serum antibodies in healthy adults outside the RSV-season. We found that the exploratory study and FIH vaccine trial yielded overall comparable results. The relative cumulative distributions of serum VNTs were similar in both studies, especially up to a VNT of 9.9 log_2. There was no significant difference in mean serum VNT between the two studies, indicating consistency of VNTs in two separate cohorts. In addition, we found that mean log_2 serum VNT were quite similar for most month screening period of the FIH vaccine trial. Interestingly, a considerable lower mean log_2 serum VNT was observed in August 2018 compared to the other months. This difference could be due to cross-sectional sampling and small sample sizes when months were compared. We did not observe an apparent trend of increasing or decreasing log_2 VNT during these months.

Previous studies have shown that subjects with relatively low VNTs are more susceptible to RSV-infection and high serum VNTs have a protective effect against RSV infection in healthy adults.[13-15] Similarly, high serum VNTs might also prevent shedding and the subsequent immune response of RSV LAV in healthy adults. However, there is no established absolute threshold for protection against RSV infection for serum VNT in healthy adults. In fact, a study by Hall *et al.*[14] showed that even healthy adults with relatively high antibody levels could be (re)infected when challenged with a wild-type RSV. Because there is no absolute threshold of protection, we suggest that the distribution of RSV-specific serum VNT in the healthy adult population should be taken into account when determining a cut-off value for VNT to be used as an eligibility criterion. For example, if only the lower third of the population is to be included, then a cut-off serum VNT of 9.3 log_2 or lower should be used (*Figure 2*). In addition, *Figure 2* can be used to estimate the amount of subjects in the healthy adult population with a suitable serum VNT. For the previous example (cut-off: VNT≤9.3 log_2) 54% of the population will have a suitable serum VNT. These estimations will help investigators to anticipate recruitment rates accordingly.

Devincenzo *et al.*[11] determined serum microneutralization titers against a RSV (A) Memphis 37 strain as part of a screening procedure for a challenge study. Similar to our study, they showed a normal distribution of serum neutralizing antibodies. Interestingly, lower titers were observed in comparison to our studies. The timing of blood sampling in relation to the RSV-season was not mentioned in this study.[11] Assay variability, such as the differences in readout and the use of the Memphis 37 strain compared to the RSV-A2 in this study could be a possible explanation for the observed difference. International standardization of RSV neutralization assays and subsequent availability of International Standard reference sera is recommended to improve comparison between studies.[19] Recently, a World Health Organization (WHO) International standard antiserum has become available for RSV-A.[20] The neutralization assay used in this study was included in the collaborative study to establish this WHO International Standard.[20] Unfortunately, this standard was not yet available during the execution of this study.

We performed both studies outside of the Dutch RSV-season because this would reduce the risk of concurrent wild-type RSV infections. Concurrent wild-type RSV infections can potentially interfere with the assessment of immunogenic endpoints in LAV trials, as natural infection can cause a significant increase in serum neutralizing antibodies.[14,21] We therefore hypothesize that lower serum VNTs can be observed outside the

RSV-season, due to the decreased incidence of RSV infection.[22] However, there is insufficient data on RSV-specific serum VNTs of the healthy adult population throughout the year to test this hypothesis. Nonetheless, the timing of sampling – and study conduct in general – outside the RSV-season could be beneficial, especially to prevent concurrent wild-type RSV infection during trials.

Some limitations should be noted. The ULOQ of the virus neutralization assay was set to the highest observed titer that was initially observed during the validation of the assay. However, in a later stage, serum VNTs above the ULOQ were observed in a small percentage of subjects. These titers were set equal to the ULOQ (approximately 12.0 $\log_2$) of the validated range of the neutralization assay. The mean and range of VNTs are therefore likely to be slightly underestimated. However, this should not interfere with the interpretation of the presented results, since screening should be based on inclusion of the lower percentiles of the presented VNT distribution. We expect that the values above the ULOQ would have followed the downslope of the normal distribution (*Figure 1*). There were relatively more female subjects included in both studies; however, we are not aware of any male-female differences regarding the prevalence of neutralizing antibodies or their protective effects. Due to variable screenings rates during the FIH vaccine trial, there were considerable differences in the amount of subjects screened per month. Therefore, no formal statistical tests were performed to compare mean $\log_2$ serum VNTs between months for the FIH vaccine trial.

In conclusion, this article describes the prevalence, distribution and relevance of predetermining serum VNTs in the healthy adults outside the RSV-season in NL. The presented results will help future RSV LAV and CHIM studies to determine cut-off values for VNT to be used as eligibility criteria. This, in turn, could improve the chance to detect a meaningful immune response in healthy adults after vaccination with a RSV LAV or increase the rate of successful inoculation after inoculation with a wild-type virus in RSV CHIM studies. Furthermore, the presented results that will facilitate investigators to more accurately estimate recruitment rates when VNT is used as eligibility criterion. Further research is needed to optimize the assessment of immunogenic endpoints in early clinical research with healthy adult volunteers.

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TABLE 1 SERUM VIRUS NEUTRALIZATION TITER PER MONTH.

	Serum virus neutralization titer (log_2)		
Month	Sample size (n)	Mean (SD)	Range
April	13	9.9 (1.4)	7.0–11.4
May	40	10.2 (1.3)	7.3–12.0
June	17	10.2 (1.2)	7.9–12.0
July	46	10.1 (1.4)	6.4–12.0
Augustus	50	9.4 (1.0)	7.5–11.7
September	24	9.9 (1.3)	7.1–10.4

FIGURE 1 RELATIVE FREQUENCY HISTOGRAM OF LOG2 SERUM VNTS OF THE EXPLORATORY STUDY (BLACK BARS) AND THE FIH VACCINE TRIAL (OPEN BARS).

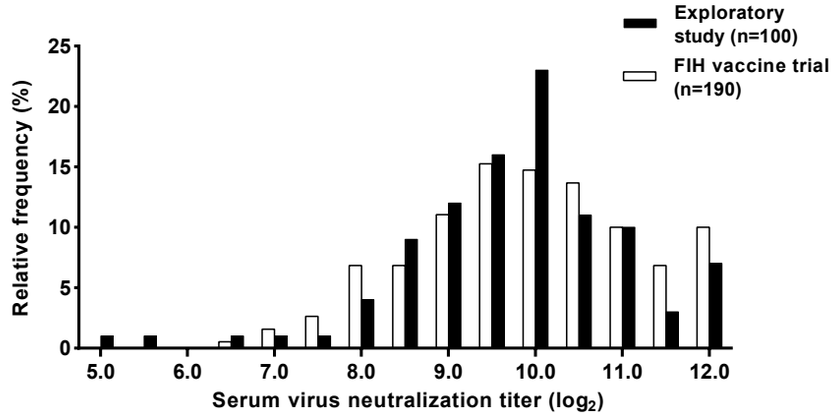
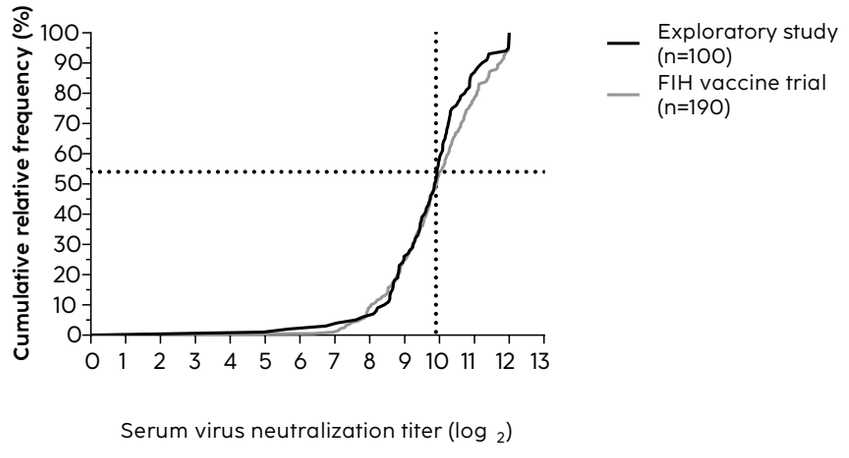


FIGURE 2 CUMULATIVE RELATIVE FREQUENCY DISTRIBUTION OF LOG2 SERUM VNTS OF THE EXPLORATORY STUDY (BLACK CURVE) AND THE FIH VACCINE TRIAL (GRAY CURVE).



Dotted line marks the 54% of subjects in both studies with a VNT ≤ 9.9 log2

CHAPTER 3

FIRST-IN-HUMAN ADMINISTRATION OF A LIVE-ATTENUATED RSV VACCINE LACKING THE G-PROTEIN ASSESSING SAFETY, TOLERABILITY, SHEDDING AND IMMUNOGENICITY: A RANDOMIZED CONTROLLED TRIAL

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SUPPLEMENTARY FIGURES AND TABLES

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

Abstract

BACKGROUND Human respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections in early infancy and in elderly. A pediatric vaccine against RSV would not only prevent morbidity and mortality amongst infants and young children but could also reduce transmission to elderly. The RSVΔG vaccine consists of a live-attenuated RSV that lacks the G attachment protein. RSVΔG is severely impaired in binding to host cells and exhibits reduced infectivity in preclinical studies. Intranasal immunization of cotton rats with RSVΔG vaccine protected against replication of wildtype RSV, without inducing enhanced disease.

METHODS We performed a first-in-human trial with primary objective to evaluate safety and shedding of RSVΔG (6.5 $\log_{10}$ $CCID_{50}$) after intranasal administration. Healthy adults aged between 18 and 50, with RSV neutralizing serum titers below 9.6 $\log_2$, received a single dose of either vaccine or placebo (n=48, ratio 3:1). In addition to safety and tolerability, nasal viral load, and systemic and humoral immune responses were assessed at selected time points until 4 weeks after immunization.

RESULTS Intranasal administration of RSVΔG was well tolerated with no findings of clinical concern. No infectious virus was detected in nasal wash samples. Similar to other live-attenuated vaccines, neutralizing antibody response following inoculation was limited in seropositive adults.

CONCLUSIONS A single dose of 6.5 $\log_{10}$ $CCID_{50}$ of RSVΔG was safe and well-tolerated in seropositive healthy adults. RSVΔG was sufficiently attenuated but there were no signs of induction of antibodies. Safety and immunogenicity can now be explored in children and eventually in seronegative infants.

Introduction

Respiratory syncytial viruses (RSV) are negative-sense, single-stranded, enveloped RNA viruses of the species *Human orthopneumovirus*.[1] RSV can cause acute respiratory tract infections in persons of all ages.[2] RSV-related acute lower respiratory tract infection accounts for approximately 3.2 million hospital admissions per year worldwide and is a major cause of mortality in children younger than 5 years.[3,4] Globally, RSV is estimated to be second to malaria as a cause of death in infants aged between 1-12 months due to a single pathogen.[5] By the age of two years almost all infants have been exposed to RSV.[6] However, immunity against RSV is incomplete and re-infections are common throughout life.[7]

Currently there is no effective licensed treatment for ongoing RSV infections. Passive immunization with humanized F-specific monoclonal antibodies (palivizumab) is limited to high-risk infants only and its application is primarily reserved to high-income countries due to its high cost. Despite the clear unmet medical need for a safe vaccine and ongoing vaccine development since the 1960s, there is still no effective vaccine available. This is partly due to a failed clinical trial in which a formalin-inactivated RSV vaccine caused enhanced disease following subsequent exposure to natural RSV infection, resulting in hospitalization of vaccine recipients and two fatalities.[8,9] However, multiple novel vaccine strategies against RSV are currently in development. The resurgence of RSV vaccine development is driven by innovations in biotechnology, such as reverse genetics.

With reverse genetic techniques recombinant RSV can be developed for use as a live-attenuated vaccine (LAV). Development of a LAV candidate against RSV has several advantages. Previous studies did not show enhanced RSV-related disease following LAV administration.[10] Live attenuated vaccines can be administered intranasally, thus mimicking the natural route of infection and thereby priming both local mucosal-and systemic immunity. In addition, intranasal inoculation is non-invasive and easy to administer.

Respiratory syncytial virus has two major surface glycoproteins, the attachment (G)- and fusion (F) protein. Both G- and F proteins contain neutralizing antibody binding sites.[11] Unlike the F protein, the presence of the surface protein G is not required for viral replication. Previous research showed that replication competence is reduced in absence of the G-protein.[12] A RSV lacking the G-protein is expected to be attenuated but

still capable of inducing an effective immune response due to presence of the surface protein F as the major antigen site and the remaining infectivity. Using reverse genetics Intravacc (NL) constructed a LAV against RSV from which the coding sequence for the attachment (G) protein was deleted from the RSV genome (RSVΔG).[13] Preclinical studies confirmed that recombinant RSV lacking the G protein was highly attenuated when administered intra-nasally and single dose administration conferred long lasting protection against wild type RSV challenge in a cotton rat model.[13] Here we present the first-in-human (FIH) study aimed to assess the safety, tolerability, viral shedding and immunogenicity of RSVΔG in healthy adult volunteers.

Material and methods

STUDY DESIGN

This was a double-blinded, randomized, placebo-controlled, parallel-group, single-dose study in 48 healthy adult volunteers. The primary objective of this study was to assess safety and tolerability of the vaccine candidate RSVΔG. Secondary endpoints were related to the viral load and shedding of RSVΔG, as well as the immunogenicity. The trial was conducted at the Centre for Human Drug Research (CHDR) in Leiden, NL. The clinical trial was performed outside the Dutch RSV season to prevent concurrent RSV wild-type infection during the trial.[14] Participants were randomized in blocks of four, one placebo and three RSVΔG treatment. Randomization codes were generated in SAS 9.4 for Windows (SAS Institute, Cary, NC, USA) by a study-independent statistician at the start of study. Participants were sequentially assigned to the intervention. Investigators, study staff and subjects were blinded to the allocated treatment.

Subjects were inoculated with a single intra-nasal dose of 0.2 ml (0.1 ml per nostril) of either RSVΔG (dose: 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$) or placebo. Subjects completed follow-up visits on 4, 7, 14 and 28 days after inoculation and received a follow-up phone call after six months. Blood and nasal wash samples were collected on follow-up visits indicated in *Figure 1*. Nasal washes were collected using the Naclerio method.[15] Into each nostril 4 mL of 0.9% NaCl was instilled. The solution was kept in the nostril for at least 20 seconds to allow sufficient dwelling time. The study was approved by the Central Committee on Research Involving Human Subjects (CCMO; The Hague, NL) and was registered in the European Clinical Trials Database (EUDRACT number: 2016-002437-30) and the Dutch trial register (NTR: NTR7173). All subjects provided written informed consent prior to participation in the study. All study related procedures were performed in accordance with the Declaration of Helsinki and the Dutch Act regarding Medical Research involving Human Subjects. As RSVΔG is a genetically modified organism, the environmental permit on 'deliberate release into the environment' (according to the directive 2001/18/EC of the European parliament and of the council) had been granted before the start of the study.

PARTICIPANTS

Eligible participants were non-smoking healthy volunteers, aged 18 to 50 years inclusive, with a body mass index between 18 and 30 kg/m². Subjects were invited for a full medical screening if they had relatively low levels of pre-existing RSV-specific neutralizing antibodies (nAbs) (≤9.6 $\log_2$).[16] Eligibility was further assessed on subject's medical history, physical examination (including anterior rhinoscopy, blood- and urine laboratory analyses including pregnancy test for women of childbearing potential), vital signs and electrocardiogram. Exclusion criteria included close contact with infants (<2 years of age) and immunocompromised individuals for 14 days following vaccine administration, any immune deficiency or use of immunomodulatory drugs, airway infection in the period of 14 days before vaccine administration, (active) allergic rhinitis or other allergies involving the airway, chronic airway diseases or history of frequent epistaxis. Participants received oral and written instructions on hygiene rules to prevent transmission of RSVΔG in the case viral shedding would occur.

VACCINE AND INTRANASAL ADMINISTRATION

The investigational vaccine was a non-sterile live-attenuated recombinant RSV (RSVΔG, Intravacc, Bilthoven, NL, batch number 100046). Details on the construction of the RSVΔG vaccine candidate have been described previously.[13] A total of 0.2 mL (0.1 mL per nostril) was administered intranasally using a spraying device (Teleflex VaxiNator™). The inoculated dose consisted of a virus titer of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$. This dose provided 100% protection against wild-type RSV in a cotton rat challenge model without inducing enhanced respiratory disease and was safe in a repeated dose toxicity and

local tolerance study in Wistar rats. Placebo treatment consisted of the formulation buffer only and was indistinguishable from the active treatment.

SAFETY AND TOLERABILITY ASSESSMENTS

Vital signs (blood pressure and pulse rate) were measured during every visit. Anterior rhinoscopy was performed by a physician prior to dosing and during every visit to examine the nasal mucosa. If symptoms were present during the visit, a symptom limited physical examination was performed. Blood chemistry and hematology tests were performed prior to inoculation and on day 7 and 14 post-inoculation at the Central Laboratories of Leiden University Medical Center (Leiden, NL). Tolerability was assessed by asking subjects to rate naso-oropharyngeal pain on a visual analogue scale (VAS), range: 0-100 millimeter, immediately after intra-nasal vaccine administration and approximately 5 minutes after administration. Subjects reported solicited adverse events by completing a daily questionnaire for 14 days following inoculation on a custom designed mobile application (E-diary).[17] Solicited adverse events consisted of cold-like symptoms and/or reaction to the vaccine such as: sore throat, epistaxis, nasal congestion, rhinorrhea, sneezing, dyspnea, coughing, malaise, myalgia or arthralgia, headache, earache, eye irritation/complaints. Severity of symptoms were scored by the participant on an ordinal scale: 0 = not present, 1 = mild (easily tolerated, light complaints), 2 = moderate (bothersome but tolerable, able to perform daily activities), 3 = severe (difficult to tolerate, withholding daily activities). Symptom severity scores (range: 0-36) were calculated by summing up the scores (0-3) for each question in the E-diary per day. Participants recorded their oral temperature twice daily in the E-diary to assess the development of febrile temperature during 14 days post-inoculation.

Non-solicited adverse events were assessed by the study physician throughout the study (until day 28). Follow-up phone calls were conducted six months after inoculation to assess late non-solicited adverse events, SAEs and concomitant medication use. For each non-solicited adverse event the relationship to inoculation was judged by the study physician as probable, possible, unlikely or unrelated. In addition, a diagnosis of upper respiratory tract infection (URTI) was given if several solicited (cold-like) adverse events coexisted at the same time and respiratory infection was clinically apparent in the opinion of the study physician. Cold-like symptoms (identical to the solicited adverse events) that were reported after 14 days were recorded in the same manner as non-solicited adverse events.

VIRAL SHEDDING

Viral replication was assessed by quantitative culture (qCulture) and quantitative PCR (qPCR) in nasal wash samples on day -1, 4, 7, 14 and 28 after inoculation. All samples were analyzed by Viroclinics B.V. (Rotterdam, NL). After addition of Phocine distemper virus type 1 (PDV) as a universal internal control, nucleic acid was isolated from nasal wash samples using the MagNA Pure 96 instrument and MagNA Pure 96 kits (Roche Applied Science).[18] A quantitative RT-PCR was performed for RSV-A on the purified nucleic acid using a Fast Virus Master Mix (Applied Biosystems, 4444436) on a 7500 Real Time PCR machine (Applied biosystems).The PCR target sequence was within the N gene.

Quantitative virus culture was performed by making serial dilutions of the nasal wash material and using these dilutions to infect Vero cells (ATCC® CCL-81™) with four replicates in a 96-well plate format. After 6 days of culture, the cells were fixed and immunostained with a murine monoclonal antibody directed against RSV F protein (Millipore, MAB858), followed by horseradish peroxidase conjugated goat-anti-mouse antibody (Life technologies, A16072) and TrueBlue (KPL, 50-78-02) to detect virus positive wells. The virus titer was calculated according to the Reed and Muench method for $TCID_{50}$.[19]

IMMUNOGENICITY MEASUREMENT

Immunogenicity was assessed in blood and nasal washes on day -1, day 7 and 28 after inoculation. Virus neutralization assays for serum and mucosal RSV-specific nAbs were performed as previously described.[16] For the palivizumab competing antibody (PCA) assay serum samples were mixed with biotin-labelled palivizumab.[20] Competitive binding was performed in 96-well microtiter plates pre-coated with purified RSV-F. Serial 2-fold dilutions of serum samples were spiked with biotinylated palivizumab and added to RSV-F-coated plates. Unbound material was washed from the wells, and a peroxidase-conjugated streptavidin was added to the plates to determine antigen bound biotinylated palivizumab.[20] Competitive binding titers were expressed as the 50% inhibition titers and were calculated as described by Zielinska et al.[21] Titers were reported as the reciprocal value of the serum dilution that resulted in 50% inhibition of biotinylated palivizumab binding. For the determination of IgA antibodies against RSV in nasal washes, a commercial ELISA kit was used (IBL International, RE56871) according to the manufacturer's instructions. The IgA concentrations were calculated

by linear regression of the OD 450 nM values using the kit internal controls as reference. RSV F-specific antibodies in serum were determined similarly as described previously.[22] In short, ELISA plates (Nunc MaxiSorp; Thermo Scientific) were coated with 25 ng of RSV F protein and incubated with 5-fold serial dilutions of serum samples.[23] After extensive washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Pierce) diluted 1:1,000. Detection of HRP reactivity was performed using tetramethylbenzidine substrate (Sigma) and an ELISA plate reader (EL-808 [from Biotek]). The IgG titer for RSV F protein was determined by calculating the end-point dilution with Gen5 software.

STATISTICAL ANALYSES

SAS 9.4 for Windows (SAS Institute, Cary, NC, USA) was used to perform the statistical analysis. Safety measures were analyzed using descriptive statistics. Prior to analysis serum RSV-specific nAbs and F-specific antibodies were transformed to $\log_2$ and $\log_{10}$ titers, respectively. Mean and standard deviation (SD) were determined for RSV-specific nAbs at baseline (day -1) day 7 and 28 and for F-specific antibodies at baseline (day -1) and day 28. RSV-specific nAbs were further analyzed with a mixed model analysis of variance (ANCOVA) with treatment, day, and treatment by day as fixed factors and subject as random factor and the baseline measurement (at day -1) as covariate. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. Contrasts were calculated within the model for RSVΔG versus placebo (overall [day 7, 28] and on day 28 separately). F-specific antibodies were analyzed with a general linear model of covariance with fixed factor treatment and baseline F-specific antibodies as covariate and same contrast as mentioned previously. The general treatment effect and specific contrasts were calculated.

Results

STUDY POPULATION

Subjects were recruited from April 2018 until September 2018. A total of 190 volunteers were screened for levels of RSV-specific nAbs. Of these volunteers 102 (53.6%) had pre-screening nAbs titers >9.6 $\log_2$ and were excluded. Forty-eight subjects were found eligible to participate in the study based on in- and exclusion criteria. All 48 subjects completed the 28-day observation period and were analysed per protocol. See *Figure 2* for the CONSORT subject flow diagram. Subject characteristics were similar for vaccine and placebo recipients (*Table 1*).

SAFETY AND TOLERABILITY EVALUATION

Intranasal administration of RSVΔG was well tolerated. Naso-oropharyngeal pain VAS scores were similarly in both the RSVΔG group (t= 0 min: mean =1.4, SD=6.1; t=5 min: mean=0.9, SD=2.2) and placebo group (t= 0 min: mean =0.6, SD=1.7; t=5 min: mean=0.1, SD=0.3). Examination by anterior rhinoscopy revealed no abnormalities related to vaccine administration. There were no findings of clinical concern in blood chemistry and hematology assessments and no clinically significant values or trends were observed in vital signs (data not shown). There was no apparent increase in body temperature following inoculation with RSVΔG compared to placebo. Two subjects reported a febrile temperature of 38.4°C (RSVΔG) and 38.2°C (placebo) on day 10 after inoculation that coincided with complaints of URTI.

In both the RSVΔG and placebo group the majority of subjects reported at least one solicited adverse event in the E-diary during the first 14 days after inoculation (*Table 2*). Sneezing and rhinorrhea had the highest relative incidence in the RSVΔG group and sore throat and malaise had the highest relative incidence in the placebo group. Epistaxis and eye irritations/complaints were reported in the RSVΔG but not in the placebo group, however, few subjects reported these symptoms. Overall, the relative frequencies of solicited adverse events were similar in both groups. Severity of solicited adverse events was comparable in both groups (mild: 75% RSVΔG versus 78% placebo, moderate: 18% RSVΔG versus 20% placebo, severe 7.3% RSVΔG versus 2.2% placebo). Total symptom scores were also similar for both treatment groups (*Figure 3*). Highest total symptom scores were observed on day 12 in the placebo group.

A summary of all possible and probable related non-solicited adverse events is provided in *Table 3*. Adverse events related to the respiratory tract were most frequently reported. The diagnosis of upper respiratory tract infection was made in 9 subjects (25%) in the RSVΔG and in 4 subjects (33%) in the placebo group (*Table 3*). All non-solicited adverse events were mild except for three events that were of moderate severity. These three adverse events consisted of a urinary tract infection (placebo group) and two cases of URTI (RSVΔG group). All adverse events, solicited and non-solicited, resolved without sequelae before the last visit (approximately 28

days after inoculation). At the six month follow-up phone call no SAEs or non-solicited adverse events were reported by the subjects (n=46). Two subjects (placebo group) could not be contacted for the six-month telephone follow-up interview. No serious adverse event (SAE) occurred during the study and no adverse events resulted in the withdrawal of subjects during this trial.

Concomitant medication to treat adverse events predominantly consisted of the use of paracetamol. Ibuprofen was used in one instance for complaints of URTI. One subject (placebo) was treated with nitrofurantoin to treat a urinary tract infection. One subject (RSVΔG) was treated with acyclovir and valacyclovir to treat a herpes simplex infection (the subject was familiar with herpes simplex re-activations) following the onset of a URTI. The same subject was later also treated with topical fucidic acid for impetigo and edema of the lip following the herpes simplex infection.

VIRAL LOAD

In the RSVΔG group, 3 of 36 subjects (8.3%) had quantifiable qCulture results of nasal wash samples compared to 3 of 12 (25%) subjects in the placebo group. All positive qCulture results were observed on single time points only and in different subjects. Two positive qCulture results were found prior to inoculation (RSVΔG=1, placebo=1) and single positive results on day 4 (placebo), day 7 (RSVΔG), day 14 (RSVΔG) and day 28 (placebo). All qCulture results were equal to the lower limit of quantification (LLOQ) (0.75 $\log_{10}$ $TCID_{50}$/mL) except for the single day 14 sample (RSVΔG) with a titer of 1.0 $\log_{10}TCID_{50}$/mL.

The presence of RSV-specific RNA, determined by qPCR, was only detected on day 4 post-inoculation in nasal wash samples of three (8.3%) subjects in the RSVΔG group. All of these samples had qPCR titers below the LLOQ (2.23 $\log_{10}$ vp/mL) and did not coincide with quantifiable qCulture results.

IMMUNOGENICITY

RSV neutralizing antibody titers in serum

All subjects were seropositive for RSV neutralizing antibodies at baseline. Mean $\log_2$ titers of RSV-specific nAbs of RSVΔG and placebo group were similar prior to inoculation (*Table 4*). The overall fold change in nAbs titers following inoculation was <2 (*Figure 4*). The highest individual observed seroresponse was a 2-fold increase in nAbs titer on day 7 and day 28 in one subject after inoculation with RSVΔG. No treatment effects on RSV-specific nAbs were observed at day 28 and overall.

Palivizumab competing antibodies (PCA) in serum

At baseline, 23% (n=11) of all subjects had positive serum samples for PCA. Subjects with positive samples on day 7 and 28 also had positive samples at baseline. No evident changes in PCA titers were observed following inoculation with RSVΔG. In the group vaccinated with RSVΔG, the number of PCA seropositive subjects declined from 9 (25%) on baseline, to 7 (19%) on day 7, and 5 (14%) on day 28. In the placebo group there were two (17%) subjects with PCA seropositive samples on baseline, these subjects remained seropositive throughout the follow-up visits.

RSV F-specific antibodies in serum

There were no evident increases in F-specific antibody titers following inoculation. Mean titers of F-specific antibodies of RSVΔG and placebo were similar on baseline (day -1) and day 28 (*Table 4*). No treatment effects on F-specific antibodies were observed.

RSV neutralizing antibodies and IgA in nasal wash

Titers of mucosal RSV neutralizing antibodies in nasal wash samples were all below the LLOQ (<8). One subject in the RSVΔG group (2.8%) had an IgA reciprocal titer of 31.8 on day 28 only. The increase in IgA titer did not coincide with an increase in other immunogenicity endpoints or with viral shedding. No IgA was detected in the placebo group.

Discussion

The results of this first-in-human study showed that a single dose of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$ RSVΔG is safe and well tolerated. Solicited and non-solicited adverse EVENTS were generally of mild to moderate severity, of short duration and resolved without sequelae. Symptom scores of the RSVΔG group were similar to those in the placebo group and showed no substantial rise in the first two weeks following inoculation, confirming the full attenuation phenotype of RSVΔG.

The majority of adverse events were related to the respiratory tract, however, RSV-infection was not confirmed by qPCR or culture in subjects with upper respiratory tract symptoms. Because of the lack of confirmation of RSV infection and the fact that the frequency of these symptoms was equally distributed amongst inoculated and placebo volunteers, it is likely that they were caused by concurrent infections with other respiratory pathogens. The observed incidence of respiratory complaints is in line with subjects being biased to recall solicited adverse events (cold-like symptoms) more often, leading to higher reporting rates.[24,25]

We did not observe clear evidence of viral shedding of RSVΔG based on qCulture and qPCR results in nasal wash samples. The presence of viral RNA determined by qPCR was only observed in 8.6% of the subjects (3/36) inoculated with RSVΔG, occurred on day 4 post inoculation and was below the LLOQ. These results further confirm that the RSVΔG vaccine candidate is sufficiently attenuated for testing in the pediatric population. However, the timing and frequency of sampling of nasal washes were tailored towards capturing the viral kinetics of wild-type RSV and other RSV LAVs.[26-28] Although we expected RSVΔG to exhibit similar kinetics, we cannot fully rule out that transient shedding of the RSVΔG occurred in between the pre-determined sampling days. Alternatively, the low incidence of viral shedding could also be due to the presence of pre-existing neutralizing antibodies in healthy adults. Even though we selected adult volunteers with relatively low levels of RSV-specific nAbs, all subjects had pre-existing nAbs due to previous exposure to RSV. Finding low to absent levels of viral shedding after intranasal inoculation with a LAV have been described previously.[29,30] A study investigating a similar vaccine concept *cp-52* (a cold passaged RSV B1 LAV lacking a large part of the coding sequence for both SH and G surface proteins) showed that only 6% (1/17) of healthy adults and ultimately only 13% (2/16) of seronegative children shed virus in nasal washes.[31] The authors concluded that *cp-52* was restricted in replication and appeared to be overattenuated.[31] Many more live attenuated RSV concepts have been evaluated since and the general conclusion is that LAV face the challenge of achieving sufficient attenuation to be safe, while remaining immunogenic enough to induce a protective immune response.[32,33] Live-attenuated RSV vaccines that showed viral replication and immunogenicity in seronegative infants were overattenuated in seropositive children and adults.[28,34,35] Minimal or absent viral shedding in adults and RSV-non naïve children is a prerequisite to proceed to safe vaccine evaluation in RSV-naïve children.[33] To further assess the attenuation phenotype and replication-competence, RSVΔG should be evaluated through age de-escalation in the pediatric population.

Analysis of immunogenicity endpoints showed no apparent signs of induction of local or system immune responses in healthy adults following inoculation with RSVΔG. For many live-attenuated vaccines a minimal level of replication is needed to reach adequate immunogenicity. The poor immunogenicity of RSVΔG in this study may be related to the limited viral replication in healthy adults with pre-existing neutralizing antibodies. Absent and low immune responses in healthy adults volunteers have been described previously for other live-attenuated RSV vaccines candidates.[31,36] It is also possible that the dose of 6.5 $\log_{10}$ $CCID_{50}$ was insufficient to overcome natural immunity and induce an immune reaction in adults.

