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## **Advances in clinical development for vaccines and therapeutics against respiratory virus infections**

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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.<sup>1-3</sup> RSV-associated ALRI is a major cause of pediatric mortality worldwide.<sup>4</sup> 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.<sup>4,5</sup> 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.<sup>6</sup> A large proportion of these vaccines use a live-attenuated vaccine (LAV) concept.<sup>7</sup> 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.<sup>8,9</sup>

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.<sup>6</sup> 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.<sup>10-15</sup> 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.<sup>14</sup>



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 F1H 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 F1H 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.<sup>16,17</sup> The exploratory study was approved by the Medical Review and Ethics Committee Foundation BEBO (Assen, NL). The F1H 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 2000g 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.<sup>18</sup> 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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 F1H 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.<sup>13-15</sup> 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.*<sup>14</sup> 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 $\leq$ 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.*<sup>11</sup> 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.<sup>11</sup> 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.<sup>19</sup> Recently, a World Health Organization (WHO) International standard antiserum has become available for rsv-A.<sup>20</sup> The neutralization assay used in this study was included in the collaborative study to establish this WHO International Standard.<sup>20</sup> 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.<sup>14,21</sup> We therefore hypothesize that lower serum vNTs can be observed outside the





rsv-season, due to the decreased incidence of rsv infection.<sup>22</sup> 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<sub>2</sub>) 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 FIN 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<sub>2</sub> serum vNTs between months for the FIN 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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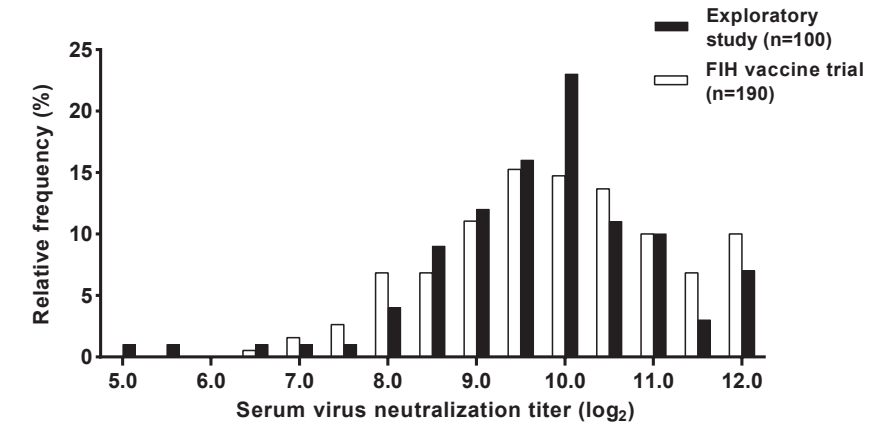
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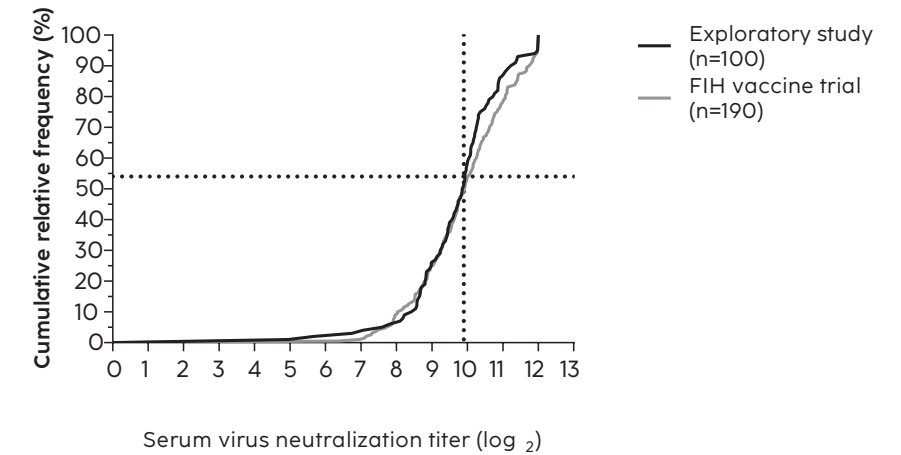
**TABLE 1 SERUM VIRUS NEUTRALIZATION TITER PER MONTH.**

Month	Serum virus neutralization titer ( $\log_2$ )		
	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 LOG<sub>2</sub> SERUM VNTS OF THE EXPLORATORY STUDY (BLACK BARS) AND THE FIH VACCINE TRIAL (OPEN BARS).**



**FIGURE 2 CUMULATIVE RELATIVE FREQUENCY DISTRIBUTION OF LOG<sub>2</sub> 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  $\leq 9.9 \log_2$*



**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.*