During this trial we applied the commonly used Naclerio method of nasal washing.[15] This method has proven to be effective for determining mucosal IgA after intranasal inoculation with a live-attenuated influenza vaccine.[37] For this reason, we also expected to detect induction of IgA antibodies following intranasal inoculation with RSVΔG (if it were to occur). However, some trials apply a more stringent method for nasal wash collection. In a study by Ascough et al. the nasal cavity was washed by alternatively withdrawing and advancing the plunger of the syringe 10 times. This study showed detectable levels of mucosal IgA prior to inoculation with a RSV subunit vaccine.[38] Although there are no studies comparing the different nasal wash techniques and the extraction of mucosal antibodies, the mucosal immune response in our trial could potentially be underestimated by our nasal wash approach.

In conclusion, a dose of 6.5 $\log_{10}$ $CCID_{50}$ of RSVΔG was safe and well-tolerated in healthy adults. In this first-in-human study, the live-attenuated genetically modified RSV variant RSVΔG did not shed following inoculation, confirming its attenuation in adults. However, with the tested dose there were no clear signs of induction of an immune response in seropositive adult subjects. Safety and immunogenicity of RSVΔG in a dose of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$ should be further explored in seropositive children and eventually in seronegative infants. In addition, dose-escalation studies may be performed in adults to test whether higher doses of RSVΔG would yield higher rates of immunogenicity, while still having a favourable safety profile.

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TABLE 1 SUBJECT CHARACTERISTICS.

	Groups	
Subject characteristics	RSVΔG (n=36)	Placebo (n=12)
GENDER, N (%)		
Female	33 (91.7)	10 (83.3)
Male	3 (8.3)	2 (16.7)
AGE, YEARS, MEDIAN (IQR)		
	23.5 (20.3, 26.0)	23.5 (21.0, 26.5)
BMI, KG/M², MEAN (SD)		
	22.5 (3.7)	22.8 (3.1)
RACE (N, %)		
White	29 (80.6)	9 (75.0)
Black or African American	1 (4.2)	1 (8.3)
Mixed	5 (10.4)	0 (0)
Asian	1 (4.2)	1 (8.3)
Other	0 (0)	1 (8.3)

BMI= Body Mass Index; IQR: interquartile range.

TABLE 2 SOLICITED ADVERSE EVENTS DURING FIRST 14 DAYS AFTER INOCULATION.

	RSVΔG n=36	Placebo n=12
Symptoms	Number of subjects (%)	Number of subjects (%)
≥1 symptom	29 (80.6)	9 (75.0)
Nasal congestion	11 (30.6)	5 (41.7)
Sneezing	15 (41.7)	5 (41.7)
Rhinorrhea	16 (44.4)	4 (33.3)
Epistaxis	4 (11.1)	–
Coughing	11 (30.6)	2 (16.7)
Sore throat	11 (30.6)	7 (58.3)
Dyspnea	2 (5.6)	2 (16.7)
Eye irritation/complaints	4 (11.1)	–
Earache	2 (5.6)	1 (8.3)
Myalgia/arthralgia	12 (33.3)	4 (33.3)
Malaise	13 (36.1)	6 (50.0)
Fever	1 (2.7)	1 (8.3)

TABLE 3 SUMMARY OF POSSIBLE OR PROBABLE RELATED NON-SOLICITED ADVERSE EVENTS.

	RSVΔG (n=36)	Placebo (n=12)
Adverse events	Number of Subjects (%)	Number of Subjects (%)
Subjects with at least one adverse event	19 (52.8)	7 (58.3)
GENERAL DISORDERS		
Fatigue	1 (2.8)	–
Feeling hot	1 (2.8)	–
INFECTIONS AND INFESTATIONS		
Impetigo	1 (2.8)	–
NERVOUS SYSTEM		
Headache	1 (2.8)	–
RESPIRATORY TRACT		
Nasal congestion	3 (8.3)	1 (8.3)
Sneezing	1 (2.8)	–
Throat lesion	–	1 (8.3)
Upper respiratory tract infection	9 (25)	4 (33)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS		
Dermatitis	–	1 (8.3)
Lip edema	1 (2.8)	–
Herpes simplex	1 (2.8)	–

TABLE 4 MEAN (SD) TITERS OF SERUM RSV-SPECIFIC NEUTRALIZING ANTIBODIES AND F-SPECIFIC ANTIBODIES.

	RSV-specific neutralizing antibodies				F-specific antibodies		
Treatment	n	Baseline (Day −1)	Day 7	Day 28	n	Baseline (Day−1)	Day 28
RSVΔG	36	8.50 (0.87)	8.49 (0.96)	8.45 (1.07)	35	5.42 (0.31)	5.49 (0.36)
Placebo	12	8.28 (1.38)	8.22 (1.40)	8.22 (1.35)	12	5.36 (0.46)	5.49 (0.37)

Mean (SD) RSV-specific neutralizing antibodies are expressed as log_2 titer, mean (SD) F-specific antibodies expressed as log_{10} titer. RSV = respiratory syncytial virus.

FIGURE 1 STUDY DESIGN.

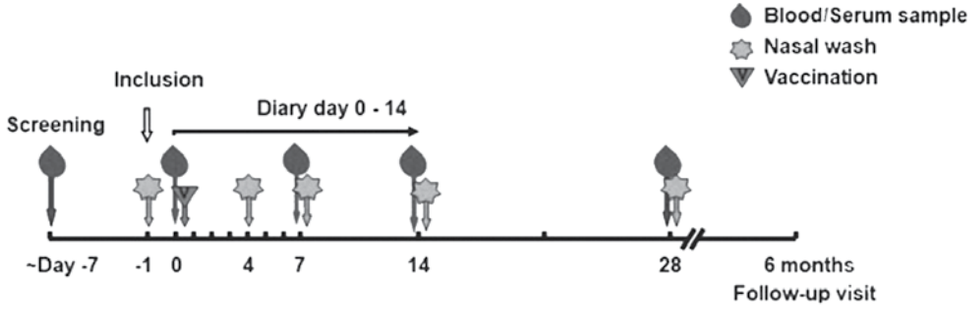


FIGURE 2 CONSORT SUBJECT FLOW DIAGRAM.

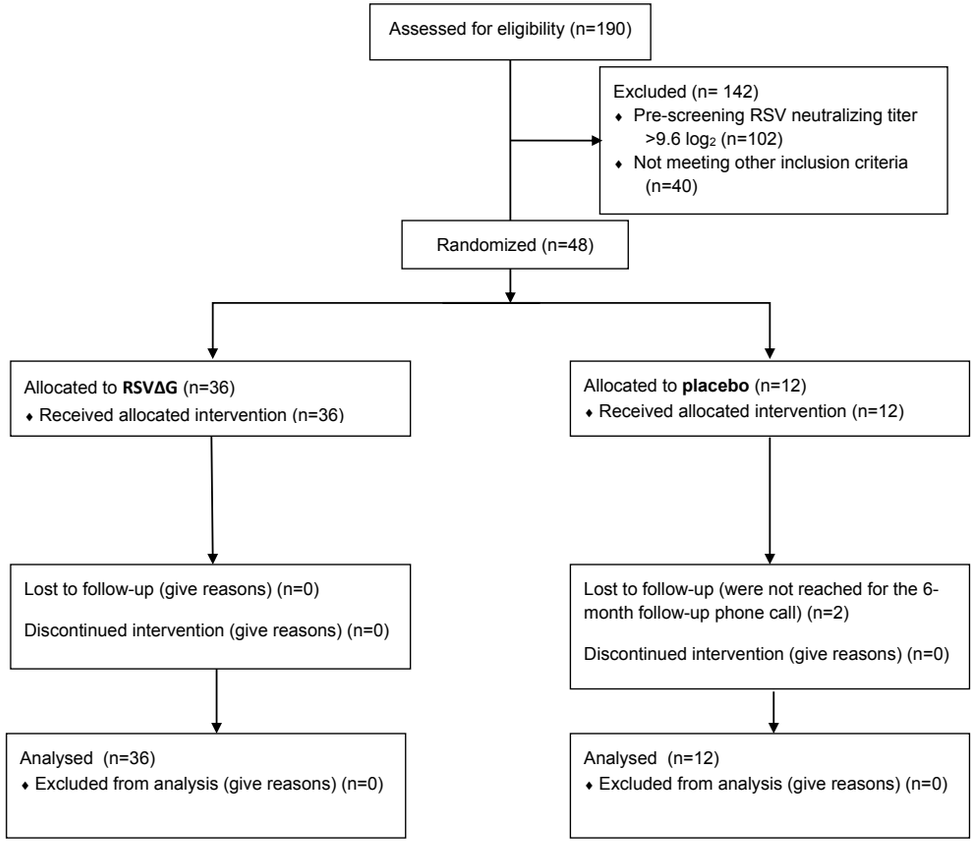
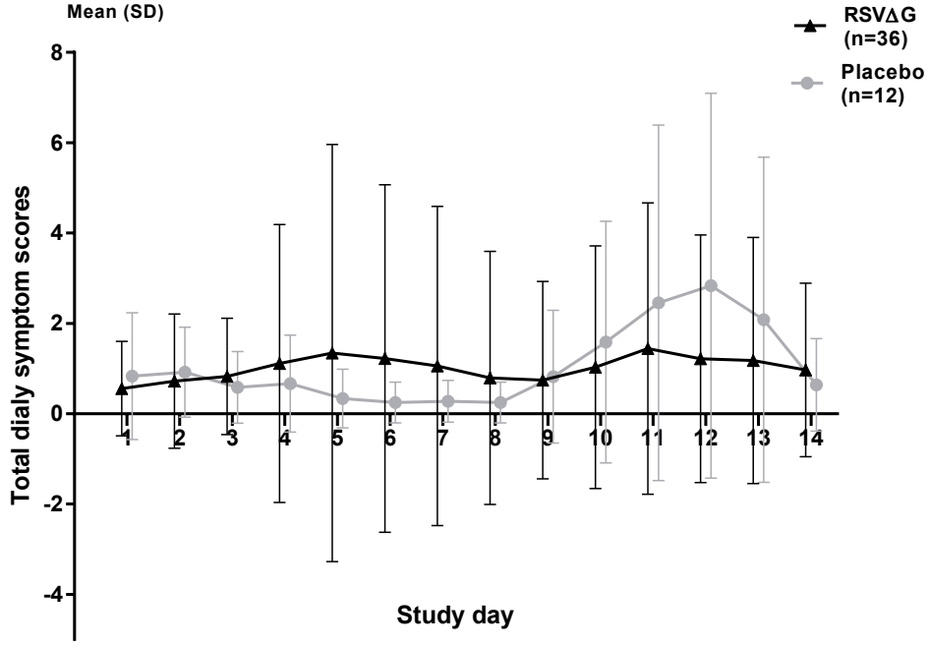
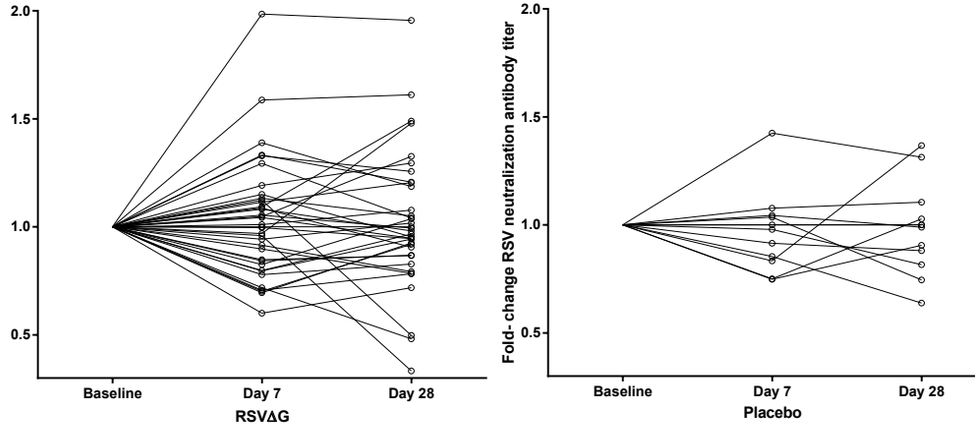


FIGURE 3 MEAN AND SD OF TOTAL SYMPTOM SCORES (RANGE 0-32) DURING DAYS 1-14 FOR RSVΔG AND PLACEBO TREATMENT.



SD = standard deviation.

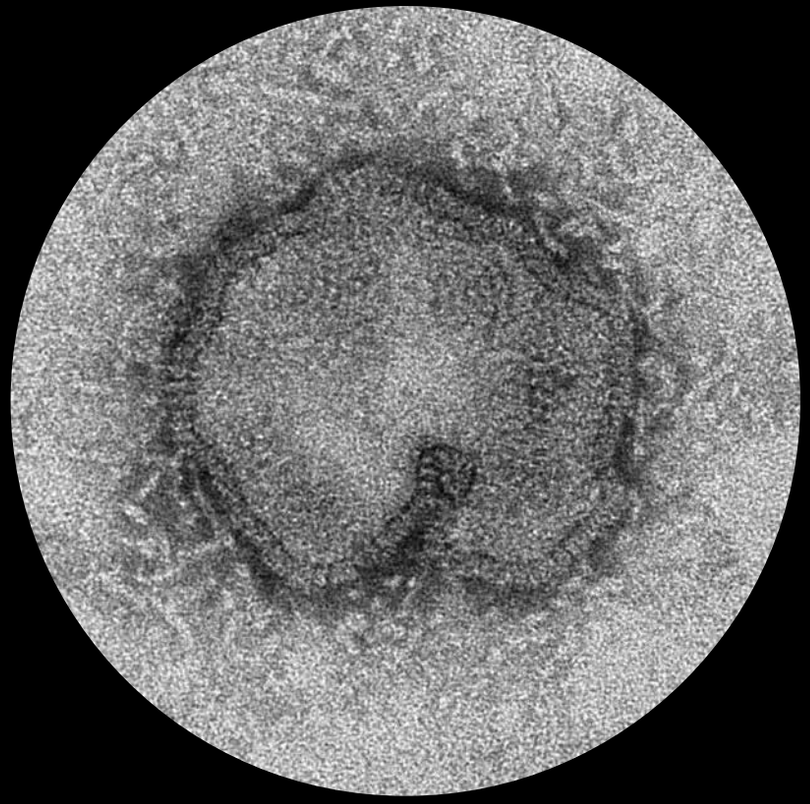
FIGURE 4 FOLD-CHANGE IN RSV NEUTRALIZATION ANTIBODY TITER, DAY 7 AND DAY 28 POST-INOCULATION VERSUS BASELINE. (a) Fold- change in RSVΔG group (n=36). b) Fold- change in placebo group (n= 12).



RSV = respiratory syncytial virus.

SUPPLEMENTARY FIGURES AND TABLES

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.



Section 2

INFLUENZA VIRUS

CHAPTER 4

SAFETY, REACTOGENICITY AND IMMUNOGENICITY OF AN INTRANASAL SEASONAL INFLUENZA VACCINE ADJUVANTED WITH GRAM-POSITIVE MATRIX (GEM) PARTICLES (FLUGEM): A RANDOMIZED, DOUBLE-BLIND, CONTROLLED, ASCENDING DOSE STUDY IN HEALTHY ADULTS AND ELDERLY

submitted

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Abstract

BACKGROUND Intranasal administration of respiratory vaccines offer many advantages such as eliciting both systemic and mucosal immunity at the point of viral entry. Immunogenicity of current intranasal vaccines can be improved by using novel adjuvants. However, there is a shortage of safe and immunogenic adjuvants for intranasal vaccination. Bacterium-like particles (BLP) derived from Lactococcus lactis have the potential to serve as a vaccine adjuvant. Preclinical studies indicated safe immune boosting properties. This clinical study investigated the safety, reactogenicity and immunogenicity of intranasal seasonal influenza vaccine adjuvanted with Gram-positive matrix particles (FluGEM).

METHODS This was a first-in-human, randomized, double-blind, controlled, dose-escalation study performed at the Centre for Human Drug Research, Leiden, NL. Participants aged 18-49 years were randomized in a 3:1 ratio to receive FluGEM in either 1.25, 2.5 and 5.0 mg BLP in a two-dose regimens (Day 1 and 21) together with standard trivalent inactivated influenza vaccine (TIV: B, H1N1 and H3N2) or unadjuvanted TIV only (control group). Primary outcomes were safety and tolerability of intranasal FluGEM assessed by solicited (up to Day 7) and unsolicited (up to Day 42) adverse events, vital signs and laboratory safety tests (biochemistry, hematology and urinalysis). Secondary endpoints were serum hemagglutination inhibition (HI) titers and IgA in nasal fluids. Descriptive statistics were used to summarize data.

RESULTS Sixty participants were included. All intranasal doses of FluGEM were safe and well tolerated. Adverse events were rated as mild (97.4%), moderate (1.3%) and severe (1.3%). There were no signs of dose-limiting toxicities, neurotoxicity or serious adverse events. The occurrence of adverse events (solicited and unsolicited) was comparable across all dose levels (range 86.7-100%) and control group (93.3%). All groups showed ≥ 2.5-fold HI geometric mean increases. Seroconversion rates were highest in the 1.25 mg and 2.5 mg group (>40% of participants, *Table 1*). Seroprotection was best achieved by the 2.5 mg dose across for three vaccine strains (≥70%). All groups showed increased IgA levels following vaccination. Highest geometric means were observed in the 1.25 mg group on day 21 (146), this effect was more pronounced in subjects with no pre-existing IgA levels (187).

CONCLUSIONS Intranasal vaccination of FluGEM® was safe and tolerable in healthy adult volunteers aged 18-49 years. Interestingly, the highest immunogenicity was observed for 1.25 mg and 2.5 mg doses (compared to 5 mg) suggesting a potential non-linear dose-response relationship.

Introduction

It has been estimated that seasonal influenza causes roughly between 290.000-650.000 deaths annually worldwide.[1] Risk of serious flu-related complications and mortality are highest in children younger than 5 years of age and adults above 65 years.[2,3] While there have been vaccines available against influenza for many decades there are still challenges to overcome. Eliciting effective and lasting vaccine-induced immune responses in high-risk groups may be complicated; in young children caused by immature immune systems and in elderly people by immunosenescence.[4-6]

Immunogenicity of currently available vaccines can potentially be improved through the use of adjuvants. Most currently licensed seasonal vaccines consist of trivalent or quadrivalent inactivated influenza viruses and are commonly intramuscularly administered to elicit a systemic immune response. Since the outbreak of the covid-19 pandemic there has been a renewed interested in intranasal vaccines for respiratory viruses as immunity at the mucosa might better prevent transmission since the infection may be halted at the point of entry.[7]

Especially for influenza, due to the constant antigenic drift and pandemic threat associated with antigenic swift, there is a urgent need for vaccination strategies that improve cross-protection against heterologous strains. Mucosal IgA elicited from intranasal immunization has the potential to provide cross-protection against heterologous and drifted strains.[8,9] In addition, intranasal vaccination has the advantages of patient-friendly needle-free administration with greater capacity for mass immunizations compared to the traditional intramuscular route.[10]

Currently, only live-attenuated vaccines are licensed for intranasal administration (Fluenz Tetra/Flumist Quadrivalent, MedImmune, Gaithersburg, us), however, its use is limited to 2-18 year olds due to higher incidence of hospital admission and wheezing in children <2 years and lower efficacy in adults compared to injected influenza vaccines.[11] Adjuvants can help to boost immunogenicity of intranasal vaccination approaches.[12] Enterotoxin proteins, including *Escherichia coli* heat-labile toxin and derivatives, have previously shown to be potent and efficacious mucosal adjuvants but their use have been associated with facial nerve paralysis.[13,14]

Alternative safe and potent mucosal vaccine adjuvants are thus highly needed and Gram-positive Enhancer Matrix (gem) may be a candidate. gem adjuvant is composed of non-living bacterium-like particles (BLPs) derived from the lactic acid bacterium (lab) *Lactococcus lactis*, a food-grade non-pathogenic Gram-positive bacterium that does not produce endotoxins and does not colonize the human mucosal cavities. gem are BLPs that consist of a peptidoglycan outer surface.[15,16] Peptidoglycan is known to have immunostimulant properties and is presumed to play an important role in the observed adjuvant properties of gem.[17,18] Studies in mice showed that intranasal administration of gem mixed with influenza antigen (FluGEM) was safe and elicited hemagglutination inhibition (hi) titers equivalent to intramuscular vaccination after one booster dose.[19] Moreover, intranasal FluGEM administration yielded a strong mucosal IgA response, was fully protective in homologous and heterologous influenza challenge models in mice, with better protection rates compared to non-adjuvanted influenza vacation.[19]

Here, we present the results from a first-in-human clinical trial that had the objective to assess the safety, tolerability, reactogenicity and immunogenicity of intranasal vaccination with FluGEM in healthy adults and elderly (aged 65 and older).

Material and methods

STUDY DESIGN

This was a first-in-human, randomized, double-blind, controlled, dose-escalation study performed at the Centre for Human Drug Research (chdr), Leiden, nl. Participants were randomized in a 3:1 ratio to receive nasally FluGEM (gem-adjuvant with trivalent inactivated influenza vaccine [tiv]: B, A/h1n1 and A/h3n2 strains) or tiv only (control group). Randomization codes were generated by a statistician in sas V9.1.3 for Windows (sas Institute, Cary, nc, usa). Both study staff and subjects were blinded for the treatment allocation.

A staggered-enrollment strategy was used for the dose-escalation part of the study (age 18-49 years). In every dose level 4 subjects (3 active: 1 control) were vaccinated and followed-up until at least 7 days post-vaccination after which a preliminary safety assessment was made and predefined halting rules (*Supplementary Appendix*) were checked prior to the enrollment of an additional 16 (12 active: 4 control) participants. An independent safety monitoring committee (smc) was to be consulted if one of the halting criteria was met. In addition, the smc decided upon the selection of the intranasal doses to be tested in a subpopulation of elderly subjects aged 65 years or older.

The study was approved by the Central Committee on Research Involving Human Subjects (CCMO), NL (EudraCT 2010-024346-30). All study-related procedures were performed in accordance with the Declaration of Helsinki and the Dutch Act regarding Medical Research Involving Human Subjects. All subjects provided informed consent in writing prior to study activities.

PARTICIPANTS

All participants underwent a full medical screening prior to enrollment. Male and female volunteers were included if they were 18-49 years of age (*part 1*), or 65 years or older (*part 2*) and overtly healthy according to the medical screening procedure. Participants were excluded if they received an influenza vaccine that same year, had HI titers >1:10 against two or more vaccine strains (B, H1H1 or H3H2) or suffered from moderate or severe illness 72 hours prior to the planned nasal FluGEM or TIV vaccination (fever ≥ 38°C or determined by the investigator). A full list of eligibility criteria is provided as *Supplementary Material*.

VACCINE FORMULATIONS AND NASAL ADMINISTRATION

FluGEM was administered intranasally and consisted of 1.25 mg, 2.5 mg and 5.0 mg doses of the GEM-adjuvant in conjunction with a standard TIV antigen dose (VAXIGRIP, 15 µg of A/California/7/2009 [H1N1], 15 µg of A/Perth/16/2009 [H3N2] and 15 µg of B/Brisbane/60/2008). FluGEM was administered as a two-dose regimen with a 21-day interval between first and second dose. The control group received nasally the TIV antigen only, diluted in phosphate buffered saline solution, in the same dose regimen as the FluGEM groups. FluGEM and control vaccine formulations were indistinguishable. Study treatments were administered by a trained physician using a disposable pipette to instill droplets of the vaccine in both nostrils while the subject remained in supine position.

SAFETY AND TOLERABILITY ASSESSMENT

The primary objective of the study was to assess safety and tolerability of intranasal doses of FluGEM. Routine laboratory safety assessments (blood biochemistry, hematology and urinalysis) were performed at screening, day 21 and day 42. Vital signs were measured prior to vaccination administration, 30 and 60 minutes after vaccination. Subjects remained in the clinical unit for at least 60 min for monitoring of any untoward medical event and were subsequently discharged if they had no adverse events, events were resolved or per discretion of the study physician. During the first 7 days post-vaccination, subjects recorded the occurrence of any solicited local or systemic solicited adverse events (*Supplementary Appendix*) and measured body temperatures daily on a diary card. Unsolicited adverse events were monitored up to day 42 and afterwards subjects were monitored for serious adverse events only (until day 210).

IMMUNOGENICITY ASSESSMENTS

Systemic immunity: hemagglutination inhibition assays

Sampling times throughout the study are depicted in *Figure 1*. Presence of antibodies against hemagglutinin (of each of the respective vaccine components) were assessed in sera by a HI assay performed by Viroclinics Biosciences B.V., NL. In short, serial two-fold dilutions of serum samples (pre-treated to remove non-specific anti-HI activity) and quality control sera were incubated with the Haemagglutin antigen suspension (previously titrated to adjust the dilution to 4 Haemagglutination Units / 25 µl). After 30 minutes incubation at 37±1°C, 25 µl of 1% (v/v) turkey erythrocytes were added in each plate, and further incubated for 1 hour at 4±1°C. Duplicate plates were scored independently by two technicians. The serum titer was defined as the highest dilution that showed complete inhibition.

Mucosal immunity: IgA in nasal fluid

Nasal fluid was collected by gently instilling 4 ml of sterile solutions of phosphate buffered saline (PBS), at a temperature of 37°C, into each nostril. Subjects were instructed to keep the solution in the nose for at least 20 seconds (with their neck extended approximately 45°) after which the nasal fluid was collected on a Petri dish. Nasal fluid material was subsequently transferred to conical polystyrene tubes and centrifuged for 10 minutes (360 g) at 4°C. Nasal IgA concentrations were measured by ELISA at Texcell, France. Six three-fold dilutions of the first 1:75 dilution of the nasal washes (or control samples) were added to empty wells that were previously incubated for 18 hours at 5±3°C to coat with influenza ANTIGENS matching the TIV hemagglutinins (50 µl of a 1 µg/ml solution). After 1 hour incubation at 37±2°C, 50 µl of anti-human IgA peroxidase substrate was added and incubation was continued for 95 min at 37±2°C. A peroxidase substrate solution (50 µl) was added and the reaction was stopped after 20 minutes at 37±2°C by adding H_2SO_4. Optical density at 450 nm (OD_{450}) was measured and IgA values were read against the standard curve of the ELISA.

Exploratory: serum IgG and subclass determination

As exploratory endpoint antigen-specific serum concentrations of total IgG and IgG subclasses were determined (age group 18-49 only). Serum IgG, and IgG1 and IgG3 subtypes were measured by ELISA at Texcell, France. Six three-fold dilutions of the first 1:50 dilution of the serum (or control samples) were added to wells coated with influenza antigens matching the TIV haemagglutinins as described above. After 1 hour at 37±2°C, 50 µl of anti-human IgG (or IgG1 or IgG3) peroxidase substrate was added and incubated for 95min at 37±2°C. A peroxidase substrate solution (50 µl) was added and the REACTION was stopped after 20 minutes at 37±2°C by adding H_2SO_4. The OD_{450} was measured and IgG, IgG1 and IgG3 values were read against a standard curve of the ELISA.

STATISTICAL ANALYSES

This phase I study utilized group sizes that were conventional for early phase trials but was not powered to test a pre-defined hypothesis. Descriptive statistics were used for safety data. For immunogenicity parameters, geometric mean titers (GMT) and associated 95% Confidence Intervals (CI), standard deviation and/or coefficient of variation and ratio's (GMR) were calculated. The following correlates of seroprotection were determined: 1) the proportion of subjects in each group exhibiting seroconversion on days 21 and/or 42, defined as either a four-fold rise in post-vaccination antibody HI titers compared to baseline HI, or a postvaccination titer ≥ 1:40 in subjects with baseline titer <1:10; 2) the proportion of subjects in each group exhibiting seroprotection defined as HI ≥ 1:40; GMT increase defined as GMT ratio compared to baseline (GMR ≥2.5).[20] SAS for windows V9.1.3 (SAS Institute, Inc., Cary, NC, USA) was used for data analysis.

Results

STUDY POPULATION AND BASELINE CHARACTERISTICS

The study was executed from 2011 till 2012. Sixty eligible subjects were included for the initial 18-49 years age group (*part 1*). All subjects completed the two-dose regimen of FluGEM or TIV, except for 3 individuals. Two subjects in the 1.25 mg group did not receive the second dose and a third was lost in the follow-up. Another subject in the 1.25 mg group had an intermittent severe adverse event (food allergy, also see 3.2) 6 days after the first administration and was withdrawn per protocol. In the 2.5 mg group a subject did not receive a second dose due to recurrent epistaxis. Baseline characteristics (*Table 1*) were overall similar for the dose groups. Subsequently, 30 elderly subjects were included to receive a selected dose of 1.25 mg FluGEM (n =15) or TIV only as control group (n=15). In the elderly group there was was a slight predominance of female subjects in the control group (53.3%) compared to the FluGEM group (33.3%). All elderly subjects received two intranasal doses of FluGEM or control treatment.

SAFETY AND TOLERABILITY EVALUATION

Age group 18-49 years

All doses of FluGEM were well tolerated and there were no signs of increased reactogenicity following the second dose of FluGEM. The percentage of subjects reporting ≥1 treatment emergent adverse events (either solicited or unsolicited) was comparable across all FluGEM groups (86.7-100%) and this was similar in the control group (93.3%). The vast majority of adverse events were mild (97.4%), there were two cases of moderate influenza-like illness in the 1.25 mg group that were self-limiting, two subjects had severe adverse advents: one subject had a concussion following an unrelated traumatic injury, another subject developed an anaphylactic reaction shortly after eating Thai food, 6 days after the first vacation (1.25 mg group). Per protocol halting rules, the subject did not receive a second vacation due to an intermittent severe adverse event and was withdrawn. The most frequent unsolicited adverse events were respiratory complaints, most frequently being throat irritation (22% in active groups; 27% in the control group). Epistaxis was reported with a low incidence (6.7%–13.3%) in both control, 1.25 mg and 2.5 mg groups, but not in the highest 5.0 mg dose group, suggesting no apparent dose-related effect. There were no serious adverse events (SAE) observed.

Solicited adverse events did not increase with increasing doses of FluGEM (*Table 2*). The frequency of solicited adverse events following FluGEM administration was comparable to the control group, with headache being reported most frequently (67%) in both the control and the 2.5 mg group. There were no signs of dose-limiting toxicities or neurotoxicity. No findings of clinical concern in blood chemistry, hematology and urinalysis assessments were observed.

Age group 65 and older

The frequency of treatment emergent adverse events in the elderly group was comparable to that of the 18-49 age group (86.7 %) as was the nature of the adverse events. Two unrelated SAEs occurred in the FluGEM (1.25 mg) dose group: one subject (age: 67) had a myocardial infarction (8 days following the second vaccination) and needed percutaneous coronary intervention with stent placement due to atherosclerosis; another subject (age: 67) developed sinus node dysfunction (3 months after the second vaccination) for which pacemaker implementation was needed. All other adverse events reported in the elderly group were mild, except for a single case of moderate gastro-enteritis that was unrelated to the vaccination.

Solicited local and systemic adverse events in the FluGEM group were similar to that in the control group by nature and frequency. Notably, sneezing was reported more often in the elderly age group (60% and 73.3%, control and FluGEM 1.25 mg group, respectively) compared to the 18-49 years cohorts. Overall, FluGEM was well tolerated in participants aged 65 and older.

IMMUNOGENICITY

Systemic antibody response (18-49 years)

Baseline HI titers against B, H1N1 and H3N2 showed a broad distribution across treatment groups (*Supplementary Table S1*) in the 18-49 years age group. Seronegativity (HI titer < 1:40) at baseline varied per strain, with percentages comparable across dose groups (range: 80-93% [B], 53-67% [H1N1], and 60-73% [H3N2]). FluGEM doses of 1.25 mg and 2.5 mg showed a more rapid increase and higher magnitude of HI titers for the B strain and H1N1 strain compared to the control group (*Table 3*).

All treatment groups had GMT fold increases ≥ 2.5 post-vaccination for all three influenza strains (Table 3). For the B strain GMRs were highest following vaccination with 1.25 mg and 2.5 mg FluGEM (approximately 2 times higher than the GMRs of the control). For the H1N1 strain the increase in GMRs following FluGEM vaccination on day 21 and 42 were comparable to the control group. However, GMRs were markedly higher at day 210 in the 1.25 mg and 2.5 mg FluGEM dose groups for both the B strain and A/H1N1 strain (GMR of 7.7 and 5.7 [1.25 mg] and 8.8 and 4.9 [2.5 mg] versus 4.8 and 3.9 [control group] for the B and A/H1N1 strain, respectively). All formulations, including the non-adjuvanted control group, showed very strong HI responses to the H3N2 strain. FluGEM adjuvanted doses did not elicited higher GMRs compared to the non-adjuvanted control group for the A/H3N2 strain on the investigated time points.

In the study population as a whole, seroconversion in ≥ 40% of subjects was achieved at both day 21 (single dose) and day 42 (two-dose) in the 1.25 mg for all tested strains and at day 42 (two-dose) only for the 2.5 mg dose (*Table 4*). The non-adjuvanted control group fulfilled this criterion for the H3N2 strain only. Seroconversion rates were higher for seronegative subjects. In the seronegative subpopulation both the 1.25 mg and 2.5 mg (but not the 5 mg group) and the unadjuvanted control group had seroconversion rates ≥ 40% for all strains (*Supplementary Table S2*).

Seroprotection was highest for the influenza A strains (H1N1 and H3N2) with all treatment groups reaching protection rate of ≥ 70% (*Table 5*). The highest seroprotection rate for the B strain was achieved with the 1.25 mg FluGEM dose level with a seroprotection rate of 73% on day 21 and 64.3 on day 42. Seroprotection rates were consistently lower for subjects seronegative at baseline (*Supplementary Table S3*).

Nasal IgA response (18-49 years)

In the control group, 2.5 mg and 5 mg FluGEM group, the majority of subjects had pre-vaccination IgA titers below the detection limit (>73% of subjects per group). However, in the 1.25 mg dose group only six subjects had IgA below this limit (*Table 6*). All groups showed increased nasal IgA levels following vaccination. Highest IgA GMRs were observed in the 1.25 mg FluGEM dose group on day 21 (ratio: 1.8). This effect was more pronounced in subjects with non-detectable IgA levels at baseline (GMR: 3.7 and 3.2 on day 21 and 42, respectively). At day 210, IgA returned to baseline levels in all treatment groups.

Total IgG, IgG1 and IgG3 subclasses (18-49 years)

Total influenza specific IgG in serum and IgG1 and IgG3 subclasses are listed in *Table 7*. Total IgG, IgG1 and IgG3 increased in all treatment groups following the first vaccination, FluGEM-adjuvanted dose groups showed a relatively faster peak titer following the first vaccination, effects on total IgG following a second vaccination were less pronounced in the FluGEM-adjuvanted groups. All treatment groups showed a IgG1 dominant response following vaccination, with highest IgG1/IgG3 ratio's being observed in the 1.25 mg dose group (data not shown).

Immunogenicity: age group 65 and older

Following interim-analysis of safety and immunogenicity data of the previous dose levels in 18-49 year old's, the 1.25 mg dose was selected by the SMC to be assessed in elderly subjects, as this dose showed overall the best immunogenicity profile. In general, systemic immunogenicity to both the non-adjuvanted inactivated trivalent vaccine (control group) and 1.25 mg of FluGEM was less pronounced than in the 18-49 year groups (*Supplementary Table S4*). Seroconversion and protection rates were similar for both control and 1.25 mg FluGEM (*Supplementary Tables S5* and *S6*). Criteria for 40% seroconversion and 70% seroprotection were not fulfilled in both dose groups. At baseline there was a difference with more subjects (93%) having IgA titers below the lower limit of detection in the control group compared to the 1.25 mg FluGEM group (64%). No apparent treatment effects on nasal IgA GMTs and ratios were observed in subjects ≥ 65 years (*Supplementary Table S7*).

Discussion

In this study FluGEM – trivalent inactivated influenza vaccine adjuvanted with GEM particles – was administered for the first time in humans via the intranasal route. We found that all explored intranasal doses (up to 5 mg in the 18-49 years group and 1.25 mg in elderly) were well tolerated. The frequency and intensity of adverse events following FluGEM vaccination were comparable to that of the unadjuvanted TIV.

In the 18-49 years age group, favorable effects of FluGEM on the humoral systemic immune response were observed compared to unadjuvanted TIV, particularly the 1.25 mg and 2.5 mg dose levels yielded the highest HI titers overall against multiple influenza strains. Several parameters for serological protection were used for the evaluation of vaccine immunogenicity. Geometric mean titer (GMT) increases ≥ 2.5-fold were observed in all treatment groups (including the control group). However, in the 1.25 mg FluGEM group seroconversion (≥ 40%) for all strains was achieved after only a single dose. Seroconversion against all strains was also achieved in the 2.5 mg FluGEM group, but after two doses. Seroprotection (≥70%) for all strains was only observed for the 2.5 mg dose (day 21 only). Historically, one out of three of these criteria should be met for annual vaccination with seasonal inactivated vaccines.[20] FluGEM doses performed better for both seroconversion and seroprotection criteria compared to the unadjuvanted intranasal trivalent vaccine (control), signifying the potential adjuvant function of FluGEM for these endpoints. In addition, FluGEM appears to elicit a more persistent systemic humoral response for both B and H1N1 strains (but not for H3N2) when HI GMT ratios at day 210 are compared to the control group.

Adjuvant effects of FluGEM on mucosal IgA were most pronounced in subjects in the 1.25 mg dose group with no pre-existing nasal IgA titers, with peak IgA titers following the first vaccination (GMT ratio: 3.7). Although there is no well-established correlate of protection for mucosal IgA, especially against currently circulating influenza strains, the magnitude of IgA responses (GMT and fold-increases in IgA) in the present study were in a similar-to-higher range than IgA levels that were considered protective in previous human challenge studies with influenza.[21,22] Eliciting sufficient IgA responses is essential for a mucosal influenza vaccine candidate, as mucosal IgA can neutralize virus at the mucosal interface before viral entry and clear the virus from respiratory epithelial cells, preventing downstream adverse host immune responses and possibly direct transmission.[8,23] These effects are not readily expected from intramuscular vaccines that do not elicit mucosal IgA responses.

We found that in the 18-49 year population FluGEM intranasal doses of 1.25 mg and 2.5 mg were more immunogenic than the highest dose level of 5 mg. In a first-in-human study of SynGEM – an intranasal vaccine candidate based on the same bacterium-like particle platform with recombinant RSV F-protein as primary antigen attached to the BLP – higher serum IgG titers were achieved following boosting of the low dose group compared to a high-dose regimen.[24] However, for other endpoints the higher dose appeared more immunogenic. It is assumed that the mode of action of GEM is through toll-like receptor (TLR)2 signaling.[25] While TLR2 activation most often shows dose-dependent downstream effects, non-linear 'bell-shaped' dose-response relationships have been described for other TLR2 agonists.[26] The exact immunological basis for the dose-response relationship of FluGEM remains unknown and to be explored in future studies.

Intranasal administration of FluGEM was also well tolerated in elderly subjects (≥ 65 years). The explored 1.25 mg dose did not have as pronounced immune effects in elderly compared to the 18-49 years population. Mucosal immunization of the elderly population remains a well-known

challenge for mucosal vaccine candidates and is likely due to immunosenescence. Animal data suggest that nasal IgA responses to immunization are likely to be diminished by age-related decline of the immune system.[27,28] Even following controlled human infection challenge with a respiratory virus, nasal IgA production can be defective in the elderly population.[29] In addition to immunosenescence, anatomical age-related changes to the nose such as mucosal atrophy and increase size of the nasal cavity may also challenge the adequate delivery of intranasal vaccines.[30] Further dose-exploration in the elderly, optimalization of vaccine formulation for instance using mucoadhesive agents to enhance the vaccine residence time and increase uptake of active compounds, or delivery systems could be explored to improve immunogenicity in this specific population.[31]

The study's eligibility criteria allowed only for inclusion of volunteers with relatively low HI serum titers against the influenza strains that were present in the trivalent vaccine. High levels of pre-existing antibodies may negatively impact the magnitude of the fold-increase in serum antibody titers following vaccination.[32] In this study, pre-existing levels of mucosal antibodies were not implemented as eligibility criteria. However, pre-existing mucosal immunity could, analogous to serum responses, impair the magnitude of the mucosal vaccine response. In subjects without pre-existing IgA titers, the 1.25 mg dose of FluGEM had a markedly higher magnitude of increase. Although limited by the sample size, these results suggest a potential for the GEM-adjuvanted intranasal vaccine platform to induce mucosal immunity against drifted strains or novel pathogens for which there is no pre-existing immunity.

Some limitations of the study should be noted. The study population had a highly immunogenic response the H3N2 strain, illustrated by already high HI titers following vaccination with the plain trivalent vaccine. Interestingly, influenza A/H3 strains did not circulate on large scale during the preceding annual flu epidemic in The Netherlands, nor in the year before.[33] Adjuvant effects of FluGEM on the H3/N2 strain in this study could be blunted by the already pre-existing strong immunogenic response to the primary vaccine antigen. While the group sizes in this study were conventional for phase I vaccine trials, the study was not powered to test pre-defined hypothesis on immunogenic endpoints, larger immunogenicity trials are needed for formal statistical interference on the observed adjuvant effects of FluGEM. Since the completion of this study, newer editions of vaccine guidelines have suggested a more elaborate assessment of immunogenicity including functional antibodies determined by virus neutralization assays and cellular immunity for influenza vaccine.[34] Future studies will need to be performed to further investigate the immunogenicity of FluGEM.

In conclusion, this study described the first-in-human administration of FluGEM, all explored intranasal doses were safe and well-tolerated with highest immunogenicity observed for the 1.25 mg and 2.5 mg doses in subjects between 18-49 years of age. Further research is warranted to assess immunogenicity of intranasal FluGEM in next phase clinical trials and in targeted subpopulations.

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TABLE 1 BASELINE CHARACTERISTICS.

	Age group 18-49				Age group 65 years and older	
Characteristics	Control group n=15	1.25 mg FluGEM n=15	2.5 mg FluGEM n=15	5.0 mg FluGEM n=15	Control group n=15	1.25 mg FluGEM n=15
AGE–YEARS						
Mean (SD)	30.0 (10.4)	27.5 (9.0)	28.5 (8.3)	25.6 (7.9)	71.1 (3.3)	69.9 (4.1)
Range	18-47	20-47	19-46	20-46	67–78	65–78
SEX–N (%)						
Female	9 (60.0)	10 (66.7)	10 (66.7)	9 (60.0)	8 (53.3)	5(33.3)
BMI, kg/m², mean (SD),	24.1 (2.9)	23.4 (2.2)	23.8 (3.3)	22.9 (2.64)	25.1 (2.6)	26.7 (2.5)
RACE OR ETHNIC GROUP–N (%)						
Asian	0	1 (6.7)	0	0	0	0
Black	1 (6.7)	1 (6.7)	1 (6.7)	0	0	0
Hispanic	0	1 (6.7)	0	0	0	0
White	13 (86.7)	11 (73.3)	13 (86.7)	14 (93.3)	15 (100)	15 (100)
Mixed	1 (6.7)	1 (6.7)	0	1 (6.7)	0	0
Other	0	0	1 (6.7)	0	0	0

TABLE 2 SOLICITED LOCAL AND SYSTEMIC ADVERSE EVENTS.

	Age group 18-49				Age group 65 years and older	
Adverse events, n (%)	Control group n=15	1.25 mg FluGEM n=15	2.5 mg FluGEM n=15	5.0 mg FluGEM n=15	Control group n=15	1.25 mg FluGEM n=15
Nasal discomfort	1 (6.7)	1 (6.7)	1 (6.7)	2 (13.3)	2 (13.3)	1 (6.7)
Sneezing	2 (13.3)			2 (13.3)	9 (60.0)	11 (73.3)
Nasal congestion	4 (26.7)	4 (26.7)	5 (33.3)	4 (26.7)	3 (20.0)	2 (13.3)
Runny nose	6 (40.0)	2 (13.3)	3 (20.0)	5 (33.3)	6 (40.0)	4 (26.7)
Loss of smell					1 (6.7)	
Red eyes					1 (6.7)	
Lacrimation			1 (6.7)		1 (6.7)	2 (13.3)
Facial swelling						
Nasal pain						
Headache	10 (66.7)	7 (46.7)	10 (66.7)	7 (46.7)	4 (26.7)	5 (33.3)
Malaise		1 (6.7)	1 (6.7)		3 (20.0)	2 (13.3)
Myalgia	1 (6.7)	2 (13.3)	2 (13.3)	1 (6.7)	3 (20.0)	3 (20.0)
Chills		1 (6.7)	1 (6.7)		1 (6.7)	
Nausea			3 (20.0)		3 (20.0)	1 (6.7)
Vomiting		1 (6.7)			1 (6.7)	1 (6.7)

TABLE 3 HEMAGLUTININ INHIBITON GEOMETRIC MEAN TITERS AND RATIO TO BASELINE TITERS IN SUBJECTS WITH DATA AVAILABLE AT ALL TIME POINTS.

FluGEM dose	Study Day	N	GMT B strain	GMR B strain	N	GMT A/H1N1 strain	GMR A/H1N1 strain	N	GMT A/H3N2 strain	GMR A/H3N2 strain
TIV only (control)	0	13	10.5	1.0	11	10.4	1.0	15	13.2	1.0
	21	13	29.9	2.8	11	49.5	4.8	15	197.2	14.9
	42	13	32.4	3.1	11	53.8	5.2	15	243.7	18.5
	210	13	50.6	4.8	11	40.8	3.9	15	179.5	13.6
1.25mg FluGEM	0	10	8.5	1.0	11	23.2	1.0	12	23.1	1.0
	21	10	48.1	5.7	11	118.0	5.1	12	261.9	11.3
	42	10	47.8	5.6	11	109.9	4.7	12	287.0	12.4
	210	10	63.9	7.6	11	131.7	5.7	12	244.3	10.6
2.5mg FluGEM	0	14	8.6	1.0	12	15.9	1.0	14	18.3	1.0
	21	14	54.0	6.3	12	78.7	5.0	14	152.5	8.3
	42	14	56.9	6.6	12	88.7	5.6	14	179.8	9.8
	210	14	75.9	8.8	12	78.0	4.9	14	141.3	7.7
5mg FluGEM	0	13	7.5	1.0	12	15.5	1.0	14	17.5	1.0
	21	13	28.7	3.8	12	59.0	3.8	14	177.3	10.1
	42	13	30.3	4.0	12	82.3	5.3	14	228.5	13.0
	210	13	53.7	7.2	12	83.0	5.4	14	187.2	10.7

GMR=geometric mean titer ratio to baseline; GMT= geometric mean titer; TIV=trivalent inactivated influenza vaccine.

TABLE 4 SEROCONVERSION RATES, 18–49 YEARS.

FluGEM dose	B strain				A/H1N1 strain				A/H3N2 strain			
	Day 21		Day 42		Day 21		Day 42		Day 21		Day 42	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
TIV only (control)	5/13	38.5	5/13	38.5	5/14	35.7	5/14	35.7	12/15	80.0	13/15	86.7
1.25mg FluGEM	5/12	41.7	6/12	50.0	6/13	46.1	6/13	46.1	7/13	53.8	7/13	53.8
2.5mg FluGEM	8/15	53.3	6/14	42.8	5/15	33.3	6/14	42.8	8/15	53.3	8/14	57.1
5mg FluGEM	4/13	30.8	5/14	35.7	4/14	28.6	5/14	35.7	9/14	64.3	10/14	71.4

TIV=trivalent inactivated influenza vaccine.

TABLE 5 SEROPROTECTION RATES, 18–49 YEARS

FluGEM dose	B strain				A/H1N1 strain				A/H3N2 strain			
	Day 21		Day 42		Day 21		Day 42		Day 21		Day 42	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
TIV only (control)	8/13	61.5	8/13	61.5	10/14	71.4	10/14	71.4	14/15	93.3	15/15	100
1.25mg FluGEM	7/12	58.3	7/12	58.3	10/13	76.9	10/13	76.9	13/13	100	13/13	100
2.5mg FluGEM	11/15	73.3	9/14	64.3	12/15	80.0	13/14	92.8	13/15	86.7	12/14	85.7
5mg FluGEM	5/13	38.5	6/14	42.8	10/14	71.4	10/14	71.4	12/14	85.7	13/14	92.8

TIV=trivalent inactivated influenza vaccine.

TABLE 6 NASAL IGA TITERS FROM SUBJECTS WITH DATA AVAILABLE AT ALL TIME POINTS.

		All subjects			Subjects with baseline IgA titers below detection level	
FluGEM dose	Study Day	N	GMT	GMR	N	GMT	GMR
TIV only (control)	0	14	57.7	1.0	12	50.0	1.0
	21	14	78.3	1.4	12	76.3	1.5
	42	14	86.1	1.5	12	83.5	1.7
	210	14	53.7	0.9	12	50.0	1.0
1.25mg FluGEM	0	13	77.3	1.0	6	50.0	1.0
	21	13	145.7	1.9	6	186.9	3.7
	42	13	140.4	1.8	6	161.6	3.2
	210	13	61.9	0.8	6	67.2	1.3
2.5mg FluGEM	0	14	57.0	1.0	11	50.0	1.0
	21	14	61.5	1.1	11	53.9	1.1
	42	14	105.1	1.8	11	75.0	1.5
	210	14	51.6	0.9	11	50.0	1.0
5mg FluGEM	0	15	50.0	1.0	14	50.0	1.0
	21	15	79.4	1.6	14	79.4	1.6
	42	15	85.0	1.7	14	85.0	1.7
	210	15	55.0	1.1	14	55.0	1.1

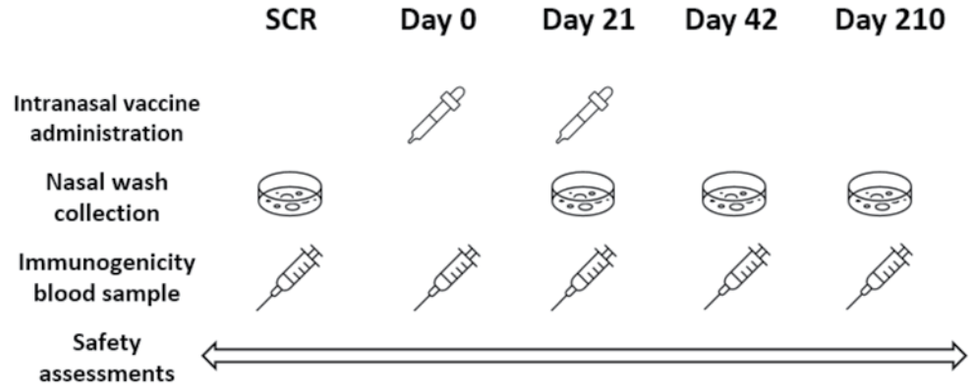
GMR= geometric mean titer ratio to baseline; GMT=geometric mean titer; TIV=trivalent inactivated influenza vaccine

TABLE 7 INFLUENZA-SPECIFIC IGG, IGG1 AND IGG3 SUBCLASSES FROM SUBJECTS WITH DATA AVAILABLE AT ALL TIME POINTS.

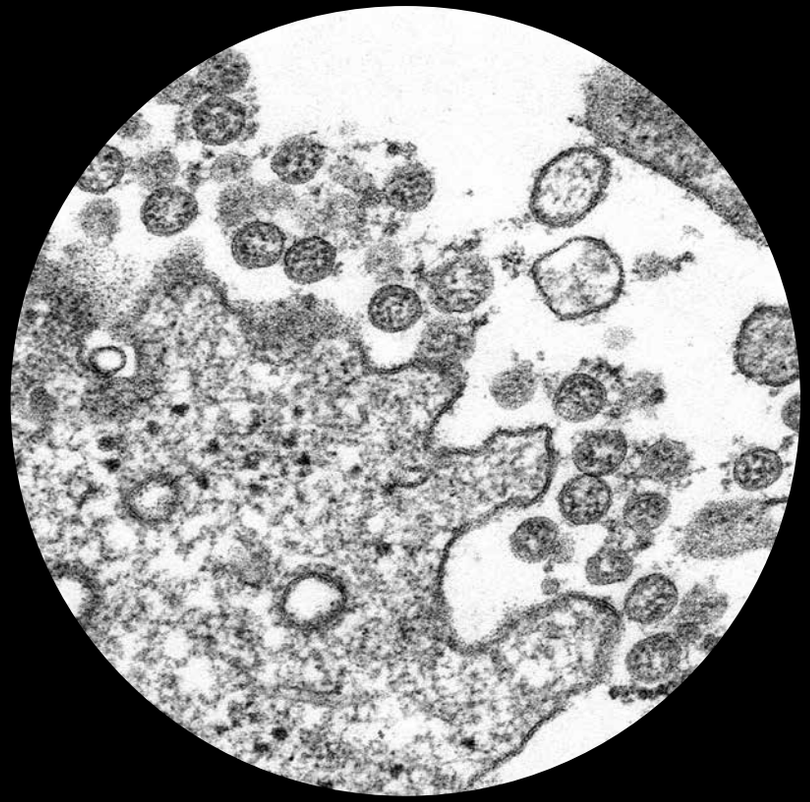
			GMT				
FluGEM dose	Study Day	N	Total IgG	N	IgG1	N	IgG3
TIV only (control)	0	15	15,349	15	2,858.5	15	80.7
	21	15	37,853	15	7,261.3	15	203.5
	42	15	53,358	14	7,582.0	14	168.2
	210	15	39,820	14	7,129.4	15	122.8
1.25mg FluGEM	0	15	19,031	15	4,269.2	15	62.4
	21	14	57,754	13	7,136.4	14	125.7
	42	13	60,839	11	8,983.7	12	181.6
	210	13	51,463	11	7,450.1	12	126.5
2.5mg FluGEM	0	15	15,613	15	2,612.7	15	68.6
	21	14	52,834	15	8,401.7	15	362.6
	42	14	45,451	13	8,022.5	14	339.2
	210	14	40,591	14	7,306.5	14	160.0
5mg FluGEM	0	15	22,729	15	2,860.5	15	124.5
	21	15	59,815	15	6,574.2	15	287.9
	42	15	55,098	15	6,174.4	14	213.0
	210	15	52,730	15	5,819.7	15	164.9

TIV=trivalent inactivated influenza vaccine.

FIGURE 1 SIMPLIFIED STUDY SCHEDULE BLOOD SAMPLES FOR THE ASSESSMENT OF HEMAGGLUTININ INHIBITION (HI). Titers were collected at screening (up to 7 days prior to study start) or baseline (day 0) prior to receiving the first vaccination and on day 21 prior to the second vaccination.



On day 210 ± 15 final blood samples were collected for assessment of persistence of antibodies. Nasal washes for IgA determination were collected at screening, day 21 (prior to the second vaccination), day 42 and day 210 ± 15. Safety was assessed throughout the whole follow-up period until day 210. Solicited adverse events were recorded for 7 days following each vaccination. SCR = screening.



Section 3

SARS-COV-2 AND CLINICAL DEVELOPMENT DURING PANDEMICS

CHAPTER 5

VIRAL CLEARANCE, PHARMACOKINETICS AND TOLERABILITY OF ENSOVIBEP IN PATIENTS WITH MILD TO MODERATE COVID-19 — A PHASE 2A, OPEN-LABEL, SINGLE DOSE ESCALATION STUDY

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Abstract

AIM Assessing viral clearance, pharmacokinetics, tolerability and symptom evolution following ensovibep administration in symptomatic COVID-19 outpatients.

METHODS In this open-label, first-in-patient study a single-dose of either 225 mg (n=6) or 600 mg of ensovibep (n=6) was administered intravenously in outpatients with mild-to-moderate COVID-19 symptoms. Pharmacokinetic profiles were determined (90-day period). Pharmacodynamic assessments consisted of viral load (qPCR and cultures) and symptom questionnaires. Immunogenicity against ensovibep and SARS-CoV-2-neutralizing activity were determined. Safety and tolerability were assessed throughout a 13-week follow-up.

RESULTS Both doses showed similar pharmacokinetics (first-order) with a mean half-life of 14 (SD: 5.0) and 13 days (SD: 5.7) for the 225 and 600 mg group, respectively. Pharmacologically relevant serum concentrations were maintained in all subjects for at least two weeks post-dose, regardless of possible immunogenicity against ensovibep. Viral load changes from baseline at Day 15 were 5.1 (SD: 0.86) and 5.3 (SD: 2.2) $\log_{10}$ copies/mL for the 225 and 600 mg dose, respectively. COVID-19 symptom scores decreased from 10.0 (SD: 4.1) and 11.3 (SD: 4.0) to 1.6 (SD: 3.1) and 3.3 (SD: 2.4) in the first week for the 225 and 600 mg group, respectively. No anti-SARS-CoV-2 neutralizing activity was present pre-dose, all patients had SARS-CoV-2 antibodies at Day 91. Adverse events were of mild-to-moderate severity, transient and self-limiting.

CONCLUSION Single-dose intravenous administration of 225 and 600 mg ensovibep appeared safe and well tolerated in patients with mild-to-moderate COVID-19. Ensovibep showed favourable pharmacokinetics in patients and the pharmacodynamic results warrant further research in a larger phase 2/3 randomized-controlled trail.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has affected over half a billion people since it was first identified in December 2019.[1] Therapies targeting the receptor binding domain (RBD) of the SARS-CoV-2 Spike glycoprotein (S) – thereby preventing virus-host interaction via the ACE-2 receptor – have proven successful in a clinical setting. Several monoclonal antibodies (mAbs) targeting this RBD have shown to reduce hospitalization and death in high risk COVID-19 patients with mild-to-moderate disease,[2-4] confirming the clinical benefits of early initiation of virus blocking therapy. As an RNA-virus that is transmitted by millions of people worldwide, new SARS-CoV-2 variants will likely continue to emerge.[5] Virus susceptibility to vaccine-induced antibodies and mAbs may be (partially) reduced in new variants.[6] It is therefore of utmost importance to bolster the arsenal of therapeutic viral blocking agents.

Ensovibep is a recombinant multispecific DARPin® molecule that was engineered to neutralize SARS-CoV-2 with high potency. While the mechanism of action of ensovibep – neutralization of the S protein by binding to its RBD – is comparable to (monoclonal) antibodies, there are inherent differences in the binding pattern that differentiates ensovibep from currently available antibody therapies. Ensovibep is a single molecule consisting of five designed ankyrin-repeat protein (DARPin) modules that are covalently linked. Three DARPins bind to an overlapping epitope of the RBD, but with different antigen-binding sequences (paratopes). This allows for cooperative binding of the tri-specific molecule with high avidity and could limit the development of mutations under therapeutic pressure from ensovibep.[7] In addition, ensovibep contains two human serum albumin binding domains to extend its systemic half-life.

In vitro studies confirmed the high potency neutralization by ensovibep of all SARS-CoV-2 variants of concern described to date.[7] Moreover, ensovibep was able to neutralize an omicron pseudovirus variant with high potency, signifying that ensovibep's neutralizing potential endures for the currently prevalent highly mutated variant of concern.[7] Studies performed in a SARS-CoV-2 hamster infection model showed *in vivo* efficacy with a significant reduction in viral load and pathogenesis after administration of ensovibep compared to placebo.[8]

Recently a phase 1, randomized, placebo-controlled, single ascending intravenous (IV) dose study was completed in healthy volunteers and showed a favorable safety and pharmacokinetic (PK) profile (dose range: 3-20 mg/kg) (manuscript in preparation).

Ensovibep is anticipated to provide benefit to COVID-19 patients at the early stages of infection, when virus replication should be halted to limit downstream immune-related damage and improve clinical outcomes. The pharmacological properties of ensovibep in combination with its high yield production process using an *Escherichia coli* fermentation-based process could provide a needed diversification of the current treatment arsenal against COVID-19.

In this article, we present the results of a phase 2a, first-in-patient, IV single-dose escalation study that assessed the viral clearance, PKs, tolerability and evolution of COVID-19 symptoms following ensovibep administration in early symptomatic COVID-19 patients.

Methods

STUDY DESIGN AND PATIENTS

This was an open-label, IV single-dose escalation, phase 2a study conducted at the Leiden University Medical Center in non-hospitalized COVID-19 patients. The protocol was reviewed and approved by the Medical Ethical Committee Leiden, Den Haag, Delft (NL76642.058.21) and was registered at ClinicalTrials.gov (NCT04834856). The trial was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice Guidelines and the principles of Dutch law on clinical experiments in humans. Written informed consent was obtained from patients prior to study-related activities. The Dutch Municipal Healthcare Services assisted in the recruitment of individuals with positive SARS-COV-2 polymerase chain reaction (PCR) tests who expressed an interest in study participation. Male and female patients were eligible if they were 18-70 years of age with symptomatic mild-to-moderate COVID-19, defined as experiencing at least one mild-to-moderate symptom (fever, cough, sore throat, malaise, fatigue, headache, muscle pain, gastrointestinal symptoms or shortness of breath with exertion) and a positive SARS-COV-2 rapid antigen test (Panbio™ COVID-19 Ag Rapid Test, Abbott) on the day of ensovibep administration. The main exclusion criteria were a high risk for COVID-19 related complications or mortality including immunodeficiency, need for hospitalization prior to screening or anti-SARS-COV-2 treatment initiation. The protocol did not allow a prior history of SARS-COV-2 infection (or vaccination), concurrent or previous use of antiviral medication (including antibodies) or convalescent plasma therapy. Vaccination was not allowed during the study until Day 29.

PROCEDURES

Patients received a single dose of either 225 mg (cohort 1) or 600 mg (cohort 2) of ensovibep administered as a 250 mL IV infusion over 60 minutes. Clinical dose and regimen projections for ensovibep were based on an integrated analysis of pre-clinical pharmacology results, available clinical safety, tolerability and PK results from the phase 1 dose-escalation first-in-human study (NCT04870164), and PK/pharmacodynamic (PD) modeling.[9] The current study had a dose-escalation design, meaning that the 600 mg dose was administered after the data review committee assessed Day 15 safety and tolerability data of the 225 mg dose. Patients remained in the clinical unit for two hours after ensovibep administration to monitor any direct untoward effects. Nasopharyngeal swabs (viral load), blood samples (PK, immunogenicity, blood chemistry and hematology) and questionnaires (14 Common COVID-19-related symptoms and Long-Covid-syndrome questionnaire) were obtained prior to ensovibep administration and on selected time points post-dose (*Figure 1*).

A validated electrochemiluminescence assay (Molecular Partners AG, Switzerland), which uses the RBD of SARS-COV-2 spike glycoprotein as a capture reagent, was used to quantify free ensovibep levels in serum. The limit of quantification (LLOQ) of this assay was 0.02 mg/mL. PK profiles of ensovibep were determined for both dose levels. Descriptive PK parameters included the maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), area under the drug serum concentration-time curve (AUC), half-life ($t_{1/2}$), volume of distribution (V_D) and clearance (CL). As an exploratory assessment outcome, anti-drug antibodies (ADAs) in human serum were measured using an electrochemiluminescence assay with acid dissociation sample pretreatment followed by neutralization (Molecular Partners AG, Switzerland). Antibodies were specifically captured via biotinylated ensovibep and detected with anti-human IgG/IgM SulfoTag detection antibodies. The assay was validated according to the Food and Drug Administration guide for industry: immunogenicity

testing of therapeutic protein products developing and validating assays for anti-drug antibody detection.[10]

Viral load was assessed by quantitative real-time PCR (qPCR) and quantitative virus cultures at Viroclinics Biosciences B.V. (Rotterdam, NL). SARS-CoV-2 qPCR analysis was performed according to a fully validated proprietary assay that is based upon the U.S. Centers for Disease Control and Prevention N1 assay,[11] but with different dyes-quenchers and a PCR program internally optimized by Viroclinics Biosciences B.V.

Determination of infectious SARS-CoV-2 virus titers was performed according to a validated proprietary assay (Viroclinics Biosciences B.V.). Briefly, in this assay VeroE6 cells were grown to subconfluent density, after which a serial dilution of an upper respiratory sample in infection medium was added to the cells in quadruplicate and incubated for 6 days. Cells were then fixed using a formalin solution and the presence of viral plaques was detected following immunostaining with an anti-nucleoprotein antibody, a peroxidase conjugate and TrueBlue staining. Virus titers were calculated as median Tissue Culture Infectious Dose 50% ($TCID_{50}$)/mL using the Spearman & Kärber method.[12,13]

Whole virus next generation sequencing (NGS) for SARS-CoV-2 was performed from a separate aliquot of the same nasopharyngeal swab used for viral load assessment by qPCR. Next-generation sequencing analysis was performed at baseline and at the last positive qPCR timepoint above the cut-off value of ≥ 4.0 log_{10} copies/mL, which was defined by the assay's capacity for successful amplification.

Serum anti-SARS-CoV-2 neutralizing activity and anti-SARS-CoV-2 IgG antibody levels were determined prior to dosing and at the final follow-up visit to assess the endogenous neutralizing immune response to SARS-CoV-2. In the virus neutralization activity assay, performed according to validated proprietary assay (Viroclinics biosciences B.V.), a serial dilution in triplicate of a serum sample in infection medium was mixed with a fixed amount of Isolate Germany/BavPat1/2020 and incubated for 1 hour. The mixture was added to VeroE6 cells at subconfluent density and incubated for 1 hour, after which the inoculum was removed and replaced by infection medium. Cells were incubated for 16-24 hours, then fixed using a formalin solution, and the presence of viral plaques was detected following immunostaining with an anti-nucleoprotein antibody, a peroxidase conjugate and TrueBlue staining. Microplaques were imaged and counted in a sx Ultimate-V Analyzer (Cellular Technology Limited). The neutralization titers were calculated from these data according to the method described by Zielinska et al.[14] Quantification of SARS-CoV-2 IgG antibodies was performed with a multiplex serology Meso Scale Discovery (MSD) assay (V-PLEX SARS-CoV-2 Panel 2, IgG kit, K15383U). Serum samples were added in duplicates in 96-wells assay plates coated with specific antigens. Following binding of serum antibodies to the respective antigens, anti-human IgG antibodies conjugated to MSD SULFO-TAG™ were used for subsequent detection. The emmitted light was measured with an MSD© instrument (Meso SECTOR 600 device). Anti-SARS-CoV-2 antibody levels were reported as international standard unit Binding Antibody Units (BAU)/mL.

Serum cytokines concentrations (IFN-γ, IL-1b, IL-6, IL-8, IL-10, and TNF-α) were determined using a multiplex electrochemiluminescent sandwich immunoassay from MSD validated in human serum by BioAgilytix Labs (Durham, NC, US). Additional information on bio-analytical assays used in this trial is provided as *Supplementary Material*.

Safety was assessed at each follow-up visit by assessment of treatment emergent adverse events (TEAEs), vital signs, physical examination, routine blood chemistry and hematology testing. Local tolerability at the infusion site was determined by the Visual Infusion Phlebitis (VIP) scale.[15] Patients were monitored throughout the study for adverse events of special interest, defined as infusion-related reactions, hypersensitivity reactions and cytokine release syndrome, and serious AEs (SAEs). Adverse events were coded according to the Medical Dictionary for Regulatory Activities (MEDDRA), version 24.0.

Symptoms related to COVID-19 were assessed daily (pre-dose until Day 15) and on Day 22 and 29 using the 14 Common COVID-19-Related Symptom questionnaire.[16] Symptoms were rated on either a three- or four-point ordinal scale and a total symptom score was calculated as the sum individual symptoms (range: 0-40). Assessment of long-term COVID-19 symptoms was performed using an experimental 'Long-Covid-syndrome' questionnaire on days 29 and 91 (*Supplementary Materials*).

STATISTICAL ANALYSIS

As this was an exploratory study, no formal power calculation was performed. Instead, a conventional (for early phase studies) group size per dose level was used. The study protocol included prespecified criteria to expand the cohort size to a maximum of 20 patients per dose level, in case of high inter-individual PK variability or signification deviation from expected

viral clearance. No formal hypothesis tests were planned nor performed. Data were summarized using descriptive statistics and graphically presented using GraphPad Prism for Windows (version 6.05). Viral load measurements <LLOQ were considered negative for the analysis. Slope of decline of viral load was estimated by the mean difference of viral load between successive study days divided by the interval (days) between measurements. Repeated measures correlations were calculated for viral load (independent variable) and the COVID-19 related total symptom scores to preliminary assess the relation between virus shedding and symptomatology within subjects. Non-compartmental PK analysis was performed using R 3.6.1 for Windows or newer (R Foundation for Statistical Computing/R Development Core Team, Vienna, Austria, 2019) using all PK samples collected according to protocol (until Day 91).

NOMENCLATURE

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology, and are permanently archived in the concise Guide to Pharmacology 2019/20.

Results

PATIENT CHARACTERISTICS

Between April and June 2021, twelve (12) COVID-19 patients who met the inclusion criteria were enrolled and received either 225 mg (n=6) or 600 mg ensovibep (n=6) (*Figure 2*). No patients were vaccinated against COVID-19 at baseline. Three patients received their first dose of SARS-COV-2 vaccine (BNT162B2, Pfizer-BioNTech) approximately 43, 52 and 69 days, respectively, after ensovibep administration. Baseline characteristics are described in *Table 1*. Median time from onset of symptoms was 5 days in both groups (total range 2-8 days). All patients were symptomatic on baseline and median COVID-19 related symptom scores were similar for both dose groups.

VIRAL CLEARANCE

All patients had a quantifiable SARS-COV-2 viral RNA load in upper respiratory tract samples determined by qPCR at baseline, collected before the administration of ensovibep (*Table 1*). The alpha virus variant (B.1.1.7) was detected in all patients. The mean viral load determined by qPCR was 7.3 ± 1.0 $\log_{10}$ copies/mL in the 225 mg group and 6.6 ± 1.6 $\log_{10}$ copies/mL in the 600 mg group at baseline. Viral RNA in nasopharyngeal swabs decreased rapidly in both dose groups during the first two weeks with mean changes from baseline of 5.1 and 5.3 $\log_{10}$ copies/mL for the 225 mg and 600 mg dose, respectively (*Figure 3*). The rate of viral load decline was highest in the first week (*Supplementary Table 1*), with an estimated daily decrease of 0.72 and 0.67 $\log_{10}$ copies/mL (Day 1-3), 0.41 and 0.71 $\log_{10}$ copies/mL (Day 3-5) and 0.45 and 0.39 $\log_{10}$ copies/mL (Day 5-8) for the 225 mg and 600 mg dose group, respectively. Results for time to PCR negativity are summarized in *Supplementary Figure 1*. No virus mutations that could potentially trigger resistance to ensovibep were identified in post-dose nasopharyngeal samples. Viral load in saliva samples was lower compared to nasopharyngeal samples but showed a similar reduction over time (data not shown). Three patients in the 225 mg group had positive viral culture results at baseline and one patient had a baseline titer equal to the LLOQ (0.75 $\log_{10}$ $TCID_{50}$/ML) of the assay. By Day 5, all viral cultures were negative. Viral cultures were negative for all analyzed samples (baseline and follow-up) in the 600 mg group.

PHARMACOKINETICS

Pharmacokinetic parameters are summarized in *Table 2*. Mean concentration-time profiles showed log-linear monophasic elimination of ensovibep (*Figure 4*). Volume of distribution (V_D) was 2844 mL (SD: 34.3 mL) in the 225 mg group and 2735 mL (SD 37.2 mL) in the 600 mg group. Drug elimination rates were similar for both doses, with a mean $t_{1/2}$ of approximately 14 days (SD: 4.9 days) and 13 days (SD: 5.7 days) for the 225 mg and 600 mg dose group, respectively. Dose escalation to 600 mg resulted in a proportional increase of C_{max} and AUC compared to 225 mg. Two individual patients (one in each cohort) showed an accelerated elimination of ensovibep at Day 22 (*Supplementary Figure 2*).

Anti-drug antibodies (ADAs) at baseline were detected in one patient in the 225 mg group. Treatment–induced ADAs were detected in 5/6 (83%) patients in each dose group (time of onset, range: 14-91 days). For most patients who developed ADAs, the elimination of ensovibep remained unaffected. The two patients who showed increased elimination had a relatively early peak of ADA titers at day 29 compared to other patients.

SAFETY AND TOLERABILITY

No SAEs, infusion site reactions, hypersensitivity, cytokine release syndrome or worsening of COVID-19 (such as immune enhancement) were observed. At least one (≥1) TEAE was reported by 4/6 (66%) patients in the 225 mg group and 3/6 (50%) patients in the 600 mg group (*Table 3*). All TEAEs were transient, resolved without intervention and were of mild-to-moderate severity. Out of 16 reported TEAEs, 5 were deemed related to the treatment and all 5 occurred in the 225 mg group. These events consisted of diarrhea (n=2) and elevated liver tests (alanine aminotransferase [ALT], aspartate aminotransferase [AST] and bilirubin, n=3). One of the two patients with transient liver enzyme increase had pre-existing elevated ALT tests. Elevated liver tests were below two times the upper limit of normal.

PATIENT-REPORTED COVID-19 SYMPTOMS

For both groups, an overall decrease in symptoms scores (range of total symptom score 0-40) was observed. Total symptoms scores showed a relative fast decline in the first week after ensovibep administration, from 10.0 and 11.3 (baseline) to 1.6 and 3.3 (Day 8) in the 225 mg and 600 mg dose group, respectively (*Supplementary Figure 3*). Mean total symptom scores were <1 in both treatment groups on Day 29, indicating minimal symptomatology at the end of the observation period. Only tiredness (n=2), myalgia (n=1), loss of smell/taste (n=1) were reported by individual patients on Day 29. Within patient, there was a linear correlation between viral load and total symptom score (r=0.77, p <0.0001).

Compared to their pre-COVID status, the majority of patients reported either no change on all items or only mild worsening on one single item of the Long-Covid-syndrome questionnaire (16 items in total). Mild fatigue compared to pre-COVID status was reported most frequently (2/6 [33%] in the 225 mg and 3/6 [50%] in the 600 mg group). On Day 91, 1/6 (17%) patients in each dose group reported mild worsening of ≥2 items on the Long-Covid-syndrome questionnaire. One patient reported an incidental severe change from the pre-COVID status at Day 91 for the domain chest pain/tightness. There were no clinical abnormalities that could explain the self-reported complaints, however, the complaints could be due to excessive exercise as the patient visited the gym frequently. Daily occupational activities of patients were not affected by Long-Covid symptoms.

ANTI-SARS-COV-2 SERUM NEUTRALIZING ACTIVITY AND CYTOKINE PRODUCTION

Patients neither had anti-SARS-COV-2 neutralizing activity nor anti-SARS-COV-2 antibodies at baseline, in serum prior to ensovibep administration (data not shown). At Day 91 (final follow-up visit), virus neutralizing activity was detected in 3/6 (50%) patients in each dose group. All three patients who received COVID-19 vaccinations had positive microneutralization titers at Day 91. All patients had developed various levels of endogenous anti-SARS-COV-2 antibodies with the highest values observed in vaccinated subjects. Decreases in serum levels of IFN-gamma, TNF-alpha, IL-8 and IL-10 cytokine were observed during the study in most patients (data not shown). No apparent changes in IL-6 and IL-1β were observed.

Discussion

In this exploratory phase 2a study ensovibep was administered for the first time to non-hospitalized symptomatic COVID-19 patients. The time of symptom onset and high viral load, in combination with absent SARS-COV-2 neutralization activity at baseline, confirmed that enrolled patients were in their initial phase of infection. All patients showed reduction in viral load after ensovibep administration. The change from baseline of viral load by qPCR was comparable for both 225 mg and 600 mg doses, suggesting no dose-dependent difference on viral clearance in this study population. Jones et al proposed a model of natural SARS-COV-2 infection with a linear increase and then decline of approximately 0.17 $\log_{10}$ units per day after a peak of viral load was reached (estimated on approximately day 4 after onset of shedding).[18] In our study we did not observe an initial increase in viral load, suggesting that most of the subjects surpassed their initial peak viral load. This is also expected as the study enrolled symptomatic patients and peak viral load is expected to occur 1 to 3 days before symptom onset.[18] The viral load decline in our study was relatively high in the first week following infection (3.6 $\log_{10}$ copies/mL in the 225 mg group and 3.4 $\log_{10}$ copies/mL in the 600 mg group on Day 8). Although the viral load dynamics in this first-in-patient study does not permit comparison to a placebo group, the observed viral load reduction, in comparison to the model of Jones et al, suggests a potential signal that ensovibep has an effect on viral clearance in

a population with low risk of COVID-19 related complication, consistent with the results obtained after monoclonal antibody treatments.[3,19] Ensovibep displayed first-order kinetics with a long systemic half-life in COVID-19 patients, confirming the *in vivo* half-life extension properties of its anti-human serum albumin DARPin modules in the presence of the compound's main binding target (SARS-COV-2 S-protein RBD).

Both doses showed consistent PK profiles with dose escalation from 225 mg to 600 mg resulting in a proportional increase of serum concentration and exposure. Due to the low variability of the PK data and consistent PD results, the expansion of the cohort was not needed. Non-compartmental PK analysis showed a relatively low V_D of approximately 2.8 liter (in the range of systemic circulation) and a long half-life of approximately 13 days. These characteristics can be attributed to ensovibep's albumin-binding domains. It is anticipated that ensovibep will distribute through tissues alongside with albumin. Albumin (like many other proteins) distributes across the epithelial lining of the lungs, despite a low V_D.[20] Monoclonal antibodies exhibit a similar V_D because they are presumed to be relatively confined to the vascular space, however, they still distribute to a sufficient degree to exert local effects. Moreover, several mAbs with a similar V_D as ensovibep have shown to be effective as treatment or prophylaxis for COVID-19.[21,22]

All patients, including the two patients with an increased elimination rate, had similar ensovibep exposures (combined with a slow elimination rate during the first two weeks following dosing). Neutralization of SARS-COV-2 will be most important during this initial phase of infection, as prolonged viral shedding of high viral quantities is associated with poor outcomes.[23]

Like other protein therapeutics, including mAbs, immunogenicity has been described previously.[24,25] In this study ADA formation was observed at various timepoints in most patients, however, mono-exponential elimination appeared to remain unaffected in most patients (83%). More importantly, immunogenicity did not appear to alter ensovibep concentrations in the first two weeks post-dose, the time interval where antiviral efficacy is anticipated to be most relevant.

Ensovibep was well tolerated in COVID-19 patients. There were no SAEs, infusion site reactions, hypersensitivity or clinical worsening of COVID-19 (such as immune enhancement-like phenomena described for antibody-based drugs and vaccines targeting coronaviruses).[26] Adverse events were of mild-to-moderate severity. Related TEAEs consisted of diarrhoea and transient mild liver enzyme increases and were only observed in the low dose (225 mg) group. A possible relationship of these TEAEs and ensovibep could not be ruled out based on the timing of onset. However, SARS-COV-2 can cause gastrointestinal symptoms and can lead to (transient) hepatocyte injury in various degrees of severity and via various mechanisms.[27-29] Therefore these adverse events could also be attributed to COVID-19.

Assessment of common COVID-19-related symptoms indicated an overall decrease in COVID-19 symptoms during the 29-day follow-up period, however, the study population already had a paucity of symptoms at baseline, which makes the interpretability of results difficult. Similar to viral load decline, there were no apparent differences between the 225 mg and 600 mg dose in the resolution of COVID-19 symptoms. Heterogeneity of clinical outcome measures in literature and timing of participant inclusion in relation to symptom onset makes it difficult to compare the observed symptom resolution with natural COVID-19 disease course. A study by Bliddal et al. in non-hospitalized PCR-positive COVID-19 patients showed a median time until cessation of symptoms of 12 to 14 days, with persistence of symptoms ≥ 4 weeks in approximately 36% of patients.[30] In our study a subset of subjects (4 out of 12 [33%]) reported symptoms at Day 29 (fatigue, myalgia, smell/taste loss). These symptoms were also most prevalent in the study population of Bliddal et al.[30]

At the time of the study, there was no standardized clinical case definition of Long-Covid. The Long-Covid questionnaire was used as exploratory tool, to gain preliminary insights on the occurrence of long-term post COVID-symptoms after ensovibep administration. Case identification of Long-Covid according to the current World Health Organisation (WHO) definition could therefore not be made.[31] Patients reported predominantly no or only mild symptoms on Day 91 compared to their pre-COVID status and no patients reported impact of symptoms on daily occupational functioning.

SARS-COV-2 serum neutralizing activity and endogenous antibody formation were assessed as an exploratory endpoint. All patients had detectable levels of SARS-COV-2 antibodies and half of the patients had SARS-COV-2 serum neutralizing activity at Day 91. Because ensovibep levels were

predicted to be very low at Day 91, any neutralizing activity in serum was initially expected to be due to the endogenous response to SARS-CoV-2 infection. However, the protocol allowed for COVID-19 vaccination after Day 29. Anti-SARS-CoV-2 antibody titers were highest in vaccinated patients (n=3). These preliminary results indicate that administration of ensovibep does not prevent an endogenous immune response to SARS-CoV-2 antigens.

Our study had some limitations. This study was intended to assess the feasibility of IV ensovibep administration in ambulatory COVID-19 before initiation of larger phase 2/3 randomized controlled trials, while exploring the first-in-patient PK and pharmacodynamic effects. The patients in this study showed clear improvement, both in symptoms and viral clearance. However, to fully determine the clinical efficacy and effect size, a comparison with an unexposed and representative control group must be made to differentiate from a natural disease course. The sample size in this exploratory study in combination with relatively young Caucasian adults, and the relatively mild disease manifestation limit the extrapolation of the results to a broader population. Lastly, COVID-19 can cause many clinical abnormalities, which made it difficult to discriminate between disease and treatment-related adverse effects.

In conclusion, this study provides the first clinical data of ensovibep in symptomatic, non-hospitalized, COVID-19 patients. Single IV administration of ensovibep (225 mg and 600 mg) was safe and well tolerated in ambulatory COVID-19 patients. Both explored doses had similar effects on preliminary pharmacodynamic outcome measures, such as viral load and symptoms, suggesting that low doses of ensovibep could be targeted in the future. The results of this study support the continued development of ensovibep as a potential treatment for COVID-19 in a follow-up randomized, double-blind, placebo-controlled phase 2/3 trial (NCT04828161).

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TABLE 1 BASELINE CHARACTERISTICS.

	Cohort 1 225mg (n=6)	Cohort 2 600mg (n=6)
Age, years	23 (21–26)	24 (22–44)
Sex, n female (%)	2 (33)	2 (33)
Race or ethnicity*, n (%)		
Mixed	0 (0)	1 (17)
White	6 (100)	5 (83)
BMI	26 (24–30)	25 (22–30)
Days between symptom onset and dosing	5 (2–8)	5 (3–5)
Positive viral culture, n (%)	4 (67)	0 (0)
Positive qPCR result, n (%)	6 (100)	6 (100)
Viral load by qPCR**, mean (SD)	7.3 (1.0)	6.7 (1.7)
COVID-19–Related Symptom score***	10.5 (4–15)	11.0 (7–18)

*Data are presented as median (range) unless indicated otherwise. *self-reported race or ethnicity of patients, who could choose from multiple categories, **viral load expressed as log_{10} copies/mL, ***possible range of aggregated COVID-19-Related symptom score: 0-40. BMI, body mass index; qPCR, quantitative polymerase chain reaction.*

TABLE 2 SUMMARY OF PHARMACOKINETIC PARAMETERS.

	MP0420 225 MG			MP0420 600 MG		
Parameter	n	Mean[a]	CV (%) or SD[a]	n	Mean[a]	CV (%) or SD[a]
AUC_{inf} (h*ug/mL)	5[b]	37170	18.1	6	100068	37.9
V_D (mL)	5[b]	2844	34.3	6	2735	37.2
CL (mL/h)	5[b]	6.13	1.02	6	6.39	2.81
C_{max} (ug/mL)	6	88.8	20.3	6	233	19.3
$t_{1/2}$ (h)	5[b]	326	119	6	303	136
T_{max} (h)	6	1.42	1.40, 2.70	6	2.04	1.37, 2.68

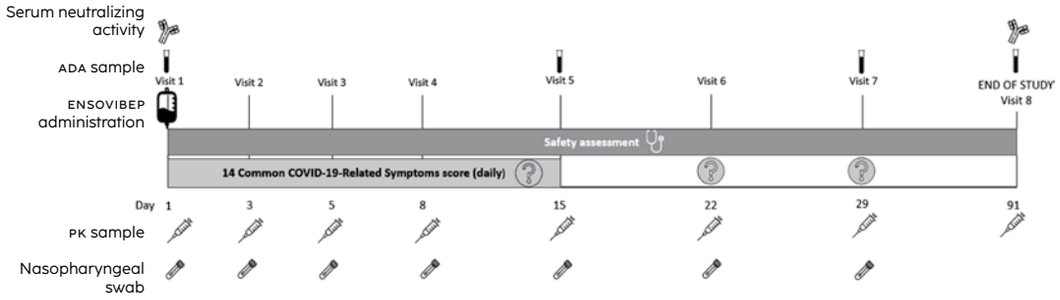
CV, coefficient of variation; AUC_{inf}, area under the concentration-time curve from time 0 to infinity; CL, clearance ;C_{max}, maximum concentration; $t_{1/2}$, half-life; T_{max}, time to maximum concentration following start of infusion; V_D, apparent volume of distribution.
a) C_{max} and AUC_{inf} are reported as geometric mean and coefficient of variation (%); CL,V_D and $t_{1/2}$ are reported as arithmetic mean and standard deviation (SD); Tmax is reported as median (minimummaximum).
b) $t_{1/2}$ could not be accurately estimated in one patient. PK parameters related to $t_{1/2}$ estimation (including V_D, AUC_{inf} and CL) are not reported for this patient.

TABLE 3 NUMBER OF RELATED ADVERSE EVENTS CLASSIFIED BY MEDDRA SYSTEM ORGAN CLASS (SOC) AND PREFERRED TERM, AND INVESTIGATOR-ASSIGNED RELATIONSHIP TO STUDY MEDICATION.

	Cohort 1, 225mg (N=6)		Cohort 2, 600mg (N=6)	
	Not related to drug administration	Related to drug administration	Not related to drug administration	Related to drug administration
Ear and labyrinth disorder	0	0	1 (17%)	0
Ear pain	0	0	1 (17%)	0
Gastrointestinal disorders	1 (17%)	2 (33%)	0	0
Diarrhoea	1 (17%)	2 (33%)	0	0
General Disorders and Administration Site conditions	1 (17%)	0	0	0
Alcoholic hangover	1 (17%)	0	0	0
Investigations	0	3 (33%)	0	0
Alanine aminotransferase increased	0	1 (17%)	0	0
Aspartate aminotransferase increased	0	1 (17%)	0	0
Blood bilirubin increased	0	1 (17%)	0	0
Metabolism and nutrition disorders	1 (17%)	0	0	0
Hypophosphatemia	1 (17%)	0	0	0
Musculoskeletal and connective tissue disorders	2 (33%)	0	0	0
Back pain	1 (17%)	0	0	0
Myalgia	1 (17%)	0	0	0
Nervous system disorders	0	0	4 (50%)	0
Headache	0	0	4 (50%)	0
Respiratory, thoracic and mediastinal disorders	0	0	1 (17%)	0
Cough	0	0	1 (17%)	0

n = number of adverse events (percentage of subject)

FIGURE 1 SCHEDULE OF ASSESSMENT.



ADA = antidrug antibodies

FIGURE 2 CONSORT DIAGRAM.

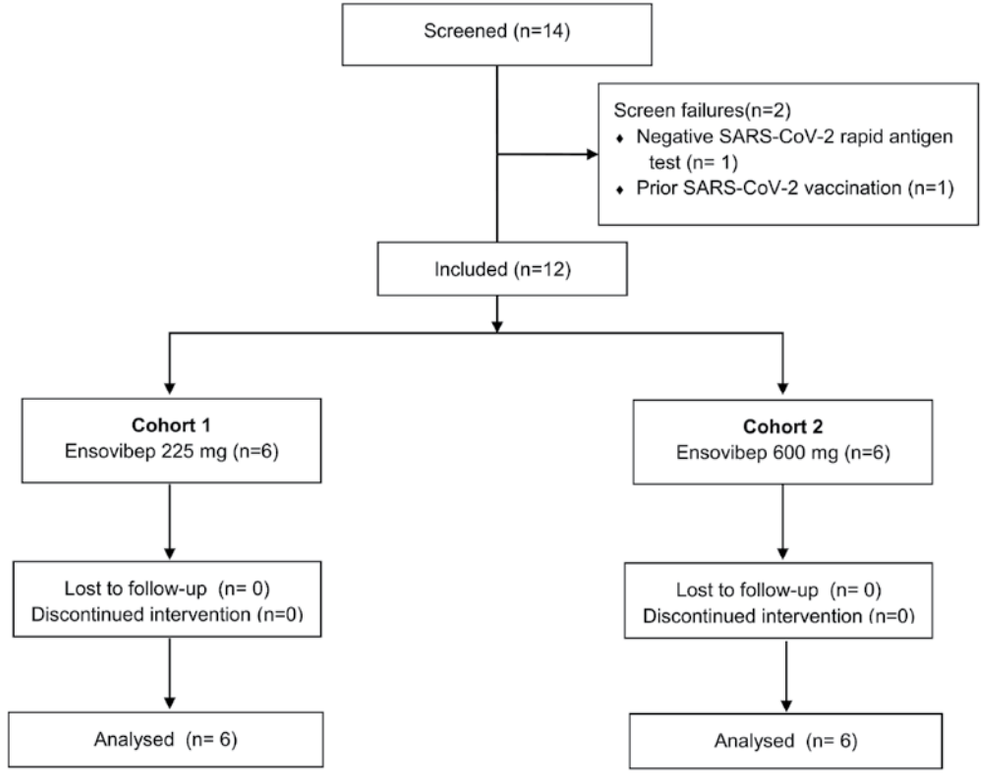


FIGURE 3 MEAN CHANGE FROM BASELINE IN SARS-COV-2 VIRAL LOAD DETERMINED BY QPCR ON UPPER RESPIRATORY TRACT SAMPLES.

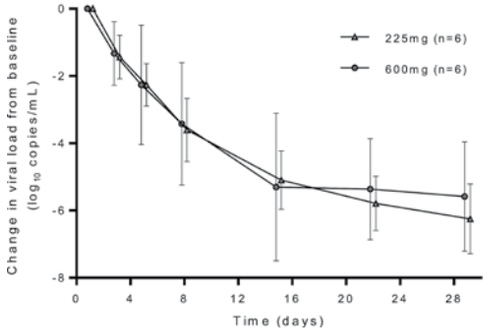
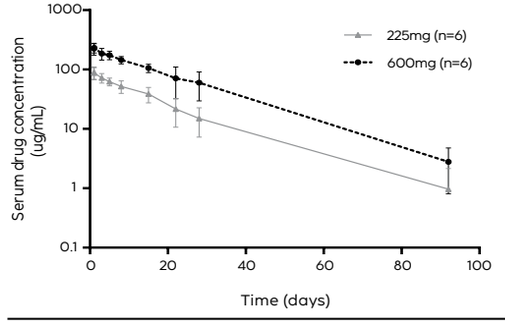


FIGURE 4 SEMI-LOG MEAN SERUM CONCENTRATION VERSUS TIME PROFILES OF ENSOVIBEP FOLLOWING 225- AND 600-MG ADMINISTRATION.



SUPPLEMENTARY FIGURES AND TABLES

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

CHAPTER 6

IMMUNOSUPPRESSION BY HYDROXYCHLOROQUINE: MECHANISTIC PROOF IN IN VITRO EXPERIMENTS BUT LIMITED SYSTEMIC ACTIVITY IN A RANDOMIZED PLACEBO-CONTROLLED CLINICAL PHARMACOLOGY STUDY

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Abstract

Based on its wide range of immunosuppressive properties, hydroxychloroquine (HCQ) is used for the treatment of several autoimmune diseases. Limited literature is available on the relationship between HCQ concentration and its immunosuppressive effect. To gain insight in this relationship we performed i*n vitro* experiments in human PBMCs and explored the effect of HCQ on T and B cell proliferation and Toll like receptor (TLR)3/TLR7/TLR9/RIG-I-induced cytokine production. In a placebo-controlled clinical study these same endpoints were evaluated in healthy volunteers that were treated with a cumulative dose of 2400 mg HCQ over 5 days. *In vitro*, HCQ inhibited TLR responses with IC50s >100 ng/mL and reaching 100% inhibition. In the clinical study, maximal HCQ plasma concentrations ranged from 75 to 200 ng/mL. No *ex vivo* HCQ effects were found on RIG-I-mediated cytokine release, but there was significant suppression of TLR7 responses and mild suppression of TLR3 and TLR9 responses. Moreover, HCQ treatment did not affect B cell and T cell proliferation. These investigations show that HCQ has clear immunosuppressive effects on human PBMCs, but the effective concentrations exceed the circulating HCQ concentrations under conventional clinical use. Of note, based on HCQ's physico-chemical properties, tissue drug concentrations may be higher, potentially resulting in significant local immunosuppression. This trial is registered in the International Clinical Trials Registry Platform (ICTRP) under study number NL8726

Introduction

Hydroxychloroquine (HCQ) is a broad immunosuppressive drug, initially developed as an anti-malarial drug. However, due to its anti-inflammatory properties, HCQ is now widely used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA)[1], systemic lupus erythematosus (SLE)[2] and Sjögren's syndrome.[3] The use of HCQ in other diseases has been under investigation, a pilot trial investigating the use of HCQ in patients after myocardial infarction showed a decrease in plasma IL-6 levels compared to placebo, and a larger trial studying the effect on recurrent cardiovascular events is currently ongoing.[4] Furthermore, HCQ was under investigation for use in moderate to severe COVID-19 patients during the COVID-19 pandemic.[5]

The exact mechanisms behind HCQs immunosuppressive functions remain unclear. HCQ accumulates in the lysosomes and inhibits lysosomal function by autophagosome fusion with lysosomes,[6] thereby inhibiting antigen presentation.[7,8] In addition, HCQ inhibits pro-inflammatory cytokine production by myeloid cells, possibly via the inhibition of endosomal Toll-like receptor (TLR) signalling.[9] It has been shown that HCQ treatment is associated with decreased interferon (IFN)α serum levels in SLE patients.[10] Furthermore, several studies investigating the effect of HCQ on peripheral blood mononuclear cells (PBMCs) or cell lines show that HCQ treatment reduces phorbol 12-myristate 13-acetate (PMA) and ionomycin or lipopolysaccharide induced cytokine production.[11-13]

Besides effects on the innate immune system, HCQ affects the adaptive immune response as well. It has been shown that HCQ inhibits differentiation of class-switched memory B cells into plasmablasts and thereby decreases IgG production in response to TLR9 stimulation or inoculation with inactivated virus.[14,15] HCQ inhibits T cell activation as well, via the inhibition of T cell receptor induced calcium mobilization and dysregulation of mitochondrial superoxide production.[16-18]

However, the concentrations used in such *in vitro* experiments studying the immunomodulatory effects of HCQ largely exceeded obtainable clinical concentrations in patients. A study in cutaneous lupus erythematosus patients receiving HCQ in clinical doses showed that higher HCQ blood levels corresponded with lower *ex vivo* IFNα responses after TLR9 stimulation, but not after TLR7/8 stimulation.[13] Moreover, influenza antibody titers

after vaccination in Sjögren's syndrome patients receiving HCQ were lower compared to HCQ naïve patients.[15] Unfortunately, little additional literature is available on the *in vivo* immunomodulatory effects of HCQ and comparing it to *in vitro* experiments.

We aimed to assess and quantify the immunomodulatory effects of HCQ on primary human immune cells, both *in vitro* and *ex vivo* in a randomized clinical trial. We assessed the effect of HCQ on cytokine production after endosomal TLR stimulation in isolated PBMCs and on T and B cell proliferation (*in vitro* as well as *ex vivo*). In the clinical trial, healthy subjects were dosed with HCQ in the standard dosing regimen for moderate-to-severe COVID-19 that was advised in the Netherlands when the study was conceived. In the study design, we accounted for a potential age effect on the study outcomes, since general immunocompetence and drug metabolism has been reported to be age-dependent.[19,20] Here we present the outcomes of the *in vitro* experiment and the randomized clinical trial.

Methods

IN VITRO EXPERIMENTS

Blood was collected by venipuncture using Sodium Heparin vacutainer tubes or Cell Preparation Tubes (CPT, Becton Dickinson, Franklin Lakes, NJ, USA) from healthy volunteers after written informed consent, in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Blood was used for the evaluation of the *in vitro* immunomodulatory activity of hydroxychloroquine (10-10,000 ng/mL, Sigma-Aldrich, Deisenhofen, Germany). All experiments were started within one hour after blood withdrawal, and incubations were performed in duplicate. Hydroxychloroquine and stimulant were added simultaneously. Per experiment, blood of 6 donors was used.

CLINICAL STUDY

We conducted a single-blind, randomized, placebo-controlled multiple dose study in forty healthy male volunteers, comprising twenty young (18-30 years) and twenty elderly (65-75 years) subjects. The study was conducted at the Centre for Human Drug Research in Leiden, the Netherlands, between June and September 2020, during the COVID-19 pandemic. All subjects in the clinical trial gave written informed consent according to Declaration of Helsinki recommendations, prior to any study-related activity. The study was approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, the Netherlands) and registered in the Toetsingonline Registry (study number NL73816.056.20), and in the International Clinical Trials Registry Platform (NL8726).

Volunteer selection

To avoid sex-related inter-individual variability in immune responses, only male subjects were included.[21] Subjects were included if they were overtly healthy. The health status of subjects was assessed by medical screening, including medical history, physical examination, vital signs measurements, 12-lead electrocardiography (ECG), urine analysis, drug screen and safety chemistry, coagulation, and hematology blood sampling. Body mass index of study participants had to be between 18 and 32 kg/m^2. Subjects with a known hypersensitivity reaction to chloroquine, HCQ or other 4-aminoquinolines, abnormalities in the resting ECG (including QTcF-interval >450ms), evidence of any active or chronic disease or condition (including long QT syndrome, retinal disease, G6PD deficiency, autoimmune diseases, diabetes mellitus type I or II, psychiatric disorders) or a positive SARS-COV-2 PCR test were excluded from study participation. Use of concomitant medication was not permitted during the study, and 14 days (or 5 half-lives) prior to the study drug administration, except for paracetamol.

Study design

Subjects were randomized to receive either HCQ sulphate (plaquenil®) or placebo tablets, in a 1:1 ratio. Tablets were dispensed by the pharmacy, according to a randomization list generated by a study-independent statistician. Plaquenil® and placebo tablets were packaged in the same way but the tablets were not indistinguishable, study drug administration was therefore performed by dedicated unblinded personnel not involved in any other study tasks. Subjects received HCQ or placebo by a loading dose of 400 mg twice daily (t = 0h and t = 12h) followed by a 400 mg once daily dose regimen (t = 24h, t = 48h, t = 72h, and t = 96h), giving a cumulative dose of 2400 mg. This reflected the standard dosing regimen for moderate-to-severe COVID-19 patients in NL when the study was conceived (total dose between 2000 and 3800 mg).

Pharmacokinetic evaluation

For pharmacokinetic (PK) assessments, blood was collected in 3 mL Vacutainer® K_2EDTA tubes (Becton Dickinson) on study day 0 (baseline and 3 hours post-dosing), and day 1, 4 and 9 (3 hours post-dosing). Hydroxychloroquine plasma concentrations were measured by Ardena Bioanalytical Laboratory (Assen, NL) using a validated LC-MS/MS method. The lower limit of quantification (LLOQ) of the analysis was 5 ng/mL.

Whole blood stimulation

Whole blood was stimulated with 10 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich) for 6 hours and 24 hours. After 6 hours, activation markers on T-cells were measured using CD69-APC (clone: REA824), CD71-FITC (clone: REA902), CD154-VioBlue (REA238) and CD25-PE (clone: 3G10), CD3-VioGreen (REA613), CD4-APC-Vio770 (REA623) and CD8-PE-Vio770 (REA734) antibodies and propidium iodide as viability dye (all Miltenyi Biotec, Bergisch-Gladbach, Germany) using a MACSQuant 16 analyzer (Miltenyi Biotec). After 24 hours, culture supernatants were collected for cytokine analysis.

PBMC isolation and TLR stimulation

PBMCs were isolated from CPT after centrifugation at 1800 x g for 30 minutes, and washed 2x using phosphate buffered saline (PBS, pH 7.2, Gibco, Thermo Fisher, Waltham, MA, USA). PBMCs were stimulated with endosomal TLR ligands PolyI:C (TLR3, 50 µg/mL), imiquimod (TLR7, 1 µg/mL), CpG class A (TLR9, oligodeoxynucleotides [ODN] 2.5 µM) and PolyI:C/lyovec (RIG-I, 1 µg/mL; all Invivogen, Toulouse, France). Supernatants were collected after 24 hours for cytokine quantification.

Proliferation assay

PBMCs were stained with 2.5µM cell trace violet (CTV, Thermo Fisher) according to user's manual. T cells were stimulated with 5 µg/mL phytohemagglutinin (PHA), and B cells with a monoclonal CD40 antibody (5 µg/mL; clone: G28.5, BioXCell) and CpG class B (2.5 µM; ODN Invivogen). After 5 days of stimulation PBMCs were stained using, CD4-PE (clone: OKT4), CD8-APC (clone: HIT8a), CD19-PE (clone: HIB19, all Biolegend, San Diego, CA, USA) and fixable viability dye eFluor780 (Thermo Fisher) and proliferation was quantified by flow cytometry, using the MACSQuant 16 analyzer.

Flow cytometry

Circulating leukocyte subsets were analyzed using flow cytometry. Red blood cell lysis was performed on sodium heparinized blood using red blood cell lysis buffer (Thermo Fisher Scientific). After washing with PBS (pH 7.2), leukocytes were incubated with fluorochrome-labeled antibodies for 30 minutes on ice. After a final washing step, leukocytes were measured on a MACSQuant 16 analyzer (Miltenyi Biotec). See *Supplemental Table 1* for a full list of antibodies used.

Cytokine measurements

IFNγ and IL-2 were quantified using the Vplex-2 kit (Meso Scale Discovery). IFNα and IL-6 were quantified using the pan-specific IFNα ELISApro HRP kit and the IL-6 ELISApro HRP kit (both Mabtech, Nacka Strand, Sweden).

STATISTICAL ANALYSIS

In vitro data are reported as mean ± standard deviation (SD). The IC50 was calculated using a inhibitory sigmoid Emax function where applicable. Analyses were performed using Graphpad Prism version 6.05 (Graphpad, San Diego, CA, USA). Repeatedly measured pharmacodynamic data were evaluated with a mixed model analysis of variance with fixed factors treatment, age group, time, treatment by time, age group by time, treatment by age group and treatment by age group by time and a random factor subject and the average pre-value as covariate. If needed, variables were log transformed before analysis. Contrasts between the placebo and HCQ treatment groups were calculated per endpoint. In addition, a potential age-specific HCQ effect was evaluated by comparing the 18-30 years with the 65-75 years age group. For the contrasts, an estimate of the difference (back-transformed in percentage for log transformed parameters), a 95% confidence interval (in percentage for log-transformed parameters), Least Square Means (geometric means for log transformed parameters), and the p-value were calculated. A p-value ≤ 0.05 was considered to be statistically significant. All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

HYDROXYCHLOROQUINE SUPPRESSED ENDOSOMAL TLR-INDUCED IFNA AND IL-6 RELEASE *IN VITRO*

PBMCs were stimulated with endosomal TLR ligands in the presence of a dose range of HCQ for 24 hours, and supernatants were analyzed for IRF-mediated IFNα and for NFκB-mediated IL-6 secretion. PBMCs were stimulated with different endosomal TLR ligands: poly I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and poly I:C lyovec (RIG-I). Hydroxychloroquine dose-dependently inhibited endosomal TLR-induced IFNα and IL-6 secretion (Figure 1). Poly I:C-induced IFNα and IL-6 release was strongly suppressed at 10.000 ng/mL (IFNα: -83.9%, IL-6: -96.6%, IC50 IL-6 = 637.2 ng/mL). Imiquimod (IMQ)-induced cytokine release was completely suppressed at the highest concentration (IFNα: -96.3%, IL-6: -96.3%, IC50 IFNα: 695.8 ng/mL, IL-6: 237.9 ng/mL). The same was observed for stimulation with CpG class A, IFNα was suppressed by 99.6% with an IC50 of 145.3 ng/mL, and IL-6 was suppressed by 96.4%, with an IC50 of 86.9 ng/mL. The RIG-I response to poly I:C/lyovec was less affected by HCQ, while IFNα release was suppressed by 66.1% at 10,000 ng/mL HCQ, IL-6 release was not significantly altered.

HCQ INHIBITED B CELL PROLIFERATION BUT NOT T CELL PROLIFERATION *IN VITRO*

PBMCs were stimulated with phytohaemagglutinin (PHA) or monoclonal anti-CD40 with CpG-B to induce T cell and B cell proliferation respectively, in the presence of a dose range of HCQ. No effect of HCQ was seen on T cell proliferation (*Figure 2A*). Also, no effects were observed on T cell activation markers after PHA stimulation for 6 hours (*Figure S1*). At HCQ concentrations >100 ng/mL, a decrease in B cell proliferation was observed, with an IC50 of 1138 ng/mL (*Figure 2B*).

CLINICAL STUDY

Demographics and safety

Of the 40 enrolled and randomized healthy subjects, 20 received a cumulative dose of 2400 mg HCQ in 5 days and 20 received placebo (*Figure 3*). The different age groups (18-30 and 65-75 years) were of equal size. Baseline characteristics are described in *Table 1*. All subjects completed their study treatment. One subject in the 65-75 years group erroneously took an additional 400 mg dose of HCQ on study day 2, after which the subject received 400 mg doses (once daily) for two consecutive days to not exceed the cumulative dose of 2400 mg.

Treatment-emergent adverse events were transient, of mild severity and did not lead to study discontinuation. Adverse events were reported more often by subjects in the active treatment arm (50%) compared to placebo (35%). Gastrointestinal complaints (20%) and dizziness (15%) were the most frequently reported adverse events in the active group. There were no findings of clinical concern following assessments of urinalysis, hematology and chemistry laboratory tests, vital signs, physical examination and ECGs.[22]

Pharmacokinetics

Mean HCQ concentration time profiles in plasma are depicted in *Figure 4A*. Individual concentration profiles have been published previously.[22] There were no significant differences in HCQ exposures between age groups (*Figure 4B*). Mean concentrations measured 27 hours after starting the treatment course (day 1, 121.0 ± 40.54 ng/mL) were in a similar range to those measured on the last day of the treatment course (day 4, 109.2 ± 35.59 ng/mL).

Pharmacodynamics

HYDROXYCHLOROQUINE DID NOT AFFECT CIRCULATING IMMUNE CELLS

The effects of HCQ on different circulating cell populations, both absolute as relative, were evaluated using flow cytometry. No apparent effects were seen on absolute values of total leukocytes, lymphocytes, monocytes or neutrophils (*Table S2*), as well as $CD14^+$ monocytes, $CD19^+$ B cells, $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells (*Table S3*). Furthermore, no effects were seen on relative T cell populations ($CD3^+$) in general, nor on subpopulations of T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and regulatory T cells ($CD4^+CD25^+CD127^-$). Similarly, no apparent treatment effects were observed in natural killer cells ($CD56^+$), B cells ($CD19^+$) and subpopulations of regulatory ($CD5^+CD1d^{hi}$), transitional ($CD24^{hi}CD38^{hi}$) and antibody secreting B cells ($CD27^+CD38^+$). Moreover, also in classical ($CD14^+$), non-classical ($CD16^+$) and intermediate ($CD14^+CD16^+$) monocytes and plasmacytoid dendritic cells (pDCs, $HLA\text{-}DR^+CD14^-CD16^-CD123^+$) no differences were found

between treatment groups. Also, between both age groups, no evident HCQ effects were observed (*Table S3*).

IN VIVO HYDROXYCHLOROQUINE SUPPRESSED IFNα SECRETION FOLLOWING TLR7 STIMULATION, BUT NOT AFTER TLR3, TLR9 OR RIG-I-LIKE RECEPTOR STIMULATION

To study the effects of HCQ on TLR/RIG-I-mediated IRF activation, PBMCs were stimulated with different endosomal TLR ligands: poly I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and poly I:C lyovec (RIG-I). Overall, no HCQ effect was observed on IFNα responses (*Figure 5*), except for a significant suppression of IMQ-driven IFNα production (inhibition of -48.2%, CI95 -72.1% – -4.0%, p = 0.038). Poly I:C-driven IFNα release also appeared to be suppressed by HCQ, but not significantly (inhibition -34.2%, CI95 -57.7% – 7.5%, p = 0.091). No differences in HCQ effect on IFNα responses were observed between the young and elderly population (*Figure S3*).

IN VIVO HYDROXYCHLOROQUINE SIGNIFICANTLY SUPPRESSED IL-6 SECRETION AFTER TLR7 STIMULATION, BUT NOT FOLLOWING TLR3, TLR9 OR RIG-I-LIKE RECEPTOR STIMULATION

Activation of NFKB signaling via endosomal TLR and RIG-I-like ligands was assessed by measuring downstream IL-6 production (Figure 6). HCQ significantly suppressed IMQ-driven IL-6 production (inhibition of -71.3%, CI95 -84.7% – -46.1%, p = 0.0005). No significant HCQ effects were observed on IL-6 production driven by CpG A (TLR9) and poly I:C (TLR3) stimulations (inhibition of -35.9%, CI95 -60. 3% – 3.6%, p = 0.068 and -37.7%, CI95 -62.6% – 3.7%, p = 0.067, respectively). No differences in HCQ effect on IL-6 responses were observed between the young and elderly population (*Figure S3*).

IN VIVO HYDROXYCHLOROQUINE DID NOT ALTER T CELL ACTIVATION

To further investigate the potential immunomodulatory effect of HCQ on T cell activation, whole blood samples were incubated with PHA, which is known to induce a general T cell response.[23] Hydroxychloroquine treatment did not modulate expression of T cell activation markers (CD25, CD69, CD71, CD154) following PHA-stimulation (*Figure S3*). In addition, PHA-induced secretion of IL-2 and IFNγ was assessed, no apparent differences were observed between HCQ and placebo (*Figure S4*).

HYDROXYCHLOROQUINE DID NOT ALTER EX VIVO B AND T CELL PROLIFERATION AFTER IN VIVO ADMINISTRATION

Proliferative capability of B cells was assessed by stimulating PBMCs *ex vivo* with anti-CD40 mAbs + CpG B ODNs, a known stimulus for human B cell activation.[24]

Following stimulation of PBMCs, the percentage of proliferative B cells in the HCQ-treated group was similar to that of the placebo group (70.47% at day 4 for placebo, 70.03% for HCQ) (*Figure 7*). In addition, PBMCs were stimulated with PHA to induce T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) proliferation. Proliferation of both $CD4^+$ and $CD8^+$ cells was comparable between the HCQ- and placebo-treated group (>95% for both groups for all time points for CD4, >92% for both groups for all time points for CD8). No differences were observed for B and T cell proliferation in the separate age groups (*Figure S5*).

Discussion

Although HCQ is widely used for the treatment of autoimmune diseases, the exact mechanism behind its immunomodulatory properties remains unclear. In this study we therefore aimed to quantify the immunosuppressive effect of HCQ by studying the endosomal TLR response and lymphocyte proliferation and activation both in *in vitro* experiments and *in vivo* in a randomized placebo-controlled trial in healthy volunteers.

In our *in vitro* experiments, HCQ dose-dependently inhibited TLR3-, 7- and 9-driven IL-6 and IFNα production, with profound effects at concentrations >100 ng/mL. These findings are in line with literature on TLR signaling modulation by chloroquine.[9,25] Limited data are available on the immunomodulatory effect of HCQ/chloroquine on RIG-I signaling.[26] RIG-I functions as a cytosolic sensor of nucleic acids, inducing a type I IFN response after activation. HCQ inhibited the IFN responses in THP-1 cells transfected with RIG-I ligands,[27] but this effect was not confirmed in cultures of human bronchial smooth muscle and epithelial cells.[28,29] This is in line with the observations in the current study, which shows that HCQ only mildly modulated RIG-I-mediated IFNα production in PBMCs, without affecting IL-6 release. Our results suggest that HCQ has a profound effect on endo-lysosomal TLR functioning *in vitro* but affects the cytosolic RIG-I-mediated

pathway to a lesser degree. This could be explained by HCQ's excessive affinity to the lysosomal intracellular compartment (expected to be 56,000-fold higher than cytosol).[30]

Hydroxychloroquine did not affect T cell activation *in vitro*. Although a dose-dependent inhibition of T cell proliferation by chloroquine following stimulation with anti-CD3/CD28 has been described,[31-33] we did not see any inhibitory effect of HCQ on T cell proliferation or expression of activation markers in our *in vitro* experiments. This may be explained by the fact that a different and more potent stimulus was used in this study (PHA), which might be more difficult to suppress. For B cell proliferation, on the other hand, a dose-dependent HCQ-mediated inhibition was observed *in vitro*, confirming previous research.[34] Although the HCQ-mediated inhibition was not as strong as the inhibition of cytokine production (IC50 of 1138 ng/mL for B cell proliferation vs 145-696 ng/mL for cytokine production), at concentrations > 100 ng/mL a clear HCQ-mediated decrease in B cell proliferation was found.

While HCQ had strong immunosuppressive effects *in vitro*, especially at high concentrations, less pronounced *ex vivo* effects of the compound were observed in our clinical study. Compared to placebo, 5-day HCQ treatment did not significantly suppress B cell proliferation or *ex vivo* TLR-driven IFNα and IL-6 secretion in PBMC cultures, except for a suppressive effect on TLR7-driven responses. The most likely explanation for this discrepancy between *in vitro* and *ex vivo* is that there was insufficient drug exposure at the evaluated HCQ dose and regimen in the clinical study. By using a 5-day dose regimen of HCQ (the recommended off-label dose for COVID-19 at the time of study conduct), an average maximum plasma concentration of 121 ng/mL was reached. This concentration is considerably lower than plasma levels found in rheumatoid arthritis patients receiving HCQ treatment of 200 mg daily for a longer time period, which ranges from 200-500 ng/mL.[35-37] Peak exposures of 100-150 ng/mL from the clinical study translate into a maximal inhibitory effect of 20 to 50% in most cellular assays. In combination with the observed variability of the endpoints, such effects remain easily undetected. However, whole blood concentrations are expected to be approximately 2-to-7-fold higher than plasma concentrations due to intracellular uptake in blood components,[38-40] which would make the concentrations more in range with the *in vitro* experiments. Also, due to the large volume of distribution, and the high HCQ tissue concentrations as compared to plasma , immunosuppressive effects in specific tissues may be significant.[39-42] Moreover, HCQ has a gradual onset of action for HCQ, and is biologically active even after drug discontinuation.[8] This would mean that the five-day treatment that was used in the current study is insufficient to detect *ex vivo* drug effects. Other studies, for example investigating HCQ effect in HIV patients, showed a discrepancy between plasma levels and drug efficacy.[43]

The widespread use of HCQ following the onset of the COVID-19 pandemic was the reason to initiate our experiments. The initial off-label use of HCQ was primarily based on studies that assessed *in vitro* antiviral activity against SARS-COV-2.[44] However, there is also a longstanding hypothesis that the immunomodulatory properties of chloroquine and HCQ could dampen immunopathology caused by viral infections such as influenza, Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) and COVID-19 by suppressing the host immune response.[45-47] Use of HCQ in COVID-19 patients did not show evident favorable effects for clinical endpoints such as mortality and mechanical ventilation for both prophylaxis and treatment.[48] Our study provides mechanistic insight in the immuno-modulatory effects of a HCQ dosing regimen that was used to treat COVID-19. We found that a 5-day treatment course of HCQ did not have extensive immuno-modulatory effect in healthy individuals. Hydroxychloroquine treatment only significantly inhibited TLR7 responses. In theory, inhibition of the TLR7-mediated innate response to viral agents may be disadvantageous during the initial stages of viral infection.[49,50] However, recent COVID-19 trials did not show an effect of HCQ treatment on disease incidence, and long-term HCQ use in rheumatoid arthritis is not associated with higher incidence of upper respiratory tract infections.[51,52]

In conclusion, we showed extensive and profound immunomodulation by HCQ *in vitro*, however in a clinical study in healthy volunteers, the overall immunomodulatory effects of a 5-day HCQ treatment regimen of 2400 mg were limited. The pharmacological activity of HCQ in autoimmunity remains to be studied in greater detail, based on the assays as presented in our studies and at a therapeutic dose and regimen relevant for the condition of interest.

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TABLE 1 BASELINE CHARACTERISTICS.

	Hydroxychloroquine		Placebo	
	Age group 18–30 yrs (n=10)	Age group 65–75 yrs (n=10)	Age group 18–30 yrs (n=10)	Age group 65–75 yrs (n=10)
Age, median (range)	23 (20–26)	68 (65–70)	23 (18–25)	68 (65–71)
BMI, mean (SD)	21.8 (1.5)	25.8 (2.0)	24.4 (1.9)	24.2 (3.0)
RACE OR ETHNICITY*, N (%)				
White	10 (100)	10 (100)	10 (100)	10 (100)
Other	0 (0)	0 (0)	0 (0)	0 (0)

**Self-reported race or ethnicity of subjects. BMI = body mass index; SD = standard deviation.*

FIGURE 1 HCQ DOSE-DEPENDENTLY INHIBITED ENDOSOMAL TLR INDUCED IFNA AND IL-6 RELEASE IN VITRO.
PBMCs were 182 stimulated with 50 µg/mL PolyI:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5 µM CpG-A (TLR9) or 1 µg/mL Poly I:C/lyovec (RIG-I) for 24 hours in the presence of a dose range of HCQ. IFNα and IL-6 release were measured by ELISA. The mean ± SD of the change from baseline of 6 subjects is shown. The IC50 was calculated using a four parameter non-linear regression fit where applicable.

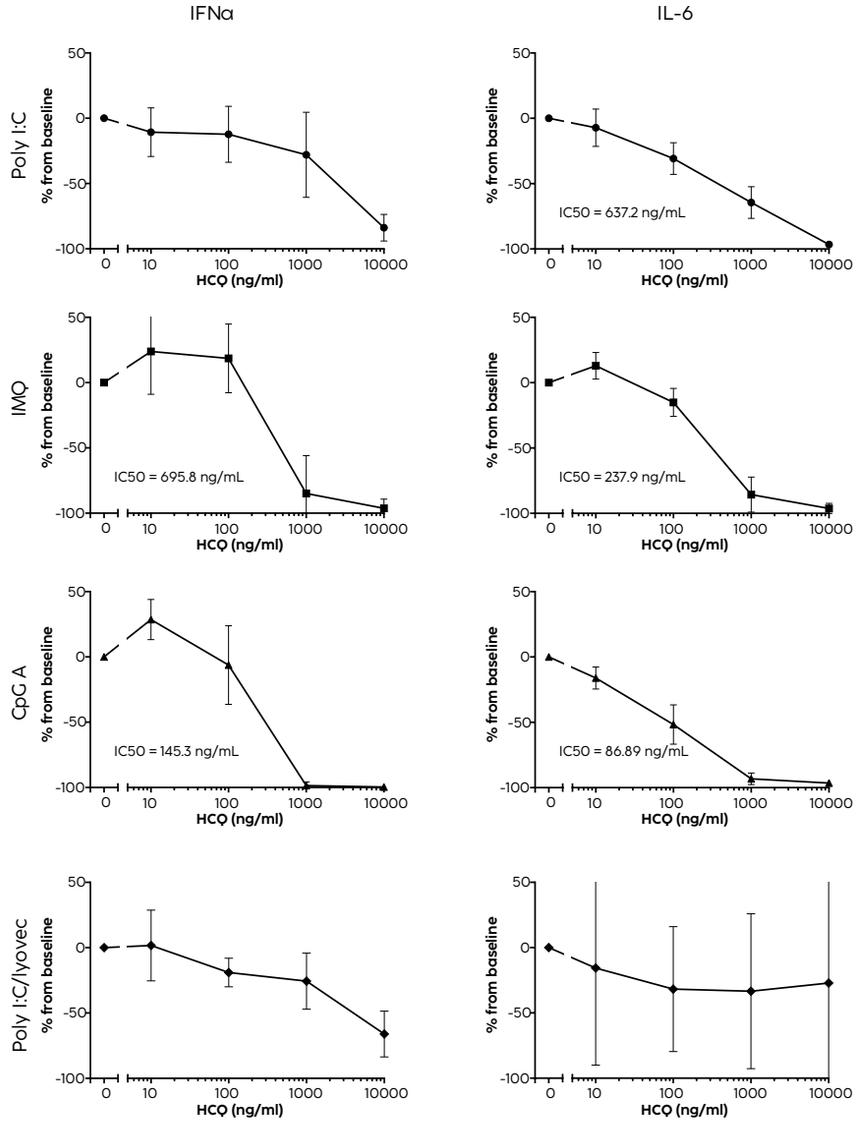


FIGURE 2 HCQ DOSE-DEPENDENTLY INHIBITED B CELL, BUT NOT T CELL PROLIFERATION IN VITRO. PBMCs from 6 healthy donors were stained with CTV and stimulated for 5 days with 5mg/ml PHA for T cell proliferation (A), or 5 MG/mL anti-195 CD40 mAb + 2.5 MM CpG B for B cell proliferation (B). Proliferation was measured by flow cytometry. The mean ± SD of the change from baseline are shown. The IC50 was calculated using a four-parameter non-linear regression fit where applicable.

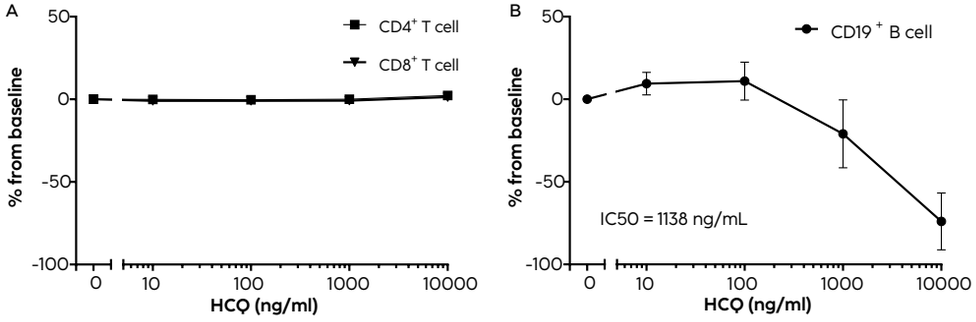
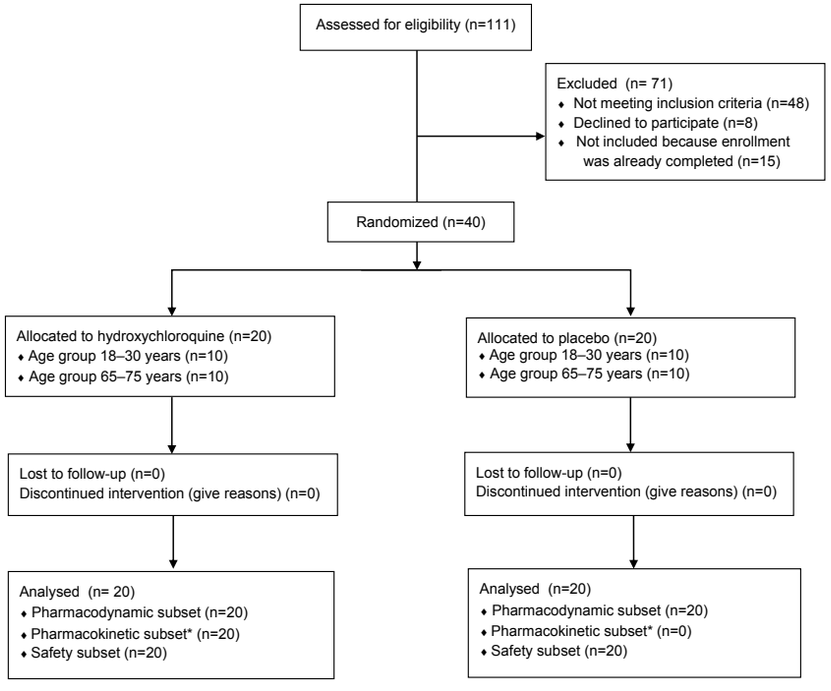
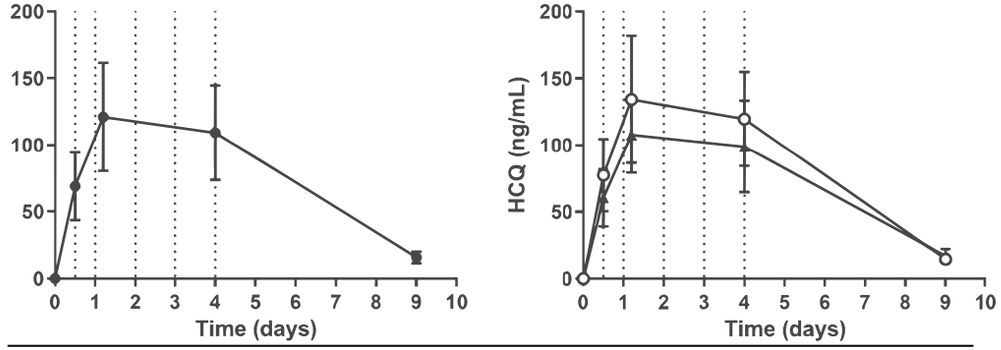


FIGURE 3 TRIAL FLOW CHART (CONSORT DIAGRAM).



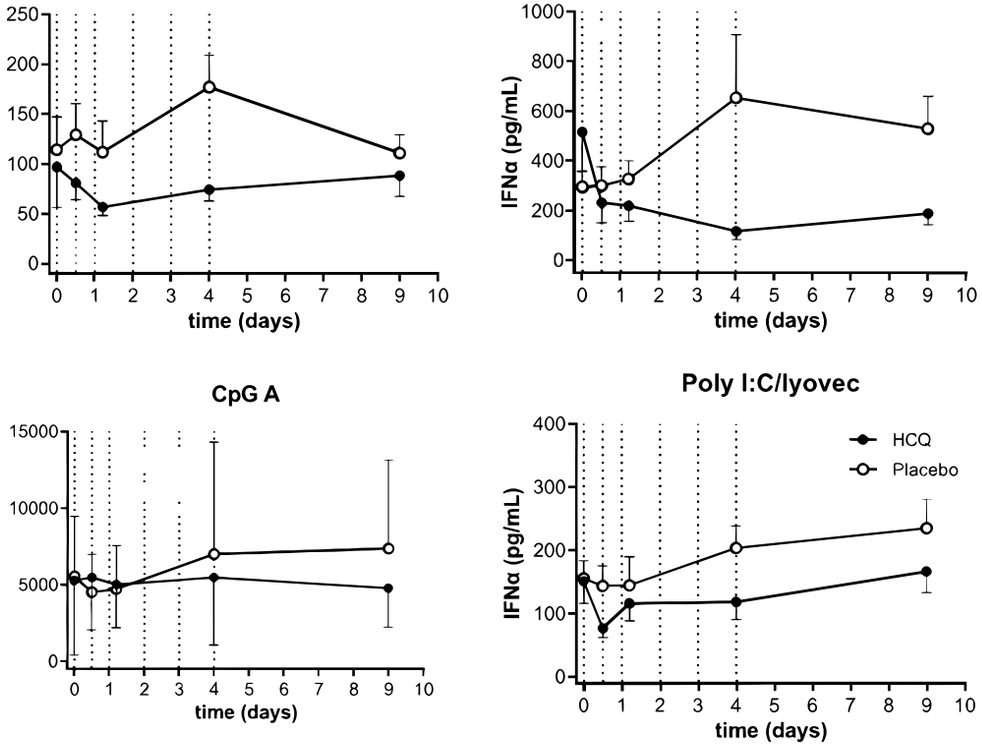
**Drug concentrations were only analyzed in the active treatment group.*

FIGURE 4 PHARMACOKINETIC PROFILE OF HCQ. Mean and standard deviation of hydroxychloroquine plasma concentrations for HCQ treatment group (left), and split for young and elderly volunteers (right).



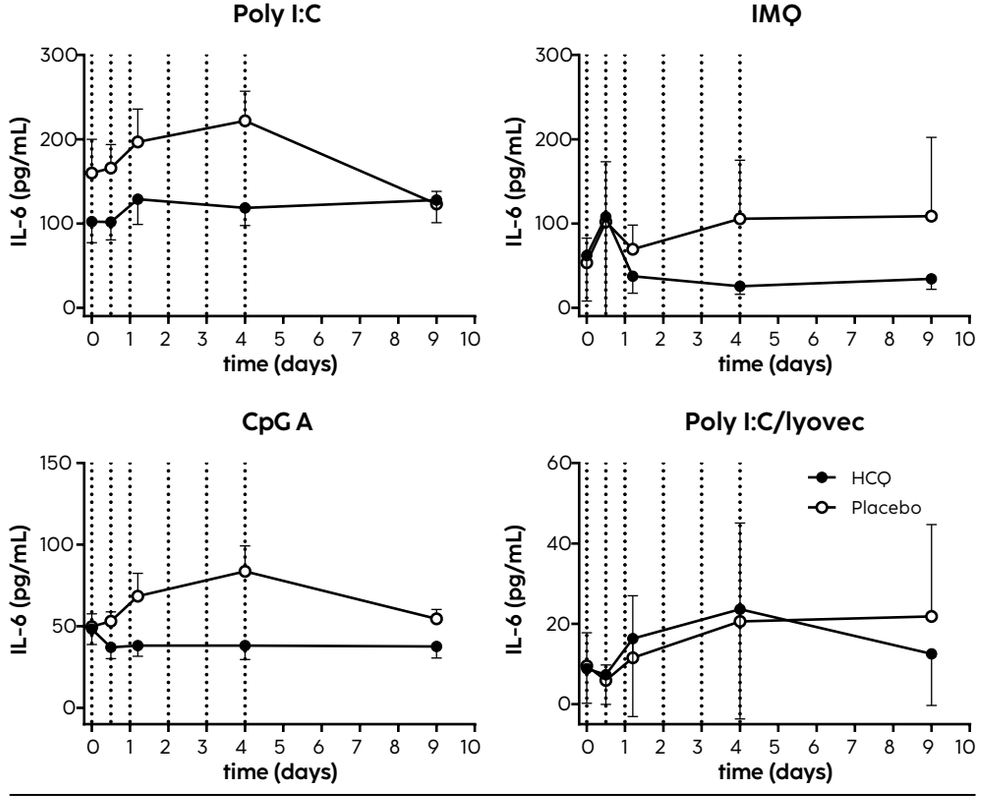
Dotted vertical lines indicate timing of HCQ dosing (0, 12, 24, 48, 72, 96 hrs).

FIGURE 5 IN VIVO HCQ INHIBITED IMQ-INDUCED IFNα RELEASE, BUT NOT TLR3, TLR9 AND RIG-I. PBMCs were stimulated with 50 µg/mL Poly I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CpG A (TLR9) or 1 µg/mL poly I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFNα release was measured by ELISA.



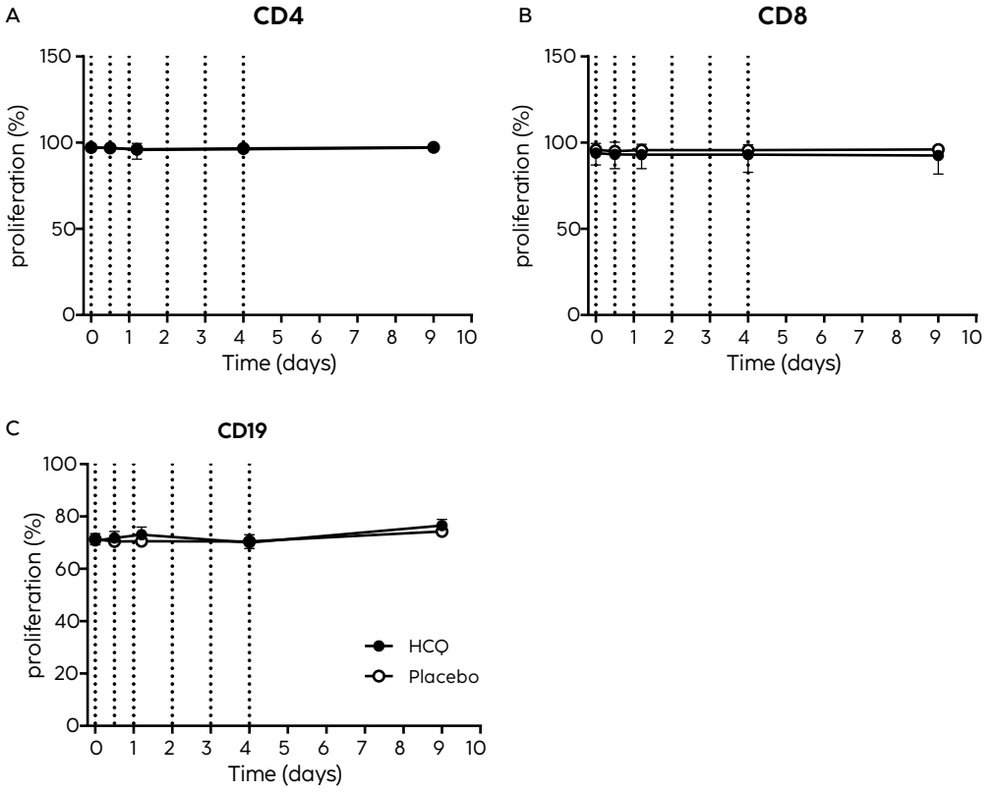
Data is shown as mean + SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.

FIGURE 6 IN VIVO HCQ INHIBITED IMQ-INDUCED IL-6 RELEASE, BUT NOT TLR3, TLR9 AND RIG-I. PBMCs were stimulated with 50 µg/mL Poly I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CpG A (TLR9) or 1 µg/mL poly I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFNα release was measured by ELISA.



Data is shown as mean + SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.

FIGURE 7 IN VIVO HCQ DID NOT AFFECT T AND B CELL PROLIFERATION.PBMCS WERE STAINED WITH CTV AND STIMULATED FOR 5 DAYS WITH 5µG/ML PHA FOR T CELL PROLIFERATION (A), OR 5 µG/ML ANTI-CD40 MAB + 2.5 µM CPG B FOR B CELL PROLIFERATION (B). Proliferation was measured by flow cytometry.



DATA IS SHOWN AS MEAN + SD. Dotted vertical lines indicate HCQ dosing times

SUPPLEMENTARY FIGURES AND TABLES

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

CHAPTER 7

ACCELERATING VACCINE TRIAL CONDUCT IN A PANDEMIC WITH A HOT SPOT-BASED INCLUSION STRATEGY USING

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Abstract

Clinical development of vaccines in a pandemic situation should be rigorous but expedited to tackle the pandemic threat as fast as possible. We explored the effects of a novel vaccine trial strategy that actively identifies and enrolls subjects in local areas with high infection rates. In addition, we assessed the practical requirements needed for such a strategy. Clinical trial simulations were used to assess the effects of utilizing the so-called 'hot spot strategy' compared to a traditional vaccine field trial. We used pre-set parameters of a pandemic outbreak and incorporated realistic aspects conducting a trial in a pandemic setting. Our simulations demonstrated that incorporating a hot spot strategy shortened the duration of the vaccine trial considerably, even if only one hot spot was identified during the clinical trial. The active hot spot strategy described in the paper has clear advantages compared to a 'wait-and-see' approach that is used in traditional vaccine efficacy trials. Completion of a clinical trial can be expedited by adapting to resurgences and outbreaks that will occur in a population during a pandemic. However, this approach requires a speed of response that is unusual for a traditional phase III clinical trial. Therefore, several recommendations are made to help accomplish rapid clinical trial set-up in areas identified as local outbreaks. The described model and hot spot vaccination strategy can be adjusted to disease-specific transmission characteristics and could therefore be applied to any future pandemic threat.

Introduction

The viral genome of the causative pathogen of coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome-coronavirus 2 (SARS-COV-2) was published on National Center for Biotechnology Information (NCBI)/ Gen Bank on January 11, 2020, about 2 weeks after the identification of the first patient with this disease that has since overwhelmed the world. The subsequent development and marketing approval of several vaccines for SARS-COV-2, took approximately a year from the identification of the viral genome. This speed of development is clearly extraordinary and has no precedent in the development of any therapeutic or preventive intervention, using modern quality standards. However, in the course of this year, 1.5 million people died and countless others became ill and the personal and economic consequences were dire. Any strategy to reduce the development time of a vaccine, even by days or weeks, would be of enormous benefit.[1] This realization has led to several initiatives to speed up the process that subsequently clearly paid off.[1] However, the question remains whether further gains could be made by better preparedness for a new pandemic, that will without any doubt occur again.[1]

After identification and construction of the vaccine compound, the development trajectory of a vaccine moves from the establishment that the prophylactic intervention *works* to that it *helps*. By this we mean that for a vaccine to work, it first needs to be established that vaccination leads to a potent and specific immunogenic response.[2,3] This can be assessed relatively quickly in clinical trials with a relatively small number of subjects. Indeed, the first studies indicating initial safety and immunogenicity of vaccines appeared approximately 6 months after a vaccine candidate was identified.[4] Although it could be argued that this is about the maximum speed possible for this phase, we have identified several bottlenecks that could be addressed to speed up clinical development.[5]

In the case of a novel pathogen it cannot be assumed that a neutralizing immune response automatically prevents clinical disease and the regulatory position about this is unequivocal.[6] Therefore, the establishment that a vaccine helps, in that it successfully prevents disease or even transmission, requires evidence from large field studies. These trials have the primary objective to establish efficacy, but also gather sufficient data on vaccine safety and therefore require a size of approximately 15.000-20.000

volunteers vaccinated with the active compound to detect rare side effects that occur with a frequency of about 1:10.000 with reasonable certainty. To put it concisely, thousands of people are vaccinated in a short period and investigators wait to see how many volunteers become infected in the group receiving the vaccine compared to the placebo (or other comparator) group. However, such trials will only reliably demonstrate efficacy when the number of infections in the studied population is sufficiently high. Consequently, these trials will not reach the efficacy objective when the caseload is low. In a pandemic, this will inevitably happen after an outbreak has been identified, as governmental control interventions will be put in place to prevent further transmission, change population behavior and reduce the caseload.

When trials are executed in areas with a less than expected caseload, the trial will take much longer to complete, or be less reliable. This problem is illustrated in ebola vaccine trials impacted by decline in cases.[7] In an urgent pandemic situation, such loss of time is directly related to increased suffering. In some cases, this problem could be solved by using a controlled human infection model.[8] Unfortunately, such models remain controversial in the case of a severe infection without adequate treatment, that has a widely varying severity in different risk groups. Additionally, these models are currently not regulatory acceptable as a surrogate for field trials. We therefore attempted to explore if the use of a "naturalistic infection model," provided by an area with a rapidly increasing infection rate, such as local outbreaks or alternatively called "hot spot," would shorten the time to determine vaccine efficacy and could consequentially further expedite clinical development. Such local outbreaks could be identified on the level of communities, districts, cities, or even states/provinces.

Because the study protocols and design for large-scale efficacy trials are largely standardized, we considered if there could be strategies used to shorten study duration by actively identifying and deploying trial activities deliberately in areas where an increase or resurgence of infections occurs, even after the study was started in another location. Such a dynamic approach may lead to faster identification of disease cases in both the active and placebo arms of the efficacy trial, as opposed to the traditional 'wait-and-see' approach. This novel strategy is clearly dependent on many factors and therefore ideally suited for trial simulation to study its feasibility. In this study, we performed a simulation of such a strategy. The model we developed is intended to have applications beyond the search for a COVID-19 vaccine and should also be applicable for future pandemics and pandemic preparedness. To execute this strategy, dedicated mobile clinical trial teams should be formed and kept operational for a rapid response once an outbreak has been identified. We also supply suggestions for the needed equipment, composition and organization of such clinical trial teams that can quickly respond after the identification of an infection hot spot to further boost the feasibility of this strategy.

Methods

SIMULATION METHODOLOGY

The potential improvement of utilizing an active hot spot vaccination strategy was quantified in a clinical trial simulation performed in R V3.5.3. Details about the model selection, definitions, simulations and script can be found as *Supplementary File*. Infections over time were simulated and the commonly used 'wait-and-see' strategy was compared with the proposed active hot spot vaccination strategy. *Table 1* shows the parameters used for the simulation of the infections over time in the general population and all parameters for the infections over time and identification criteria in a hot spot. A mean hot spot growth rate of 3% was chosen for the baseline scenario, resulting in a doubling of the number of infections after ~ 23-24 days. After a certain duration (40, 60, or 90 days) stringent government measures were put in place that immediately reduced the growth rate.

Furthermore, *Table 1* provides information on logistics related characteristics, such as the maximal number of vaccinations per day that can be given and the percentage of the total vaccinations given in a hot spot. If a hot spot-based vaccination strategy was applied, the total number of vaccinations in the general population was set to N_{total}-$N_{hot\ spot}$. Therefore, the total number of administered vaccinations (the total sample size) was identical in both strategies. In the scenario where no hot spot was simulated or could be identified, the $N_{hot\ spot}$ vaccines were randomly distributed over the total population at day 100.

In order to explore the effect of both strategies on the study duration, the following simulation methodology was applied:

1 Simulate infection profile in the general population and in a hot spot, with a hot spot occurring after X days since start of study
2 Run clinical trial simulation both on the *wait-and-see* and *active hot spot vaccination* strategy
3 Randomly vaccinate subjects and randomly infect subjects, based on the daily infection rate for the general population and the hot spot
4 Check each day if a hot spot was identified based on the hot spot identification criteria.
   a) If a hot spot was identified, start subject inclusions and additional vaccinations in the hot spot
5 Count the cumulative number of infections after the time until vaccine effectiveness in the study population (both in the placebo and active group)
6 End the study if the target level of infections has been reached in the study population and record the total trial duration

Oneach simulated infection profile, eight trial simulations were run (four per strategy), to account for the stochasticity in the random sampling procedure. Due to the variable nature and spread of (novel) pandemic infections, a local sensitivity analysis was performed to explore differences compared to the baseline scenario, in which one model parameter at a time was changed. For each scenario, 20 different infection profiles were simulated and analyzed to determine the mean and standard error of the study duration and the difference (Δ) between the two strategies.

Results

Figure 1 presents the baseline scenario of the number of infections over time in the general population and in a simulated hot spot. A clear increase in the number of infections can be observed in the hot spot with a corresponding reduction after stringent government measures were put in place. With the wait-and-see strategy, this hot spot population would only have received 5% (1000) of all vaccines in the study due to the random inclusion of subjects following this approach. The number of vaccinations in the hot spot is increased to 2.900 (receiving an additional 10% of the vaccines) at seven days after the identification of a hot spot (three days before start vaccinations and four days of administering vaccines). By using the hot spot-based inclusion strategy the number of infections in the study population increased and thereby reduced the total study duration with 15 days in the baseline scenario, a 10% reduction of the study duration compared with the baseline scenario (*Table 2*).

Table 2 and *Figure 2* show the differences between the wait-and-see versus active hot spot vaccination strategy for all the explored scenarios. These results show that in almost all the investigated scenario's a reduction of the study duration was shown when applying the hot spot-based vaccination strategy. Especially when increasing the percentage of vaccines that were deployed in a hot spot up to 20%, which resulted in a 22.7 days decrease of the study duration. The only explored scenario in which an increase in duration was present was when no hot spot occurred and the withheld vaccines were administered as late as day 100, after which the study duration showed a minor increase from 157 to 162 days. These results indicate that regardless of potential changes in infections over time or lockdown measures in a hot spot (that would reduce the number of infections back to baseline) withholding part of the total vaccine pool for an active vaccination strategy has the potential to reduce the study duration with multiple weeks and only has a limited risk of increasing the study duration. Additionally, when lower baseline infection rates in the general population are present (leading to an increase in the study duration) and faster identification of the hot spot is possible (improving the benefit of the proposed strategy) an even larger reduction in study duration could be observed.

RAPID RESPONSE TRIAL TEAM

The lead time for the formation and operationalization of such a team should be as short as possible. Therefore, clinical trial teams should be kept in readiness and mobilized as soon as the phase I trials of new vaccines start in a pandemic. Teams should ideally be managed from a central location, for instance, from national public health organizations or the World Health Organization. Ideally such a strategy should be employed across different countries. An essential component of the strategy is the possibility to have approved standardized study protocols, where only pre-specified data of the vaccine must be inserted. Pandemic preparedness arrangements with pertaining authorities and ethics committees should exist for fast tracking the final approval with expected approval times of less than a week.

We recommend that rapid response trial teams are constituted on a national level but based upon international standards for training and equipment. If this is not feasible, trial teams should be deployed for low-to-middle wage countries or countries that lack sufficient clinical trial infrastructure or experience.

When a hot spot is targeted there will be little time for communicating and therefore generic communication plans for local and social media should be prepared in advance to improve local community engagement. Software systems will be an essential asset and should be set up for multilingual use and to require minimal or no paper administration (*Table 3*).

Discussion

Developing novel vaccines in a pandemic setting requires a different paradigm for clinical development. Our simulation demonstrates that a field trial can be expedited by adapting to the changing nature of disease incidence in a pandemic, but that this requires a speed of response that is unusual in the standard phase III clinical trial. The feasibility and success of performing large scale phase III field trials is dependent on the incidence of the disease in a population. If the incidence is relatively low this means that a large group of participants needs to be followed over a long time to encounter enough cases in the active/placebo arm of the trial. On the other hand, if the reproduction rate of the pathogen is too high, stringent government measures to reduce transmission within a population can hinder the feasibility to perform a field study and previously selected areas to study the vaccine might not have been optimal. When the preparation time for a trial is too long, the outbreak may already be under control in the place where the trial was intended. As a result, clinical trials are initiated in endemic countries with relatively high incidence of disease but may be prematurely halted due to dropping disease incidence. We suggest a strategy to expedite vaccine development where recruitment of participants is performed dynamically in areas where disease incidence rates are growing fast. By identifying local outbreaks and deploying mobile ground teams to move to these areas with high infection incidence, it is possible to conduct a clinical trial in a subgroup of volunteers with a high *a priori* risk of being exposed and infected to the pathogen. We demonstrate by our model that key endpoints such as disease incidence in these so called 'hot spots' can be reached more efficiently compared to the traditional wait-and-see approach.

The hot spot vaccination strategy described in this paper utilizes a more straightforward approach compared to other case-reactive vaccination strategy such as cluster or ring vaccination, used in ebola vaccine trials.[9] The hot spot vaccination approach described in this paper simply aims to recruit, enroll and randomize subjects on an individual level, but dynamically in areas where there is a higher *a priori* risk of being exposed to the pathogen. Although a ring vaccination trial might be preferable in some outbreak situations, it has some inherent methodological drawbacks associated with cluster randomization.[10]

The vaccination approach described in the paper has several advantages compared to a passive wait-and-see vaccination approach currently used in field trials. Our model illustrates that in almost all explored scenario's active hot spot vaccination will lead to a reduction in study duration. We used realistic infection profiles over time in which growth rates of 2-5% were simulated in the hot spot, these parameters would change on a case-by-case basis in other pandemics. Lastly, the model can be adjusted to disease-specific transmission characteristics and be used for any future pandemic threat.

Identifying local outbreaks of infection requires a digital infrastructure and means of active surveillance, testing and contact tracing of novel infection cases. Most countries with developed economies already have such a system in place. During the COVID-19 pandemic multiple countries developed special testing and tracing mobile applications. Such mobile applications can also be used to identify regions where a hot spot vaccination strategy is possible. Moreover, in this digital age vaccine trials still mostly rely on paper source data, visits to the research center for measurements of vital signs and face-to-face meetings with the investigators. COVID-19 has shown that electronic alternatives such as electronic questionnaires and digital informed consent are possible[11] and vital signs can be measured using wearable technology.[12,13] Use of these modern technologies will further improve the feasibility to conduct a hot spot vaccine strategy during an acute outbreak with sufficient speed.

The suggested hot spot approach has a few limitations that have to be noted. As with every clinical trial it is important to recruit and engage participants. Moreover, as the hot spot vaccination approach will be deployed in local outbreaks it is important to create local community engagement to participate in clinical research. Much of this will have to be done on a regular basis before a pandemic is identified and yearly pandemic

preparedness simulation exercises can be a good way to keep communities engaged. Our model had as the key outcome the proportional reduction of the incidence of disease. Other outcomes, such as reduction of infectivity or duration of protection do obviously require longer trials with more intensive sampling, but these outcomes could also be studied further after initial or conditional authorization of a vaccine to limit a pandemic. Last, as mentioned previously, the described strategy requires that sophisticated contact tracing for the pathogen is readily available.

For future pandemic preparedness, maintaining a mobile hot spot vaccination trial approach will require continuous financing for potentially long periods. Even if no local outbreak is identified, a study team needs to be on stand-by mode, ready to be deployed as soon as a hotspot is identified. The pharmacoeconomic evaluation of utilizing and maintaining this strategy falls beyond the scope of this paper. However, we feel that this investment is worthwhile given the merits of expediting the generation of efficacy data and accelerating vaccine development which ultimately has profound societal and economic impact. Such costs must be borne by funds from a dedicated pandemic defense budget, analogous to funding of military national defenses.

The applied simulation methodology was performed as a proof-of-concept, in which a combination of realistic baseline parameters for infection rates and hot spot parameters were applied. However, the performed local sensitivity analysis only shows the results of modifying one parameter at a time based on the baseline scenario. Changes in multiple parameters at the same time or scenarios with a completely different set of parameters are more likely depending on study logistics and infection characteristics. Clinical trial simulations in the future should therefore be adapted on a case-by-case basis. Furthermore, the current infection model treated the general population and the hot spot as independent populations in which growth rates were randomly sampled from uniform and normal distributions. The exponential growth rate in the hot spot switched to an exponential decline rate after a fixed number of days in this simulation. In reality, this switch would be more subtly caused by the stepwise introduction of governmental measures which would broaden the hot spot peak, and would further improve the benefit of a hot spot-based vaccination strategy. As this simulation was primarily focused on clinical trial design and execution, this model oversimplifies the complex epidemiological components of disease outbreaks and an extension with the modelling of mixing patterns could improve the precision of this simulation.[14]

In conclusion, by investigating vaccine efficacy in clusters of subjects with a high risk of infection, efficacy data can be generated more efficiently, as is shown in our model. Our suggested hot spot-based vaccination approach may reduce clinical development time and thus, expedite clinical development of new prophylactic interventions in emergent pandemic situations and thus may save considerable opportunity costs and, above all, lives.

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TABLE 1 BASELINE PARAMETERS USED FOR THE SIMULATION OF INFECTION RATES OVER TIME, THE VACCINE EFFECTIVENESS, AND THE STUDY DESIGN (LOGISTICAL) COMPONENTS IN THE GENERAL POPULATION AND IN THE HOT SPOT.

Description	Value
Total population pool size	10 million
Population size of general population	9.5 million (95% of total population)
Population size of hot spot	500.000 (5% of total population)
INFECTION PARAMETERS	
Minimal number of infections per day in population over time*	6 / 100.000
Day–to–day reproduction rate (not during hot spot)	−5% to 4.5% (uniform distribution, sampled at random per day)
HOT SPOT PARAMETERS	
Start of hot spot since start of trial	20 days
Daily growth rate in hot spot	Mean growth of 3% (normal distribution, standard deviation of 2%) per day
Duration of growth period	60 days
Daily decline rate after growth period until baseline is reached	− 3%
Duration of lockdown period	40 days or until minimal number of infections was reached
VACCINE AND STUDY INFORMATION	
Total number of vaccinations given and subjects included (N_{total})	20.000
Number of random vaccinations given in total population per day	2.500
Time until effectiveness of vaccine (days)	21
Target total number of infections in study population for completion of study (% of study population)	100 (0.5%)
Effectiveness of vaccine	80%
Hot spot threshold value for identification	3 days of > 1.5x the infection rate of general population (infections/100.000)
Time until start vaccination in hot spot after identification	3 days
Number of vaccinations given in hot spot population per day	500
Total number of vaccinations given in hot spot ($N_{hot\ spot}$)	2.000 (10% of total)

**Infections are constrained to not go below the baseline level of 6/100.000 to simulate an ongoing pandemic.*

TABLE 2 DIFFERENCE IN TRIAL DURATION USING AN ACTIVE HOT SPOT VACCINATION STRATEGY COMPARED TO A WAIT AND SEE APPROACH. Each row shows 1 component of the simulation that was altered and the resulting change between strategies. Δ hot spot vaccination strategy is the mean difference in study duration of 20 iterations for each scenario.

Scenario	Value	Δ hot spot vaccination strategy (days)*
Baseline simulation		−15.36 (1.79)
Hot spot duration	40 days	−2.54 (2.09)
Hot spot duration	90 days	−17.29 (1.09)
Hot spot growth rate per day	2%	−3.64 (2.07)
Hot spot growth rate per day	5%	−27 (1.6)
Hot spot percentage of vaccinations	20%	−22.7 (2.04)
Hot spot percentage of vaccinations	5%	−5 (2.09)
Hot spot start vaccinations	5 days	−10.28 (1.9)
Hot spot start vaccinations	9 days	−13.29 (2.16)
Hot spot vaccinations per day	1000	−12.6 (1.65)
Hot spot vaccinations per day	2000	−13.9 (2.92)
Population size of hot spot	0.50%	−15.09 (2.16)
Population size of hot spot	1%	−13.85 (1.94)
Population size of hot spot	10%	−9.62 (1.79)
Onset of hot spot after start of study	60 days	−3.51 (1.6)
Onset of hot spot after start of study	Never	4.72 (2.15)
Time to vaccine effectiveness	14 days	−14.93 (2.21)
Time to vaccine effectiveness	28 days	−7.91 (1.91)

**Mean (standard error)*

TABLE 3 PRACTICAL AND PERSONNEL REQUIREMENTS FOR MOBILE TRIAL UNITS AND CENTRAL COORDINATING CENTER.

RAPID RESPONSE PERSONEL

- At central coordinating center:
  Infectious disease specialist
  Clinical epidemiologist/modeler
  Logistic expert
  Modeler/metrician
- In mobile units:
  Technical staff (location management, security)
  Pharmacy technicians
  Nursing staff and trial physician

KEY FACILITIES COORDINATING CENTER

- Communication facilities to mobile center.
- Continuous access to epidemiological data.

OTHER

- Public (or access to) up-to-date data on disease incidence per region.
- Home-monitoring equipment and software.

KEY FACILITIES HOT SPOT SITE

- Mobile vaccination center(s) (e.g. portacabin,
- repurposed existing community facilities.
- Transportable laboratory or infrastructure to centralize laboratory assessments.
- Mobile pharmacy and refrigeration units.

IT INFRASTRUCTURE

- Mobile software applications for digital contact tracing.
- Dependent on location: GSM and satellite communication equipment and internet connections.
- Reliable power supply.
- Digital infrastructure for informed consent procedure, recording of participant reported outcome measures and vital signs (home monitoring) and electronic case report forms.

COMMUNICATION KITS

- Participants information text.
- Public media campaigns.

FIGURE 1 SIMULATED INFECTION PROFILES IN GENERAL POPULATION AND IN HOT SPOT OVER TIME (DAYS). The onset of the hot spot in the baseline scenario is 20 days since start study and continues up until 80 days since start study. Grey area shows 90% prediction interval of the baseline scenario. Black dashed lines show 20 random iterations of the baseline infection profiles.

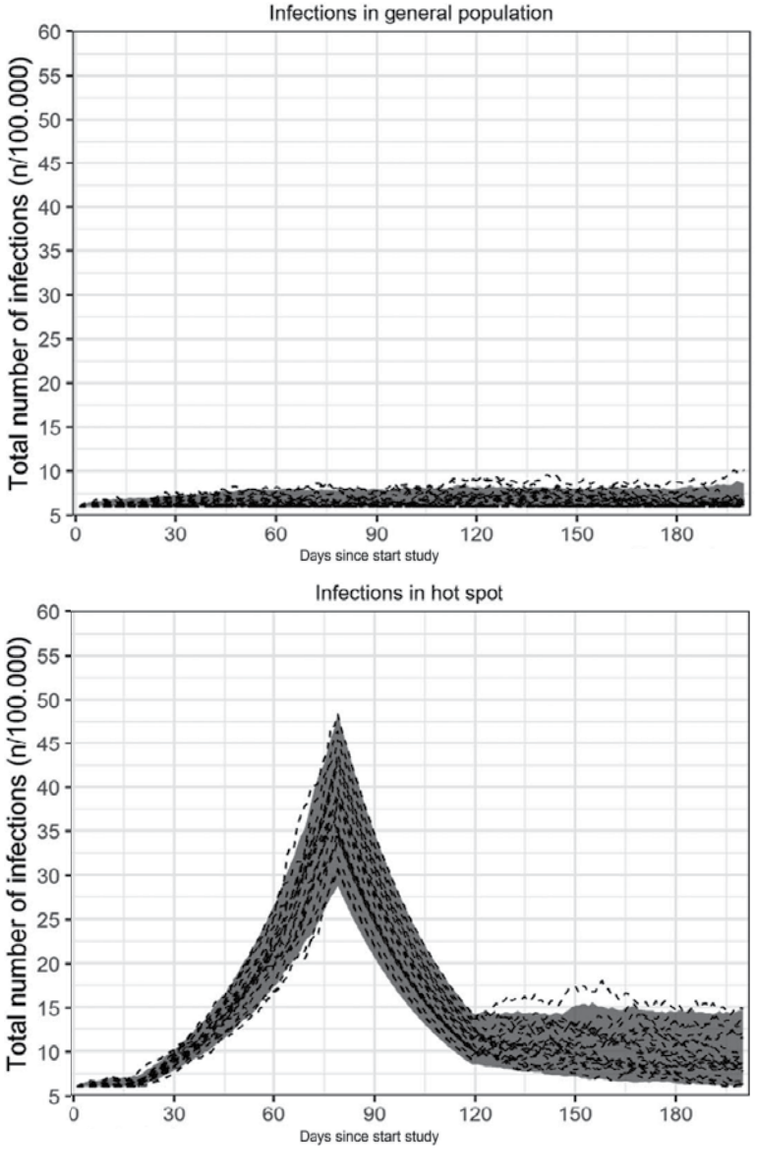
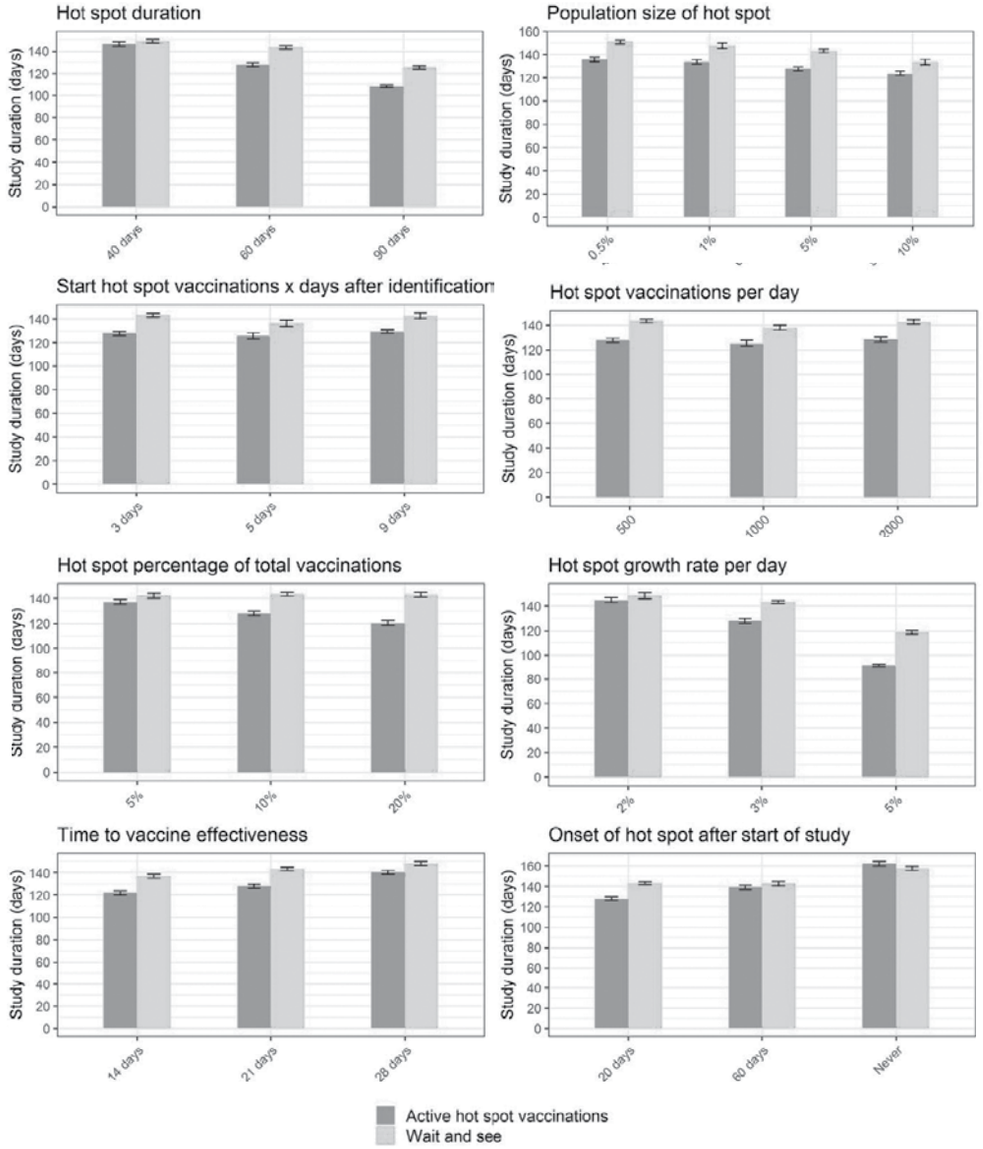


FIGURE 2 MEAN OF THE STUDY DURATION FOR ALL EXPLORED SCENARIO'S AND BOTH STRATEGIES.ERROR BARS PRESENT THE STANDARD ERROR OF ALL ITERATIONS (N=20). The baseline scenario is included in each facetted labeled with the default parameter combination (e.g hot spot duration of 60 days, hot spot size of 5%, etc.).



CHAPTER 8

HOW TO EXPEDITE EARLY-PHASE SARS-COV-2 VACCINE TRIALS IN PANDEMIC SETTING — A PRACTICAL PERSPECTIVE

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SUPPLEMENTARY FIGURES AND TABLES

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

The COVID-19 pandemic is the biggest global health crisis of this century, straining health care capacity worldwide and bringing nations to a standstill with an unprecedented social and economic impact. A safe and effective vaccine is needed to help counter the COVID-19 pandemic. Vaccination development, however, is a complex and lengthy process and attrition rates are high.[1] It is not unusual for a new vaccine to take up to 10 to 15 years from antigen discovery to licensing. These timelines become problematic when acute outbreaks demand immediate interventions for novel infectious agents. This has been exemplified in outbreaks with severe-acute-respiratory syndrome (SARS) (2003), H1N1/09 influenza (2009), Middle East respiratory syndrome (MERS) (2012), ebola virus disease (2013), zika virus disease (2015). For H1N1/09 a vaccine was approved only after the outbreak reached its peak. A vaccine for ebola was approved six years after the major outbreak in West-Africa. No vaccines against zika and the coronaviruses causing SARS and MERS have been licensed to date. Several vaccine candidates are currently in development for COVID-19, either in the preclinical or early clinical phase. However, previous outbreaks show us that vaccine development typically does not keep up with the speed of a pandemic.

Several bottlenecks can be identified in the vaccine development process.[2] After preclinical studies and manufacturing processes, a rate-limiting step in vaccine development is the conduct of clinical trials. In addition, the majority of vaccine candidates fail in clinical development and never reach market authorization.[1] Only 77% of the vaccine candidates will successfully transition from phase I to phase II and only 58% will successfully transition from phase II to phase III.[3] These early phase clinical trials (classical phases I and II) are essential to assess safety, tolerability and immunogenicity of the vaccine candidate, as well as preliminary information on its efficacy, before progressing further to large scale phase III trials. In the current COVID-19 pandemic it is more important than ever to identify and select the most promising vaccine candidates as early as possible and stop clinical development for failing candidates to avoid wasting valuable time and resources.

Here, we discuss several practical suggestions that could accelerate early phase vaccine trials in the COVID-19 pandemic.

1 COLLABORATION AND COMMITMENT OF ETHIC COMMITTEES AND REGULATORY AUTHORITIES TO REDUCE APPROVAL TIMELINES

Thorough and critical appraisal of a clinical trials submissions by medical ethics committees (MECs) and competent authorities (CAs) is essential to safeguard the health and wellbeing of participants in medical research. However, approval for a clinical trial can take up to several weeks or even months. These timelines are simply too long to allow rapid development of a pandemic vaccine. Fortunately, there are several options to expedite the clinical trial submission process.

Timelines for clinical trial approval can be substantially reduced if dedicated and mandated (sub)commissions are established to prioritize the review of COVID-19-related research files. Expert members can be identified beforehand to participate in these dedicated sub-commission and provide the necessary background knowledge to facilitate a high quality and effective review. Also, investigators and sponsors spent a tremendous amount of time drafting documents and assembling a trial application dossier that is submitted as a complete dossier to the MEC and CA. Time can be further reduced if applicant and reviewer work in parallel. For instance, the key documents (e.g. study protocol, investigator's brochure and investigator's medicinal product dossier) should be submitted immediately once available for initial assessment by the MEC. Other key documents, such as subject information leaflets, consent forms and recruitment materials can be prepared while awaiting the first assessments of the clinical trial application and should be submitted once finalized. The clinical trial submission process can be accelerated if applicant and evaluator are willing and able to work in tandem. Optimization of submission procedures will require good communication and flexibility of both investigators, MECs and authorities to minimize timelines in pandemic setting.

We had discussions with the Central Committee on Research Involving Human Subjects (CCMO) in the Netherlands to explore the possibilities for such an accelerated review procedure for COVID-19 vaccine research on a national level. Already early on in the COVID-19 pandemic the committee implemented a national *Fast Track* procedure for COVID-19 vaccine research to reduce approval timelines.[4] Following this decision several accredited local MECs and competent authorities initiated similar fast track procedures pertaining to COVID-19 related research.

2 CLINICAL TRIALS APPLICATIONS FOR GENETICALLY MODIFIED VACCINE CANDIDATES

Progress in biomolecular insight of pathophysiology combined with innovation in biotechnology has led to an increase in genetically modified organisms (GMO) based vaccine platforms. These platforms are currently deployed to develop novel vaccines against SARS-COV-2. GMO-containing vaccines candidates for SARS-COV-2 mainly consist of viral vector-based vaccines and viruses that have been genetically modified to become attenuated.[5] Performing vaccine trials with GMOs is inherently more complex due to additional legal requirements. Obtaining environmental permits for trials with GMO interventions can be difficult. In Europe, clinical trials involving a GMO must be compliant with the European GMO legislation; authorization needs to be obtained from national authorities besides the 'regular' approval of the competent authority and MEC.[6] Legislation pertaining to the use and deliberate release of GMOs is not fully harmonized between countries. Therefore, regulation by national authorities can differ substantially between countries. In addition, regulatory bodies often work independently from each other. Obtaining the environmental permit for a clinical trial with a GMO-based vaccine can take up to several months, depending on the country-specific regulatory framework for the use of GMOs.[7] These timelines impose a significant hurdle for rapid clinical development of a vaccine against SARS-COV-2. However, temporary legal exceptions can be made to the application process when there is a clear and urgent need for human health develop a GMO for the purpose of a vaccine. In NL, an accelerated licensing procedure has been implemented for GMO-based vaccine candidates. Following discussions between researchers and regulators, an emergency regulation has been implemented in NL by use of a ministerial decree. In short, the emergency regulation means that permits can be issued immediately, even before the clinical trial approval. This emergency regulation allows to process permit application through the regular licensing procedure, but drastically shortens the decision period of the application from 120 days to maximum of 28 days.[8]

The GMO application process can be further expedited if the applicant consults the competent authorities and advisory bodies in an early stage and the review board has previous experience with the vaccine platform. In the Netherlands, the authorities can be consulted to provide a pre-advice (before the formal clinical trial application) to help streamline the application process. Pro-active and transparent communication between applicant, sponsors and regulators are essential to complete the mandatory GMO applications in an expedited manner.

3 ALLOW INVESTIGATORS TO START PREPARING FOR TRIALS BEFORE THE FORMAL CLINICAL TRIAL APPROVAL

Another possibility to expedite the start-up phase is to allow investigators to start recruitment and screening of potential participants before formal approval of the clinical trial. Recruitment and screening of participants are time consuming activities in early phase clinical trials. Significant time can be saved if potential participants can be identified, counseled and general health status assessed before the formal clinical trial approval. This can be achieved by a conditional approval of the clinical trial submission or by submitting a separate research protocol that solely aims to identify eligible participants for COVID-19 vaccine trials. This will allow the investigator to maintain an ongoing pool of (pre)screened healthy participants that are ready to be enrolled in vaccine trials. It is imperative that participants are again counseled and consented for the final, approved, study protocol prior to enrollment in the clinical trial. However, the majority of recruitment and screening activities will than already be completed and will allow for a rapid start of the clinical trial.

4 CENTRALIZATION OF FACILITIES TO PERFORM IMMUNOGENICITY ASSAYS FOR COVID-19 VACCINES

Another rate-limiting step in clinical trial start-up is the validation of immunogenicity assays. The relevant immune assays in vaccine trials will typically depend on the mechanism of action of the vaccine candidate and possible known correlates of protection. Unfortunately, for SARS-COV-2 such correlate of protection have not yet been identified. However, most current COVID-19 vaccine trials use some form of virus neutralizing assay to assess immunogenicity. For biosafety reasons, such assays which involve propagation of SARS-COV-2 should be performed at biosafety level grade 3 facilities. Centralized availability of facilities where such assays have been standardized, validated and implemented would accelerate initiation of trials and enhance comparability of trial results.

5 CONSIDERATIONS WHEN DESIGNING EARLY PHASE CLINICAL TRIALS FOR COVID-19 VACCINES

Vaccines are a heterogeneous group of medicinal products. A myriad of vaccine technologies are currently deployed to develop a prophylactic vaccine against SARS-CoV-2.[5] Route of administration, dosing regimens and study endpoints are all dependent of the type of vaccine technology used. Consequently, clinical trials need to be tailored to the vaccine candidate. There are guidelines for clinical evaluation of vaccines, however, these are not developed for outbreak situations where accelerated development is key. The European Medicines Agency (EMA) is providing guidance and early support for COVID-19 treatments and vaccine development.[9] The United States Food & Drug Administration (FDA) has created a similar emergency program (Coronavirus Treatment Acceleration Program).[10] Input from regulators is needed to facilitate effective data collection for safety, immunogenicity and efficacy, needed for regulatory approval. Seeking early regulatory advice to improve early clinical trial design may pay off in the long run of vaccine development.

Another practical approach to expedite early phase clinical trials is to use combined phase I and II study protocols. Several developers have already registered phase I/II protocols for COVID-19 vaccine candidates (NCT04324606, NCT04352608, EudraCT 2020-001038-36). Combining classical phase I and II studies in a single trial protocol with clear go/no-go criteria enable researchers to rapidly move forward to pivotal trials if safety and efficacy endpoints appear favorable. Performing combined phase I/II research protocols requires greater monetary investment. Smaller biotech companies may need to acquire funding or engage in partnerships with larger biopharmaceutical companies to perform these kinds of study protocols. It is imperative that these combined phase I/II trials should incorporate sufficient safeguards such as independent data safety monitoring board reviews (DSMB) to identify safety signals at an early stage and prevent unnecessary exposure of participants. Nonetheless, a well-designed combined phase I/II clinical trial may save valuable time in early clinical development as this reduces the administrative burden and review processes by combining research protocols for each classical drug development phase.

In conclusion, the conventional vaccine development paradigm is not equipped to allow rapid vaccine development in view of pandemic crisis such as COVID-19.[11] This perspective gives some practical suggestions and examples that could help investigators, developers and authorities to accelerate early clinical vaccine development for COVID-19. Precious time can be saved during the initiation phase of early clinical trials, through fast track application pathways, by allowing investigators and authorities to work in parallel rather than a sequential order and by identifying, validating and centralizing immunogenicity assays as soon as possible. Early discussions with authorities and regulators about study design may also facilitate guided and rapid drug development. Vaccine development in a pandemic setting requires flexibility of both investigators, developers and authorities. In these trying times we need to find practical solutions and make joint efforts to expedite vaccine development for COVID-19.

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SUPPLEMENTARY FIGURES AND TABLES

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CHAPTER 9

DISCUSSION AND FUTURE PERSPECTIVES

In this dissertation

The work for this thesis has been executed in a turbulent time for the fields of respiratory viral diseases, vaccinology and clinical pharmacology. The research for this thesis initially began with work on clinical trials investigating novel vaccines and adjuvants for respiratory syncytial virus (RSV) and Influenza. Then, half-way through this PhD-project that set out to investigate innovations in clinical development for respiratory viruses, the COVID-19 pandemic broke out.

The COVID-19 pandemic posed an enormous challenge for the entire world including medicine. However, driven by an unprecedented societal demand for treatment and vaccines for COVID-19, the pandemic also led to many innovations in clinical and translational medicine. With limited evidence on possible treatments against SARS-COV-2, existing compounds were first experimentally administered to treat COVID-19. In parallel, new vaccines and drugs were expeditiously brought from bench to bed. The pandemic also illustrated that the traditional linear development paradigm for therapeutic interventions was not suitable to meet the quest for necessary innovation.

This thesis consists of studies investigating vaccines and therapeutics against respiratory viruses. The first sections focus on novel vaccines and adjuvants for RSV and influenza (pre-pandemic). The last section focusses both on a new and repurposed compound against SARS-COV-2, explores novel methods of accelerating vaccine trials during a pandemic and concludes with an overview of several procedures that could expedite (early) clinical development during a pandemic.

RESPIRATORY SYNCYTIAL VIRUS (RSV)

Developing a safe and effective needle-free mucosal RSV vaccine will have many benefits. By boosting local innate and humoral immunity at the site of potential virus entry – the nasal mucosa – this approach may help to protect against infection and possibly prevent subsequent transmission of the virus. Live-attenuated vaccines have historically been safe platforms for intranasal vaccination and have not been associated with vaccine-induced disease enhancement.[1] The genetically modified vaccine candidate RSVΔG was designed using reverse genetics and is characterized by the deletion of the attachment protein (G) of the wild-type virus. The vaccine was expected to be attenuated but still able to replicate by the intact fusion protein (F).[2]

As described in the introduction, RSV-vaccine candidates are tested sequentially in adults (who have had multiple RSV infections throughout their lives), seropositive children and finally in seronegative children. It was hypothesized that high pre-existing levels of neutralizing serum antibodies in adults could pre-emptively neutralize RSVΔG and thereby prevent a successful vaccine immune response.[3] An observational study was performed to characterize the levels and distribution of off-season serum neutralizing antibodies against RSV in healthy adults (*Chapter 2*). This study aimed to identify a threshold titer to be used as eligibility criterion for healthy adult participants in first-in-human studies. The distribution of antibody titers in this population also enables researchers to predict screen failure rates when selecting a threshold titer for clinical trial inclusion. Selecting lower titers of neutralizing antibody may facilitate low-grade replication of the vaccine virus, thereby increasing the chance of successful vaccination with a live-attenuated virus but also observing viral shedding (in adults). However, the prevalence of the antibody titers should be considered when selecting a threshold to avoid screening unrealistically large numbers of subjects. A threshold of 9.6 $\log_2$ neutralizing antibody titer was selected as eligibility criterion for our randomized clinical trial.

Intranasal administration of RSVΔG was shown to be safe and well tolerated in healthy adult volunteers (*Chapter 3*). Minimal signs of viral shedding further confirmed the full attenuated phenotype of RSVΔG. Substantial and prolonged replication in adults is an indicator for under-attenuation in RSV-naïve infants.[1] The safety and viral load results thus paved the way for further investigation in seropositive children. Immunogenicity analysis, however, showed no apparent induction of systemic or mucosal immunity in adults. Previous studies with live-attenuated vaccines have shown that vaccines that are highly attenuated in adults can be immunogenic in seronegative children and even under-attenuated.[4] The immunogenicity data obtained from adults with pre-existing immunity against RSV are therefore not fully predictive for the target pediatric population. The immunogenicity data from this trial also suggest that the pre-established cut-off of 9.6 $\log_2$ for neutralizing antibodies should be reconsidered, such a level of pre-existing humoral immunity may still prevent the full immune response against live-attenuated viruses in healthy adults.

The lack of immunogenicity signal in adults could also indicate that the selected single dose was too low to induce sufficient immunogenicity. A follow-up dose-selection study could further assess the dose-immune

response relationship. Alternatively, it can be attempted to alter the vaccine concept to improve immunogenicity. Both the genome and the outer surface of the recombinant RSVΔG lack the G-protein. A variant of the vaccine concept would be to complement the outer surface of RSVΔG with G-proteins. The resulting vaccine variant (G-RSVΔG) will be able to attach to the host cell via its G-protein, thereby increasing the initial infection potency.[5] The progeny virions will be identical as RSVΔG (lacking the G-protein) and highly attenuated. Lastly, altering the confirmation state of the F-protein antigen to sustain a pre-confirmation state might also improve immunogenicity of the vaccine candidate.[6]

INFLUENZA VIRUS

Mucosal vaccine candidates could have additional benefits for seasonal influenza vaccination compared to traditional intramuscular vaccination. Live-attenuated intranasal vaccines are currently available but their use in the European Union is limited to the group between 2 and 18 years of age. Elderly are at risk for influenza-related complication and hospitalization and immune response to influenza vaccines weakens at higher age. There is therefore a high need for improved vaccine strategies for this high-risk group. As stated previously, intranasal vaccines have the potential to not only prevent disease but also prevent or reduce transmission of viruses due to eliciting mucosal immune responses. Such vaccine attributes would be especially beneficial for preventing outbreaks among high-risk elderly living in long-term care facility.

An intranasal trivalent virosomal-subunit vaccine adjuvanted with *Escherichia coli* (*E. coli*) heat labile enterotoxin (NasalFlu, Berna Biotech) was withdrawn from the market after epidemiological association with facial nerve paralysis.[7] The role of the *E. coli* enterotoxin adjuvant in the development of facial nerve paralysis has never been fully elucidated, but further use has been abandoned.[8-10] Therefore, the need for alternative safe and potent mucosal adjuvants with sufficient immunogenicity of intranasal influenza vaccines remains. Bacteria like particles (BLP) derived from gram-positive bacteria containing a peptidoglycan outer surface could function as such immunostimulant. BLPs are assumed to activate toll-like receptor (TLR)-2, which subsequently leads to a cascade of events that stimulate innate immune responses and ultimately the potentiation of the adaptive immune response. The randomized control clinical trial described in *Chapter 4* assessed the safety and immunogenicity of BLPs derived from *Lactococcus lactis*, a non-pathogenic gram-positive bacterium combined with inactivated trivalent seasonal influenza vaccine (FluGEM®) in different age groups. Intranasally administered FluGEM showed a favorable safety profile for all explored doses in the age group of 18 to 49 years. Lower doses of FluGEM appeared to elicit higher IgG-titers compared to high doses. The exact immune mechanism of this dose-response relationship remains unknown, but non-linear dose immune response relationships have been described previously for other TLR2 agonists.[11] The immunogenic low dose that was selected for assessment in subjects aged over 65 years (target population) was safe and well tolerated but failed to elicit a strong immune response. The addition of a separate cohort with subjects aged over 65 years in this early clinical study gave valuable insight for the development of this adjuvant for the potential target population. Further research is needed to improve immunogenicity of the BLP-based platform for mucosal vaccination in elderly (for instance dose-optimization or adaptation of a BLP-based delivery system).

SARS-COV-2 AND CLINICAL DEVELOPMENT DURING PANDEMICS

Therapeutics often serve as a first-line defense against an emerging novel pathogen. For the treatment of COVID-19 several antiviral, immunomodulatory and anticoagulant drugs have been approved. Many of these therapies were repurposed compounds or antiviral therapies already in late-stage clinical development for other RNA viruses. Novel compounds with pathogen-specific targets needed to be development in parallel to improve the therapeutic arsenal. One of these novel compounds is ensovibep, a Designated Ankyrin Repeat Protein (DARPin) with cooperative tri-specific binding capability to the SARS-COV-2 Spike-protein. For the clinical development program of ensovibep it was needed to assess the overall feasibility of administering ensovibep in an ambulatory setting. A smaller, open label, first-in-patient study was performed in ambulatory patients with mild-to-moderate COVID-19 (target population) (*Chapter 5*). Administration of ensovibep in an ambulatory setting was well tolerated and no antibody-dependent enhancement of infection was observed. Pharmacokinetic analysis confirmed the relatively long half-life of ensovibep in patients. Interpretation of pharmacodynamic parameters was limited due to the small group size

and non-controlled design. However, the magnitude of the decline in viral load was comparable to monoclonal antibodies that received emergency authorization for COVID-19.[12,13] There was no apparent difference between high or low doses for pharmacodynamic outcomes. Outcomes of this study facilitated the next-stage clinical development of ensovibep. It was later shown that ensovibep, like many other antivirals administered later in the disease course, did not improve clinical outcomes in hospitalized COVID-19 patients compared to patients receiving standard care (including remdesivir).[14-16] However, preliminary top-line data from the ambulatory population suggest a possible reduction of COVID-19 hospitalization and death in patients treated with ensovibep (press communication), highlighting the need for early treatment initiation of antivirals in respiratory virus infections.[16] Further data from late-stage clinical development is required by regulators for the market authorization of ensovibep.

Off-label use of 4-aminoquinolines (chloroquine and hydroxychloroquine) for the treatment and prophylaxis of COVID-19 occurred on a large scale in the early phase of the COVID-19 pandemic.[15] The *in vitro* antiviral activity against SARS-COV-2, its well-characterized safety profile from auto-immune and malaria indications and widespread availability made these compounds candidates for repurposing for the treatment and prophylaxis of COVID-19. In addition, it was hypothesized that the immunomodulatory effects of hydroxychloroquine could also treat or prevent adverse immune reactions (such as cytokine storms) that occurred in critically ill COVID-19 patients. The exact mechanism of hydroxycholoroquine's immunomodulation is not completely understood. In short, hydroxychloroquine is believed to have multiple effects on both innate and adaptive immunity, including endosomal TLR signalling, inhibition of T cell activation, and altered differentiation of memory B cells. However, *in vitro* experiments assessing the immunomodulatory effects often used hydroxychloroquine concentrations that far exceded clinical concentrations observed in patients.[17-20] To better assess and quantify the immunomodulatory properties of clinically relevant doses of hydroxychloroquine, we conducted a study (*Chapter 6*) that combined both *in vitro* and *ex vivo* experiments on human peripheral blood mononuclear cells (PBMCs). For the *ex vivo* part of this study, a randomized clinical trial was performed in healthy volunteers that received a 5-day treatment course of hydroxychloroquine with a cumulative dose of 2400 mg. This was the dose that was recommended

(off-label) regimen by national guidelines for the treatment of moderate-to-severe COVID-19 at the time of the study. The *in vitro* part of this study showed that hydroxychloroquine had strong dose-dependent inhibitive effects on TLR responses and to a lesser extent inhibited B-cell proliferation but had no effects on T cell activation. Strong immunosuppressive effects were observed at high (>1000ng/mL) concentrations of hydroxychloroquine. Such concentrations, and thus immune effects, were unlikely attained in PBMCs from our clinical study that used a 5-day course of hydroxychloroquine with peak plasma levels of 100-150 ng/mL. The discrepancy between *in vivo* and *in vitro* experiments suggests that the dose-regimen used for off-label treatment of COVID-19 resulted in insufficient drug exposure of hydroxychloroquine to reach clinically relevant concentrations. A slow clinical onset (3-6 months) of immunomodulatory therapeutic effects of hydroxychloroquine is observed in patients with auto-immune conditions that use comparable daily dosing regimen.[21,22] This can in part be attributed to hydroxychloroquine's high volume of distribution, possibly explained by sequestration to lysosomes.[21] Steady-state concentrations are only reached after months while the drug is likely to further accumulate intracellularly. One of the limitations of this study was that we did not measure the intracellular concentration of hydroxychloroquine. This study exemplifies that a *reverse translation approach* may provide mechanistic insights that further oppose the use of hydroxychloroquine for COVID-19 based on functional immunological effects. It corroborates and explains the clinical evidence that there is no role for (short-term use of) hydroxychloroquine for prevention or treatment of COVID-19.[23]

Next to innovation in drug and vaccine development, a pandemic also demands innovation in research methodology, organization and regulations. Large scale deployment of COVID-19 vaccines depended on the evaluation of pivotal phase III field trials. In these trials, thousands of participants are vaccinated in endemic countries to eventually compare cases in the control group to the active group in order to evaluate the efficacy of the vaccine. However, if infection rates drop, for instance due to governmental measures to prevent spread of the disease (such as social distancing, quarantine, promoting hygiene etc.), it will take longer to establish vaccine efficacy or the study can become underpowered. In *Chapter 7*, we therefore explored a more agile approach to conducting vaccine field trials, namely by identifying local surges of infection spread

in a population, so-called hot-spots. *In silico* experiments were performed by modelling a pandemic outbreak and simulating vaccine trials using the proposed *hot-spot* identification approach versus a traditional vaccine trial. Our experiments showed that the key endpoints (such as achieving a certain number of cases) can be reached more efficiently using the *hot-spot* approach and duration of vaccine trials can be reduced accordingly. The model parameters can be adjusted to fit disease characteristics for any future pandemic threat. This chapter also highlights the need to prepare clinical trial infrastructure for future pandemics. Several organizational requirements are provided to improve vaccine trial conduct for a more rapid and agile response to a pandemic.

During a pandemic it is important to select the most promising compounds as early as possible and stop clinical development for less promising candidates to avoid wasting valuable time and resources. Early phase clinical trials play a vital role in this. Next to safety and tolerability assessment in healthy individuals, these trials can also provide preliminary insight in the intended effects and should utilize a cyclical translational approach (e.g. forward and backward translation). Next to a rational 'content-driven' approach, pandemic drug development also requires acceleration of the 'administrative' part of the drug developmental trajectory. *Chapter 8* identifies five organizational and regulatory bottlenecks specific for early-stage vaccine clinical development and provides recommendations to expedite clinical development in a pandemic setting.

Medical ethics review committees and competent authorities should utilize accelerated review programs for pandemic clinical development. Fortunately, many committees and authorities have quickly adapted fast-track procedures for COVID-19 clinical trials which reduced time from submission of study-protocol to first-dose significantly.[24] Several new vaccines platforms such as reverse engineered live-attenuated, chimeric and recombinant vector vaccines are classified as genetically modified organisms (GMO). Clinical trials that investigate GMO vaccines need to additionally comply with European GMO-legislation relating to biosafety and possible introduction of the product into the environment. Time before approval for use of GMOs in clinical trials varies greatly. It takes several months to years depending on the country reviewing the application. In the Netherlands special exemptions for COVID-19 clinical research were implemented for GMO-based vaccines.[25]

Recruitment and screening of potential participants are costly and time-consuming activities for (early-phase) clinical trials. Conditional approval of study protocols may allow the investigator to identify eligible participants for clinical trials. Alternatively, a pool of healthy and willing participants may be identified, screened for eligibility and kept on stand-by before the trial commences. At CHDR the *beReady* protocol was designed to identify and (pre-)screen healthy potential participants based on the common standard eligibility criteria for COVID-19 clinical trials. Participants that were found to be potentially eligible for study participation were pooled in a database and kept on stand-by, participants were subsequently invited to partake in COVID-19 clinical trials. This approach reduced recruitment time and screen failures substantially. Validation of laboratory assays may be a rate-limiting step for clinical trial start-up. Harmonization and centralization of laboratory is needed to enable better comparison of results between various vaccine trials. The Coalition for Epidemic Preparedness Innovations (CEPI) has since established a centralized laboratory network that support COVID-19 vaccine development and the World Health Organization has issued international standards for immune assays.[26,27]

Developers are advised to seek regulatory advice early on to improve early-phase clinical trial design. Clinical trials can then be tailored to address key questions needed for market licensing. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have set-up mandated task forces of (in)formal consultation and advice.[28]

Future perspectives

The COVID-19 pandemic has led to several breakthroughs for the treatment and prevention of respiratory virus disease. A real gamechanger was the utilization of mRNA and adenovector-based platforms, that were developed decades earlier. These platforms differ from previous vaccine technologies in that it uses the recipient's cell own translational system to generate antigens, much like natural occurring virus infections. In addition, it is relatively simple to adapt these vaccines to include new antigens against novel mutations of concern. As these novel vaccine technologies have come into use, we are only starting to understand their potential. Longevity of immune responses may be further improved, and heterologous prime-boost regimens could utilize a potential synergistic effect between mRNA and adeno

vector-based vaccines. The current successes of these platforms may be a starting point for the development of novel vaccines for other viruses or pathogens.

There has also been a renewed interest for needle-free mucosal vaccination because locally elicited immunity could help to prevent infection, reduce transmission of respiratory viruses and reduce vaccine hesitancy due to injection-related fears.[29,30] Evoking potent systemic and long-lasting mucosal immune response will be difficult to achieve in all age groups, as was illustrated in *Chapters 3 and 4* in this thesis. Novel delivery technologies and safe adjuvants are needed to improve immunogenicity of mucosal vaccines. Development of intranasal vaccines may be the next step to prevent not only disease but also spread of respiratory viruses.

For RSV there may be a breakthrough imminent with several vaccines candidates in late-stage clinical development.[31,32] Immunogenicity and efficacy data from controlled human challenge studies show promising results for these compounds.[33,34] The majority of these late-stage vaccines utilize the prefusion configuration of the fusion protein (F) as main antigen.[35] All current vaccines in late-stage development use vaccination strategies that target either the elderly or maternal population (to subsequently immunize newborns).[35]

Advances have also been made in the development of a universal influenza vaccine, which is considered a holy grail in vaccinology. With many new pan-influenza vaccine candidates attempting to direct an immune response to more conserved regions on the hemagglutinin protein. A universal vaccine could also serve as an important defense against pandemic influenza. Similar initiatives are ongoing to develop pan-corona vaccines.

Major challenges for the development of therapeutic drugs against respiratory viruses will remain. The clinical benefits of current antiviral therapies are limited, especially during late-stage infection. In addition, the risk will remain that drug-resistant virus strains emerge under therapeutic pressure. Ideally, new antiviral therapies should have broad effectivity against multiple strains of viruses, exhibit more potent pharmacodynamic effects such as viral load reduction and decreased duration of shedding with drug formulations that enable outpatient use (e.g. oral or inhalation formulations). Easy-to-use formulations that can be used at home, early in the disease course, could improve efficacy and clinical benefit. Use of antiviral combination preparations could also improve efficacy and reduce the development of antiviral resistance.

Demonstrating pharmacodynamic effects in early clinical trials that involve healthy volunteers is often difficult for compounds targeting infectious diseases. These compounds generally bind to targets that are only present on the pathogen itself or depend on host-pathogen interaction and are of course not present in non-infected healthy volunteers. Controlled human infection models (also known as *human challenge studies)* can provide researchers with the unique opportunity to assess pharmacodynamics or vaccine efficacy relatively early in the development trajectory. That is, if there is a sound scientific and ethical justification for exposing healthy volunteers to a pathogen. Although human challenge studies cannot replace large phase 3 studies, they can provide important insight about the potential of a new therapy or vaccine before field trials are initiated. Information on dose-(immune)response relations from challenge study will also improve outcomes in subsequent phase 3 trials. Even more importantly, the unique circumstances created by controlled human infection models could solve knowledge gaps in the pathogenesis of respiratory viruses and may help to identify new correlates of protection. In recent years human challenges have been frequently used for the clinical development of vaccines and drugs against RSV, a self-limiting disease in healthy adults, and may also play a role in the development of other seasonal respiratory viruses. It is a prerequisite that virus challenge stocks used in these studies are updated to represent the most prevalent and clinically relevant virus strains.

Lastly, the COVID-19 pandemic showed that equitable access to new therapies and vaccine can be significantly improved. Sharing of intellectual property, technology and know-how could enable more diverse geographical spread of production facilities and help to better distribute and give access to vaccines and medicines. In addition, investing in supply (cold) chain facilities is needed for low-to-middle income countries to ensure equitable access to healthcare products.

Conclusion

This thesis exemplifies several innovative approaches for the clinical development of vaccines and therapeutics against respiratory viruses. However, there still there remains an urgent need for further innovation to prevent and treat respiratory viruses. Due to the mutagenic capability of these viruses and natural selection we will need to adapt to keep up with the viruses. We are essentially aiming at a moving target.

The COVID-19 pandemic has shown that the traditional development paradigm for vaccines and therapeutics can be altered. This paradigm change was the key to success to dampen a pandemic threat. It is inevitable that new pandemic threats will emerge. Globalization, overpopulation, intensive life-stock farming and climate change is likely to increase the risk of new epidemics. We should reflect and learn lessons from the response to COVID-19 to be better prepared for the next pandemic. Pandemic preparedness and investing in further innovation could prevent future emerging infectious diseases from becoming the next global health disaster.

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CHAPTER 10

NEDERLANDSE SAMENVATTING

Dit proefschrift is tot stand gekomen tijdens een turbulente periode op het gebied van (luchtweg)virusonderzoek, vaccinologie en klinische farmacologie. Het onderzoek voor dit proefschrift begon in eerste instantie met klinisch onderzoek naar nieuwe vaccins en adjuvans voor respiratoir syncytieel virus (RSV) en influenza. Echter brak halverwege dit PhD-onderzoek naar luchtwegvirussen de COVID-19 pandemie uit. De pandemie vormde een enorme uitdaging voor de hele wereld, in het bijzonder voor de geneeskunde. De ongekend grote maatschappelijke vraag naar nieuwe behandelingen en vaccins voor COVID-19 leidde tot vele soorten innovatie in klinisch en translationeel onderzoek. Noodgedwongen werd eerst op basis van schaarse wetenschappelijke informatie over het virus reeds bestaande geneesmiddelen in onderzoeksverband gegeven aan patiënten om COVID-19 te behandelen. Tegelijkertijd werd in laboratoria geprobeerd om nieuwe vaccins en geneesmiddelen te ontwikkelen om uiteindelijk toe te dienen aan patiënten. De pandemie liet zien dat de traditionele 'lineaire' manier om medicijnen te ontwikkelen niet voldeed aan de vraag naar noodzakelijke innovatie.

Dit proefschrift bestaat uit verschillende onderzoeken naar vaccins en geneesmiddelen voor luchtwegvirussen. Het eerste gedeelte van dit proefschrift focust op nieuwe vaccins en adjuvans voor RSV en influenza (verricht vóór de pandemie). Het tweede gedeelte richt zich op onderzoek naar nieuwe medicijnen, als ook de herbestemming van bestaande geneesmiddelen (*repurposing*) tegen SARS-COV-2. Daarnaast worden nieuwe methoden geëxploreerd om vaccinonderzoek te versnellen tijdens een pandemie en wordt tenslotte een overzicht gegeven van verschillende procedurele processen die versneld kunnen worden in (vroege fase) klinische ontwikkeling.

RESPIRATOIR SYNCYTIEEL VIRUS (RSV)

Het ontwikkelen van een veilig, effectief en naaldloos mucosaal vaccin heeft veel potentiële voordelen. Door het versterken van zowel de humorale als de lokale afweer op de plek waar het virus binnendringt – op het niveau van het neusslijmvlies – kan een vaccin beschermen tegen infectie en mogelijk ook de verspreiding van het virus. Vaccins bestaande uit levend verzwakte virus bleken in het verleden veilige vaccinatietechnologieën voor intranasale toediening en zijn niet geassocieerd met vaccin geïnduceerde ziekte-ergergering. RSVΔG, een genetisch gemodificeerd kandidaat-vaccin, werd ontworpen door gebruik te maken van omgekeerde genetica (*revers genetics*) om het virushechtingseiwit (G) te verwijderen. Hierdoor werd verwacht dat het vaccinvirus verzwakt was, maar nog steeds in staat om te repliceren doormiddel van het intacte fusie-eiwit (F).

RSV-vaccins moeten eerst in volwassen worden getest. Deze populatie heeft al meerdere keren RSV-infecties doorlopen. Hierna kan een vaccin achtereenvolgens worden onderzocht in seropositieve kinderen en vervolgens in seronegatieve kinderen (die nog geen afweerstoffen hebben tegen RSV). Hypothetisch kunnen de circulerende pre-existente antistoffen in volwassenen vroegtijdig het levend verzwakt vaccin (RSVΔG) neutraliseren en zo een succesvolle afweerreactie voorkomen. Daarom werd een observationele studie verricht om de hoogte en verdeling van serum antistofspiegels tegen RSV in volwassenen te karakteriseren (*hoofdstuk 2*). Deze studie was gericht op het identificeren van een antistoftiter dat gebruikt kon worden als geschiktheidscriterium voor vroege fase klinisch onderzoek in volwassenen (waar het onderzoek voor het eerst in mensen wordt toegediend). Het kennen van de verdeling van antistoffen in de onderzoekspopulatie maakt het ook mogelijk voor onderzoekers om te voorspellen hoeveel deelnemers gescreend moeten worden om een geschikt aantal kandidaten te identificeren. Het selecteren van deelnemers met lage antistoftiters kan er mogelijk voor zorgen dat het verzwakte virus in mindere mate toch kan repliceren. Een lage mate van replicatie zou ervoor kunnen zorgen dat een succesvolle afweerreactie op gang komt, maar het vergroot ook de kans op uitscheiding van virusdeeltjes. Het is hierbij belangrijk om de prevalentie van antistoffen mee te wegen wanneer een drempelwaarde wordt gekozen als inclusiecriterium voor klinisch onderzoek. Bij een te lage drempelwaarde moeten onrealistisch grote groepen deelnemers worden gescreend op antistoffen. Een drempelwaarde van 9.6 $\log_2$ werd geselecteerd als inclusiecriterium voor het klinische vaccinonderzoek met RSVΔG.

De intranasale toediening van het kandidaatsvaccin RSVΔG bleek veilig en goed getolereerd in gezonde volwassenen. Het volledig geattenueerde fenotype van RSVΔG werd bevestigd door de zeer lage mate van virusuitscheiding. Substantiële en langdurige replicatie van het vaccinvirus bij volwassenen is een indicatie dat het virus niet voldoende is verzwakt voor de pediatrische populatie. De uitkomsten van vaccinveiligheid en uitscheiding maken het mogelijk om het vaccin verder te onderzoeken in seropositieve kinderen. De analyse van immunogeniciteit liet geen

duidelijke inductie zien van systemische of mucosale afweer. Eerdere onderzoeken met levend verzwakte vaccins lieten zien dat vaccins met een sterk verzwakte werking in volwassenen toch immunogeen kunnen zijn in seronegatieve kinderen en in sommige gevallen zelfs niet genoeg verzwakt zijn. De verkregen immunogeniciteitsdata uit dit onderzoek zijn dus niet volledig voorspellend voor de beoogde pediatrische populatie. De uitkomsten van deze studie suggereren ook dat de eerder geselecteerde antistoftiter van 9.6 $\log_2$ moet worden heroverwogen. Deze mate van rondcirculerende antistoffen in gezonde volwassenen zou een volledige immuunreactie tegen het verzwakte virus mogelijk nog kunnen hinderen.

Het ontbreken van een signaal van immunogeniciteit bij volwassenen zou er ook op kunnen wijzen dat de geselecteerde vaccindosis te laag was om een goede afweerreactie te induceren. Een vervolgstudie zou verschillende oplopende doseringen kunnen onderzoeken om de dosis-effect relatie te beoordelen. Als alternatief zou het vaccinconcept kunnen worden aangepast om de immunogeniciteit te verbeteren. Het hechtingseiwit (G) ontbreekt zowel in het genoom als in het virusoppervlakte van RSVΔG. Als variant op het vaccinatieconcept kan het buitenoppervlak van het RSVΔG viruspartikel worden gecomplementeerd met G-eiwitten (terwijl het genoom hetzelfde blijft). De variant G-RSVΔG zou initieel kunnen binden aan de gastheercel door het hechtingseiwit (G). Hierdoor wordt de potentie van het virusvaccin om te infecteren vergroot. Het nageslachtvirion van het vaccin zal identiek zijn aan RSVΔG en zal dus sterk verzwakt zijn. Tenslotte zou de confirmatiestatus van het F-eiwit aan het vaccin kunnen worden aangepast naar de pre-confirmatie staat om de immunogeniciteit van het vaccin verder te verbeteren.

INFLUENZA VIRUS

Mucosale vaccins kunnen ook extra voordelen hebben voor seizoensgebonden griepvaccinatie vergeleken met de huidige intramusculaire vaccinatietechniek. Levend verzwakte intranasale vaccins zijn beschikbaar, maar het gebruik hiervan binnen de Europese Unie is beperkt tot 2 tot 18-jarigen. Ouderen lopen met name risico op griep gerelateerde complicaties en ziekenhuisopnames. Afweerreacties op griepvaccins nemen af op hogere leeftijd. Daarom bestaat er een grote behoefte aan verbetering van vaccinatiestrategieën voor deze hoogrisicogroep. Zoals eerder beschreven hebben intranasale vaccins de potentie om niet alleen ziekte voorkomen, maar ook virustransmissie te verminderen door het opwekken van afweer op het niveau van het neusslijmvlies. Dergelijke vaccineigenschappen zouden bijzonder gunstig zijn om griepuitbraken te voorkomen onder ouderen met een hoog risico op complicaties (zoals bewoners van verpleeghuizen).

Een intranasaal trivalent virosomaal subunit vaccin met als adjuvans *E. coli* hittelabiel enterotoxine (NasalFlu, Berna Biotech) werd van de markt gehaald nadat een epidemiologische associatie met aangezichtsverlamming (Bellse parese) werd gevonden. De rol van het enterotoxine adjuvans bij het ontwikkelen van aangezichtsverlamming is nooit volledig opgehelderd, maar het gebruik van het toxine als adjuvans is verlaten. Om die reden bestaat er behoefte aan een alternatief mucosaal adjuvans, dat voldoende immuniteit opwekt en veilig is. Bacterie-achtige partikels afgeleid van grampositieve bacteriën met een peptidoglycaan buitenoppervlakte kunnen mogelijk immuunstimulerend werken door activatie van toll-like receptor (TLR)-2. Activatie van TLR2 leidt tot een cascade van reacties dat het aangeboren immuunsysteem activeert en uiteindelijk ook het adaptieve immuunsysteem stimuleert. Het gerandomiseerde, gecontroleerde, klinisch onderzoek in *hoofdstuk 4* onderzocht de veiligheid en immunogeniciteit van bacterie-achtige partikels afkomstig van de *Lactococcus lactis*, een niet-pathogene grampositieve bacterie, gecombineerd met geïnactiveerd trivalent seizoensgebonden griepvaccin (FluGEM®) in verschillende leeftijdsgroepen.

Intranasaal toegediend FluGEM liet een gunstig veiligheidsprofiel zien voor alle geteste doseringen in de leeftijdscategorie van 18 tot 49 jaar. Lagere doses van FluGEM leken hogere IgG antistoftiters op te wekken in vergelijking met de hoge doseringen. Het exacte immuunmechanisme achter deze dosis-effect relatie is nog onbekend. Niet-lineaire dosis-effect relaties zijn eerder beschreven voor TLR2 agonisten. De meest immunogene dosering werd vervolgens onderzocht bij vrijwilligers ouder dan 65 jaar en bleek veilig en goed verdragen. Deze dosering slaagde er echter niet in om een sterke afweerreactie op te wekken. De toevoeging van een cohort met oudere patiënten bleek een waardevol inzicht te geven voor de verdere ontwikkeling van dit adjuvans voor één van de mogelijke doelpopulaties (ouderen). Verder onderzoek, zoals bijvoorbeeld dosisoptimalisatie of aanpassingen in de manier hoe het vaccin aankomt in het lichaam, zijn nodig om de immunogeniciteit van bacterie-achtige partikels te verhogen voor de oudere doelpopulatie.

SARS-COV-2 EN KLINISCHE ONTWIKKELING TIJDENS EEN PANDEMIE

Medicijnen dienen vaak als eerstelijnsverdediging tegen een opkomende nieuwe ziekteverwekker (vóórdat een vaccin is ontwikkeld). Voor de behandeling van COVID-19 zijn verschillende middelen met antivirale, immunomodulerende of anticoagulante werking goedgekeurd. Veel van deze therapieën waren herbestemde (*repurposed*) geneesmiddelen of antivirale middelen die al in een laat stadium van klinische ontwikkeling waren voor andere RNA-virussen. Tegelijkertijd werden in parallel nieuwe geneesmiddelen met voor het pathogeen specifieke aangrijpingspunten ontwikkeld. Een van deze nieuwe geneesmiddelen is ensovibep, een zogenoemde 'Designated Ankyrin Repeat Protein' (DARPin) met een coöperatief tri-specifiek bindingsvermogen voor het SARS-COV-2 S eiwit. Voor het klinisch ontwikkelingprogramma van ensovibep was het nodig om vast te stellen of het haalbaar was om het geneesmiddelen toe te dienen in een ambulante populatie. Daarom werd in een open onderzoeksopzet (open label) in een kleine groep ambulante patiënten met mild tot matige COVID-19 het onderzoeksmiddel voor het eerst toegediend (*hoofdstuk 5*). Ensovibep werd goed verdragen in ambulante COVID-19 patiënten zonder tekenen van antistofafhankelijke verergering van infectie. Farmacokinetische analyses bevestigden de relatief lange halfwaardetijd van ensovibep in patiënten met SARS-COV-2. De interpretatie van farmacodynamische parameters werd beperkt door een relatief kleine groepsgrootte en onderzoeksopzet zonder controlegroep. De omvang van de geobserveerde afname van de virusuitscheiding was echter vergelijkbaar met het effect van de eerdere geautoriseerde monoklonale antilichamen voor COVID-19. Voor de farmacodynamische uitkomstmaten was er geen duidelijk verschil tussen het gebruik van lage of hoge dosering van ensovibep. De uitkomsten van deze studie faciliteerde de verdere klinische ontwikkeling van ensovibep. In later onderzoek bleek dat ensovibep geen verbetering gaf in gehospitaliseerde patiënten met COVID-19 vergeleken met de standaard behandeling, net zoals veel andere antivirale middelen die relatief laat in het ziektebeloop worden toegediend. Echter suggereert voorlopige data afkomstig uit de ambulante populatie een mogelijk reductie in ziekenhuisopnames door COVID-19 en sterfte in patiënten behandeld met ensovibep (perscommunicatie). Dit benadrukt de noodzaak van een vroege start van behandeling met antivirale middelen bij luchtwegvirusinfecties. Meer gegevens afkomstig uit latere fase klinische onderzoek zijn nodig voor een eventuele marktautorisatie van ensovibep.

Offlabel gebruik van 4-aminoquinolonen (chloroquine en hydroxychloroquine) voor de behandeling of profylaxe van COVID-19 vond op grote schaal plaats in de eerste fase van de coronapandemie. De redenen hiervoor waren *de in vitro* antivirale activiteit van het middel tegen SARS-COV-2, een goed omschreven en bekend veiligheidsprofiel afkomstig van het gebruik bij malaria en auto-immuun aandoeningen en de initiële wijdverspreide beschikbaarheid van het middel. Daarnaast bestond de hypothese dat de immunomodulerende effecten van hydroxychloroquine de nadelige gastheerafweerreactie op SARS-COV-2 (zoals cytokinestormen) kon voorkomen of behandelen. Het exacte immunomodulerende mechanisme van (hydroxy)chloroquine wordt echter nog niet volledig begrepen. Hydroxychloroquine zou meerdere effecten hebben op zowel het aangeboren als de adaptieve afweer, waaronder endosomaal TLR signalering, inhibitie van T-cel activering en veranderde differentiatie van geheugen B-cellen. *In vitro* experimenten naar immunomodulerende effecten van hydroxychloroquine gebruikten echter veel hogere geneesmiddelconcentraties dan de klinische concentraties gezien bij patiënten. Een studie werd daarom verricht om de immunomodulerende eigenschappen van klinisch relevante hydroxychloroquine doseringen beter te kunnen beoordelen (*hoofdstuk 6*). Dit onderzoek combineerde zowel *in vitro* als *ex vivo* experimenten met humane perifeer bloed mononucleaire cellen (PBMCs). Voor het *ex vivo* gedeelte van het onderzoek werd een gerandomiseerd klinisch onderzoek uitgevoerd in gezonde vrijwilligers die een vijfdaagse hydroxychloroquine kuur ontvingen (cumulatieve dosis van 2400 mg). Dit was de off-label dosering die werd aanbevolen door de nationale richtlijnen voor de behandeling van matig tot ernstige COVID-19 ten tijden van de studie. Het *in vitro* gedeelte van de studie liet zien dat hydroxychloroquine sterke dosisafhankelijke remmende effecten had op TLR-responsen en in mindere mate remde het B-cel proliferatie. Hydroxychloroquine had echter geen duidelijk effect op T-cel activatie. Sterke immunosuppressieve effecten werden waargenomen bij hoge (>1000 ng/ml) hydroxychloroquine concentraties. Dergelijke concentraties (en daarmee samenhangende immuuneffecten) werden zeer waarschijnlijk niet bereikt in de PBMCs afkomstig uit onze klinische studie

waarin piek plasmaconcentraties van 100-150 ng/ml werden gezien. De discrepantie tussen *in vivo* en *ex vivo* experimenten suggereert dat het doseringschema dat werd gebruikt voor de off-label behandeling van COVID-19 niet voldoende was om klinisch relevante concentraties van hydroxychloroquine te bereiken (om significante immuuneffecten waar te nemen). Het therapeutisch immunomodulerend effect van hydroxychloroquine wordt bij patiënten met auto-immuun aandoeningen vaak pas laat gezien (3-6 maanden). Dit kan deels verklaard worden door het grote verdelingsvolume van hydroxychloroquine, dit komt waarschijnlijk door de intracellulaire sekwestratie van hydroxychlorquine naar lysosomen. Steady-state concentraties worden pas na maanden bereikt terwijl het geneesmiddel waarschijnlijk verder intracellulair accumuleert. Eén van de limitaties van deze studie was dat er uiteindelijk geen intracellulaire concentratie van hydroxychloroquine was gemeten. Echter illustreert dit onderzoek dat een omgekeerde translationeel wetenschappelijke benadering mechanistische inzichten kan verschaffen die het gebruik van hydroxychloroquine voor COVID-19 weerleggen, gebaseerd op functionele immunologische effecten. Het onderzoek bevestigt en verklaart het eerder gevonden klinische bewijs dat er geen rol is voor (kortdurend gebruik van) hydroxychloroquine voor de preventie en behandeling van COVID-19.

Naast innovatie op het gebied van medicijn– en vaccinontwikkeling is tijdens een pandemie ook innovatie nodig in onderzoeksmethodiek, organisatie en regelgeving. Grote fase III veldonderzoeken moeten worden geëvalueerd voordat COVID-19 vaccins op grote schaal konden worden goedgekeurd en ingezet. In klinische onderzoeken worden duizenden gevaccineerde deelnemers vergeleken met een controle groep (die vaak geen vaccin heeft gekregen) om zo de effectiviteit van het vaccin vast te stellen. Als het aantal infecties echter daalt, bijvoorbeeld door rigoureuze overheidsmaatregelen om verspreiding tegen te gaan (zoals *social distancing*, quarantaine, hygiënemaatregelen etc.), zal het langer duren voordat de werkzaamheid met de juiste mate van nauwkeurigheid kan worden beoordeeld. In *hoofdstuk 7* werd daarom een meer adaptieve manier van klinisch vaccinatieonderzoek onderzocht. Namelijk door lokale incidentiepieken binnen een populatie te identificeren, zogenaamde *hot-spots*. Hiervoor werd een pandemische uitbraak gemoduleerd en vervolgens een klinische onderzoek gesimuleerd (*in silico experiment).* In dit experiment werd de voorgestelde methode met *hot-spot* identificatie vergeleken met de traditionele methode van vaccinatieonderzoek. Het onderzoek liet zien dat belangrijke uitkomstmaten, zoals het behalen van een bepaald aantal ziektegevallen in een groep, efficiënter kunnen worden bereikt doormiddel van de *hot-spot* methodiek. Ook kan de totale duur van het vaccinatieonderzoek worden verkort met deze methode. De parameters van het model kunnen tevens worden aangepast voor specifieke ziektekenmerken bij toekomstige uitbraken en pandemieën. *Hoofstuk 7* benadrukt ook de noodzaak om een klinische trial infrastructuur op te zetten voor toekomstige pandemische dreigingen. Meerdere organisatorische vereisten worden omschreven voor de verbetering van vaccinatieonderzoek en een snellere en adaptieve respons op een pandemie.

Om ten tijde van een pandemie te voorkomen dat kostbare tijd en middelen worden verspild is het belangrijk om zo vroeg mogelijk veelbelovende onderzoeksmiddelen te identificeren en de ontwikkeling van teleurstellende kandidaatsmiddelen vroegtijdig te stoppen. Vroege fase klinische onderzoeken spelen hierin een cruciale rol. Behalve het beoordelen van de veiligheid kunnen deze onderzoeken een voorlopig inschatting geven van de werkzaamheid van een onderzoeksmiddel. Naast een versnelde inhoudelijke evaluatie van een onderzoeksmiddel moet ook de regelgeving en de verplichte administratieve vereisten omtrent klinisch onderzoek een versnelde evaluatie mogelijk maken tijdens een pandemie. *Hoofdstuk 8* identificeert vijf organisatorische en regulatoire knelpunten voor vroege fase klinische vaccinontwikkeling en geeft verschillende aanbevelingen om dit soort onderzoek te versnellen ten tijde van een pandemie.

TOEKOMSTPERSPECTIEF

De COVID-19 pandemie heeft geleid tot verschillende doorbraken voor de behandeling en preventie van luchtwegvirussen. Een grote doorbraak was het gebruik van mRNA en op adenovectoren gebaseerde vaccinatieplatformen (welke enkele decennia geleden zijn ontdekt). Deze platformen verschillen van eerdere vaccinatiemethoden omdat ze het translationele systeem van de gastheercel gebruiken om zelf antigenen te produceren, zoals dit ook deels gebeurt bij een natuurlijke virusinfectie. Daarnaast kunnen vaccins door het gebruik van deze platformtechnieken relatief makkelijk worden aangepast voor zorgwekkende virusmutaties. Deze nieuwe soorten vaccins hebben veel potentie en we zullen in de toekomst meer begrijpen over de aanvullende werking en toepassing van soort vaccins. De duur van de

afweerreactie zou bijvoorbeeld kunnen worden verbeterd en heterologe vaccinatieschema's met mRNA en adenovirusvectors kunnen mogelijk synergistisch werken. De geboekte successen met deze soorten vaccins kunnen een beginpunt zijn voor de ontwikkeling van nieuwe vaccins tegen andere micro-organismen.

Daarnaast bestaat er een hernieuwde interesse in naaldloze mucosale vaccinaties. Het opwekken van plaatselijke immuniteit (in het luchtwegepitheel) kan helpen om infectie te voorkomen en de overdracht van respiratoire virussen te verminderen. Daarnaast kan een pijnloze vaccinatie zonder naald uitkomst bieden voor mensen met prikangsten en zo ook de vaccinatiegraad vergroten. Zoals duidelijk werd uit *hoofdstuk 3 en 4* zal het moeilijk blijven om in bepaalde leeftijdsgroepen zowel systemisch als mucosaal een potente en langdurige afweerreactie op te weken. Nieuwe vaccinatiemethoden en veilige adjuvans zijn daarom nodig om de immunogeniciteit van mucosale vaccins te verbeteren.

Een doorbraak voor RSV is mogelijk aanstaande, met meerdere kandidaatvaccins momenteel in de laatste fase van het klinisch onderzoekstraject. Eerder werd al goede immunogeniciteit en effectiviteit van RSV-vaccins gezien in gecontroleerde humane infectiestudies. Het merendeel van deze vaccins gebruiken de pre-fusie confirmatie van het fusie-eiwit als antigeen. De bovengenoemde vaccins richten zich zowel op de oudere als maternale populatie als doelgroep.

Voortgang is er ook in het ontwikkelen van een mogelijk universeel griepvaccin (in de literatuur vaak aangeduid als de heilige graal van de griepvaccins). Veel nieuwe paninfluenzavaccins proberen een immuunrespons te richten op nieuw ontdekte geconserveerde gebieden van het hemagglutinine eiwit. Een universeel vaccin zou ook kunnen dienen als belangrijke verdediging tegen een mogelijke influenzapandemie. Soortgelijke initiatieven zijn momenteel ook gaande voor de ontwikkeling van een pancoronavaccin.

Grote uitdagingen blijven bestaan voor de ontwikkeling van geneesmiddelen om respiratoire virusinfecties te behandelen. De klinische voordelen van behandeling met de huidige antivirale middelen zijn beperkt, vooral tijdens de latere stadia van infectie. Daarnaast blijft het risico bestaan dat resistente virusstammen ontwikkelen onder (persisterende) therapeutische druk. Idealiter hebben de nieuwe generatie antivirale middelen een brede effectiviteit tegen meerdere virusstammen, met meer potente farmacodynamische effecten op bijvoorbeeld het reduceren van de duur en hoeveelheid van virusuitscheiding. Daarnaast zullen er formuleringen van medicijnen moeten komen die patiënten ook thuis kunnen gebruiken (zoals orale middelen of inhalatie). Het combineren van verschillende antivirale middelen kan ook de kans op resistentieontwikkeling voorkomen en mogelijk de antivirale effectiviteit vergroten.

Het aantonen van farmacodynamische effecten in het vroege fase onderzoek, dat gebruik maakt van gezonde vrijwilligers, is vaak lastig voor geneesmiddelen gericht tegen micro-organismen. Deze middelen binden doorgaans aan doelwitten die enkel aanwezig zijn op de ziekteverwekker of zijn afhankelijk van een gastheer-pathogeen interactie. Deze aangrijpingspunten zijn niet aanwezig in niet-geïnfecteerde gezonde vrijwilligers. Gecontroleerde humane infectiestudies, waarbij gezonde vrijwilligers worden geïnfecteerd met een ziekteverwekker, kunnen een unieke kans geven om de farmacodynamiek of effectiviteit vroeg in het ontwikkelproces te onderzoeken. Zulke onderzoeken moeten aan strikte voorwaarden voldoen, er moet immers een degelijke wetenschappelijke en ethische rechtvaardiging bestaan om gezonde vrijwilligers doelbewust bloot te stellen aan een ziekteverwekker. Hoewel in veel gevallen gecontroleerde humane infectiestudies een grote fase III studie niet kunnen vervangen, kan het wel belangrijke inzichten geven over het potentieel van een nieuwe therapie of vaccin (voordat een veldstudie wordt verricht). Informatie over dosis-(immuun)respons relaties uit dit soort onderzoeken biedt ook informatie waarmee het ontwerp van fase III studies verbeterd kan worden. Daarnaast bieden deze onderzoeken unieke omstandigheden om belangrijke kennishiaten in de pathogenese van ziekten op te lossen en kunnen nieuwe correlaten van bescherming worden geïdentificeerd. Gecontroleerde humane infectiestudies zijn eerder gebruikt voor de ontwikkeling van geneesmiddelen en vaccins voor RSV en kunnen ook uitkomst bieden voor andere luchtwegvirussen. Het is hierbij belangrijk dat relevante en actuele virusstammen worden gebruikt voor de gecontroleerde infectie.

Tenslotte liet de COVID-19 pandemie zien dat er wereldwijd niet altijd eerlijke toegang was tot nieuwe therapieën en vaccins. Het delen van intellectuele eigendommen, technologieën en kennis kan helpen bij het geografisch verspreiden van productiefaciliteiten en zo de toegang tot en distributie van vaccins en medicijnen verbeteren. Daarnaast is het nodig

om te investeren in de (koude) keten faciliteiten voor lage- en middeninkomenslanden om eerlijke toegang tot gezondheidszorgproducten te waarborgen.

Conclusie

Dit proefschrift behandelt verschillende innovatieve benaderingen voor de klinische ontwikkeling van vaccins en geneesmiddelen voor respiratoire virussen. Er blijft echter een dringende noodzaak bestaan voor verdere innovatie om respiratoire virusinfecties te voorkomen en te behandelen. Vanwege de sterk mutagene eigenschappen van luchtvirussen en natuurlijke selectie zullen we de middelen die we hebben moeten aanpassen op nieuwe varianten. De COVID-19 pandemie heeft aangetoond dat het traditionele paradigma voor de ontwikkeling van vaccins en geneesmiddelen kan worden verlaten in tijden van nood. Deze paradigmaverschuiving was de sleutel tot succes voor het beperken van COVID-19 en de gevolgen van de pandemie. Dat een volgende pandemische bedreiging weer zal opduiken is helaas onvermijdelijk. Globalisering, overbevolking, intensieve veehouderij en klimaatverandering verhogen de waarschijnlijkheid op nieuwe epidemieën. We moeten daarom reflecteren en lessen trekken uit de COVID-19 pandemie om beter voorbereid te zijn op een volgende pandemie. Pandemische paraatheid en investeren in innovatie kunnen voorkomen dat een opkomende infectieziekte zich ontwikkelt tot een wereldwijde gezondheidsramp.

CURRICULUM VITAE

Johan Leendert van der Plas was born on the 3rd of September 1991 in Katwijk aan Zee, the Netherlands. After graduating pre-university education (Andreas College – Pieter Groen) in 2010 he obtained a propaedeutic diploma in psychology at the Leiden University while undertaking extracurricular philosophy courses at the Faculty of Humanities. In 2011, he switched to study medicine and obtained his medical degree from the Leiden University at the end of 2017. Johan's professional career started at the Centre of Human Drug Research, CHDR in 2018, where he was employed as research physician and project leader. After completing several early phase clinical trials across different therapeutic areas, Johan's primary research focus shifted towards Infectious Diseases. This led to a thesis in the clinical development of vaccines and therapeutics for respiratory virus infections under the supervision of prof. dr. Jacobus Burggraaf and dr. Ingrid de Visser-Kamerling. During the first wave of the COVID-19 pandemic, Johan was seconded to the Infectious Diseases department of the Leiden University Medical Centre to help set-up and coordinate two phase 3 studies for remdesivir. Right after this he started to work on the phase 2 trial for the Janssen COVID-19 vaccine. Still in a experimental setting, he administered the first COVID-19 vaccine (Ad26.COV2-S) in the Netherlands on the morning of 14 September 2020. Whilst at CHDR, Johan helped to lay out the ground for the start of a dedicated Infectious Diseases research group, conducting multiple clinical trials in RSV, influenza, COVID-19 and malaria. During his PhD Johan also trained to become a Clinical Pharmacologist. In 2022 Johan started a clinical residency at the Internal Medicine department of the Amstelland Hospital. He briefly returned as interim Clinical Study Manager at the CHDR in 2023, before commencing his specialist training in Medical Microbiology. He registered as a board-certified Clinical Pharmacologist in 2023.

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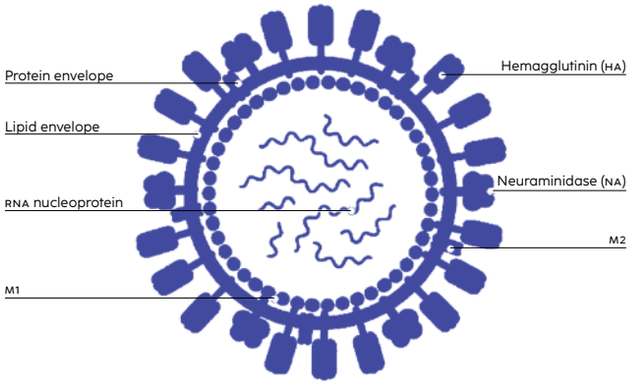
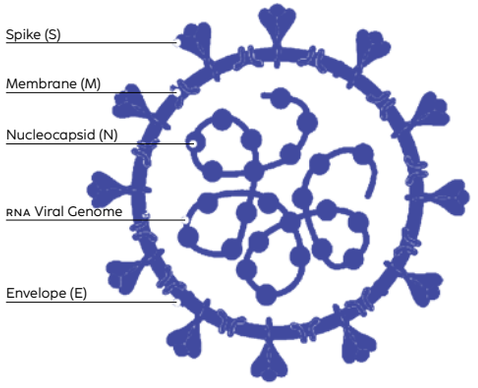
**contributed equally as first authors.*

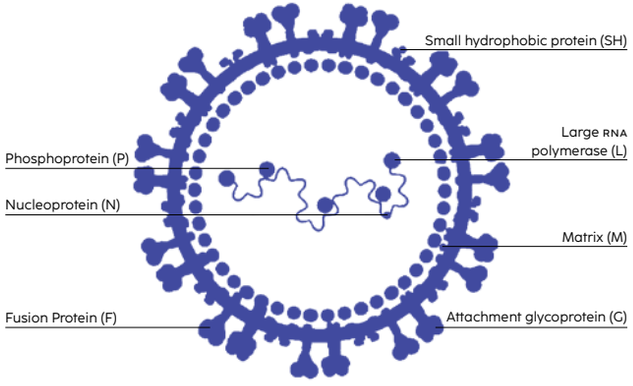
Influenza Virus



SARS-CoV-2



Respiratory Syncytial Virus



Source: https://nonacus.com/covid-19-flu-rsv-multiplex/